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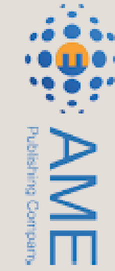

LUNG CANCER PRECISION MEDICINE

EDITORS: JIE HE, RAFAEL ROSELL, ERIC Y. CHUANG

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Room 604 6/F Hollywood Center, 77-91 Queen's road, Sheung Wan, Hong Kong

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Preface

Advances in science and cancer medicine are growing ceaselessly, augmenting the complexity of cancer biology and posing new problems to medical practitioners. However, there are still many tantalizing therapeutic approaches available to improve cancer curability, including lung cancer, which is one of the most frequent causes of cancer mortality. The Lung Cancer Precision Medicine book provides many opportunities for readers to satiate their curiosity for burning issues. Readers will find searched for answers in multiple facets of lung cancer. The distribution of chapters permits a reader to begin the book at any point which is a very novel aspect. The more we know about cancer and the more advanced the anticancer therapies, the more hurdles we face, especially in lung cancer. In recent years many promising targetable genetic alterations have been identified in non-small cell lung cancer (NSCLC), opening the era of oral targeted therapies (1). Nevertheless, single targeted therapy is becoming recognized as insufficient since it immediately leads to cancer signaling pathway compensation in order to escape the anti-cancer therapy effect, leading to further tumor growth and metastases. Despite a wealth of knowledge, the design of most therapeutic strategies requires more understanding of cell type-specific cross-talks of different pathways (2).

The reader will find many intriguing aspects on modern approaches of surgery and radiotherapy as well as cancer biology and other forms of diagnosis and treatment. The TNM Staging classification in NSCLC could be further improved to also include immunological markers. Stromal CD8+ tumor-infiltrating lymphocytes have been shown to be strong determinants of predicting survival (3). What do we do when a patient asks about markers of response to immunotherapy? Cytotoxic CD8+ T cells are of increasing interest in lung cancer and help to predict response to programmed death-1 (PD-1) and PD-ligand 1 (PD-L1) antibodies. What is the usefulness of biomarkers? Mechanistically, PD-L1 is only active when expressed on the cell membrane, either through dynamic IFNγ expression or through constitutive oncogene activation.

The book is outstanding in transmitting the quest for lung cancer curability and incorporates multiple collaborations of internationally renowned investigators, including expert multidisciplinary teams in surgery, radiotherapy, cancer biology, early diagnosis, new diagnostic techniques, biomarkers and novel forms of targeted therapy and immunotherapy. Many innovative aspects can also be found regarding clinical trials, adjuvant therapy studies, statistical analysis and circulating biomarkers.

AME has made a great contribution to the field of lung cancer with its initiative of this book. Throughout the numerous sections, different readers and experts in various fields will find what they expect and more. Importantly, the information retrieved from the book can be useful in the clinical practice or to reinforce self-esteem and confidence in laboratory research. The book has been edited splendidly and its long list of chapters and authors is unique. We congratulate the authors for their dedication and hard work as well as for sharing their findings and experience with the scientific community.

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Rafael Rosell, MD, PhD

Cancer Biology and Precision Medicine Program, Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Barcelona Spain

Jie He, MD, PhD

Department of Thoracic Surgical Oncology, Cancer Institute & Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College; National Cancer Center, Beijing 100021, China.

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Preface

Lung cancer is the leading cause of cancer-related death worldwide that makes up 27% of all cancer deaths and brings significant socioeconomic impact to patients, their families, and society. Non–small cell lung carcinoma (NSCLC) accounts for the majority of lung tumors. Among NSCLCs, adenocarcinoma and squamous cell carcinoma are the two major histological types, representing 60–70% of all lung cancers. Even though numerous research efforts have been devoted to the development of lung cancer treatment over the past few decades, the overall five-year survival rate is still about 17% according to American Society of Clinical Oncology (ASCO). The high mortality of lung cancer worldwide is largely attributable to the difficulty of obtaining an early diagnosis and the lack of effective therapeutic methods. With the expansion of available high-throughput genomics technologies such as DNA microarray and next generation sequencing (NGS) in the past two decades, many studies have performed high throughput screening to better elucidate lung cancer etiology. Based on genomic analyses, researchers can further analyze and investigate possible regulatory mechanisms of human genes and diseases in order to discover potential therapeutic targets or predictive biomarkers. To improve survival rates in lung cancer patients, a comprehensive analysis of the molecular signature of the carcinogenic processes in NSCLC is needed to identify better predictive biomarkers for diagnosis and prognosis, and new molecular targets for drug development or radiation treatment. In this book, we have edited to put together many papers published by AME journals on various topics including "Genetic Changes, Screening for Lung Cancer, Diagnosis of Lung Cancer, Treatment of Lung Cancer, Prognosis of Lung Cancer, New Drug Development, and Design and Statistical Principles of the Trial. Moreover, on January 20, 2015, President Obama announced the Precision Medicine Initiative (PMI) that was planned to lead Americans into a new era of medicine in which researchers, health-care providers and patients work together to develop individualized care through research and technology. The US President asked for $215 million to support the Initiative in 2016. Of this total proposed budget, $130 million was scheduled to build a national, large-scale research cohort, and $70 million was scheduled to lead efforts in cancer genomics with the National Cancer Institute. Furthermore, a similar initiative has been announced in China. The government of China will invest $10 billion on Precision Medicine before 2030. Therefore, precision medicine is an emerging and important field for improving treatment modality for many different diseases. Since the AME Publishing Company has been making a great effort to publish many top quality papers focusing on lung cancer, I am sure that this book will be serving as a good beginning for advancing precision medicine of lung cancer treatment.

Eric Y. Chuang
Graduate Institute of Biomedical Electronics and Bioinformatics
NTU YongLin Biomedical Engineering Center
National Taiwan University, Taipei, Taiwan

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Foreword

Lung cancer remains one of the most challenging malignancies encountered by clinicians and specialists around the world. The enormity of the human costs are staggering. It is the cause of death in over 1.5 million individuals globally and is the greatest source of overall cancer mortality. At nearly every aspect, regarding this disease, there is difficulty. On a molecular level, the heterogeneity and complexity of aberrations reflect the myriad neoplastic transformative pathways that lead to the development of lung cancer and provide numerous avenues within which these tumors can develop resistance towards treatment; collectively presenting a moving target for novel therapies. The central location of these tumors and quiescent early clinical course often prevent early detection and diagnosis, as the majority of these patients present with advanced disease. The lungs themselves are a critical organs, with physical and physiologic limitations that oftentimes preclude aggressive surgery. Further, it is comprised of delicate tissues that limit the extent of radiotherapy and chemotherapy that can be given to a patient. Lastly, the co-morbidities that often accompany lung cancer provide yet another barrier to the maximal care that can be provided to this population. Given the scope of this global problem, therefore, the editors and authors of *Lung Cancer Precision Medicine* and the efforts of the AME Publishing Company are to be praised and congratulated, for they have provided a cogent, comprehensive, and superbly structured book on this complicated subject.

The sections of the book follow in a logical order, starting first at the molecular level. Discussed are the well-known aberrant pathways (EGFR and ALK rearrangement), but of equal importance is an overview and update on the wealth of knowledge that has been gained regarding the molecular pathogenesis of lung cancer, as we move through the Genomic Era. This first section provides the foundation for the remaining sections, as the molecular information is deftly woven into the discussions of screening/diagnosis, prognosis, and treatment strategies (including both the development of novel, targeted biologic therapies and improvements within the traditional surgical, radiotherapeutic, and chemotherapeutic modalities). The future directions of clinical trial design, which ties all of these concepts together, comprise the final section of this book and provide a road map for the ongoing struggle against this difficult disease.

Regardless of the experience or background of the reader, *Lung Cancer Precision Medicine*, is an embodiment of the multidisciplinary approach. The reader will easily add to their own cache of knowledge within their own area of expertise. However, given the manner in which this book is constructed and how the chapters are written, it will invite readers to learn considerations and insights regarding this disease from perspectives and fields that are quite different than their own, thus enriching their own understanding of lung cancer.

Jiade J. Lu, MD, MBA
National Distinguished Expert, Executive Vice President, Shanghai Proton and Heavy Ion Center

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School of Medicine Shanghai Jiaotong University

Lung Cancer Precision Medicine
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EDITORS

Jie He
Department of Thoracic Surgical Oncology, Cancer Institute & Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College; National Cancer Center, Beijing 100021, China

Rafael Rosell
Cancer Biology and Precision Medicine Program, Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Ctra Canyet, Badalona (Barcelona), Spain

Eric Y. Chuang
Graduate Institute of Biomedical Electronics and Bioinformatics, NTU YongLin Biomedical Engineering, Center National Taiwan University, Taipei, Taiwan

AUTHORS

Batoul Y. Abdallah
Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, USA

Mansoor M. Ahmed
Radiation Research Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Ryan S. Alden
Dana-Farber Cancer Institute, Boston, MA, USA

Asim Amin
Divisions of Immunotherapy and Medical Oncology, Carolinas HealthCare System, Levine Cancer Institute, Charlotte, NC 28204, USA

Anthony Bell
Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia

Allen Berry
Department of Pathology, St. Francis Hospital, Memphis, TN, USA

Matthew J. Bott
Division of Thoracic Surgery, Washington University School of Medicine, St. Louis, Missouri, USA

Graham Brock
Exosome Diagnostics, Cambridge, MA 02139, USA

Stuart H. Burri
Department of Radiation Oncology, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Elena Castellanos-Rizaldos
Exosome Diagnostics, Cambridge, MA 02139, USA

Joe Y. Chang
Department of Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Alice P. Chen
Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD, USA

Indrin J. Chetty
Henry Ford Health Systems, Detroit, MI, USA

C. Norman Coleman
Radiation Research Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health,Rockville, MD, USA

Daniel B. Costa
Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

Christine Coticchia
Exosome Diagnostics, Cambridge, MA 02139, USA

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Christina M. Coughlin
Novartis Oncology, East Hanover, NJ 07936, USA

Traves Crabtree
Division of Thoracic Surgery, Washington University School of Medicine, St. Louis, Missouri, USA

Thomas A. D'Amico
Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA

Khanh Do
Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

Jennifer L. Ersek
Department of Solid Tumor Oncology and Investigational Therapeutics, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

Nick Faris
Thoracic Oncology Research Group, Baptist Memorial Health System, Memphis, TN, USA

Andrew Fellowes
Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia

Renata Ferrarotto
Department of Thoracic and Head and Neck Medical Oncology, University of Texas, MD Anderson Cancer Center, USA

Clara N. Finch-Cruz
Pathology and Laboratory Medicine Service, Department of Veterans Affairs, VA Medical Center Memphis, TN, USA

Erik Folch
Division of Thoracic Surgery and Interventional Pulmonology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

Mei Ka Fong
Department of Pharmacy, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

Stephen Fox
Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia; Department of Pathology, University of Melbourne, Victoria 3002, Australia

David R. Gandara
University of California Davis Cancer Center, Sacramento, CA, USA

Ryan D. Gentzler
Division of Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

Christopher S. Giampapa
Medical Center Laboratory, Jackson-Madison County General Hospital, Jackson, TN, USA

Carri K. Glide-Hurst
Henry Ford Health Systems, Detroit, MI, USA

Daniel R. Gomez
Department of Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Nicolas Goossens
Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, USA; Division of Gastroenterology and Hepatology, Geneva University Hospital, Geneva, Switzerland

Axel M. Grothey
Department of Oncology, Mayo Clinic, Rochester, MN, USA

Daniel E. Haggstrom
Levine Cancer Institute, Carolinas Healthcare System, Charlotte, NC, USA

Richard D. Hall
Division of Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

John H. Heinzerling
Department of Radiation Oncology, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Henry H. Q. Heng
Center for Molecular Medicine and Genetics, Department of Pathology, Wayne State University School of Medicine, Detroit, USA

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Roy S. Herbst
Yale Cancer Center, Yale School of Medicine, New Haven, CT, USA

Holly L. Hilsenbeck
Duckworth Pathology Group, Memphis, TN, USA

Steven D. Horne
Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, USA

Yujin Hoshida
Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, NY, USA

Lan Hu
Exosome Diagnostics, Cambridge, MA 02139, USA

Robert W. Jarrett Jr
Pathology and Clinical Laboratories, North Mississippi Medical Center, Tupelo, MS, USA

Feng Ju
Department of Industrial and Systems Engineering, University of Wisconsin, Madison, WI 53706, USA

Christine Khoo
Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia

Edward S. Kim
Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Feng-Ming (Spring) Kong
Department of Radiation Oncology, GRU Cancer Center, Georgia Regents University, Augusta, Georgia, USA

Tri M. Le
Division of Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

J. Jack Lee
Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Hyo Kyung Lee
Department of Industrial and Systems Engineering, University of Wisconsin, Madison, WI 53706, USA

Michael J. Leukam
Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL, USA

Jingshan Li
Department of Industrial and Systems Engineering, University of Wisconsin, Madison, WI 53706, USA

Gangning Liang
Department of Urology, University of Southern California, Norris Comprehensive Cancer Center, Los Angeles, CA 90089, USA

Suyu Liu
Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Li Liu
Illumina, San Diego, CA 92122, USA

Guo Liu
Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, USA

Bo Lu
Department of Radiation Oncology, Kimmel Cancer Center and Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA, USA

Sumithra J. Mandrekar
Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN 55905, USA

Shigeki Nakagawa
Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, NY, USA

Geraldine O'Sullivan Coyne
Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD, USA

Raymond U. Osarogiagbon
Multidisciplinary Thoracic Oncology Program, Baptist Cancer Center, Memphis, TN, USA

Geoffrey R. Oxnard
Dana-Farber Cancer Institute, Boston, MA, USA

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Joshua D. Palmer
Department of Radiation Oncology, Kimmel Cancer Center and Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA, USA

Herbert Pang
School of Public Health, Li Ka Shing Faculty of Medicine, Pok Fu Lam, Hong Kong SAR, China; Department of Biostatistics and Bioinformatics, Duke University School of Medicine, Durham, NC, USA

Vassiliki A. Papadimitrakopoulou
Department of Thoracic and Head and Neck Medical Oncology, University of Texas, MD Anderson Cancer Center, USA

Sandip Pravin Patel
Division of Hematology and Oncology and Center for Personalized Cancer Therapy, UC San Diego Moores Cancer Center, San Diego, California 92093-0987, USA

Jai N. Patel
Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Charles N. Prabhakar
Department of Respiratory Medicine, Royal Prince Alfred Hospital, NSW 2050, Australia

Anish Prasanna
Radiation Research Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health, Rockville, MD, USA

Mary W. Redman
Clinical Statistics, Clinical Research Division, Fred Hutchinson Cancer Center, Seattle, WA, USA

Lindsay A. Renfro
Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN, USA

Toni-Maree Rogers
Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia

Michael C. Sachs
National Cancer Institute, Biometric Research Branch, MSC 9735, Bethesda, MD 20892-9735, USA

Elizabeth W. Sales
Doctors Anatomic Pathology, Jonesboro, AR, USA

Daniel J. Sargent
Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN 55905, USA

Richard Simon
Biometric Research Branch, National Cancer Institute, Bethesda, MD, USA

Charles B. Simone II
Department of Radiation Oncology, Hospital of the University of Pennsylvania, Philadelphia, PA, USA

Lindsey Sirianno
Department of Solid Tumor Oncology, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

Johan Skog
Exosome Diagnostics, Cambridge, MA 02139, USA

Alex Yick-Lun So
Illumina, San Diego, CA 92122, USA

David Spencer
Trumbull Laboratories, LLC/Pathology Group of the Mid-South, Memphis, TN, USA

Ellen S. Story
Endocrine Center, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

Xiaochen Sun
Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, NY, USA

Scott J. Swanson
Department of Thoracic Surgery, Brigham and Women's Hospital, Boston, Massachusetts, USA

Paul A. VanderLaan
Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

Jeryl Villadolid
Department of Pharmacy, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

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Victoria M. Villaflor
Department of Internal Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, USA

Nestor Villamizar
Department of Thoracic Surgery, Brigham and Women's Hospital, Boston, Massachusetts, USA

Xiaofei Wang
School of Public Health, Li Ka Shing Faculty of Medicine, Pok Fu Lam, Hong Kong SAR, China; Department of Biostatistics and Bioinformatics, Duke University School of Medicine, Durham, NC, USA

Yixin Wang
Ventana Medical Systems, Inc., Tucson, Arizona 85755, USA

Daniel J. Weisenberger
Department of Biochemistry and Molecular Biology, Los Angeles, CA 90089, USA

Mia C. Weiss
Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL, USA

Matthew Witek
Department of Radiation Oncology, Kimmel Cancer Center and Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA, USA

Jeffrey Wright
Baptist Memorial Hospital, Memphis, TN, USA

Chi-Fu Jeffrey Yang
Department of Surgery, Duke University Medical Center, Durham, NC, USA

Christine J. Ye
Division of Hematology/Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, USA

Po Yee Yip
Department of Medical Oncology, Chris O'Brien Lifehouse, Camperdown, NSW, Australia; Department of Medical Oncology, Macarthur Cancer Therapy Centre, Campbelltown, NSW, Australia

Xinhua Yu
School of Public Health, University of Memphis, Memphis, TN, USA

Nicholas G. Zaorsky
Department of Radiation Oncology, Kimmel Cancer Center and Jefferson Medical College of Thomas Jefferson University; Department of Radiation Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA

Executive Copy Editor

Emily M. Shi

Executive Typesetting Editor

Maple S. Zhu

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Cancer genome evolution

Steven D. Horne[1], Christine J. Ye[2], Batoul Y. Abdallah[1], Guo Liu[1], Henry H. Q. Heng[1,3]

[1]Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, USA; [2]Division of Hematology/Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, USA; [3]Department of Pathology, Wayne State University School of Medicine, Detroit, USA

Correspondence to: Henry H. Q. Heng, PhD. Center for Molecular Medicine and Genetics, Department of Pathology, Wayne State University School of Medicine, 3226 Scott Hall, 540 E. Canfield, Detroit, MI 48201, USA. Email: hheng@med.wayne.edu.

Abstract: Cancer genome sequencing efforts have resulted in high amounts of diverse, conflicting genomic data, which ultimately challenge the gene mutation theory of cancer. Since each tumor is different, and the genomic landscape of cancer is highly dynamic, a new evolutionary framework and approaches are required to unify the field. While many recent publications of cancer genome sequencing data have addressed cancer genome evolution, there is no clear definition and a lack of systematic analyses, as the true evolutionary meaning of these massive genetic and epigenetic changes has been largely ignored in the search for specific drivers. Furthermore, there is a common confusion between traditional, Darwinian stepwise evolution and stochastic, punctuated cancer evolution. Herein, the genome theory, an emerging holistic theory that covers multiple levels of genetic and non-genetic alteration by shifting emphasis onto genome-defined (sequence and three-dimensional topology) system inheritance, is presented as a refreshing and promising new framework. Specifically, following introduction of the importance of the genetic information defined by the genome, the newly discovered pattern of cancer evolution (including the two phases of cancer evolution) and its genetic basis (including fuzzy inheritance) are described, which were recently confirmed by single-cell level DNA sequencing and explain how genome and gene/epigene level heterogeneity drive macro- and micro-cellular evolution. From these new understandings, both the limitations of current methodologies and new strategies are discussed. Finally, we suggest that understanding cancer evolution holds the key to understanding other complex diseases and evolutionary theory in general.

Keywords: Genome chaos; punctuated cancer evolution; genome theory; macro-cellular evolution; system inheritance

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Introduction

Like any scientific advancement, new generation sequencing technologies have brought both excitement as well as challenges for cancer research. On one hand, the newly discovered, highly dynamic genetic and epigenetic landscapes of the cancer genome have finally explained why it is so hard to identify common gene mutations in clinical samples, as these dynamics are against the key prediction of the current gene mutation theory of cancer. On the other hand, massive amounts of data from these technologies have also generated confusion in the field (1). For example, despite the clinical reality that the majority of cancer cases display high heterogeneity, most basic researchers have focused on identifying commonly shared genetic patterns. This strategy is largely influenced by results generated over decades from various *in vitro* and *in vivo* experimental models, despite the fact that many model systems of cancer come with drastically reduced heterogeneity. However, the gap between basic research and clinical reality is rapidly increasing, and this is one of the key rationales for pushing the cancer genome sequencing project and unbiasedly map the cancer genome landscape and identify these common gene mutations once and for all (1,2). Unexpected by

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many, the cancer genome sequencing project has forcefully denied such rationale by presenting the highly complicated reality to the research community where every cancer is different, and there is no fixed genomic landscape (3,4). To face this daunting challenge, a new conceptual framework is needed that accounts for the abundant genetic/epigenetic diversity observed. This sentiment has also been shared by some leading researchers, who have admitted that it is not enough to simply continue collecting more sequencing data and suggested that a new paradigm is urgently needed to understand cancer in this age of massive quantities of diverse data (5,6). In contrast, others continue promoting the strategy of sequencing more samples. They are convinced that, by sequencing more samples and using more powerful mathematical and bioinformatics models, the mystery of cancer will ultimately be solved.

To compare these conflicted strategies and reconcile different schools of thought, if possible, we need to understand cancer in the framework of cancer evolution. In particular, genetic/epigenetic variation revealed by sequencing needs to be discussed using the evolutionary mechanism of cancer.

Cancer progression represents an evolutionary process due to its multiple levels of variation (genomic, genetic, epigenetic), inheritance of this variation during progression, and the selective advantages resulting from this heterogeneity. Traditionally, cancer evolution is considered to be a stepwise, Darwinian process, where gene mutations accumulate during waves of clonal expansion. Under this framework, each wave is driven by specific gene mutations, which provide a proliferative advantage to the disease. These powerful molecular drivers are necessary for cancer to progress, and it is believed that key drivers are shared among most patients. Stepwise accumulation is understood as the general pattern of cancer evolution, and any diversification that may occur happens during clonal expansion. Like the concept of Darwinian evolution, cancer evolution is believed to be a continuous, traceable process and is similar to natural selection in the wild.

The evolutionary model of clonal expansion is well accepted in the field of cancer research and is supported by patterns of gene mutations within experimental populations, as well as some exceptional cancer cases such as chronic phase chronic myeloid leukemia (CML-CP) (7). Unfortunately, despite wonderful examples in many experimental systems, the cancer gene mutation theory fails when translated to most clinical cases, and so do stepwise evolutionary explanations of how gene mutations cause cancer. In order to solve this paradox, not only do we need to treat cancer as an evolutionary issue, but we must also search for the correct framework of cancer evolution. Key questions in this search include: Why has the cancer evolutionary concept thus far failed to solve the mystery of cancer? What is the true pattern of cancer evolution? Is current cancer genome sequencing telling us something new regarding cancer evolution? What are the roles of genes, epigenes, and genomes in cancer evolution? What is the new conceptual framework of cancer evolution that takes high levels of genetic heterogeneity into account? Finally, could cancer progression offer a special window to study evolutionary theory in general? One emerging holistic framework, the genome theory of cancer evolution, could serve to answer these questions and clarify confusion in the field.

To address these questions under this proposed framework, we will briefly review the concept of the genome and its importance in cancer evolution, in particular, genome defined system inheritance and its ultimate function in cancer evolution. The genome theory of cancer evolution will be discussed by describing the features of the two phases of cancer evolution and how genome and gene mediated heterogeneity drive macro- and micro-cellular evolution respectively. We will also suggest technologies that focus on genome-level heterogeneity, and we will point out the limitations of current methodologies and statistical approaches that are currently implemented to understand cancer. Finally, the potential applications of cancer genome evolution for understanding organismal evolution will be described.

What is cancer evolution, and why is understanding cancer evolution crucial?

In order to understand cancer evolution, we must first briefly review elementary evolutionary concepts. First, there are three key features that define bio-evolution. These are the following: (I) variations exist within the population; (II) these variations are inheritable and passed on among generations; and (III) these variations provide selective advantages in processes such as competition for space, nutrition, and other resources. Over time, the population will be enriched with certain genetic variations, which are responsible for some dominant features. Second, evolution is traditionally considered as a Darwinian, stepwise process, where the accumulation of small advantages over long periods of time lead to big changes, such as the formation and emergence of new systems or species.

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Application of organismal evolutionary concepts in understanding cancer is a logical approach. After all, the cancer process fits well with these three key criteria for evolution, and normal and mutated cells do compete with one another for resources and space in order to successfully grow and dominate cellular populations. The concept of studying cancer evolution dates back to the 1970s (8-12). Classical molecular evolutionary study focuses largely on gradual gene-level change over time, and cancer evolution research has followed this same paradigm. It was believed that a few sequential gene mutations are ultimately needed to transform normal, healthy somatic cells into cancer cells. Clonal expansion thus provides opportunities for cell populations to accumulate the gene mutations necessary to present cancer phenotypes. The reasoning behind this is that molecular pathway change through individual genetic or epigenetic alteration would result in increased fitness, and this would drive cancer growth and progression. Under gene mutation theory, cancer is the result of a stepwise accumulation of small changes in its evolutionary process. Thus, the logical approach would be to look for specific gene mutations that drive cancer evolution. In accordance with this logic, the majority of cancer research is focused on the identification of shared genetic aberrations (e.g., universally common chromosomes or key gene mutations), which would in turn serve as potential diagnostic and therapeutic targets to eradicate cancer. It is important to note that molecular geneticists have identified many gene mutations and pathways, giving the impression that molecular approaches alone would solve the mystery of cancer even without the framework of somatic cell evolution.

Surprisingly, as history and current efforts show, however, this simple concept is difficult to apply to the reality of diverse cancer cases. Aside from exceptional cases including CML-CP, this molecular approach as well as evolutionary explanations have been unsuccessful for the majority of cancers (7). Specifically, solid tumors are, by and large, marked by high degrees of intra- and inter-tumor genome heterogeneity at multiple genetic and non-genetic levels (13,14). This was recently confirmed with high-throughput sequencing (15-17). The high degrees of heterogeneity observed coupled with a lack of common driver mutations have posed a challenge to the general strategy of cancer research and even question the stepwise concept of cancer evolution (1,3,18). What is more troubling is that the efforts to identify shared drivers have resulted in massive amounts of varying and even conflicting data, which have generated confusion and frustration in the field, as these results would suggest that individual genes and pathways offer a minimal contribution to the overall cancer patient population, therefore only holding limited clinical value.

On one hand, we know so much about individual gene mutations, pathways, and the molecular basis for all hallmarks of cancer. On the other hand, the massive sum of diverse data does not make sense under the popular gene mutation theory of cancer. To solve this paradox, we need a new, holistic evolutionary framework that accounts for and unifies this diversity. As we will discuss, shifting research focus from a lower gene level to a higher genome system level embraces this observed multi-level heterogeneity at a single cell resolution while accounting for often neglected large-scale alterations.

Why is it crucial to study cancer at the genome level?

Influenced by gene-centric thinking, cancer research has traditionally focused on the identification and characterization of cancer gene mutations (1). The overwhelming heterogeneity illustrated by current cancer genome sequencing has forced researchers to change the strategy by studying the somatic cell evolutionary process, as an individual gene mutation has limited power in understanding the clinical reality. While the field of cancer evolution research is now picking up steam, as reflected by many important publications, most publications that discuss or acknowledge genome evolution are actually only discussing cancer evolution at the gene level. In fact, very few publications have addressed the issue of cancer evolution at the genome level, despite that cancer genome evolution has become a popular term (16,19).

Genome-based study, which takes into account both overall sequence and three-dimensional topology, has been long ignored in cancer research. Part of the reasoning behind this ignorance may be due to confusion, as the common perception of the genome is that it is merely the collection of genes or the complete DNA sequence. The sequencing of all genes in cancer cells (gene mutation and copy number characterization) has been mistakenly considered as genome research. Due to its highly evolving and re-organizing features, there is no fixed cancer genome. Furthermore, the concept of the genome is not simply the two-dimensional order of nucleotides in DNA! In reality, genome topology serves as a higher level of genetic organization, which governs and defines the genetic network structure. Under

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the genome, the system can be modified (e.g., through genetic mutation, epigenetic change), however, these lower level changes impact the system at a lesser degree than chromosomal change. To illustrate this important concept, it is essential to redefine what the genome is and why the genomic topology is such an important feature of the bio-system.

Under the genome theory, the genetic information can be classified as "parts inheritance", or instruction of how to make specific proteins from genes and "system inheritance", or the directions to assemble a given bio-system. The genetic blueprint does not just provide parts inheritance, but more importantly the system inheritance to organize all parts. A given genome reflected as a new karyotype defines new system inheritance. In nuclei, three-dimensional gene interaction is defined by the order of genes along each chromosome and among different chromosomes, which occupy unique positions; this is opposed to genes, which define parts inheritance (20-22). This concept can be made clear with the following analogy. Consider each individual gene as a unit of building material (e.g., lumber, brick). These can be utilized to construct any kind of building yet, depending on their arrangement, the final results will differ drastically (e.g., cabin, skyscraper). Here, the genome serves as the blueprint that determines how the "building materials" (i.e., genes and their encoded products) will come together to form the structure of the genetic and protein networks. Despite similar gene content, simply changing the genomic topology drastically alters the gene interaction relationship. This has been supported by organismal evolution where distinctive karyotypes can separate different species, and in particular, recent studies where karyotypic alterations were shown to influence gene expression profiles, as well as by single cell sequencing of glioma (23,24). In addition, evidence from yeast studies strongly supports that aneuploidy directly affects gene expression, resulting in phenotypic alteration due to the fact that genome alteration can overcome the lost function of an individual gene (25). The relationship between phenotype and karyotype was recently supported by single cell and population based analyses where genome heterogeneity was linked to growth heterogeneity (26). Thus, the consequence of genome-level alteration is new system formation defined by new system inheritance. This is of high importance in understanding tumor growth and progression, as karyotypic change can result in the generation of an aggressive phenotype, and most cancers are driven by genome replacement coupled with high levels of gene mutation and epigenetic alterations (13). To summarize, from an evolutionary perspective, the role of stochastic genome aberrations in cancer is to increase the evolutionary potential of the disease through increased genome system heterogeneity, resulting in the generation of a wide array of phenotypes and maximized odds for survival upon selection.

One of the major contributions of cancer genome sequencing is the confirmation of previous cytogenetic findings, which have demonstrated that genome level alteration (i.e., karyotypic alteration) is a common phenomenon of most cancers. Genome sequencing and cytogenetic analyses of clinical samples have revealed high rates of chromosomal abnormalities. Subcategories of genome chaos (rapid, stochastic chromosome fragmentation and reorganization) including chromothripsis and chromoplexy have been observed in various types of cancer, and chaotic genomes have been detected in the majority of cases of certain cancer types (16,19,22,27). As discussed, these chromosomal aberrations are necessary for cancer progression as they increase tumor population heterogeneity and thus evolutionary potential. Changes of this magnitude explain the relatively small contribution that individual genes and pathways seem to have in the context of genome alteration-mediated cancer evolution. Chromosomal topology alterations can impact the tumor phenotype more than changing individual pathways by gene mutation, providing explanation why there are so many different types of non-clonal chromosomal aberrations (NCCAs) detected in various cancers and other diseases (3,28-30). This also offers the reasoning why in order to accurately study genome evolution, we must focus on the karyotype level.

To further illustrate the importance of the genome (over individual gene mutations), we have recently introduced the evolutionary mechanism of cancer (31,32). This holistic concept takes a large number of diverse factors into account that can contribute to cancer evolution, including genetic, non-genetic, internal and external factors, as long as it serves as a source of stress to the system and particularly if it induces genome instability (3). We equate the evolutionary mechanism of cancer to the sum of all molecular mechanisms, and this consists of three steps: stress-induced genome system instability, resulting heterogeneity at multiple genetic levels, and somatic cell evolution (4).

The genome theory and evolutionary mechanism of cancer can be understood with application of the multiple level adaptive landscape model (3,22,33), which directly illustrates the relationship between genome change (macro-

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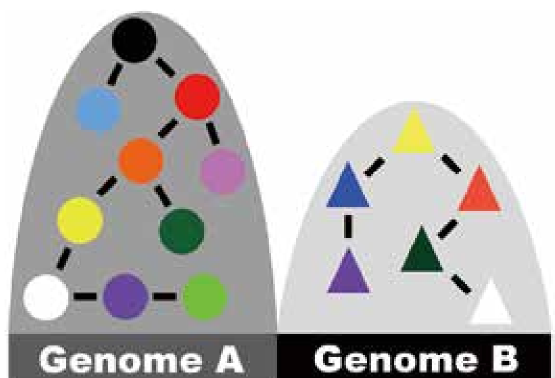

Figure 1 Landscape modeling depicts the relationship between genome-level change (macro-cellular evolution) and gene/epigene/nongenetic-level change (micro-cellular evolution). Each peak represents a separate genome-mediated system (Genome A, Genome B). Lower-level gene/epigene/nongenetic change (local landscape change), represented by different colored shapes within each peak, can be achieved and modify a particular genome system without changing the overall system in the majority of cases. Global landscape change (genome-level change) is often required for new system formation, illustrating the contribution of genome rearrangement in cancer evolution. As genome replacement is key in cancer evolution and progression, this type of modeling emphasizes the importance and power of karyotypic alteration (i.e., reshaping the global landscape) while displaying the typically small impact of lower level genetic change (local landscape alteration).

evolution) and gene changes (micro-evolution) (*Figure 1*). Here, pathway switching within a cell represents micro-cellular evolution, or small adaptations by local landscape change. Genome switching among cells, however, represents huge adaptation across the overall landscape (macro-cellular evolution). Every genome-mediated global landscape can be achieved through large numbers of pathway-mediated local landscapes. This new strategy accounts for not only the fitness landscape (micro-cellular evolution), but the survival landscape as well (macro-cellular evolution). The key to appreciating the contribution of genome rearrangements lies in understanding the two phases of cancer evolution.

The evolutionary mechanism of cancer and the multiple level adaptive/survival landscape model have also addressed an important issue, which is how to integrate the massive epigenetic dynamics observed from most cancers. Yes, gene mutations and gene regulatory aberrations do not work in isolation, but rather serve to complement each other in bypassing growth controls; however, they mainly address the issue of micro-cellular cancer evolution. In fact, current genomic knowledge of the activation-inactivation relationship between driver genes and a combination of other gene mutations, epigenetic silencing, network regulation, copy number variations, and even chromosomal aberrations are mainly explained within the framework of specific gene mutations and pathways. This is the reason why we have focused mainly on the key challenge to the gene mutation theory, as all genetic/epigenetic alterations are still being linked to gene function [more discussion regarding cancer epigenome can be found in the review from this special issue, see Weisenberger and Liang, 2015 (34)]. Recently, a systems biologist has taken action for such integration (33).

It is important to note that the implications of this understanding extend well beyond cancer and provide insight on many common, complex diseases (21,30,35). Recently, a general model of common and complex diseases has been suggested, where the key is diverse causes lead to genome instability (36). Furthermore, fuzzy inheritance is the basis for such high degree of genome instability. Fuzzy inheritance is a newly identified type of inheritance, where the genetic information at the somatic cell level is much less precise than classical genetics predicted. This mechanism required for evolutionary adaptation ensures necessary variation in cancer and also explains why there is an issue of missing heritability (35-37).

What is the pattern of cancer evolution?

Based on the functional separation of gene and genome, it is important to study the pattern of cancer evolution from both gene and genome point of view. By adapting the new concept that stochastic genomic changes represent an index to measure system instability (traditionally thought of as insignificant "noise"), we performed experiments allowing us to watch cancer evolution in action to compare karyotype changes during cellular immortalization, transformation and drug resistance (10). The following are some discoveries from those studies.

Even prior to The Cancer Genome Atlas (TCGA), the pattern of cancer evolution was already demonstrated to be more complicated than Darwinian stepwise evolution alone (1). The two phases of cancer evolution were originally based on karyotypic observations from an immortalization model where a pattern of clonal and non-clonal expansions was detected, and these phases were recently confirmed in breast cancer using single cell genome sequencing (10,17,38). Cancer evolution is a series of genome-mediated system replacements

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consisting of dynamic cycles of NCCAs and clonal chromosome aberrations (CCAs) occurring within two evolutionary phases. During the stepwise phase, the majority of cells are clonal across generations, and karyotypic diversification is traceable. The punctuated phase is defined by a high frequency of NCCAs and massive genome reorganization, which break multiple system constraints (e.g., genome integrity, tissue architecture). Cancer progression thus consists of both macro-cellular (genome system replacement) and micro-cellular (genome system modification) evolution. In addition, multiple runs of evolution involve totally different pathways or gene signatures. Further, evolution involves the contributions of multiple genetic levels (genome, gene, epigene), however, their influences vary sharply. Gene-level change modifies an existing system, but genome topology change rapidly creates new systems. Recently, the concepts of macro-micro phases of evolution in cancer have received increased support (39). Interestingly, although the two phases of cancer evolution were recently confirmed with single cell genome sequencing, the punctuated phase can be identified at different genetic levels; however, the correct measures must be taken so that the punctuated phase is not an oversight. For example, the stepwise relationship detected at the sequence level can be found during either the stepwise or punctuated phase at the genome level (10,24,40).

Cancer evolution is much more complicated than traditionally accepted stepwise clonal expansion, and this realization has implications in better understanding cancer progression. First, cancer progression consists of many NCCA/CCA cycles. Second, the NCCA/CCA pattern is dependent on cellular stresses. Under high stress, the phase of cancer evolution can be quickly shifted. Third, in a time of crisis, genome chaos can rapidly change the genomic landscape of the cell population to provide cancer the opportunity to increase heterogeneity and evolutionary potential (through swift creation of drastically altered systems), putting the odds of survival back in cancer's favor (27). Outlier groups resulting from this process that can better serve niches within the evolutionary landscape could then dominate later cancer progression with new features (e.g., aggressive proliferation) (26).

Since the evolutionary pattern is associated with a wide variety of high stresses, including chemotherapeutics, accounting for macro-cellular evolution has relevance in better understanding drug resistance as well (27,41,42). The current primary standard of care for metastatic patients is application of maximum tolerated doses to eliminate as many tumor cells as possible. While there is initial success with this strategy, there is life after death, as surviving resistant subgroups rapidly repopulate the tumor cell population. With the understanding of stress-induced genome chaos, this paradox becomes clear. Regardless of the specific treatment approach applied, high treatment-related stress will eliminate cells while effectively inducing genome fragmentation and reorganization. These surviving cells with altered genome systems can swiftly recoup lost numbers from the treatment and aggressively drive cancer progression (43) (Horne *et al.*, in preparation). This new mechanism demonstrates that cancer drug resistance is an adaptive process rather than an intrinsic property that is selected for by treatment and must be taken into account in the development of treatment regimens and strategies.

What has TCGA project taught us?

The original goal of TCGA was the identification of common driving gene mutations. It was reasoned that if cancer were a common, stepwise evolutionary process, each patient would represent one snapshot of the same, shared process. By sequencing a large number of samples, the hope was that the overall process as well as main contributing factors would ultimately be identified. These results could then be combined to reveal the overall landscape of the cancer genome and precisely determine the pattern of cancer evolution.

With utilization of technological advancements and large sample sizes, there are waves of excitement that come with releases of major findings and publications from TCGA. As we detail in *Table 1*, there have certainly been discoveries and achievements from these efforts (the majority of current publications have extensively highlighted most of the achievements of TCGA, and there is no need to repeat them here), but most of these are surprising rather than expected, and they do not fit the original goals of TCGA as rather than finding new key signals in spite of the "noise" of cancer, more heterogeneity is revealed with usage of more powerful technologies. This calls for further evaluation of the limitations as well as challenges of TCGA.

What methods are needed to study cancer evolution at the genome level?

In order to properly study genome-mediated cancer evolution, focus must be directed at genome-level alterations rather than at other genetic levels. This includes

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Table 1 Some key findings from The Cancer Genome Atlas (TCGA) reports and their significance, limitations and challenges

TCGA findings	Gene theory interpretation	Genome theory interpretation	Challenges and future directions
Identification of many involved epigenetic, copy number and chromosomal alterations	Emphasis and efforts must be now focused on linking these to specific gene functions	It is crucial to link these results to stress-induced chromosome instability-mediated evolutionary potential	Which level of information (genome, gene, epigene) is the most important? New methods are needed to integrate this information and monitor the phases of evolution
Confirmation of previously identified genes in cancer	Supports the importance of key cancer genes	Reduced the importance of individual gene mutations, as the frequencies are much lower than previously believed. With so many additional changes revealed from these efforts (from epigene to genome), there is no clear causality for any given gene/pathway	Efforts have revealed an increased degree of stochasticity. Confirmation of identified genes does not offer much new information of value
Identification of new mutations	These illustrate new and sensible mechanistic explanations, such as the role of chromatin modifiers	There are many more revealed mutations that cannot be rationalized from the gene point of view. However, in the context of genome diversity and evolutionary selection, the overall variations rather than specific gene mutations are more important, and cherry picking genes does not make sense	Efforts are now needed to develop quantitative measures of total change. More attention must be devoted to the evolutionary mechanism of cancer and not just on individual molecular mechanisms. In particular, the concept of emergent properties at the genome level is the key
High level of genetic heterogeneity revealed	Solving this issue will require additional sequencing efforts, including a shift to single cell resolution sequencing, in order to identify the common key factors	Key understandings will not be identified with increased data or even single cell resolution findings, but rather by studying the emergent properties. More sequencing efforts will only reveal more heterogeneity	A shift to a more holistic or higher level framework will clarify our understanding of cancer through simplified explanations of the complexity observed. Quantifying this heterogeneity will measure the evolutionary potential. Importantly, simply sequencing all single cells and combining these data to understand heterogeneity is not going to work, as cancer heterogeneity is defined by the dynamic evolutionary process
Identification of potential drivers	Important. Gene drivers can serve as potential molecular targets for therapy	Not very important. Due to the high dynamics and stochasticity of cancer evolution, gene-level drivers are moving targets. Thus, specific targeting therapy only works for a limited time and could induce genome chaos. It is already known that the roles of “passengers” and “drivers” can switch, and there is no constant driver except in linear experimental models	Rather than aiming to maximize the elimination of cancer cells by targeting driver mutations, therapeutic success should be achieved by system constraint and controlling the speed of cancer evolution

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the selection of appropriate techniques to visualize and understand these alterations, as the wrong approach could lead to misinterpretations. For instance, genome chaos cannot be inferred by sequencing alone, as it is a highly rapid and stochastic process rather than traceable and continuous. Importantly, most of the altered genomes observed within cancer samples have already undergone many rounds of genome chaos. Because of this, it is difficult to imagine or infer the process based on end products alone. We have recently demonstrated that the end products and the initial chaotic genomes are drastically different and highly unpredictable (27). However, genome chaos can be precisely followed with *in vitro* models designed to follow evolution-in-action, and products of genome chaos can be easily defined at the single-cell level with cytogenetic techniques including spectral karyotyping (10,27).

Application of statistical analysis in cancer research warrants reconsideration as well. In light of the strong influence outliers have on the growth and progression of a cancer cell population (26,43), removal of outliers from data sets in the pursuit of "statistically significant" findings has skewed our understanding of cancer. The same goes for average profiling techniques and methodologies, as the average cancer cell likely does not exist within the population (26). Thus, in the effort to identify key cancer "drivers" (i.e., highly expressed markers of averaged results), the actual driving forces of cancer are neglected and eliminated from these analyses (i.e., genome heterogeneity and outlier contributions). To improve the current situation, new analytical platforms are needed that measure heterogeneity and complexity to achieve a true understanding of the disease rather than emphasizing gene and pathway specificity.

As evolution results in different end products with each round (10), it is important for researchers to perform multiple, parallel runs of experiments and incorporate different models in their studies. An individual run may reveal a particular driving factor at a particular time, and a specific linear model may consistently follow a similar molecular progression when repeated. However, these findings only represent conditional possibilities that are quickly muddled when coupled with additional experimental trials or models, or in particular, when compared to clinical data. A general, holistic understanding focused on overall stability and heterogeneity can remedy this confusion. In addition, experiments that follow evolution-in-action will provide greater insight into the disease process than dissecting end products, as the final results will differ with each experiment and thus offer very limited and often misleading information about the overall evolutionary process.

Since cancer is an evolutionary process, and heterogeneity is the key feature of evolution, methods should be developed to monitor the degree of heterogeneity and predict the transition between NCCAs and CCAs. We are currently developing a method to measure the degree of karyotype heterogeneity and complexity. Information from this approach will provide necessary insight for improving patient management (21,36,37,44).

Finally, comparative analysis must be performed to determine the contributions of different genetic levels (epigene, gene, genome) during cancer evolution. Based on previous studies demonstrating the impact of genome-level change on other genetic levels, we anticipate that this type of analysis will undoubtedly show that genome topology alteration drastically alters genetic and epigenetic profiles. We also expect that the role of a particular gene or pathway would change dramatically with karyotypic alteration, as this level of change impacts the entire genetic network. Equally important, quantitative methods are needed to provide improved prediction power in the clinic.

Cancer genome evolution as an ideal model to reveal evolutionary principles

Genome-mediated cancer evolution has offered valuable insight beyond the field of cancer research. For instance, the observed genome/gene dynamics of the evolution-in-action experiments solved the mystery of the main function of sexual reproduction. Traditionally, it is considered that sexual reproduction functions to increase genetic variation. However, under this new paradigm, sex primarily acts to eliminate genomic alterations despite its secondary function of mixing genes (45-47). Thus, sexual reproduction acts as a filter that effectively removes high levels of stochastic genome alterations and maintains species identity.

Cancer genome evolutionary studies have also revealed a trade-off that provides the basis for the many common diseases that lack a clear, causative molecular linkage or heritable factor (30). High-level genome alterations and elevated genome instability have been reported in a wide variety of common diseases including autism, Alzheimer's disease, Gulf War illness, chronic fatigue syndrome, celiac and Crohn's disease (36,44,48-52). Interestingly, genome alterations have also been observed in normal, healthy tissues, including the polyploidization of liver

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cells, skeletal muscle, ovary, placenta, thyroid gland, blood, urothelium, Purkinje neurons, blastocyst mosaicism and trisomy 21 mosaicism in the general population, as well as detected stochastic karyotypic changes caused by environmental and physiological challenges (29,53-57). Whole-genome sequencing of healthy individuals recently revealed an increase of genome-level alteration (58). It is understood that cells at any given time are subject to a wide variety of internal and external stress, under either normal physiological or pathological conditions. Stress, in general, results in many infrequent genome alterations (10,29). Recall that genome-level alterations are more effective at drastically changing the genetic system than gene mutation or epigenetic change. This would suggest that stress-induced genome level change could effectively provide an adaptive advantage for cells against high levels of environmental stress. In addition, genome diversity within normal, healthy tissues allows for complex organ function while providing the genome heterogeneity necessary to account for organ function-associated stress, such as liver-mediated blood detoxification. Thus, stress-induced heterogeneity is necessary for successful adaptation to occur, but the trade-off is potential disease onset (30,36,37,59). If we take into account the new function of sexual reproduction as a filter to eliminate large-scale genome aberrations from the germline, we can understand how system dynamics are promoted for short-term adaptation at the individual level while the accumulation and passing of alterations to offspring is prevented, and this provides clarification behind the "missing heritability" of many common diseases (35).

These studies also led to the realization that macro- and micro-cellular evolution are not simply bridged by time, or in other words, an accumulation of small, stepwise gene-level changes does not often result in large genome topological change over long periods of time. We have analyzed the pattern of cellular evolution by comparing multiple runs of *in vitro* evolution across several years of culture. Populations that survive always display different genomes, representing macro-cellular evolution (Heng *et al.*, in preparation). In fact, macro- and micro-cellular evolution are two different mechanisms, as evidenced by the genome chaos studies where large-scale genome alteration and new system formation occur within a very short period of time. Micro-cellular evolution, however, acts to tinker and refine the existing system at a much smaller scale (e.g., gene mutation, epigene modification). Interestingly, this distinction between the two mechanisms applies directly to organismal evolution.

The popular framework behind the pattern of organismal evolution needs reconsideration given what we know now about cancer genome evolution. After analyzing a large number of species, King concluded that distinctive karyotypes are the most important features among various species (60). Various groups including our own have promoted this view (46,52,61-63). Thus, rather than a stepwise, Darwinian progression of small changes that lead to speciation events, speciation is likely due to large-scale genome dynamics and preservation of the new species-specific genome through sexual reproduction. Recent genome sequencing of various species supports this idea, as genome alteration is the main event of speciation.

One challenge as well as an opportunity for the field of evolutionary study is to pay more attention to the information derived from cancer progression. Somatic cell evolution provides a unique window to study the interaction of gene mediated micro-cellular evolution and genome replacement mediated macro-cellular evolution. Various *in vitro* and *in vivo* systems can serve as platforms to watch evolution-in-action and compare different runs of evolution. Such research opportunities are extremely difficult to access in other systems. Even though cancer differs from many organismal systems, cancer still represents a biological system. This means that cancer should still follow the laws of evolution. Since cancer evolution study has revealed two phases of evolution and can connect the dots between sexual and asexual reproduction and between micro- and macroevolution, the messages derived from cancer evolution extend far beyond somatic cells and are applicable and essential to understanding organismal evolution. One urgent task is to quantitatively study the multiple levels of genetic heterogeneity and how fuzzy inheritance contributes to this heterogeneity (35,36). Furthermore, it is crucial that we understand the similarities and differences that separate cancer genome evolution and organismal genome evolution. The time is now to shift our focus, efforts and technologies onto a new, promising direction and take the next step.

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Cancer biomarker discovery and validation

Nicolas Goossens[1,2], Shigeki Nakagawa[1], Xiaochen Sun[1], Yujin Hoshida[1]

[1]Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, USA; [2]Division of Gastroenterology and Hepatology, Geneva University Hospital, Geneva, Switzerland

Correspondence to: Yujin Hoshida, MD, PhD. Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA. Email: yujin.hoshida@mssm.edu.

Abstract: With the emergence of genomic profiling technologies and selective molecular targeted therapies, biomarkers play an increasingly important role in the clinical management of cancer patients. Single gene/protein or multi-gene "signature"-based assays have been introduced to measure specific molecular pathway deregulations that guide therapeutic decision-making as predictive biomarkers. Genome-based prognostic biomarkers are also available for several cancer types for potential incorporation into clinical prognostic staging systems or practice guidelines. However, there is still a large gap between initial biomarker discovery studies and their clinical translation due to the challenges in the process of cancer biomarker development. In this review we summarize the steps of biomarker development, highlight key issues in successful validation and implementation, and overview representative examples in the oncology field. We also discuss regulatory issues and future perspectives in the era of big data analysis and precision medicine.

Keywords: Cancer; biomarker; drug response biomarker; prognosis; companion biomarker

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Introduction

The Precision Medicine Initiative unveiled in January 2015, included an investment of $70 million to the National Cancer Institute (NCI), to "scale up efforts to identify genomic drivers in cancer and apply that knowledge in the development of more effective approaches to cancer treatment" (1). In the field of cancer research and care, the concept of precision medicine—prevention and treatment strategies that take individual variability into account—hinges on the development of valid biomarkers interrogating key aberrant pathways potentially targetable with molecular targeted or immunologic therapies (1). Although biomarkers such as prostate-specific antigen (PSA), have been known and used for decades to attempt to guide prognostic and therapeutic decisions, the recent revolution in molecular biology, with the rise of high-throughput sequencing and increased molecular characterization of tumor tissue has led to an exponential increase in attempts to measure and target aberrant pathways at the molecular level. Nevertheless, there has been a large gap between multiple initial reports of biomarkers, often with diagnostic performance that cannot be reproduced in later studies, and full clinical implementation and validation of the biomarkers due to issues in study design, assay platforms, and availability of specimens for biomarker development (2,3).

Nevertheless, with the recent emergence of highly selective molecular targeted agents and high-throughput genomic characterization technologies, robust and well-validated cancer biomarkers are increasingly needed. For instance, more than 90% of oncological drugs that enter clinical development will not reach market approval due to failure of clinical trials to demonstrate therapeutic benefit, contributing to costly and slow cancer drug development (4). As acknowledged by the USA Food and Drug Administration (FDA), the judicious use of biomarkers is expected to play an important role in minimizing risk of clinical trial failure by enriching the trial populations with specific molecular subtypes responding better to tested therapies. In this review, we overview recent

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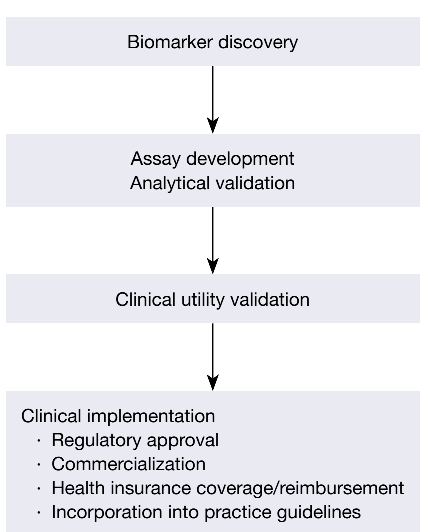

Figure 1 Schematic overview of the processes of cancer biomarker development.

trends in cancer biomarker development and discuss the issues in clinical translation of cancer biomarkers.

Biomarkers in cancer care

A biomarker is an objectively measured characteristic that describes a normal or abnormal biological state in an organism by analyzing biomolecules such as DNA, RNA, protein, peptide, and biomolecule chemical modifications (5). However, it must be acknowledged that the definition of biomarkers has been evolving over the past decade, with one especially broad definition by the World Health Organization suggesting that "A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease." (6,7). More specifically in terms of clinical utility, a cancer biomarker may measure the risk of developing cancer in a specific tissue or, alternatively, may measure risk of cancer progression or potential response to therapy. Besides providing useful information in guiding clinical decision making, cancer biomarkers are increasingly linked to specific molecular pathway deregulations and/or cancer pathogenesis to justify application of certain therapeutic/interventional strategies. The conceptual framework of cancer biomarker development has also been evolving with the rapid expansion of our omics analysis capability of clinical biospecimens based on the traditional path of biomarker deployment (5).

Cancer biomarkers can be classified into the following categories based on their usage. Predictive biomarkers predict response to specific therapeutic interventions such as positivity/activation of *HER2* that predicts response to trastuzumab in breast cancer (8-10). Similarly, *KRAS*-activating mutations predict resistance to epidermal growth factor receptor (EGFR) inhibitors such as cetuximab in colorectal cancer (11). Prognostic biomarker, on the other hand, may not be directly linked to or trigger specific therapeutic decisions, but aim to inform physicians regarding the risk of clinical outcomes such as cancer recurrence or disease progression in the future. An example of a prognostic cancer biomarker is the 21-gene recurrence score which was predictive of breast cancer recurrence and overall survival in node-negative, tamoxifen-treated breast cancer (12). Another class of biomarker, the diagnostic biomarker, is used to identify whether a patient has a specific disease condition. Diagnostic biomarkers have recently been implemented for colorectal cancer surveillance by testing for stool cancer DNA (13).

Processes of biomarker development

Biomarker development involves multiple processes, linking initial discovery in basic studies, validation, and clinical implementation (*Figure 1*) (5,14-21). The ultimate goal of the processes is to establish clinically accessible biomarker tests with clinical utility, informing clinical decision-making

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to improve patient outcomes (21,22). However, there are many hurdles as evidenced by the low estimated rate (0.1%) of successful clinical translation of biomarkers (23). Here we elaborate each of the processes, which should be designed/planned prior to the conduct of the study to ascertain validity of cancer biomarkers.

Biomarker discovery

At the start of any biomarker development, biomarkers should be "discovered" and are typically validated within the same initial report. Validation based on predefined prediction rule in an independent patient series is ideal, but it is often substituted by cross-validation-based methods when independent patient sets are not available. The research question and plan, including the fundamental use of the biomarker, should traditionally be clearly defined prior to the analysis, although this can be challenging at the very early stages of biomarker development. In this era of ever-evolving high-throughput omics technologies where thousands of individual molecules can be easily interrogated without a priori assumptions, research hypotheses are often generated in a *post hoc* manner, following often serendipitous discovery from unbiased mining of the genome-wide measurements (data-driven hypothesis generation) (20). Another relevant issue to be addressed early in biomarker development is the target population to be tested in specific clinical contexts, which will guide subsequent clinical evaluation and implementation. In general, broader target populations could lead to increased costs and risks of failure during the development stage.

Study design/setting, from which analyzed biospecimens are derived, is the major source of bias that hampers subsequent biomarker development. Ideally, the specimens should be prospectively collected based on well-defined inclusion and exclusion criteria together with accompanying clinical annotations pre-specified in the study protocol. A cohort or case-control study design is typically employed. In a cohort study, clinical characteristics of enrolled individuals as well as information of intervention and follow-up are critical in identifying molecular correlates associated with clinical outcomes of interest. In a case-control study, potential confounding factors should be properly matched between cases and controls to minimize false discovery. In practice, biomarker discovery is often based on "samples of convenience", which were incidentally available to the investigator at the time of research and collected without prior intention of specific biomarker discovery (24). This could introduce unrecognized confounding factors, which may contribute to the false positive associations of the biomarkers. The study design quality may be semi-quantitatively evaluated by using scores such as level of evidence scale proposed by Simon *et al.* (16). In general, evidence derived from large-scale well-predefined prospective trials is regarded as most reliable. Retrospective, observational studies may be affected by multiple sources of bias, which can be better identified if reporting guidelines such as Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) for prognostic studies (25), Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) for observational studies (26) and Standards for Reporting of Diagnostic Accuracy (STARD) for diagnostic studies (27) are used to determine reliability and quality of biomarkers in the initial reports.

A common cause of failure in developing robust predictive and especially prognostic biomarkers is to define them based on clinically invalid surrogate endpoints such as objective response in oncology trials as well as short-term outcomes from retrospective studies. Biomarkers trained for poorly-defined endpoints are more likely to fail in subsequent prospective evaluation. A prognostic gene-expression signature trained on long-term outcome using archived specimens has been successfully validated in a series of independent clinical and experimental studies (28-31). While the most optimal setting is prospective sample collection and follow-up based on a fully predefined protocol, this requires costly and lengthy biomarker assessment, which hampers timely deployment of cancer biomarkers. As an alternative, retrospective analysis of samples archived as part of previously completed prospective trials (prospective-retrospective design) is proposed to shorten the time frame while ascertaining quality of study design (16). Another solution is to develop a biobank in which biospecimens and complete clinical annotations are prospectively accumulated based on well-defined protocols. However, in part due to the complex and heterogeneous nature of cancer, it has become increasingly recognized, that there is a need for larger integrated biobanks (32,33) which require careful development and adherence to published biobanking guidelines (34). The practical challenges of biobanking in cancer patients has been underlined by a recent USA survey of NCI-funded cancer researchers who conduct tissue-based research showing that 39-47% reported difficulty obtaining biospecimens of adequate numbers and quality and low-quality biospecimens resulted in 60% questioning their

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findings and 81% limiting the scope of their work (35). Quality of clinical annotations is another key factor in utilizing the resources to identify reliable biomarkers and validate their clinical utility. A recent NCI joint workshop recommended improved sharing of existing specimens and data and creation of NCI-wide inventory of prediagnostic specimens and cancer diagnosis data, ongoing engagement of the clinical, translational and basic research communities, and encouraging the development of pilot projects (18).

Robustness of sample processing and data analysis procedures is another factor that influences reproducibility of biomarker studies. For example, a high diagnostic accuracy of a peptide signature for ovarian cancer was not confirmed in subsequent independent reanalysis of the original dataset possibly due to variation in sample processing (36,37). One report of proteomic biomarker discovery noted that common statistical algorithms run on data with low sample sizes can overfit and yield misleading misclassification rates and that prefiltering variables exacerbated this problem (38). Similarly, a critical review of prognostic microarray studies in cancer revealed that half of the reported prognostic gene signatures were not reproducible due to critical flaws in the data analysis methods (39). These reports highlight the importance of careful assessment of technical soundness and methodological validity and disclosure of information to the research community to enable fair evaluation of reported biomarkers and identification of candidates for further development. In addition, ensuring reproducibility of bioinformatics analysis is a critical determinant of successful clinical translation of genome-based biomarkers. There have been several efforts to develop informatics infrastructure to address this issue, including public repository of datasets with relevant annotations on biological, clinical, and experimental parameters, analysis software repository, and systems to record whole process of data analysis itself to allow anyone to rerun or modify the analysis to verify robustness of reported findings (40,41).

Biomarker assay development and analytical validation

Following the discovery phase that typically includes internal validation, candidate biomarkers are adapted to clinically applicable assay platforms, and subjected to two types of validation, namely analytical validation, i.e., how accurately and reliably does the test measure the analyte(s) of interest in the patient specimen and clinical validation, i.e., how robustly and reliably is the test result correlated with the clinical phenotype or the outcome of interest. Analytical validation is typically performed by assaying the same set of samples by both the assay used in the initial discovery and the clinical deployment platform to determine robustness and reproducibility of the measurements. Frequently used assay technologies generally used for analysis of single gene/protein anomalies include real-time polymerase chain reaction (RT-PCR) to assess gene expression or DNA mutations (e.g., *BRAF* V600E mutation in melanoma), fluorescent in situ hybridization (FISH) to assess DNA copy number or genetic translocation (e.g., *HER2* amplification, *BCR-ABL* translocation), and immunohistochemistry (IHC) to assess protein expression and subcellular localization (e.g., estrogen receptor expression in breast cancer).

More recently, several multi-gene assays classified as in vitro diagnostic multivariate index assays (IVDMIA) have been introduced into clinic (13,42,43). The implementation of gene expression-based multi-gene assays has been a challenging task due to poorer reproducibility of the measurements (44). Currently available tests, such as MammaPrint (45) and Oncotype Dx (12), are performed in centralized laboratories to minimize technical variability. Emerging technology such as direct digital counting of transcripts without target amplification could enable more robust gene expression measurements reproducible across individual laboratories (46,47). Resequencing of a targeted panel of genes (disease-specific, exome, etc.) has been tested as another option (48), identifying somatic DNA mutations potentially driving cancer in nearly 2/3 of patients with lung adenocarcinomas and linking to molecular targeted therapy in 28% of patients (49). Clinical sequencing is a promising approach, but the interpretation and reporting of incidental findings from non-targeted sequencing is still being debated (50). In addition, high demand on data analysis, referred as the "$1,000 genomic test [but] $100,000 genomic analysis", is another layer of challenge in sequencing-based approaches (51). Capability to analyze formalin-fixed, paraffin-embedded (FFPE) tissue samples greatly enhances general applicability of biomarker assays (52-54). Emergence of highly sensitive assays, e.g., single cell profiling, are expected to enable analysis of body fluid-derived specimens such as whole blood, plasma, serum, ascites, and urine to assess circulating microRNA, circulating DNA, and circulating tumor cells (CTCs)-derived biomolecules (55,56). These technologies are expected to achieve less-invasive assessment of molecular biomarkers (liquid biopsy) (55). Circulating tumor DNA was highly accurate in assessing mutation status of

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BRAF V600E mutation (100% specificity and sensitivity reported) and *KRAS* point mutations (>90% sensitivity and specificity) in subjects with metastatic colorectal cancer in one blinded prospective trial (57). Another report, assessing the role of CTCs, defined as 5 or more per 7.5 mL of whole blood in this study, in metastatic breast cancer, did not find an improvement in outcomes after changing therapy in case of persistently elevated CTCs but confirmed that CTCs were strongly prognostic for overall outcome (58). In addition to their role in diagnosis, circulating cell-free microRNAs are also being currently assessed as a predictive cancer biomarker with some encouraging preliminary reports (59,60).

Validation of clinical utility

After analytical validity is confirmed, the biomarker assay in the clinical deployment platform must be evaluated to confirm its performance in predicting or diagnosing the clinical phenotype or outcome of interest as demonstrated in the discovery and initial validation phase (5,21,61). Ideally, the biomarker should be evaluated in statistically well-powered prospective trials as performed in the TransATAC study for breast cancer recurrence prediction (62). However, it is realistically infeasible to test all candidate biomarkers in this manner due to financial constraints and/or limited availability of patient cohorts. Therefore, similar to the setting of biomarker discovery, the use of prospective-retrospective design and/or biobank/biorepository samples could be a potential alternative to overcome these obstacles. Clinical utility assessment could also include analysis of clinically meaningful outcome benefit, comparative effectiveness, cost-effectiveness of biomarker-guided clinical care, and assessment of alternatives and availability of the biomarker based on real-world clinical data or mathematical modeling (21,63).

Clinical implementation

An analytically and clinically validated biomarker assay is now ready for implementation in clinical care. This phase includes the following four key elements, which vary widely across regions: regulatory approval, commercialization, coverage by health insurance companies, and incorporation in clinical practice guidelines. In the USA, there are two paths for regulatory approval: in vitro diagnostic device (IVD) as commercial medical device with 510(k) clearance overseen by the FDA, and laboratory developed tests (LDT), home-grown assay developed and optimized at a diagnostic lab performing the test, which will likely be regulated by the FDA although current oversight is more limited (64). Clinical biomarker tests must be conducted in diagnostic laboratories certified for Clinical Laboratory Improvement Amendments (CLIA) and in accordance with state-specific regulations. Coverage by health insurance is critical for physicians to order the tests. Assignment of current procedural terminology (CPT) codes as well as incorporation into clinical practice guideline/recommendation supports payer's decision. Centers for Medicare & Medicaid Services (CMS) classifies the tests into tier 1 (CPT code-assigned, commonly performed tests) and tier 2 (less commonly performed tests grouped by complexity). CMS defers pricing for new CPT codes to the local Medicare administrative contractors in a procedure known as "gapfill", which causes delayed reimbursement for many biomarker tests (65). Post-marketing clinical utility validation will further support the use of biomarker tests, and may result in indication for additional diseases and/or clinical scenarios. Resources such as the National Comprehensive Cancer Network Biomarkers Compendium (66) are available to access the current recommendation for biomarkers in clinical guidelines (67).

Cancer biomarkers currently available in clinic

An example of a molecular biomarker in clinic is overexpression/amplification of HER2 (*ERBB2*), a member of the EGFR family, predictive of response to monoclonal antibodies such as trastuzumab and pertuzumab in breast cancer (8-10). It has been shown in pivotal phase III trials in breast cancer that subjects with HER2 overexpression (approximately 20% of patients) treated with anti-HER2 therapy have improved disease-free and overall survival (8-10). American Society of Clinical Oncology and College of American Pathologists recommend primarily IHC and in situ hybridization for assessment of HER2 status (68). Currently, the FDA has approved 10 HER2 assays as companion diagnostic devices (50% of all approved companion diagnostic devices) and 3 other HER2 assays as nucleic acid based tests cleared by the Center for Devices and Radiological Health [FDA website accessed on March 20th 2015 (69)]. HER2 overexpression is similarly predictive of response to trastuzumab in esophago-gastric adenocarcinoma (70). OmniSeq Target assay analyzes clinically actionable somatic DNA alterations in 23 known cancer-related genes, which acquired the New York state

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approval as LDT. Other major predictive biomarkers, including *BCR-ABL* in chronic myeloid leukemia and *KRAS* mutations in colorectal cancer and multiple mutations in non-small cell lung cancer (NSCLC), are listed in *Table 1*.

Despite the numerous prognostic biomarkers reported in the literature, only seven biomarkers have been approved by the FDA Center for Devices and Radiological Health (*Table 2*) (48). One of the major reasons is that prognostic prediction itself often does not directly change clinical decision making unless coupled to specific therapeutic options. Despite this, many other prognostic biomarkers are available through the LDT pathway. Mammaprint is one of the first gene expression signature-based assays based on the measurement of 70 genes to predict breast cancer recurrence after chemotherapy, which was recently adapted for use in FFPE tissue (45). Another gene expression-based assay, Oncotype Dx Breast Cancer Assay measures 21 genes predicting breast cancer recurrence in women with node negative or node positive, ER-positive, HER2-negative invasive breast cancer (12,79). Similar tests are also available for colon and prostate cancer, all of which analyze gene expression in tumor tissue (80,81). A 186-gene expression signature in non-tumor stromal liver tissue has been validated to predict hepatocellular carcinoma development and recurrence as well as liver cirrhosis progression, and was recently implemented in an FDA-approved diagnostic device (28-30).

Diagnostic biomarkers are one of the most diverse classes of biomarkers ranging from assays developed for cancer screening to diagnostic tests assessing progression of a known cancer (see *Table 2* for a list of FDA-approved diagnostic genetic tests). One recent example of a diagnostic biomarker is Cologuard, a multigene DNA (*KRAS* mutations, aberrant *NDRG4* and *BMP3* methylation) stool test combined with fecal immunochemistry designed to screen for colorectal cancer in individuals at average risk of colorectal cancer. In a recent clinical trial of nearly 10,000 participants, sensitivity of the test for detecting colorectal cancer was higher than fecal immunochemical test alone (92.3% and 73.8% respectively) although the test also had a higher rate of false positives (specificity 86.6% and 96.4% for Cologuard and fecal immunochemical test respectively) (13). These encouraging results led to the approval of this test by the FDA in August 2014. Recently, there has also been increased interest in developing minimally invasive diagnostic tumor biomarkers, using the measurement of circulating DNA or microRNA. For instance, a new technology termed cancer personalized

Table 1 Predictive biomarkers in clinical use

Organ	Cancer	Biomarker and mechanism	Assay for measurement	Associated target and drug	Approximate proportion of positive tests	Stage of clinical validation	References
Breast	Breast cancer	HER2: oncogene overexpression	ISH, IHC	HER2: trastuzumab, pertuzumab, ado-trastuzumab emtansine	18-20%	In clinical use	(8-10)
		ER/PR: suggests sensitivity to endocrine therapy	IHC, LBA	ER: endocrine therapy (tamoxifen, aromatase inhibitors)	75%	In clinical use	(71)
Gastro-intestinal	Colorectal cancer	KRAS: mutations activate RAS-RAF-MEK pathway and resistance to EGFR therapy	PCR	EGFR: cetuximab, panitumumab	40% mutated	In clinical use	(11,72)
	GIST	KIT: mutation leads to consitutional activation	IHC	BCR-ABL: imatinib	95%	In clinical use	(73)
	Esophago-gastric adenocarcinoma	HER2: oncogene overexpression	ISH, IHC	HER2: trastuzumab	7-22%	In clinical use	(70)

Table 1 (*continued*)

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Table 1 *(continued)*

Organ	Cancer	Biomarker and mechanism	Assay for measurement	Associated target and drug	Approximate proportion of positive tests	Stage of clinical validation	References
Hematological	Chronic myeloid leukemia	BCR-ABL: balanced t(9;22) leading to the formation of a constitutively active tyrosine kinase	Cytogenetics, FISH, RT-PCR	BCR-ABL: imatinib, dasatinib, nilotinib	>90%	In clinical use	(74)
	Acute promyelocytic leukemia	PML-RARa: balanced t(15;17) leading to aberrant retinoid receptor	Cytogenetics, FISH, RT-PCR	PML-RARa: All-trans retinoic acid	>90%	In clinical use	(75)
Lung	NSCLC	EGFR (HER1): mutations in tyrosine kinase domain	Sequencing, ISH	EGFR: Erlotinib, gefitinib, afatinib	15% adenocarcinomas in USA (higher in Asians, women and nonsmokers)	In clinical use	(76)
		ALK: Inversion in chromosome 2 leads to EML4-ALK fusion oncogene	FISH (IHC)	ALK: crizotinib, ceritinib (alectinib under development)	4% (mostly adenocarcinoma)	In clinical use	(77)
	Lung adenocarcinoma	Multiple genes:				Continued validation	(49)
		BRAF (V600E and non-V600E)	Multiplex sequencing	BRAF: AZD6244	2%		
		EGFR (HER1): mutations in tyrosine kinase domain		EGFR: erlotinib, gefitinib, afatinib, cetuximab	17%		
		HER2: oncogene overexpression		HER2: decinutubub, neratinib, lapatinib, trastuzumab	3%		
		KRAS: mutations activate RAS-RAF-MEK pathway and resistance to EGFR therapy		KRAS: erlotinib, tivantinib, everolimus, ridaforalimus, AZD6244	25%		
		ALK: inversion in chromosome 2 leads to EML4-ALK fusion oncogene		ALK: crizotinib, ceritinib	8%		
		MET		MET: cizotinib	<1%		
Skin	Melanoma	BRAF V600: 80-90% V600E mutation, a downstream mediator of RAS, leads to downstream activation of MEK and ERK	Sequencing	BRAF: vemurafenib, dabrafenib	40-60%	In clinical use	(78)

HER2, human epidermal growth factor 2; (F)ISH, (fluorescence) in situ hybridization; IHC, immunohistochemistry; ER, estrogen receptor; PR, progesterone receptor; LBA, ligand binding assay; MEK, mitogen-activated protein kinase kinase; EGFR, epidermal growth factor receptor; (RT-)PCR, (reverse transcription-) polymerase chain reaction; GIST, gastrointestinal stromal tumor; PML, promyelocytic leukemia gene; RARa, retinoic acid receptor-alpha; NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; EML4, echinoderm microtubule-associated protein-like 4; ERK, extracellular-signal-regulated kinases.

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Table 2 Prognostic and diagnostic nucleic-acid based tumor biomarkers approved by the Center for Devices and Radiological Health (FDA)

Organ	Cancer	Biomarker target	Biomarker name	Tissue sampled	Method
Diagnosis					
Bladder	Bladder cancer	Aneuploidy for chromosomes 3, 7, 17 loss of 9p21	Vysis UroVysion Bladder Cancer Recurrence Kit	Urine	FISH
Breast	Breast cancer	MG, CK19	BLN assay	Sentinel lymph node	RT-PCR
Gastro-intestinal	Colorectal cancer	Multi-target DNA (aberrantly methylated BMP3 and NDRG4 promoter, mutant KRAS, and β -actin). Hemoglobin assay	Cologuard	Stool	PCR immunochemical assay for hemoglobin
Hemato-logical	B-cell chronic lymphocytic leukemia	alpha satellite (centromeric) region, 12p11.1-q11	CEP 12 SpectrumOrange Direct Labeled Fluorescent DNA Probe Kit	Peripheral blood	FISH
Ovary	Ovarian cancer	BRCA1 and 2 gene mutation	BRACAnalysis CDx	Blood	PCR
Prostate	Prostate cancer	PCA3	PROGENSA PCA3 Assay	Prostate biopsy, urine	PCR
Prognosis					
Breast	Breast cancer	58 gene RNA expression profile	Prosigna Breast Cancer Prognostic Gene Signature Assay	Tumor	nCounter system
		70-gene expression profile	Amsterdam 70-gene profile (MammaPrint)	Tumor	Agendia BV
		HER2	INFORM HER2 Dual ISH DNA Probe Cocktail; HER2 CISH pharmDxTM Kit DakoCytomation HER2 FISH pharmDx™ Kit	Tumor	ISH
		TOP2A	Dako TOP2A FISH PharmDx Kit	Tumor	FISH
Prostate	Prostate cancer	tPSA	NADiA ProsVue	Blood	PCR
Hemato-logical	Acute myeloid leukemia	EGR1	Vysis D7S486/CEP 7 FISH Probe Kit	Bone marrow peripheral blood	FISH
	B-cell chronic lymphocytic leukemia	TP53, ATM, D13S319 deletion D12Z3 gain	Vysis CLL FISH Probe Kit	Peripheral blood	FISH

(F)ISH, (fluorescence) in situ hybridization; MG, mammaglobin; BLN, breast lymph node; (RT-)PCR, (reverse transcription) polymerase chain reaction; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase II alpha; PSA, prostate specific antigen; ATM, serine/threonine kinase.

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Table 3 Predictive biomarkers currently under clinical evaluation and registered in clinicaltrials.gov

Organ	Cancer	Biomarker	Associated drug	Phase	Clinicaltrials.gov identifier
Breast	Breast cancer	BRCA1/2	Olaparib	III	NCT02000622
		CTCs positive for HER2	Trastuzumab—Emtansine	II	NCT01975142
		TOP2A (in subjects with HER2 overexpression)	Anthracycline-based neoadjuvant chemotherapy	II	NCT02339532
		HER2 (negative in tumor but positive in CTCs)	Lapatinib	III	NCT01619111
Gastrointestinal	Colorectal	New biomarkers (unspecified)	Cetuximab	II	NCT01276379
		RAS (mutation-type)	FOLFOXIRI and bevacizumab	II	NCT02350530
		BRAF	LGX818, BYL719	II	NCT01719380
	Esophago-gastric	HER2	Afatinib and trastuzumab	II	NCT01522768
Head and neck	Squamous cell carcinoma	HER and KRAS	HM781-36B	II	NCT02216916
Hematological	Cutaneous and peripheral T-cell lymphomas	GATA-3	MLN9708	II	NCT02158975
Lung	NSCLC	ROS1	Crizotinib	II	NCT02183870
		BRAF V600E	Dabrafenib, trametinib	II	NCT01336634
Skin	Melanoma	BRAF V600E/K	Trametinib, binimetinib	II	NCT02196181

CTCs, circulating tumor cells; HER2, human epidermal growth factor receptor 2; TOP2A, topoisomerase II alpha; NSCLC, non-small cell lung cancer.

profiling by deep sequencing (CAPP-Seq) has been tested on circulating tumor DNA in patients with NSCLC. Levels of circulating DNA correlated with tumor volume and provided earlier response assessment than radiography in this preliminary trial while potentially allowing the non-invasive detection of actionable mutations (82). Another report, focusing on circulating microRNA serum profiles identified a microRNA profile thought to distinguish subjects with pancreatic cancer from healthy controls, even at early stages of the disease (83). This result requires further validation but may suggest a direction towards which the field of diagnostic biomarkers is moving. However, even when FDA-approved, commercialization may still be a challenge due to the high cost required for assay development.

Cancer biomarkers under evaluation in clinical trials

Multiple predictive biomarkers, mostly based on single gene/protein, are currently in phase II or III evaluation along with their companion therapeutic agents (*Table 3*). From this snapshot, the increasing importance of predictive biomarkers is apparent as is a trend to develop minimally invasive cancer biomarkers. Biomarkers validated in a certain type of cancer are undergoing discovery and validation in other cancers (for instance BRAF mutations or HER2 overexpression) underlining certain shared oncogenic drivers and less prevalent cancers are also benefitting from the rapid developments in the field. The 70-gene breast cancer signature is currently being evaluated for its recurrence-predictive capability in comparison to clinico-pathological assessment in a large prospective trial enrolling more than 6,600 subjects in nine countries (MINDACT study) with early results suggesting that the 70-gene signature added information to usual assessment (84).

Future perspectives and conclusions

In this review, we aimed to overview the current landscape of cancer biomarker development. The speed of technological development has highlighted the challenges facing

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regulatory oversight and legislation in their attempts to keep up with the rapid pace of scientific changes while allowing proper consideration to how the new biomarkers could shape the future of medicine (85,86). One of the major challenges is to manage the tradeoff between safety and speed of clinical translation. For example, regulation of LDT by the FDA will improve assay quality and safety and increase overall medical utility of the tests, while it could hamper timely deployment of the tests and benefit only large commercial laboratories with capabilities to accommodate the high requirements. The large amount of data generated by the assays have posed supplementary challenges in the analysis of "big data", which requires massive computational resources for data storage, processing, and interpretation (87). Informatics resources such as ClinGen (88) are being developed to support the process. Also, systems to integrate genomic information with electronic medical records (EMRs) are actively developed, where protection of patient privacy is a central issue such as the Electronic Medical Records and Genomics Network (eMERGE), a NIH-funded consortium aiming to develop and disseminate approaches combining DNA biorepositories with EMRs (89). However, the integration of EMRs with genomic datasets remains in its infancy, due to a number of challenges including defining optimal storage standards of genomic data, integration of rich phenotype information, interpretation of complex data in a format easily accessible to clinicians and of course ethical, legal and social issues (90). Defining unified standard for the systems and data formats is particularly challenging due to the big financial/commercial interests.

Another crucial aspect of biomarker development, especially genomic biomarkers, is the issue of intellectual property. In the USA, a recent high profile Supreme Court decision, The Association for Molecular Pathology versus Myriad, determined that isolated but otherwise unmodified genes were products of nature and therefore not patent eligible subject matter (91). This decision was a response to an ongoing lawsuit between Myriad Genetics, who owned the exclusive rights to analyze the BRCA1 and BRCA2 gene mutations, and a coalition of groups who challenged the constitutionality and validity of the BRCA1 and BRCA2 gene patents. In this context, the USA Patent and Trademark Office has recently issued new guidelines which enforce more stringent criteria to patent natural products such as antibiotics, or even nucleic acids, peptides and proteins. These new guidelines have generated considerable concern in the biotechnology world due to their far-reaching consequences that are still being considered (92). Of note, genetic sequences are currently still patent-eligible in the European Union and in Australia if certain conditions are fulfilled (93,94). It is expected to take more time to reach a solution acceptable to all relevant parties.

Despite unclear future prospects and regulatory and legislative minefields, several examples of successful clinical translation summarized above have emphasized the challenges but also the opportunities at each step of cancer biomarker development. Acknowledging these challenges and implementing them in the design of biomarker development will help streamline the whole process, and eventually transform cancer patient care by fulfilling the vision of Precision Medicine.

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Analysis of cancer genomes through microarrays and next-generation sequencing

Li Liu[1]*, Alex Yick-Lun So[1]*, Jian-Bing Fan[2]

[1]Illumina, San Diego, CA 92122, USA; [2]AnchorDx Corp., Cancer Research Institute, Southern Medical University, Guangzhou 510300, China
*These authors contributed equally to this work.
Correspondence to: Jian-Bing Fan, PhD. CEO, AnchorDx Corp., Professor, Cancer Research Institute, Southern Medical University, Guangzhou 510300, China. A. Email: jianbing_fan@yahoo.com.

Abstract: The use of microarrays and next-generation sequencing (NGS) has made significant contributions to the understanding of cancer development. These technologies have been used to profile gene expression patterns in cancer cells, identify genomic regulatory elements bound by cancer-relevant transcription factors, and determine the genetic and epigenetic landscape of cancer cells. Importantly, scientists and clinicians have begun to apply these findings to develop personalized medicine by which an individual's cancer genomic information is used to guide the selection of cancer therapy. In this article, we will review the impact that genomics technologies have had on the cancer field.

Keywords: Next-generation sequencing (NGS); microarrays; cancer genomics

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Microarray analysis of cancer

Microarray technology has been widely used in cancer research for more than a decade. The traditional solid phase DNA microarray is a collection of DNA probes attached to a solid surface such as glass, plastic or silicon chips. Alternatively, the bead array is a collection of microscopic polystyrene beads with a specific probe attached to each bead. For instance, bead arrays were applied to quantify gene expression in formalin-fixed paraffin-embedded (FFPE) tissues (1). The specific probes on the bead arrays are usually designed from short sections of the target sequences used to hybridize to DNA or cDNA samples. The relative abundance of nucleic acid sequences in the target can be detected by probe-target hybridization and quantified by detection of fluorophore or chemiluminesence signals.

Impact of microarrays on cancer biology field

Various types of microarrays have been developed for different applications. Before the development of next-generation sequencing (NGS), microarrays have had major impacts in the field of cancer biology. One of the earliest applications of microarrays was to identify differences in gene expression between cancer and normal cells (2). For instance, an early study by DeRisi *et al.* utilized a density microarray of 1,160 DNA elements to demonstrate that as high as 9% of the transcriptome change in expression upon cancer cell transformation (2). Since then, numerous studies have utilized microarray approaches to profile gene expression patterns that initiate or maintain the oncogenic state of cancer cells. The development of DNA microarrays enabled the acquisition of gene expression data of virtually the entire expressed genome. Tens of thousands of genes are simultaneously monitored to study their expression levels in tumor and non-tumor tissues, which facilitate the detection of meaningful patterns in complex gene-expression patterns in cancer research (3). From the understanding that cancer cells can undergo dramatic changes in gene expression, microarrays have also been utilized to improve tumor classification, which is crucial for selecting the appropriate course of cancer therapy. A seminal study showed that profiling gene expression patterns through microarrays

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could be applied to easily distinguish between acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), demonstrating the feasibility of cancer classification through this approach (4).

In addition to monitoring gene expression patterns, microarrays have also been broadly used to decipher signal pathways directly orchestrated by cancer-relevant transcription factors. Martone *et al.* combined the power of chromatin immunoprecipitation with microarrays, known as ChIP-chip, to demonstrate that the NF-κB transcription factor p65 generally bind genomic elements distal from the promoter of their target genes (5). Subsequently, many groups applied ChIP-chip to demonstrate that cancer-relevant transcription factors, such as p53, estrogen receptor, and androgen receptor, generally bind to regulatory elements within both intergenic and intragenic regions far from their target promoter (6-8). Thus, the application of microarrays has advanced the scientific understanding of how cancer-relevant transcription factors control gene networks and ultimately cancer development.

Furthermore, microarrays have also been widely used to understand the genetic and epigenetic makeup of cancer cells. Microarrays have been used to identify small genetic changes, such as single nucleotide polymorphisms (SNP), in tumor cells through the use of SNP arrays. Also, the use of array comparative genomic hybridization (aCGH) has been widely used to identify large genetic abnormalities associated with cancer development, which include genetic deletions of several kilobases or duplications of entire chromosomes (9). Moreover, microarrays have been widely applied to decode the epigenome of many types of cancer cells. For example, DNA methylation arrays detect global patterns of methylation in cancer and identification of cancer biomarkers (10,11). From many studies, it is now clear that the transformation of normal to cancer cells involve a large number histone modification and DNA methylation changes.

NGS process

NGS technology has revolutionized our understanding of the cancer genome (12,13). Twenty years ago, sequencing one human genome took more than ten years and cost $3.8 billion (14,15). In 2008, the cost had dropped to $2 million (16). Same year, the first cancer genome was sequenced by NGS technology (17). Today, a human genome can be sequenced for $1,000 on Illumina Hiseq X platform. The dramatic increase in throughput and the drop in cost greatly improved our capability to comprehensively understand a cancer and offers opportunities to advance cancer prevention, diagnostic, prognostics and treatment.

In first-generation sequencing technologies, genomic DNA is fragmented and individual fragments are cloned into plasmids or phage to create a library with millions of individual clones. The plasmids are introduced into bacterial cells, followed by growing individual bacterial clones and isolating plasmids from each clone. Millions of individual sequencing reactions are performed on plasmid DNA to generate sequence data for each plasmid. This is a very time-consuming process and cost ~$20 million to sequence a single human genome. In second-generation sequencing, the serial process of growing and sequencing millions of individual clones is replaced by highly parallel process in which billions of DNA fragments are amplified and sequenced simultaneously. The process is composed of four major steps: library preparation, clonal amplification, sequencing, and data analysis.

To create a DNA sequencing library, the isolated DNA is fragmented into 500 bp segments by sonication. Followed by end repair and addition of a single A base, Y-shaped adaptors are ligated to the ends of the DNA fragments. Alternatively, fragmentation and adaptor ligation can be achieved by incubating genomic DNA with a transposase that carries DNA adaptor sequences. The transposase simultaneously cleaves the DNA and ligates the adaptors to create a library.

The flowcell surface is pre-coated with oligonucleotides that are complementary to the adaptor sequences on the library. The DNA library is denatured and captured onto the flowcell by hybridization to these oligonucleotides. The library is clonally amplified by a process called bridge amplification, resulting in over one billion clusters with each cluster containing ~1,000 molecules. A sequencing primer is then added to the free ends of the DNA.

The billions of clonal clusters are sequenced simultaneously and in parallel (*Figure 1*) (18). Reversibly terminated and fluorescently labeled nucleotides are added to the sequencing primer by an engineered DNA polymerase. The reversible terminator prevents the addition of more than one base in one sequencing cycle. Each base is labeled with one of four colors that emit a fluorescent color for imaging. After recording the color and location of each cluster, the reversible terminator and the fluorescent dye are removed, allowing the incorporation of the next nucleotide. This process is called sequencing by synthesis (SBS). It is repeated 150-250 times in one direction and the

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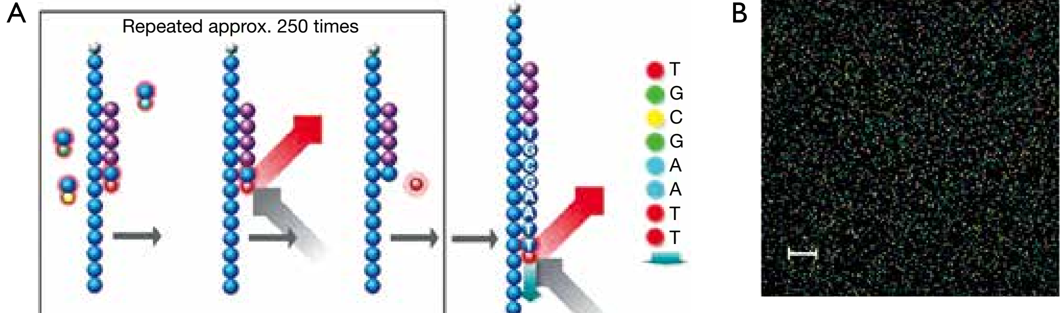

Figure 1 SBS with reversible terminators. The billions of clusters generated by clonal amplification are sequenced in parallel. Fluorescently labeled and reversibly terminated nucleotides are added to the sequencing primers by an engineered polymerase (A). Only a single base can be added to the growing DNA strand by the polymerase enzyme per cycle because the terminator blocks further polymerization. Unincorporated nucleotides are washed off and the flowcell is imaged to record the color and location of the incorporated nucleotides (scale bar 10 μm) (B). After imaging, the terminator and fluorescence dye are cleaved off, allowing the incorporation of the next nucleotide. This process is repeated 250 times to build a 250 bp sequence. Reprinted with permissions from "cancer genome sequencing" by Lakdawalla *et al.* (18). SBS, sequencing by synthesis.

DNA molecules are flipped over allowing re-synthesis of the reverse strand. The forward strands are then cleaved, leaving the reverse strands to be sequenced. The paired-end sequencing strategy produces sequence information from both ends of each DNA molecule, yielding twice the sequencing information from a library and facilitating an accurate alignment of the sequenced fragments.

The raw image data is converted to a fluorescence intensity table that records the location of each cluster and the color intensity values. These values are then converted to base calls. At the end of a sequencing run, billions of clusters have each produced a 2×[150-250] bp read, which are then aligned to a reference genome and converted to BAM file format that can be imported into genome browsers, such as UCSC and IGV genome browsers, for visualization. A genomic DNA library contains fragments from multiple copies of genomic DNA, therefore each base will be read multiple times from independent clones. The reads aligned to the same region are combined to make a high confidence base call.

Targeted DNA sequencing

Although whole-genome sequencing has been widely used in cancer research, the cost is still substantial and the majority of the sequence obtained is with no known significance in cancer. Targeted sequencing has thus emerged as a cost-effective approach to tumor genetic profiling (19). In target enrichment sequencing strategies, the genomic sequences of interest are selected for sequencing. Several target enrichment methods have been developed, including PCR, molecular inversion probes, array based or in-solution hybrid capture. The choice of target enrichment method depends on a variety of factors, such as size of the target region, DNA input, and genomic architecture of the region (20). In PCR-based approach, sequences of interest are enriched and amplified with sequence-specific primers. For example, Illumina Truseq Amplicon-Cancer Panel is a predesigned panel covering 212 mutations in 48 cancer-related genes. Each amplicon is flanked by two oligonucleotide probes with the same orientation followed by a proprietary extension-ligation step. PCR is then performed to add an index and sequencing motifs. In array-based capture, oligonucleotides complementary to the sequences of interest are synthesized on a chip and hybridization occurs on the surface of the chip, whereas in solution-based capture method, hybridization between DNA and probes occurs in solution, which allows less DNA input and smaller reaction volumes (21).

Annotation and interpretation of sequencing data

The sequence reads are assembled into a consensus sequence and compared against the reference genome to derive a list of variants. Whole genome sequencing data is generally low coverage (10-40× coverage) and suitable for the detection of constitutional variants. Target sequencing of specific genomic sequences of interest may increase the coverage

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to 1,000× or higher, permitting more sensitive evaluation of variants in cancer (22). Major structure variations, such as translocations, copy number variations (CNV), and insertion/deletion (indel) can also be detected by various algorithms (23). Translocation is usually detected by split-read method, where single reads are mapped to the genome discontinuously. Changes in read depth over large regions often indicate copy number changes. Indels can be detected using discordant paired reads or split reads.

New discoveries of cancer biology using NGS

Many applications that previously used microarrays for genomic studies have been replaced with NGS. Similar to microarrays, NGS can also be used for RNA profiling, identifying genomic elements bound by cancer-relevant transcription factors, isolating genetic changes that occur upon cell transformation, and deciphering the epigenetic makeups of cancer cells.

The invention of NGS has revolutionized the cancer biology field by providing the ability to sequence DNA in a genomic scale at unprecedented speed. NGS has essentially been used to study cancer biology in essentially every facet. Not long ago, it was thought that the human genome was made up of mostly 'junk' DNA. Since the sequencing of the human genome, the application of the NGS technology has contributed significantly towards the understanding that the genome encodes many important and previously unappreciated elements critical for normal cell function. NGS has paved the road for identifying non-coding RNAs, including microRNAs, long non-coding RNAs, and circular RNAs. It is now appreciated some of these non-coding RNAs play crucial roles in tumorigenesis and tumor suppression (9,24).

Because NGS has the potential to gather DNA sequence information of individual cells from samples that contain heterogeneous cell populations, it has become the leading platform to identify somatic mutations associated with cancer. A few years ago, a number of studies reported the use of whole genome or exome sequencing to identify recurrent somatic mutations associated with various cancer types. This led to the discovery of novel signal pathways that mediate cancer development. For instance, Wang *et al.* sequenced tumor samples from patients with chronic lymphocytic leukemia (CLL) and identified frequent somatic mutations in the coding region of SF3B1, which is a factor belonging to the spliceosome (25). In separate studies using NGS, Graubert *et al.* and Yoshida *et al.* also found recurrent mutations in other mRNA splicing factors in myelodysplasic samples (26,27). These studies led to the discovery that abnormal mRNA splicing contributes to oncogenesis in blood neoplasm. Using similar approaches, Puente *et al.* also identified recurrent mutation in the NOTCH1, XPO1, MYD88, and KLHL6 genes in patients with CLL (28). Thus, NGS technology has been critical in discovering new somatic mutations and signal pathways that are associated with cancer pathology.

Application of NGS in personalized medicine

The NGS technology has contributed to the identification of 'hotspot' somatic mutations associated with particular cancer types. Thus, clinicians have begun to test for the existence of these 'hotspots' mutations in patients to guide therapeutic selection. For instance, patients with NSCLC are often tested for somatic mutations in the kinase domain of EGFR because it has been shown that EGFR mutational status is correlated with tumor sensitivity to the kinase inhibitors gefitinib and erlotinib (29-31). Also, it has been reported that the KRAS mutational status in patients with metastatic colorectal cancer is inversely correlated with response to panitumumab therapy (32,33). Therefore, there is also an interest in identifying KRAS mutations in these cancer patients. Because of the heterogeneous and complex nature of tumors, there is a growing demand for profiling somatic mutations in a panel of 'hotspot' genes rather than just at an individual gene. Thus, the need to identify somatic mutations at a number of loci simultaneously has increased the demand of using NGS to guide cancer therapy.

As a result of collaborative efforts amongst academic institutions, industries, and hospitals, multiple NGS platforms/assays that examine mutations at a panel of candidate genes have become available for clinical use. For instance, Asuragen offers the Suraseq 500 panel for clinical trials that uses NGS platforms to assess the mutational status of 17 cancer targets and 500 genomic sites in tumor tissues. Similarly, the Oncotype DX diagnostic tests (Genomic Health Inc) were developed to use the genomic information of the patients' tumors to guide breast, colon, or prostate cancer treatment; the information can be used for assessing potential chemotherapy benefits as well as likelihood of cancer recurrence. Of note, the MiSeqDx instrument (Illumina Inc) became the first NGS platform approved by the FDA for vitro diagnostic (IVD) use. Thus, it is evident that the application of NGS in clinical settings has become more pronounced and will continue to be a key

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factor in shaping personalized medicine.

It is noteworthy to mention that although targeted panels will be useful for guiding selection of cancer therapy, all the mutations that induce cancer development and maintenance have not been identified. Moreover, cancer development is complex and includes various types of mutations, including somatic mutations in coding and non-coding regions, genetic translocations, gene amplifications, and genetic deletions. The ultimate goal of personalized medicine is to be able to sequence the entire genome of cancer patients to unbiasedly identify relevant somatic mutations, which will not only enable discovery of novel and previously unappreciated mutations but also enhance the precision in using genomics to guide cancer therapeutic selection.

Application of NGS technologies in liquid biopsies

Although assessing somatic mutation from sequencing tumor tissue is the gold standard for clinical molecular diagnosis, it is limited by the acquisition of tumor tissue samples. The development of non-invasive methods has become essential for cancer detection and monitoring. Recent studies on circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and tumor-derived exosomes highlights the potential of monitoring tumor genome evolution from a simple blood draw—an approach known as a 'liquid biopsy' (34-41).

CTCs are intact tumor cells shed into the bloodstream from both primary and metastatic tumors. They can be purified from blood by cell surface markers that distinguish them from normal blood cells (42). The major challenges of utilizing CTCs are isolating rare cells and sequencing low-input material. Lohr *et al.* reported a method to isolate, qualify and sequence whole exomes of CTCs with high fidelity using a "census-based sequencing" strategy, in which combining multiple single CTC libraries markedly reduced the false-positive rate of called somatic single-nucleotide variants (41). Using this technique, the authors demonstrated that they could detect CTC mutations that are also present in matched tumor tissues.

ctDNA is composed of small fragments of nucleic acid that are released into the bloodstream from apoptotic and necrotic tumor cells (43). Given the fact that ctDNA is significantly more abundant and easier to purify than CTCs, it is a more preferable source for molecular diagnosis. Sequencing of ctDNA has demonstrated that ctDNA is detectable in most patients with metastatic cancers, across all major cancer types (34). The biggest technical challenge of analyzing ctDNA is its low mutant allele frequency and large dynamic range. The level of ctDNA in cancer patients ranges from <0.1% to >50% out of the total cfDNA. Therefore, the technical sensitivity and dynamic range of the assay are critical to maximizing the clinical utility of cfDNA. Bratman *et al.* reported an ultrasensitive method for quantifying ctDNA called "cancer personalized profiling by deep sequencing (CAPP-Seq)" (35). They implemented CAPP-Seq for a non-small cell lung cancer (NSCLC) study with a design covering 139 recurrently mutated genes, and detected ctDNA in 100% of patients with stage II-IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to ~0.02% (35). It is believed that with the rapid development of highly sensitive and accurate NGS technologies, "liquid biopsies" will enhance patient care and play an essential role in personalized medicine.

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Molecular landscape of non-squamous, non-small cell carcinoma of the lung

Mia C. Weiss, Michael J. Leukam, Victoria M. Villaflor

Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL, USA

Contributions: (I) Conception and design: All authors; (II) Manuscript writing: All authors; (III) Final approval of manuscript: All authors.

Correspondence to: Victoria M. Villaflor. Department of Medicine, Section of Hematology/Oncology, University of Chicago, 5841 South Maryland Ave., MC 2113, Chicago, IL 60637, USA. Email: vvillafl@medicine.bsd.uchicago.edu.

Abstract: The treatment of advanced or metastatic non-small cell lung cancer (NSCLC) has undergone a major change over the past decade, from a single option of platinum-based systemic chemotherapy to an increasingly personalized approach to treatment based on specific molecular alterations within tumors. The scope of this paper is to review the literature on the treatment of non-squamous NSCLC and give a broad understanding of the current molecular targets for which therapies currently exist, as well as other targets for which therapies may soon be developed. Additionally, issues of resistance with targeted therapies will be discussed. This manuscript only summarizes the work done to date, and in no way is meant to be comprehensive.

Keywords: Non-small cell lung cancer (NSCLC); molecular; adenocarcinoma; lung; cancer

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Introduction

Lung cancer is the leading cause of cancer related mortality in the United States, with an estimated 200,000 new cases and 160,000 deaths annually (1). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers (2) and is further subtyped into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Over the past decade it has become clear that subsets of NSCLC can be further subdivided based on the driver mutations occurring in multiple oncogenes including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), kirsten rat sarcoma viral oncogene homolog (KRAS), ros oncogene 1 (ROS1), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), human epidermal growth factor receptor 2 (HER2)/NEU, Ret proto-oncogene (RET), MAPK/Erk kinase (MEK), and C-mesenchymal-epidermal transition (C-MET)/recepteur d'origine nantais (RON). Targeting therapy toward these specific genetic alterations is becoming the standard for NSCLC treatment.

This review aims to provide an overview on the genetic alternations most often seen in non-squamous NSCLC and treatments aimed at targeting these alterations. We also examine some of the mechanisms of resistance to these therapies and ways of overcoming resistance to further improve overall survival rates in these patients.

Epidermal growth factor receptor (EGFR)

EGFR is a receptor tyrosine kinase involved in cellular differentiation, proliferation, angiogenesis and apoptosis. It is estimated that 10% of NSCLC patients in the United States with NSCLC and 35% in East Asia have tumors with EGFR mutations (3,4), making this receptor an important molecular target for disease treatment. The classic activating mutations including exon 19 deletions and exon 21 L858R substitution account for approximately 45% and 40% of all EGFR mutations respectively (5). Many large studies have emerged in the last few years validating the clinical use of EGFR tyrosine kinase inhibitors (TKIs) over chemotherapy as first line treatment for NSCLC patients harboring EGFR mutations (3,4,6). These therapies initially included first line EGFR TKIs gefitinib and erlotinib, both of which work by reversibly binding and blocking the ATP binding site of EGFR's tyrosine kinase domain preventing

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homodimer formation and subsequent activation of the signaling cascade (4,6-8).

The combination of first generation TKIs and standard chemotherapy regimens have historically not shown to have any significant benefit in patients not selected for EGFR mutations (9-12). The FASTACT 2 study, which used an intercalated approach combining intermittent dosing of chemotherapy with EGFR tyrosine kinase inhibition demonstrated encouraging progression free survival and overall survival specifically when selected for EGFR mutated NSCLC (13). This trial however, did not compare results with single agent EGFR TKI's which are now standard of care for EGFR mutation positive tumors and are less toxic to the patient than combination therapy (14).

Initial responses to EGFR-TKIs are favorable, however, most patients will go on to become resistant to these treatments within 1-2 years. There are many mechanisms of resistance, the most common of which is the acquired mutation T790M, which occurs in approximately 50% of patients (15,16). The T790M mechanism of resistance prevents drug binding to the domain through steric hindrance. Other resistance mechanisms to TKIs include transformation to small cell carcinoma, emergence of HER2 amplification, and MET overexpression (17).

Afatanib is a second generation TKI that acts as an irreversible EGFR inhibitor. Phase III trials including the Lux-3 and Lux-6 studies showed a progression free survival benefit when compared with standard chemotherapy in patients with EGFR mutated tumors (18,19). A joint analysis of both trials showed that the median overall survival was not significantly increased for patients given afatanib as compared with chemotherapy. However, when the combined trial data were analyzed based upon the specific mutation present, a statistically significant benefit was observed in both overall and progression-free survival in patients with exon 19 deletions. In patients with the L858R mutation there was a significant benefit in progression-free, but not overall survival (20). The currently ongoing Lux-7 trial is a phase IIb trial comparing afatinib to gefitinib as first line treatment in patients with documented EGFR mutations (NCT01466660).

Third generation TKIs like AZD9291 and rociletinib (CO1686) have emerged as potential therapeutics in tumors harboring acquired T790M resistance mutations (21). A recently published phase I/II clinical trial evaluating patients with acquired resistance to EGFR TKI's showed favorable results with AZD9291 (22). Multiple ongoing phase III trials are examining AZD9291 compared to standard chemotherapy regimens (NCT02296125, NCT02474355). Further clinical trials under investigation are examining AZD929 in combination with novel immunotherapeutic agents such as MED 4736, a PD-L1 antibody (NCT02143466, NCT02454933). In a recently published phase I/II clinical trial Rociletinib showed favorable results in patients who progressed on previous TKI therapy (23). More data on the use of rociletinib will be examined in the ongoing phase 3 TIGER-3 study, which aims to examine Rociletinib versus single agent chemo in patients who have failed at least one previous TKI and platinum doublet chemotherapy (NCT02322281).

The N-methyl-N'-nitrosoguanidine human osteosarcoma transforming gene (*MET*) receptor kinase is involved in tumor-cell proliferation, mobilization and angiogenesis. Overexpression, amplification or aberrant signaling of the *MET* receptor tyrosine kinase has been implicated as a mechanism of erlotinib resistance in tumors with EGFR- activating mutations (24-26). MET activation increases the expression of some EGFR ligands and coactivation of EGFR and MET has been described to result in resistance (27). Small molecule inhibitors of MET have not yet demonstrated much therapeutic success. ARQ197 (Tivantinib) is a selective small molecule that inhibits MET receptor tyrosine kinase causing inhibition of cell proliferation and induction of cellular apoptosis, and has been studied in combination with EGFR TKIs. The recent phase III MARQUEE trial comparing erlotinib with or without tivatinib showed increased progression free survival but did not improve overall survival in nonsquamous NSCLC patients treated with the combination (28). The similar phase III ATTENTION trial was terminated early due to increased incidence of interstitial lung disease in the ARQ197 group (29). There is encouraging data for the role of MET inhibition using monoclonal antibodies against MET. Onartuzumab, a monoclonal antibody against the MET receptor has been shown in a recent phase II trial to increase progression free survival and overall survival in MET+ patients when combined with EGFR TKIs (30). This is being further investigated in an ongoing phase phase III trial evaluating onartuzumab in combination with erlotinib in patients With MET-Positive, EGFR mutant NSCLC (NCT01887886).

Improving progression free survival in EGFR mutated NSCLC tumors by employing synergy with other small molecules (such as VEGF inhibitors) is another goal of many ongoing trials. Although a recent phase III clinical trial showed no benefit of bevacizumab plus erlotinib

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versus erlotinib alone after failure of standard first-line chemotherapy in an unselected group of non-squamous NSCLC patients (31), further data suggests that there may be a survival benefit in patients with tumors specifically known to harbor EGFR driver mutations (32). Further clinical trials evaluating small molecules such as VEGF inhibitors or immune therapy with EGFR inhibitors are ongoing [NCT01532089 (VEGF), NCT01998126 (CTLA-4), and NCT02013219 (PDL1)].

Anaplastic lymphoma kinase (ALK)

It is estimated that 3-5% of patients with NSCLC harbor a fusion mutation involving ALK. The most common variant contains an inversion in chromosome 2 that juxtaposes the 5' end of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene with the 3' end of the anaplastic lymphoma kinase (*ALK*) gene, resulting in the novel fusion oncogene *EML4-ALK* (33). Patients with ALK-rearrangements tend to be young, never or former light smokers, and most likely to have tumors of the adenocarcinoma histologic subtype (34).

Crizotinib is an orally available ALK/MET/ROS1 TKI. The phase III PROFILE 1007 study compared crizotinib with chemotherapy as second-line therapy in ALK^+ patients. Findings demonstrated an increase in progression free survival in the experimental arm but no significant increase in overall survival (35). Similar results were seen in the phase III PROFILE 1014 study comparing crizotinib with chemotherapy in patients with ALK-arranged NSCLC who had not received prior systemic treatment. Progression free survival was prolonged in the experimental group but no significant difference was seen in overall survival in the interim analysis. Interpretation of overall survival is complicated by exposure of the patients assigned to the control arm to crizotinib during follow-up (36). To investigate this treatment option further, an ongoing study is evaluating pemetrexed with or without crizotinib for patients with advanced ALK-rearranged NSCLC that has progressed after treatment with crizotinib alone (NCT02134912).

Resistance to Crizotinib inevitably occurs within the first few years of treatment. Resistance mechanisms identified include an acquired secondary mutation within the ALK tyrosine kinase domain, most common being the gatekeeper L1196M mutation, followed by the G1269A mutation (37,38). Other resistance mechanisms include amplification of the ALK fusion gene, which is observed in about 9% of crizotinib-resistant cases (39), and a number of so-called "bypass signaling pathways" involving abnormal functioning of epidermal growth factor receptor (EGFR), KIT, and insulin-like growth factor-1 receptor (IGF1R) pathways (40-42).

Patients with ALK translocated tumors often relapse in the CNS, which is a challenge for patients who progress while receiving crizotinib (43). Relapse is common in the CNS as it acts as a sanctuary site for metastasis given the inability of most chemotherapeutic agents to cross the blood brain barrier. Ceritinib (a second generation ALK inhibitor discussed below), has blood-brain barrier penetration in preclinical studies and showed intracranial activity in the ASCEND-1 trial (44).

Certinib is a second generation ALK inhibitor that was recently approved based on a single-arm clinical trial which demonstrated durable improvement in overall response rates in patients who have failed crizotinib. It is currently undergoing phase III trials to explore the antitumor activity of this novel agent compared to reference chemotherapy in previously untreated ALK-positive, stage IIIB or IV, nonsquamous NSCLC (NCT01828099). A second study will evaluate the antitumor activity of certinib compared to chemotherapy in patients previously treated with chemotherapy (platinum doublet) and crizotinib (NCT01828112). Other second-generation ALK inhibitors in development include Alectinib, which has demonstrated an increased survival benefit in phase II studies. This drug is currently undergoing phase III trials evaluating alectinib vs. crizotinib in treatment-naive ALK-positive advanced NSCLC (NCT02075840), as well as alectinib alone in patients after disease progression on or intolerance to prior ALK TKI therapy (NCT02271139). Additionally, phase II studies are currently underway for 2nd generation TKI inhibitors brigatinib (AP26113) (NCT02094573), and PF-06463922 (NCT01970865). X-396 is a potent ALK inhibitor with a similar chemical structure to that of crizotinib, but with a 10-fold higher potency and is currently being studied in a phase I trial (NCT01625234).

Potential Therapeutic Strategies to overcome ALK TKI Resistance include the addition of heat shock protein 90 (HSP90) inhibitors. ALK fusion proteins bind to HSP90 and are thought to depend on HSP90 as a chaperone protein to form tertiary structure and stabilize the protein. A number of ongoing trials are currently testing safety and efficacy of HSP90 inhibitors in addition to ALK inhibitors. (NCT01752400, NCT01712217).

Early data suggest checkpoint inhibitor immunotherapy

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with EGFR inhibitors may improve response and survival by matching the cancer's ability to mutate and evolve, thus increasing the potential for durable response (17). These data appears to be similar with ALK translocated tumors hence there is current interest in adding checkpoint inhibitors in ALK-rearranged NSCLC patients. A current phase I study using nivolumab [an antibody which functions as a programmed cell death receptor/ligand programmed death receptor (PD1)/programmed cell death ligand (PDL1) checkpoint inhibitor] in addition to ceritinib is currently ongoing (NCT02393625). Alectinib is being evaluated with PDL-1 inhibitors in patients with tumors that are ALK translocated and treatment naïve [NCT02013219].

Kirsten rat sarcoma viral oncogene homolog (KRAS)

KRAS mutations are found predominately in the adenocarcinoma histologic subtype of NSCLC (30%) and less frequently in the squamous cell carcinoma subtype (approximately 5%) (45). Most often, these mutations are found in patients with a smoking history (46,47). Mutations in KRAS are typically mutually exclusive with aberrations of other oncogenic drivers including EGFR, BRAF, HER2 mutations and ALK and ROS1 rearrangements (15). KRAS mutations in NSCLC predominantly occur in codons 12 or 13 and with a lower frequency in codon 6 (48). Mutant Ras proteins are insensitive to GTPase activating protein (GAP), rendering the proteins constitutively GTP bound and activated, leading to stimulus-independent, persistent activation of RAS downstream effectors, in particular, the Raf (Rapidly Accelerated Fibrosarcoma)-MAPK (Mitogen-activated Protein Kinase)-ERK (Extracellular signal regulated kinases) cascade (49).

The prognostic and predictive role of KRAS mutations remains controversial. These mutations have not shown to be predictive for the use of adjuvant chemotherapy (50). In metastatic NSCLC KRAS mutations did not predict response to standard chemotherapy (51,52). KRAS mutations also seem to negatively predict response to EGFR TKIs (53-55).

At present there is no established therapy for patients with KRAS mutations. No direct inhibitor of KRAS exists, but targets downstream of KRAS, such as the MEK pathway have shown some encouraging results (56). The MEK1/2 inhibitor selumetinib has shown some promising activity in a recent phase II clinical trial comparing selumetinib versus standard chemotherapy in previously treated KRAS mutant NSCLC patients (57). Currently, a phase III trial with selumetinib is ongoing (SELECT-1, NCT01933932).

Trametinib is another inhibitor of MEK, which has not shown to improve survival outcomes of KRAS mutant patients in a phase II trial when compared to standard chemotherapy as a second line therapy (58). Positive response rates have been noted in clinical trials evaluating at Trametinib plus docetaxel or pemtrexed (59,60), but further investigation is required. Inhibition of other downstream signaling pathways such as PI3K and focal adhesion kinase (FAK), have shown benefit in KRAS positive tumors (61,62) and multiple clinical trials with FAK inhibitors (defactinib/VS-6063) and PI3K inihibitors (BKM120) are ongoing.

Ros oncogene-1 (ROS-1) translocation

The C-ros oncogene 1 (ROS1) is a receptor tyrosine kinase of the insulin receptor family that that acts as a driver oncogene via a genetic translocation between ROS1 and a number of other genes, most commonly CD74 (63). This translocation is seen in only 1 to 2 percent of NSCLC typically in younger non-smoking patients (63-65). Crizotinib, a potent inhibitor of ALK and MET has also shown activity against ROS1-rearranged NSCLC likely due to a high degree of homology between the ALK and ROS tyrosine kinase domains (66). The PROFILE 1001 phase I trial showed favorable response rates in patients with ROS1 tumors treated with crizotinib (67). Phase II trials evaluating crizotinib in pre-treated patients with ROS1 mutations is ongoing NCT02499614. Other agents are currently being investigated for ROS1-positive lung cancer patients including foretinib, ceritinib, AP26113, PF-06463922 as well as HSP90 inhibitors (68).

Resistance to crizotinib in ROS1 mutated tumors is known to occur. It has been shown that in patients harboring CD74-ROS1 fusions, resistance to crizotinib was partly mediated by the ROS1 G2032R mutation (69). Other possible mechanisms of resistance include EGFR pathway activation, epithelial-to-mesenchymal transition, and various ROS1 tyrosine kinase mutations (65,70). A number of novel TKIs with activity against ROS1 are being investigated including AP26113, Foretinib, and PF-06463922 (71-73), (NCT01970865).

BRAF

BRAF (v-Raf murine sarcoma viral oncogene homolog B) is a downstream signaling mediator of KRAS, which

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activates the MAP kinase pathway. BRAF mutations have been observed in 1 to 3 percent of NSCLC. Of NSCLC patients harboring BRAF mutations ~50% contain the classical V600E mutation (74), seen commonly in metastatic melanoma. BRAF V600E mutations are associated with light/never smoker status, micropapillary histology and occur more frequently in female patients (68). In contrast, non-V600E mutations (for example mutations within exons 5 or 11) are seen in former or current smokers and are associated with poorer outcomes (75,76).

BRAF targeting TKIs including dabrafenib and vemurafenib are being studied in the treatment of BRAF mutated NSCLC. Preliminary results from a recent phase II trial with dabrafenib in patients harboring V600E mutant NSCLC have shown positive partial response rates (77). Several case reports show responses in NSCLC patients with vemurafenib (78-80). Studies with metastatic melanoma suggest synergy with the combination of BRAF- and MEK-inhibition (81), and are now being studied in combination in a phase II clinical trial in BRAF mutant NSCLC (NCT01336634).

Human epidermal growth factor receptor 2 (HER2)/NUE

HER2 is a member of the ERBB receptor tyrosine kinase family and mutations have been detected in approximately 1 to 2 percent of NSCLC tumors (68). These mutations are more prevalent among never-smokers and women (82,83). Unlike HER2 overexpression in patients with breast cancer and GI malignancies, NSCLC tumors have mutations that have not been shown to respond to anti-HER2 therapies (84-86). Further studies have showed favorable response when combining HER2 inhibitors with chemotherapy (83,87), and with the EGFR inhibitor, afatanib (88). A recent phase I trial with neratinib (an irreversible pan HER inhibitor) combined with the mTOR inhibitor temsirolimus has also showed promising clinical activity (89) and is currently undergoing phase II trials (NCT01827267).

Currently, several clinical trials are investigating the role of HER2-directed antibodies such as trastuzumab, pertuzumab, as well as the HER2-targeting TKIs (afatinib, dacomitinib and neratinib) NCT00004883 NCT02289833, NCT00063154, NCT00818441.

RET

RET (rearranged during transfection) encodes a surface receptor tyrosine kinase found to be mutated in about 1.5% of NSCLC patients who are generally younger, light or never smokers with adenocarcinoma histology and poorly differentiated tumors (90). The most common RET translocation is the KIF5B-RET fusion variant on chromosome 10. Additional gene fusion partners including CCD6, NCOA4 and TRIM33 have also been described (68).

RET TKIs such as vandetanib, sorafenib and sunitinib, have overall not shown significant survival benefit in unselected NSCLC patients. Case reports have shown positive response rates in patients with RET translocations who were treated with vandetanib (91,92) and another inhibitor cabozantinib (93). A preliminary report from a phase II clinical trial NCT01639508 investigating cabozantinib in RET fusion positive NSCLC tumors with 16 evaluable patients showed that 7 had partial responses (38 percent) and 9 had stable disease (72 percent) (93). With a median follow-up of two years, the median progression-free survival was seven months and the median overall survival was 10 months (94). Clinical trials are ongoing going looking at different multi-kinase TKIs, which include RET as a target, including Ponatinib (NCT01813734), vandetanib (NCT01823068), and Lenvatinib (NCT01877083).

MAPK/Erk kinase1 (MEK1)

MEK1 also named MAP2K1, is a serine-threonine kinase with mutations occurring in approximately 1% of NSCLC (mostly adenocarcinoma) (95). MEK itself is not an oncogene product, but it is the focus of many of the signal transduction pathways activated by known oncogenes (including BRAF and KRAS mutations) and tyrosine kinase receptors (95). Therefore, inhibition of MEK has the potential to prevent the subsequent downstream phosphorylation and activation of MAP kinase (to pMAPK/pERK) to potentially induce tumor regression and/or stasis (96,97). A phase two study looking at PD-0325901 a small-molecule inhibitor of both MEK isoforms, MEK1 and MEK2 did not show significant survival benefit in non-selected NSCLC patients (98).

C-mesenchymal-epidermal transition (C-MET)/ recepteur d'origine nantais (RON)

Mesenchymal-epidermal transition (MET) is a receptor tyrosine kinase, which undergoes homodimerization by binding its ligand; hepatocyte growth factor (HGF) causing autophosphorylation of MET and ultimately leads to the activation of various intracellular signaling pathways

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including RAS-RAF-MAPK and PI3K-AKT-mTOR (99). MET abnormalities are most often overexpression due to gene amplification and exon 14 skip splice mutations (100). Studies have suggested that approximately 40% of lung cancer tissue overexpresses MET (101).

In general, studies of multiple MET inhibitors have not shown significant improvement in survival data. In the last two years, three landmark phase III trials investigating Met targeted agents (including HGF monoclonal antibody ornatuzumab and small molecule met inhibitor tivantinib) in combination with erlotinib (an EGFR inhibitor) in pre-treated lung cancer were suspended following interim analyses that indicated no improvement in survival and/or safety concerns (30,102-104).

Further studies with MET inhibition are ongoing, including a study of Crizotinib which is being evaluated in patients with NSCLC who have intermediate or high *MET* gene amplification (NCT00585195).

RON is a MET-related receptor tyrosine kinase. It's natural ligand is macrophage stimulating protein, but beta-1-integrins can also activate RON via c-Src-dependant signaling pathways (105). RON signaling has roles in the regulation of inflammation and the motility and activation of macrophages, and therefore contributes to tumor growth and metastasis. RON signaling activity is synergistic or additive with MET leading to transformation, cell spreading, motility and cell survival (106,107). At present no specific c-met/RON inhibitors exist. An early clinical trial for MGCD265 (a multikinase inhibitor directed against c-MET, VEGR1, 2, 3, RON, and Tie-2) has not yet reported results (NCT00975767).

PIK3CA

The phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR pathway is one of the most often deregulated signaling cascades in human cancers, including NSCLC, detected in 2% of tumors and is more commonly seen in squamous cell lung cancers (108). PIK3CA encodes the catalytic subunit of PI3K, which is an intracellular central mediator of cell survival signals (109). PIK3CA mutations can occur in combination with other known driver mutations like EGFR or KRAS mutations as well as in the setting of acquired EGFR TKI resistance (109). Pre-clinical models have shown that tumors harboring PIK3CA mutations are highly sensitive to PI3K inhibitors (110), and further clinical evaluation are ongoing with other PI3K inihbitors including BKM120 (NCT01723800), GDC0941 (NCT01493843), and XL-147 (NCT00692640).

Programmed death receptor (PD1) and programmed cell death ligand (PDL1)

PDL1 is a cell surface signaling molecule that binds to PD1 on T-cells, causing anergy and prevention of secretion of pro-inflammatory cytokines in cytotoxic T-cells and transformation of helper T-cells into immune-suppressing T-regulatory cells (111). In a healthy host, this immune checkpoint mechanism prevents over-activity or inappropriate activation of the adaptive immune system. When PDL1 is overexpressed by cancer cells, an appropriate immune response to the tumor is suppressed (111). The rationale behind PD1 and PDL1 as treatment targets is that preventing the interaction of the receptor and ligand will increase anti-tumor immune activity. PDL1 has found to be expressed on the surface of 45-50% of NSCLC cells regardless of subtype (112). PDL1 overexpression is associated with presence of EGFR mutations, solid predominant subtype, and advanced pathologic stage (113-115). The prognostic significance of elevated PDL1 in NSCLC h as been unclear, with two studies focused exclusively on the adenocarcinoma substype reporting an opposite effect on overall survival (115,116), though a recent meta-analysis in NSCLC in general (which included the adenocarcinoma studies) found overall decreased overall survival with increased PDL1 expression (117).

Immune checkpoint inhibition using PD1/PDL1 disruption has been studied in multiple malignancies, now including NSCLC. In 2012, a phase I trial of nivolumab (a monoclonal antibody against PD1) in a variety of solid tumors demonstrated an objective response in 5 of 49 patients with non-small-cell lung cancer with six additional patients with NSCLC who had stable disease lasting at least 24 weeks (118). Some responses were quite durable: in the overall cohort, responses lasted for 1 year or more in 8 of 16 patients with at least 1 year of follow-up (118). Trials of other immune checkpoint inhibitors are ongoing. Early results from a phase I/II clinical trial of MEDI4736, (an antibody against PDL1) have demonstrated a response rate of 10% in adenocarcinoma (119,120). Though overall, the response rates to PD1/PDL1 checkpoint inhibition have not been as robust as in the squamous subtype, there may be subpopulations within adenocarcinoma that may receive additional benefit. Patients with a smoking history (121), and higher levels of PDL1 expression (112,118) have been found to have a more robust response to PD1/PDL1 checkpoint

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inhibition. The EGFR positive population, with higher levels of PDL1 expression as above, may represent a subgroup of adenocarcinoma patients with a potential for benefit from PD1 inhibition.

Ongoing studies of PDL1/PD1 inhibition in NSCLC (including adenocarcinoma) include a phase III trial of pembrolizumab (an antibody against PD1) versus placebo with or without standard adjuvant chemotherapy for resected stage IB-IIIA NSCLC (Clinicaltrials.gov identifier: NCT02504372) and two phase I studies of the novel anti-PDL1 antibodies MPDL3280A, a dose-escalation study in a variety of malignancies and a tolerability study in NSCLC patients who have undergone stereotactic ablative radiotherapy (Clinicaltrials.gov identifier NCT01375842 and NCT02400814).

Conclusions

As this review article has attempted to illustrate, there are numerous molecules that have been identified as potential targets for the treatment of non-squamous NSCLC. Although not covered in this review, many novel molecular targets for the treatment of squamous cell NSCLC are on the horizon as well. A great deal of research is currently underway to further our understanding of these molecular targets and ways that they can be translated to ultimately prolong survival and improve quality of life in patients with this disease. The most promising part of this research effort is in its ability to bring us closer to a more personalized approach to patient care, which will hopefully result in overall improvement in patient outcomes.

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Footnote

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Epidermal growth factor receptor in non-small cell lung cancer

Charles N. Prabhakar

Department of Respiratory Medicine, Royal Prince Alfred Hospital, NSW 2050, Australia

Correspondence to: Charles N. Prabhakar. Department of Respiratory Medicine, Royal Prince Alfred Hospital, Missenden Rd, Camperdown, NSW 2050, Australia. Email: cprabhak@gmp.usyd.edu.au.

Abstract: Following the identification of a group of patients in the initial tyrosine kinase inhibitor (TKI) trials for lung cancer, there has been detailed focus on which patients may benefit from inhibitor therapy. This article reviews the background, genetics and prevalence of epidermal growth factor mutations in non-small cell lung cancer (NSCLC). Additionally, the prevalence in unselected patients is compared against various other reviews.

Keywords: Lung neoplasms; receptor; epidermal growth factor; carcinoma; non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer is a disease with significant burden, with nearly 2.5 million new diagnoses in 2011 contributing to almost 1.5 million deaths worldwide (1). However, no longer is lung cancer managed by distinguishing non-small cell lung cancer (NSCLC) and the associated subtypes from small cell lung cancer (SCLC), but as variety of distinct, although related, diseases each with requiring their own treatment options.

NSCLC make up approximately 85% (2) of lung cancers, which is then further broken down into three distinct histological subtypes (3); adenocarcinoma, squamous cell carcinoma and large cell carcinoma (LCC). Adenocarcinoma comprises the majority of all new lung cancer diagnosed with an associated fall in the proportion of squamous cell cancers (4,5).

Epidermal growth factor receptor (*EGFR*), is one of several somatic mutations, in NSCLC (6), which is seen more frequently in certain population groups. This population group is classically described as Asian, non-smoking females with adenocarcinoma (7-9). The interest in these mutations is due to the small molecule targeted therapies (such as erlotinib and gefitinib) available and in development, which can have significant prognostic benefits (10,11).

The role of EGFR in NSCLC

The EGFR is a 170 kdalton member of the ErbB family of cell surface tyrosine kinases (12) and is encoded on chromosome 7. The receptor belongs to the HER/erbB family of tyrosine kinases, which include HER1 (EGFR/erbB1), HER2 (neu, erbB2), HER3 (erbB3), and HER4 (erbB4) (13). The function of the receptor is to regulate both cell proliferation and apoptosis via signal transduction pathways (14).

The EGFR is a transmembrane receptor consisting of three portions; an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (15). Activation of EGFR is achieved by the binding of a ligand [such as epidermal growth factor, transforming growth factor and neuregulins (16)] to the extracellular portion. The binding of a ligand results in receptor dimerization or heterodimerisation with related receptors [especially HER2/neu (17)] (18). Without a ligand bound to the receptor and the subsequent dimerisation there is no activity at the enzymatic site of the intracellular portion (16).

Once dimerisation occurs there is disruption of the autoinhibitory activity of the intracellular domain resulting in rapid autophosphorylation at tyrosine residues located on the intracellular portion (15,19). The phosphorylated

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Table 1 List of *EGFR* mutations in NSCLC resulting in sensitivity or resistance to first generation TKIs (21)

TKI sensitivity	Exon 18	Exon 19	Exon 20	Exon 21
Sensitive	G719C, G7119S, G7119A, V689M, N700D, E709K/Q, S720P	ΔE746-A750, ΔE746-T751, ΔE746-A750 (ins RP), ΔE746-T751 (ins A/I), ΔE746-T751 (ins VA), ΔE746-S752 (ins A/V), ΔL747-E749 (A750P), ΔL747-A750 (ins P), ΔL747-T751, ΔL747-T751 (ins P/S), ΔL747-S752, ΔL747-752 (E746V), ΔL747-752 (P753S), ΔL747-S752 (ins Q), ΔL747-P753, ΔL747-P753 (ins S), ΔS752-I759	V765A, T783A	L858R, N826S, A839T, K846R, L861Q, G863D
Resistance		D761Y	D770_N771 (ins NPG), D770_N771 (ins SVQ), D770_N771 (ins G), N771T V769L, S768I, T790M	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TKIs, tyrosine kinase inhibitors.

receptor then functions to allow assembly and activation of intracellular messenger proteins (18), especially through the mammalian target of rapamycin (mTOR) (20).

Dysregulation of the EGFR leads to increased intracellular pathways activity, via tyrosine kinase autophosphorylation, resulting in directly or indirectly, cell proliferation, angiogenesis, invasion and metastasis (12).

Overexpression of the *EGFR* gene has been identified in a variety other cancers including: head and neck, ovary, cervix, bladder, oesophagus, stomach, brain, breast, endometrium, colon and lung (21). *EGFR* overexpression has been identified in between 40% to 89% of NSCLC (6,22), with highest rates seen in squamous tumours (89%) and lowest in adenocarcinomas (41%) (22).

Tyrosine kinase domain mutations

As *EGFR* was noted to be overexpressed in NSCLC, it was felt that targeting the receptor with an tyrosine kinase inhibitor (TKI), gefitinib, would be an effective treatment for NSCLC, however this was not shown to be case (23). However, during the initial trial of gefitinib, a subgroup of patients were identified that had significant improvement in their lung and metastatic lesions (6). The identification of a particular subgroup of patients with dramatic response to TKI treatment led to molecular investigation of the EGFR pathway. This subgroup of patients was analysed separately by both Lynch *et al*. and Paez *et al*. who each showed that patients who possessed mutations in the tyrosine kinase domain of the EGFR (6,24). These mutations were shown to occur in exons 18, 19 and 21.

Analysis of the tyrosine kinase domain of the EGFR of 617 unselected lung cancer specimens by Shigematsu *et al*. identified that all mutations occurred within exons 18-21, with a prevalence of 21% (7). These mutations (listed in *Table 1*) provide sensitivity to targeted therapies, known as TKIs, such as erlotinib and gefitinib (21).

The majority of mutations in exon 21 are point mutations whereas exon 19 consists of almost entirely in-frame deletions (20). The L858R point mutation and ΔE746-A750 comprise up to 86% of all EGFR mutations in some studies (25). Both the aforementioned mutations result in changes near the ATP cleft, which results in enhanced catalytic activity and autocatalysis of the tyrosine kinase when the receptor is not stimulated by EGF (or other ligands), with up to a three-fold increase in activity compared to the wild-type EGFR (6).

Whilst most tyrosine kinase domain mutations lead to sensitivity to TKIs (*Table 1*), mutations in exon 20 are associated with intrinsic resistance (26-31) which may account for up to 9% of all EGFR mutations (31).

While squamous cell carcinomas (SCC) rarely possess mutations in the tyrosine kinase domain of the EGFR receptor, about one-third of SCCs demonstrate amplification of the EGFR protein (2). Approximately 5% of SCC possess deletion mutations in exons 2-7 (EGFRvIII) which code for the extracellular domain of the protein (32). In the same series no adenocarcinomas possessed EGFRvIII mutation, however the extracellular domain mutations are frequently seen in SCCs of head and neck cancers (33).

Histology

Amongst the various forms of NSCLC, adenocarcinoma, is

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most commonly identified in all comers tested for EGFR mutation status (34-40). Bronchioloalveolar cell carcinoma (BAC), a subtype of adenocarcinoma, was associated in some of the early gefitinib studies (6,41) with response to treatment. As most NSCLCs do not respond to gefitinib, unless they have the activating mutation, then this would suggest that BAC is more commonly associated with EGFR mutation than other forms of adenocarcinoma. A retrospective audit of 139 NSCLC patients treated with gefitinib, by Miller *et al.*, revealed that significantly more patients, who experienced response to TKI therapy, possessed BAC features than those that did not receive a benefit from drug therapy (38% *vs.* 14%, P<0.001) (41).

BAC was then further divided into mucinous, non-mucinous carcinomas and mixed non-mucinous and mucinous or indeterminate in the World Health Organisation histological classification of tumour guide (3). However, since 2004, further clarification of the term BAC has occurred and subsequently recommended the discontinuation of the term BAC in preference for the following categories; adenocarcinoma *in situ*, minimally invasive adenocarcinoma (mucinous and rarely mucinous), lepidic predominant (non-mucinous), adenocarcinoma predominantly invasive with some non-mucinous lepidic components and, finally, invasive mucinous adenocarcinoma (42). The latter two are the forms of BAC formerly referred to as nonmucinous BAC and mucinous BAC respectively (42). As the studies referenced below present their data using the original nomenclature, the data will be presented using the papers author's original terms to ensure that no inappropriate interpretation is undertaken.

In analysing 141 primary NSCLC biopsies, of which 118 were adenocarcinomas from a Japanese population, Sakuma *et al*. demonstrated that 54% (P<0.0001) of the adenocarcinomas with EGFR mutations possessed histopathological features consistent with nonmucinous BAC (43). Similarly, Marchetti *et al.* found that the 56% (P=0.00002) of adenocarcinomas with EGFR mutation were BAC with all patients possessing a nonmucinous subtype (44). However, while Tam *et al*. also demonstrated that nonmucinous BAC was significantly associated with EGFR mutation (79%), only 13% of adenocarcinomas with an identified EGFR mutation were of BAC subtype (45).

Using the updated histological nomenclature from international association for the study of lung cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) (42), Yoshizawa *et al*. analysed 440 resected lung adenocarcinomas. They demonstrated that 167 cases were positive for EGFR mutation with a high rate of features consistent with adenocarcinoma *in situ* (85.7%), minimally invasive adenocarcinoma (83.3%), lepidic (71.4%) and papillary predominant (68.5%), while there were no mutations identified in mucinous subtype tumours (46). Using the same criteria, Gahr *et al*. demonstrated that of the 101 patients with EGFR positive NSCLC (from a population of 1,122), 90% were nonmucinous adenocarcinoma, with only 22% poorly differentiated. Further divided down 65.3% of EGFR positive tumours had features consistent with non-lepidic-nonmucinous adenocarcinoma and 21.8% lepidic-nonmucinous histology (35). In a population of Korean smokers (n=249), of the 51% with EGFR positive NSCLC, when classifying the tumour on the major histological subtype, the most common finding was acinar (68.5%) followed by papillary (11.8%), solid (9%), lepidic (7.5%), micropapillary (1.4%) and only 1.8 % falling into the invasive mucinous category (47).

While the vast majority of EGFR mutations in NSCLC are found in adenocarcinoma, the mutation is also seen in SCC and LCC. Comparing 15 studies (*Table 2*), the majority of which were in selected patient populations, the prevalence of EGFR mutation positive SCC lung cancer ranged between 0-14.6%, with an average of 4.9% when the 4,870 patients were combined into a single group.

Epidemiology

The conventional phenotype of patients who develop a lung cancer that is positive for an EGFR mutation is the young, Asian, non-smoking, female with adenocarcinoma (7-9). While this does certainly appear to be the case, there are very few studies that have prospectively analysed non-selected populations of patients. The vast majority of papers, that examine the predictors and prevalence of EGFR mutations, recruit patients with advanced stage disease or who have failed alternate therapies (surgical or first-line chemotherapy). Even those studies that do not select for patient populations commonly have intrinsic selection bias, by the very fact that they recruit patients from a single country with homogenous ethnic populations.

Of the eight papers identified (35,36,39,58-62), which measured the frequency of EGFR mutations in NSCLC in unselected patients, only four clearly indicated that the data was gathered in a prospective manner (35,36,60,62). There was a range of mutation testing, with the majority of papers examining for mutations in exons 18-21, but some limiting

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Table 2 Frequency of EGFR positive mutations in different histological types of NSCLC

Paper	Year	Total patients	*EGFR* positive	Adenocarcinoma, n (%)	SCC (including adenosquamous cell carcinoma), n (%)	Large cell, n (%)	Other, n (%)	Comments
Takeda *et al*. (48)	2014	68	68	67 (98.5)	1 (1.5)	0 (0.0)	0 (0.0)	
Douillard *et al*. (49)	2014	1,060	106	102 (96.2)	2 (1.9)	1 (0.9)	1 (0.9)	
Sahnane *et al*. (50)	2013	46	23	22 (95.7)	0 (0.0)	0 (0.0)	1 (4.3)	
Gahr *et al*. (35)	2013	1,122	101	93 (92.1)	5 (5.0)	3 (3.0)	0 (0.0)	
Unal *et al*. (51)	2013	48	48	32 (66.7)	7 (14.6)	4 (8.3)	5 (10.4)	
Wheler *et al*. (52)	2013	39	15	13 (86.7)	2(13.3)	0 (0.0)	0 (0.0)	
Hsiao *et al*. (53)	2013	580	124	121 (97.6)	2 (1.6)	0 (0.0)	1 (0.8)	
Cadranel *et al*. (37)	2012	307	44	32 (72.7)	4 (9.1)	0 (0.0)	7 (15.9)	One data point missing
Kim *et al*. (8)	2011	229	110	105 (95.5)	2 (1.8)	1 (0.9)	2 (1.8)	
Helland *et al*. (54)	2011	240	18	16 (88.9)	2 (11.1)	0 (0.0)	0 (0.0)	
Tanaka *et al*. (39)	2010	308	112	104 (92.9)	8 (7.1)	0 (0.0)	0 (0.0)	Adenocarcinoma included adenosquamous, SCC included other carcinomas
Wu *et al*. (26)	2008	515	23	22 (95.7)	1 (4.3)	0 (0.0)	0 (0.0)	
Tsao *et al*. (55)	2006	159	14	14 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Mitsudomi *et al*. (56)	2005	59	59	50 (84.7)	6 (10.2)	3 (5.1)	0 (0.0)	
Han *et al*. (57)	2005	90	17	14 (82.4)	1 (5.9)	0 (0.0)	2 (11.8)	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

their investigation to only the common mutations (exon 19 deletions and L858R substitution). None of the studies examined for the effect of race on the presence or absence of the EGFR mutation.

The findings of the eight studies are listed in *Table 3* (individual studies were excluded from analysis in the case of missing data).

EGFR prevalence

The prevalence of the various EGFR mutations tested for was 13.9% of the 7,595 patients with the highest prevalence (36.4%) of mutations seen in the single study conducted in Japan.

Smoking

In those patients with the EGFR mutation, nearly 60% of patients were identified as never-smokers [or less than 20 years in one study (39)]. The prevalence of never smokers with the mutation was 42%, whereas the mutation was still identified in 10.7% of current or former smokers. The variation of the mutation presence was identified as significant in 5 of the 6 studies where statistical analysis was performed.

Sex

In the EGFR mutation group, 64.9% of the patients were female, while the prevalence of the mutation overall was 25.8% for females but only 12.2% for males. This was statistically significant for all studies that tested for significance.

Age

No correlation with age and the presence or absence of the mutation was identified.

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Table 3 Studies which analysed all comers for *EGFR* mutation positive NSCLC

Study	Year	Country	Total patients	*EGFR* positive	Female		Males		Never smokers		Smokers or former smoker	
					EGFR positive	WT	*EGFR* positive	WT	*EGFR* positive	WT	*EGFR* positive	WT
Gahr *et al*. (35)	2013	Germany	1,201	118	81	385	37	698	38	118	22	506
Locatelli-Sanchez *et al*. (36)	2013	French	753	121	76	210	45	422	73	NA	48	NA
Paik *et al*. (59)	2012	United States	675	164	77	329	48	182	110	183	54	328
D'Angelo *et al*. (62)	2011	United States	2,142	503	346	969	157	670	302	278	201	1,361
Tanaka *et al*. (39)	2010	Japan	308	112	60	41	52	155	59	37	43	150
Rosell *et al*. (60)	2009	Spain	2,105	350	244	570	106	1,181	231	381	116	1,266
Fontanini *et al*. (61)	2009	Italy	411	52	37	139	15	220	14	38	16	149
Beau-Faller *et al*. (58)	2014	France	10,117	1,047	NA	NA	NA	NA	NA	NA	NA	NA

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; WT, wild type; NA, not available.

Table 4 Prevalence of *EGFR* mutation

Study	Prevalence of *EGFR* mutation			
	Males (%)	Females (%)	Never-smokers (%)	Smokers (%)
Shigematasu *et al*. (7)	14	42	51	10
Lindeman *et al*. (non Asians) population (63)	18	28	45	15
Lindeman *et al*. (Asian population) (63)	32	58	58	26
Mitsudomi *et al*. (64)	10	38	47	7
Summary of *Table 3*	12.2	25.8	42.2	10.7

The final row contains a summary of the data obtained from the studies listed in *Table 3*. *EGFR*, epidermal growth factor receptor.

Tumour type

In those studies where enrolment criteria were not limited to adenocarcinomas, only 3-9% of tumours identified did not possess histology consistent with adenocarcinoma. The analysis of exact tumour type could be limited in these studies as histological analysis can be difficult in cytology only specimens (such as obtained with fine needle aspiration). Only one of the studies indicated the source of tumour specimens used in mutation analysis.

Discussion

When Lindeman *et al*. analysed the EGFR mutation prevalence rate, divided by race from multiple previous studies, they found that amongst the Asian/Indian population the prevalence was 52% when compared to only 24% amongst Caucasians (63). Shigematsu *et al*., in multi-nationality study (primarily South East Asia and Caucasians) of patients with resectable disease found an overall mutation prevalence of 23% (7). However, when divided by race the mutation rate amongst Australians and North Americans was 7% and 14% respectively, whereas the mutation rate in Asian countries was as high as 34% in the Taiwanese population.

It is difficult to resolve the wildly varying prevalence of the EGFR mutation in the above studies. The analysis performed in this paper on an unselected population of 17,712 mainly European and American patients is close to Shigematsu *et al*. overall prevalence calculation. When Lindeman *et al*., Shigematsu *et al*. and this papers analysis are compared (*Table 4*) the overall prevalence in the unselected cohort (this study) is similar to that of the non-Asian population. The final row in *Table 4* was obtained by calculating the prevalence of each stated factor in the

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studies listed in *Table 3* (studies with incomplete data were excluded from individual analyses).

The prevalence of the individual sub-populations (males, females, never-smokers and smokers) are similar between the three papers when Caucasian population is considered. This suggests that the EGFR mutation is far more prevalent in Asian populations than Caucasians. Logistic regressions have only demonstrated that a low smoking history and adenocarcinoma histology are significant independent predictors of EGFR mutation status, but not sex nor age (39).

Conclusions

EGFR mutations are significant drivers in NSCLC, especially amongst Asian females who are never-smokers with adenocarcinoma histology. However 10% of patients with *EGFR* mutant NSCLC have some degree of smoking history and 12% are male. Simple choosing to only mutation test patients who fit a single phenotype will miss a significant proportion of suffers who may benefit from small molecule therapy.

Current studies on the prevalence of the mutation tend to focus on a single race and many do not test for the presence of the mutation in all lung cancer stages. Despite smoking remaining the highest risk for lung cancer (5,65), there is a rising incidence of adenocarcinoma in non-smokers (66). Having an accurate model of who may develop *EGFR* mutation NSCLC may allow prognostic benefits with targeted therapies.

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Beyond *ALK-RET*, *ROS1* and other oncogene fusions in lung cancer

Takashi Kohno[1,2,3], Takashi Nakaoku[1], Koji Tsuta[4], Katsuya Tsuchihara[2], Shingo Matsumoto[2,3], Kiyotaka Yoh[3], Koichi Goto[3]

[1]Division of Genome Biology, National Cancer Center Research Institute, Tokyo, Japan; [2]Division of Translational Research, Exploratory Oncology Research & Clinical Trial Center (EPOC), National Cancer Center, Tokyo and Chiba, Japan; [3]Division of Thoracic Oncology, National Cancer Center Hospital East, Chiba, Japan; [4]Division of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan

Correspondence to: Takashi Kohno. Division of Genome Biology, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo, Japan. Email: tkkohno@ncc.go.jp.

Abstract: Fusions of the *RET* and *ROS1* protein tyrosine kinase oncogenes with several partner genes were recently identified as new targetable genetic aberrations in cases of non-small cell lung cancer (NSCLC) lacking activating *EGFR*, *KRAS*, *ALK*, *BRAF*, or *HER2* oncogene aberrations. *RET* and *ROS1* fusion-positive tumors are mainly observed in young, female, and/or never smoking patients. Studies based on *in vitro* and *in vivo* (i.e., mouse) models and studies of several fusion-positive patients indicate that inhibiting the kinase activity of the RET and ROS1 fusion proteins is a promising therapeutic strategy. Accordingly, there are several ongoing clinical trials aimed at examining the efficacy of tyrosine kinase inhibitors (TKIs) against *RET* and *ROS1* proteins in patients with fusion-positive lung cancer. Other gene fusions (*NTRK1*, *NRG1*, and *FGFR1/2/3*) that are targetable by existing TKIs have also been identified in NSCLCs. Options for personalized lung cancer therapy will be increased with the help of multiplex diagnosis systems able to detect multiple druggable gene fusions.

Keywords: RET fusion; ROS1 fusion; oncogene fusion; tyrosine kinase inhibitor (TKI); multiplex diagnosis system

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Introduction

Oncogene activation is a critical step toward the development of non-small cell lung cancer (NSCLC), particularly lung adenocarcinoma (LADC); these activated genes are called driver oncogenes (1-3). Representative driver oncogenes include *EGFR*, *KRAS*, *BRAF*, and *HER2/ERBB2*, which are activated by missense and/or insertion/deletion mutations, and the *ALK* gene, which is activated by fusion to other genes (called partner genes) (*Figure 1*). Aberrations of these genes are mutually exclusively detected in LADC; therefore, they are believed to drive LADC development. Suppressing the activity of aberrant gene products inhibits the growth of LADC cells harboring oncogenic aberrations in the corresponding driver genes. Indeed, tyrosine kinase inhibitors (TKIs) have become the standard drug treatment for advanced cases of LADC harboring *EGFR* mutations and *ALK* fusions (1,4,5).

In 2012, two additional oncogenes, *RET* and *ROS1*, were added to the list of driver oncogenes that are targetable with existing TKIs (*Figure 1A*) (1,6-8) and clinical trials investigating the efficacy of such TKIs have been conducted. Furthermore, analysis of lung cancer genome and/or transcriptome has identified other gene fusions, including the *NTRK1* (9), *NRG1* (10,11), and *FGFR1/2/3* fusions (12-14), as novel targetable driver genes in a minor fraction of NSCLC cases. *In vitro* and *in vivo* experimental data show that existing TKIs are a promising therapy for lung cancer cases that are positive for these novel oncogenic fusions. Here, we review the oncogenic fusions associated with NSCLC and discuss the issues surrounding personalized therapy.

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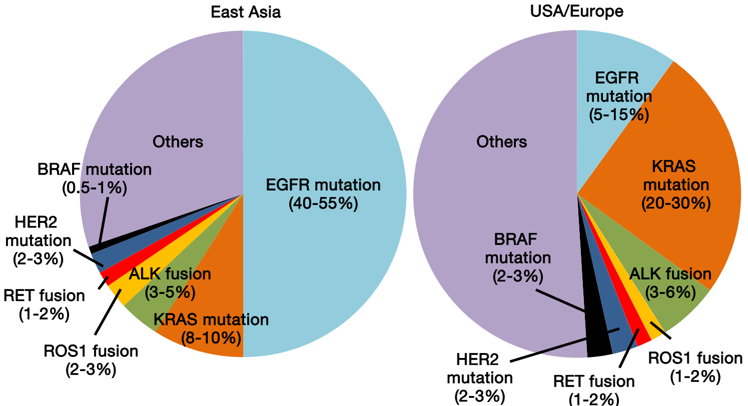

Figure 1 Pie charts showing the proportion of LADC harboring aberrations in driver oncogenes. Data from patients in East Asia (Japan, Korea, and China) and from those of European descent were generated by summarizing the results from previous reports (2-4). LADC, lung adenocarcinoma.

The *RET* fusion in LADC

The link between the oncogenic *RET* fusion and LADC was discovered by several groups (including our own) in 2012. The *RET* gene was fused to the *KIF5B* and *CCDC6* genes in 1-2% of LADC cases (6-8,15,16); none of these positive cases harbored *EGFR*, *KRAS*, *BRAF*, or *HER2/ERBB2* mutations or *ALK* fusions. The *RET* fusion is mainly detected in young, female, and/or never/light-smoker patients (6,7,17-19). Also, it occurs in adenocarcinoma but not in squamous and small cell lung cancers (SQLC and SCLC) (2,7). LADCs harboring the *RET* fusion show well- or moderately-differentiated histological features, similar to those of LADCs harboring *EGFR* mutations; however, a subset of LADCs harboring the *RET* fusion show mucinous cribriform features, similar to those of ALK fusion-positive LADCs (6,17-19).

Oncogenic *RET* variants fused to six partner genes have been identified in lung cancers (10,20,21) (*Figure 2*). In all of these variants, the coiled-coil domains of the partner proteins induce dimerization of the RET fusion proteins, resulting in constitutive activation of the RET kinase (as in the case of oncogenic *ALK* fusions). The tumorigenic activity of the *RET* fusion gene was illustrated by transformation of NIH3T3 cells (6-8) and in a transgenic mouse model in which the *KIF5B-RET* gene was specifically expressed in lung epithelial cells (22); The tumorigenic activity was suppressed by RET TKIs, indicating its dependence on the kinase activity of the RET protein. Consistent with this, a human LADC cell line derived from a Japanese patient, which carries the *CCDC6-RET* fusion gene, is sensitive to RET TKIs (23,24). Therefore, LADC cells harboring the *RET* fusion are in a state of "oncogenic addiction" to constitutive RET kinase activation. This makes the *RET* fusion a promising therapeutic target.

The US Food and Drug Administration (FDA) has approved two multi-kinase inhibitors with RET TKI activity, vandetanib (ZD6474) and cabozantinib (XL184), for the treatment of advanced medullary thyroid cancer in which activating *RET* mutations are observed in >50% of cases (16). Five phase II clinical trials are currently examining the therapeutic effects of RET TKIs against *RET* fusion-positive NSCLCs (*Table 1*). These trials have single-arm open-label designs, with response rate as the primary endpoint. Our own group is conducting one of these phase II clinical trial in Japan (UMIN00001009). This trial, designated "LURET (lung cancer with *RET* rearrangement study)", is designed to investigate the therapeutic efficacy of vandetanib against NSCLC. We are using a RT-PCR-based screening method to select patients with *RET* fusion-positive tumors. This process is being carried out in >170 hospitals via a consortium called "LC-SCRUM (lung cancer genomic screening project for individualized medicine in Japan)", and >1,000 patients with advanced NSCLC without *EGFR* mutations have been screened as of Aug 31, 2014 (2). A trial conducted at Memorial Sloan-Kettering Cancer Center (NCT01639508) reported promising responses in the first three patients treated with cabozantinib (20). In addition, another study reported that one patient with LADC harboring a *KIF5B-RET* fusion showed a positive response to vandetanib (25). Although the number of patients in these studies is small and follow-up is

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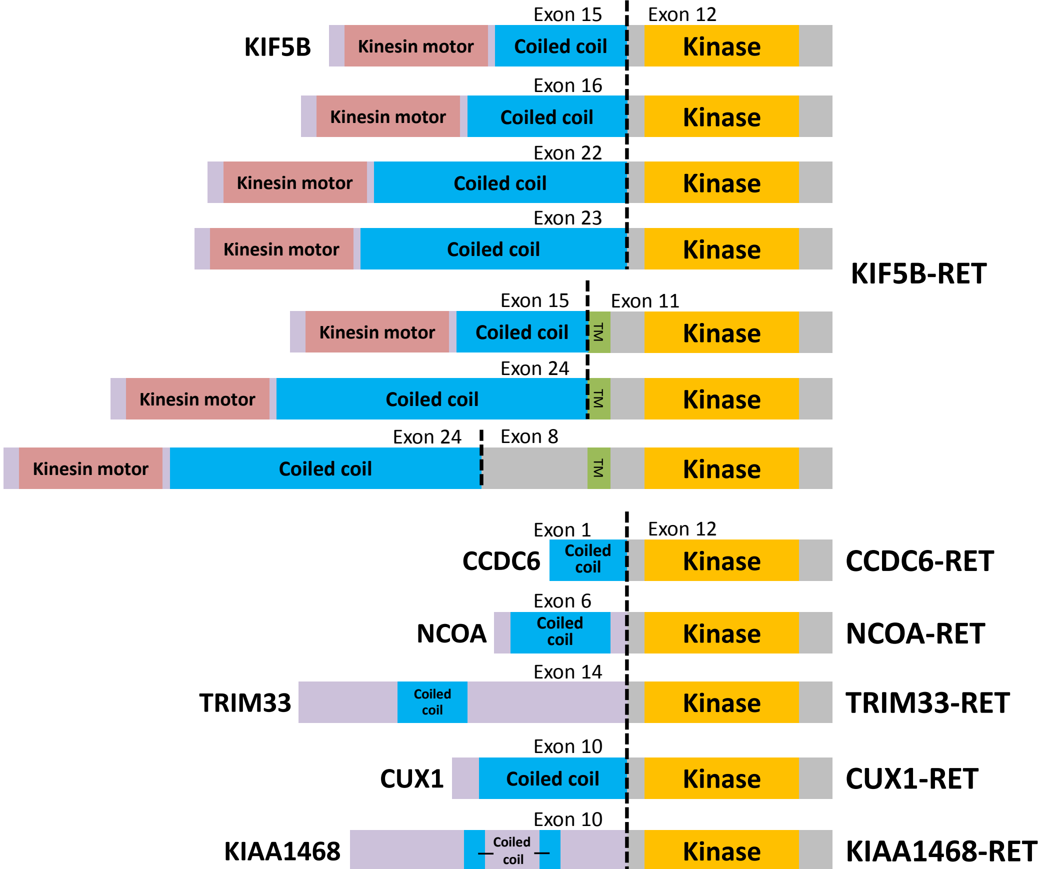

Figure 2 Schematic diagram showing *RET* fusion proteins in LADC. The domains are highlighted in different colors: RET tyrosine kinase domain (orange), RET transmembrane domain (TM; green), and coiled-coil domain (blue) in fusion partners. LADC, lung adenocarcinoma.

Table 1 Clinical trials of TKIs in patients with *RET* and *ROS1* fusion-positive non-small cell lung cancer (NSCLC)

Gene fusion	Trial number*	Drug	Pharmaceutical company	Phase	Location	Primary endpoint	Enrollment	Start date
RET	NCT01639508	Cabozantinib/XL184	Exelixis	II	USA	Response rate	25	July 2012
ROS1, *NTRK1*, and others**	NCT01639508	Cabozantinib/XL184	Exelixis	II	USA	Response rate	25	August 2014
RET	UMIN000010095	Vandetanib/ZD6474	AstraZeneca	II	Japan	Response rate	17	February 2013
RET	NCT01823068	Vandetanib/ZD6474	AstraZeneca	II	Korea	Response rate	17	April 2013
RET	NCT01877083	Lenvatinib/E7080	Eisai	II	Global	Response rate	20 or more	April 2013
RET	NCT01813734	Ponatinib/AP24534	ARIAD	II	USA	Response rate	20	June 2013
ROS1	NCT01945021	Crizotinib	Pfizer	II	Asia	Response rate	110	September 2013
ROS1	NCT01964157	Ceritinib/LDK378	Novartis	II	Korea	Response rate	32	October 2013
ROS1 and *ALK*	NCT01970865	PF-06463922	Pfizer	I/II	Global	Response rate (phase II)	200	October 2013
ROS1	NCT02183870	Crizotinib	Pfizer	II	EU	Response rate	30	June 2014

*, detailed information is available at http://clinicaltrials.gov/ or https://upload.umin.ac.jp; **, including MET (overexpression, amplification, or mutation) and AXL (overexpression, amplification, or mutation). TKI, tyrosine kinase inhibitor.

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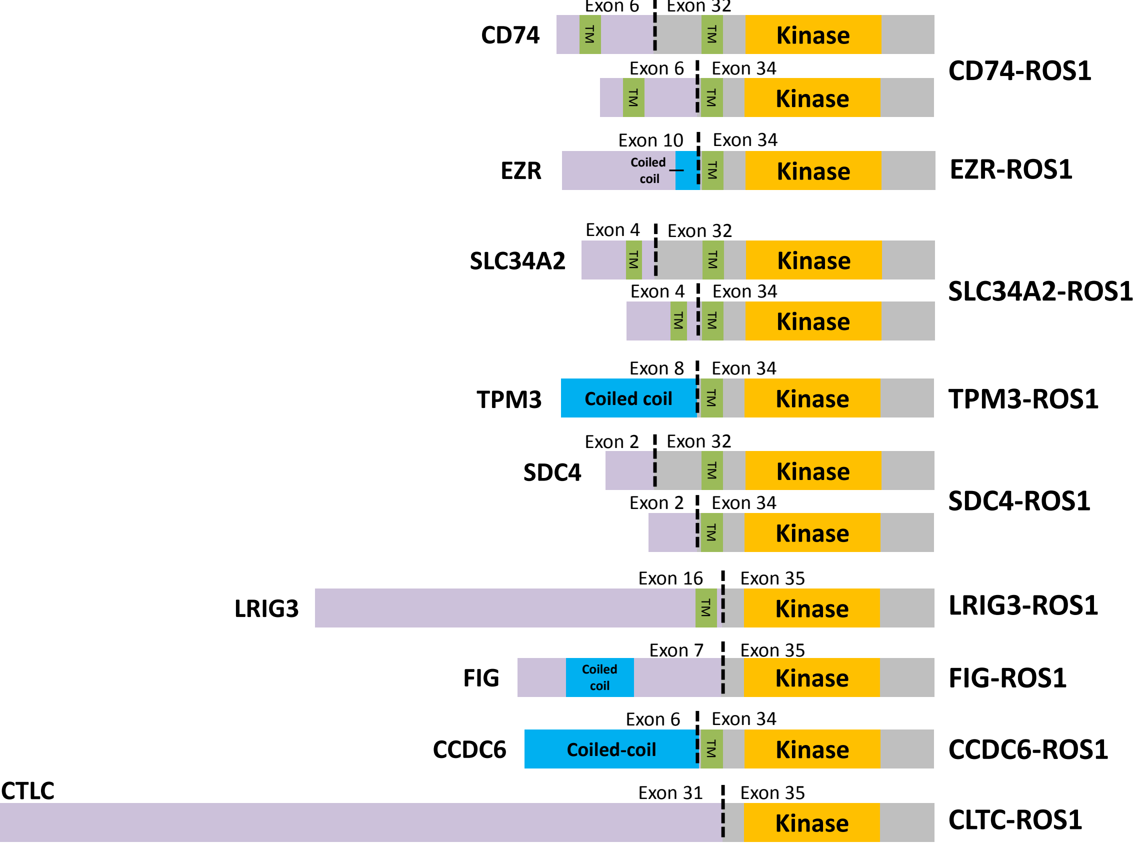

Figure 3 Schematic diagram showing ROS1 fusions in LADC. The domains are highlighted in different colors: ROS1 tyrosine kinase domain (orange), ROS1 transmembrane domain (TM; green), and coiled-coil domain (blue) in fusion partners. LADC, lung adenocarcinoma.

limited, the results provide early proof-of-principle that the *RET* fusion is targetable by existing TKIs.

The *ROS1* fusion in LADC

The oncogenic *ROS1* fusion is present in 1-2% of LADC cases (6), and is likely to be specific for adenocarcinoma (26). The *ROS1* gene fuses to several partner genes, although *CD74* is the most common (*Figure 3*) (27-29). As is the case for the *RET* fusion, the *ROS1* fusion occurs in a manner that is mutually exclusive with other known driver oncogene mutations and fusions. The *ROS1* fusion is preferentially detected in young, female, and/or never/light-smoker patients (6,18,30-32). LADCs harboring the *ROS1* fusion often show mucinous cribriform features (6,18,30,31), similar to those of *ALK* fusion-positive LADCs. The *ROS1* fusion is also likely to be specific for LADC (6,18,30,32).

The transforming activity of the *ROS1* fusion gene has been demonstrated *in vitro* using NIH3T3 cells (6,33) and *in vivo* using a transgenic mouse model in which the *EZR-ROS1* gene is specifically expressed in lung epithelial cells (33). Crizotinib, a TKI approved by the FDA for ALK fusion-positive lung cancer, also inhibits the ROS1 protein due to the structural similarity of the kinase domains of ROS1 and ALK proteins. In fact, the LADC cell line, HCC78, which harbors a *SLC34A2-ROS1* fusion, is sensitive to crizotinib (26,32). Thus, LADC cells harboring the *ROS1* fusion are in a state of “oncogenic addiction” to constitutive ROS1 kinase activation. In contrast to the *RET* and *ALK* fusions, constitutive activation of the ROS1 kinase protein is unlikely due to dimerization of ROS1 fusion proteins since the majority of ROS1 partner proteins lack dimerization domains (27) (*Figure 3*).

A phase I trial (NCT00585195) examining the efficacy of crizotinib against ROS1 fusion-positive NSCLC showed an objective response rate of 60% (27). Other studies (32,34-36)

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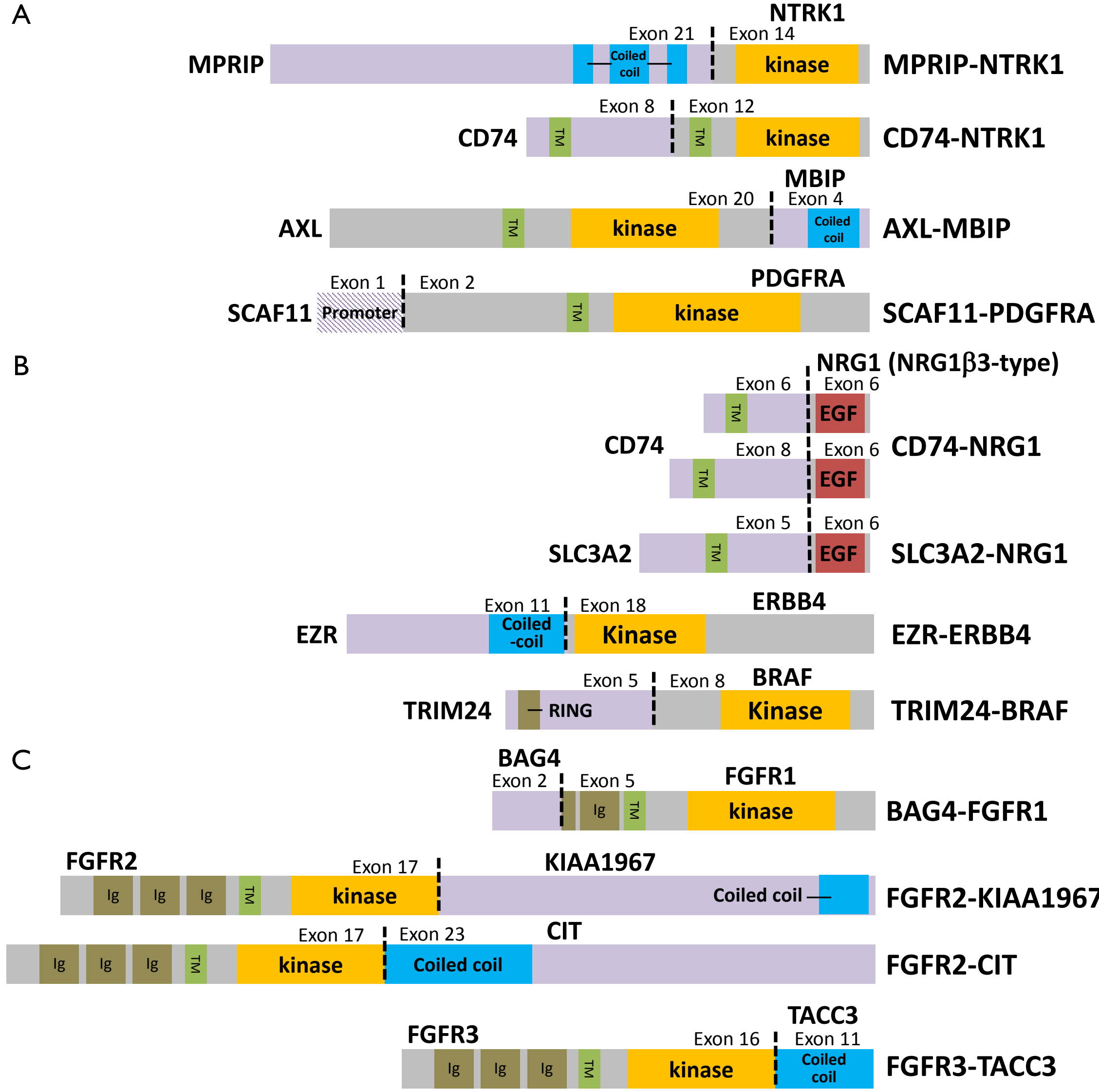

Figure 4 Schematic diagram of other fusion proteins in non-small cell lung cancer. (A) Fusion proteins in LADC. TM, transmembrane domain; (B) fusion proteins in IMAs. EGF, EGF-like domain; (C) FGFR fusion proteins in SQLC. The domains are highlighted in different colors: tyrosine kinase domain (orange), transmembrane domain (TM; green), immunoglobulin-like domain (dark green), coiled-coil domains (blue). LADC, lung adenocarcinoma.

report that patients with LADC harboring a *ROS1* fusion show a near-complete or partial response to crizotinib. Therefore, molecular-targeted therapy using crizotinib (and other ROS1 TKIs) appears promising. Five phase II or I/II clinical trials have been conducted to examine the therapeutic effects of ROS1 TKIs against *ROS1* fusion-positive NSCLCs (*Table 1*). The LC-SCRUM consortium is currently screening *ROS1* fusion-positive tumors in Japan and *ROS1* fusion-positive patients are being enrolled in a crizotinib trial (NCT01945021).

Other protein kinase fusions in LADC

Other oncogenic fusions of protein kinase genes have been detected in LADCs that are negative for known driver oncogene aberrations (*Figure 4A*). Oncogenic fusions of the *NTRK1* gene (which encodes a nerve growth factor receptor, TRKA) with the *CD74* and *MPRIP* genes were

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recently identified in 3% of patients within an American cohort (9). However, other LADC cohorts, including a TCGA-USA cohort (n=230), a Korean cohort (n=87), and our own NCC-Japan cohort (n=200, unpublished data), contained no *NTRK1* fusion-positive cases (9). Thus, the prevalence of *NTRK1* fusion remains unclear. A few TKIs (ARRY-470, CEP-701, and crizotinib) that suppress the activity of the TRKA protein kinase also suppress the NIH3T3-transforming activity of the *NTRK* fusion gene (9). Notably, a LADC patient harboring the *MPRIP-NTRK1* fusion showed a minor therapeutic response to crizotinib (9). An ongoing clinical trial (NCT01639508) includes not only patients positive for the *RET* and *ROS1* fusions, but also patients positive for the *NTRK1* fusion (*Table 1*).

The *AXL-MBIP* and *SCAF11-PDGFRA* fusions, two more protein kinase gene fusions (*Figure 4A*), were each detected in a single case of LADC in a Korean cohort of 200 patients (29). Since these fusions were not detected in either the TCGA-USA cohort (n=230) (28) or our own NCC-Japan cohort (n=200, unpublished data), they may only occur in a very small subset of LADC cases.

Multiple oncogenic fusions in invasive mucinous LADC

Invasive mucinous adenocarcinoma (IMA) of the lungs, which accounts for 2-10% of all LADC cases in Japan, the USA, and Europe, is thought to be a distinct histological type of LADC that commonly (>50%) harbors *KRAS* mutations (37,38). We recently identified multiple oncogene fusions involving the *NRG1* (*neuregulin*), *ERBB4*, *BRAF*, *ALK*, and *RET* genes as drivers for the development of IMA in the absence of *KRAS* mutations (10) (*Figure 4B*). Among these, the *CD74-NRG1* fusion was the most common (5-15%). The *CD74-NRG1* fusion has also been detected in another Japanese IMA cohort and in a Taiwanese IMA cohort (11,39). The fusion product acts as a ligand for HER2:HER3 and causes anchorage-independent growth of NIH3T3 cells (9,10,11). Its transforming activity is suppressed by HER2 inhibitors that are approved for clinical use, including lapatinib and afatinib (10), suggesting that IMAs may be amenable to personalized therapy.

FGFR1/2/3 fusions in SQLC

Amplification of the *FGFR1* gene has been identified as a major oncogene aberration in approximately 10% of SQLC cases (40), whereas activating mutations in *FGFR1*, *FGFR2*, and *FGFR3* are detected in a small subset of SQLC cases (41). Recent studies have detected fusions of the *FGFR1*, *FGFR2*, and *FGFR3* genes to several partner genes in SQLC (*Figure 4C*) (13,14,28). In particular, the *FGFR3-TACC3* fusion, which is detected in 3% of glioblastoma multiforme cases (42), was recurrently observed in a 2-3% of LSQC cases. The *FGFR3-TACC3* fusion gene induces cell transformation and accelerated growth. Both cell growth and tumorigenicity are suppressed by FGFR TKIs (13). Importantly, several clinical trials examining the efficacy of FGFR TKIs against SQLC harboring mutation/amplification of the *FGFR* genes are ongoing, although broadening the inclusion criteria for such clinical trials would be beneficial.

Diagnosis of fusion-positive cases

The findings discussed to date provide a strong rationale for developing precision medicine approaches based on targeting oncogene fusions in LADC and LSQC. Since this form of therapy is applicable only to a subset of LADC and LSQC cases, it is important that we develop suitable diagnostic methods that are able to identify fusion-positive cases (43). The diagnosis of ALK fusion-positive lung cancer is based on fluorescence *in situ* hybridization (FISH) either with or without immunohistochemistry (IHC) (44). FISH and IHC are also suitable for the diagnosis of ROS1 fusion (45,46); however, IHC is not suitable for the diagnosis of *RET* fusion (7,8,19).

Because only very small amounts of material can be obtained from biopsies, there is a need to develop diagnostic systems that enable simultaneous examination of multiple gene fusions in routine formalin-fixed and paraffin-embedded (FFPE) clinical specimens. However, because the FFPE technique damages DNA, the robustness against DNA qualities is needed for the diagnostic systems. In addition, most of the samples that are subjected to testing are small biopsies; therefore, the system must also be able to deal with limited amounts of tissue and/or extracted DNA/RNA. Accurate and sensitive profiling must be achieved, even when the proportion of tumor cells within the specimens is low.

Representative systems are currently being developed that will enable multiple, robust, and sensitive diagnoses (*Table 2*). Some employ the method of target re-sequencing of tens to hundreds of genes using DNA or RNA extracted from tumor tissues (47,48), while others employ quantitative RT-PCR or RNA molecule counting (21,49,50). Optimizing these (or other equivalent) systems for use in the clinic will

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Table 2 Multiplex diagnostic systems for gene fusions

Method	Material	Detectable fusions
Target capture followed by next-generation sequencing (8,9,47)	Genomic DNA	ALK, RET, ROS1, NTRK1, and others
Target capture followed by transcript counting (21)	RNA	ALK, RET, and ROS1
Multiplex RT-PCR followed by next-generation sequencing (48)	RNA	ALK, ROS1, and others
Multiplex ARMS RT-PCR (49)	RNA	ALK, RET, and ROS1
Anchored multiplex RT-PCR (50)	RNA	ALK, RET, ROS1, NTRK1, and others

greatly facilitate the progress toward precision medicine for lung cancer.

Perspective: issues still to be investigated

In vitro/*in vivo* experiments and the responses of the few patients examined in trials suggest that the therapies described in this review hold promise. However, innate and acquired resistance to TKIs may become a problem, as is the case for TKIs targeting the ALK and EGFR proteins. The mechanisms underlying resistance are beginning to be unraveled and several next-generation TKIs have been developed to treat resistant ALK fusion and *EGFR* mutations (5,51). This is good news because some ROS1 fusion-positive cases also have acquired resistance to crizotinib (52). Further studies should be done on the resistance of other fusions to TKIs so that lung cancers harboring novel fusions can be treated effectively.

Preventing the development of lung cancer via oncogenic fusions is another issue to be tackled by those involved in lung cancer medicine. LADCs harboring oncogene fusions are mainly observed in never/light smokers; therefore, preventive methods other than smoking cessation are necessary. We have been investigating the molecular mechanisms underlying chromosome inversions that generate oncogenic *RET* fusions in LADC by cloning genomic segments that contain breakpoint junctions (53). We found that inversions were most likely caused by the mis-repair of DNA strand breaks, which occurred in a region spanning a few Kb within the *RET* gene (the region in which DNA strand breaks leading to *RET* rearrangements in papillary thyroid tumors also frequently occur) (53). Thus, tobacco-independent DNA strand breaks are likely to trigger development of the *RET* fusion. To the best of our knowledge, no studies have elucidated the structure of the breakpoints in *ALK*, *ROS1*, and other fusions. Further examination of the molecular processes underlying gene fusion, as well as identifying the endogenous/exogenous factors that cause DNA breaks, will provide the key to preventing the development of lung cancers harboring oncogenic gene fusions.

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Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway in non-small cell lung cancer

Po Yee Yip[1,2]

[1]Department of Medical Oncology, Chris O'Brien Lifehouse, Camperdown, NSW, Australia; [2]Department of Medical Oncology, Macarthur Cancer Therapy Centre, Campbelltown, NSW, Australia

Correspondence to: Po Yee Yip. Chris O'Brien Lifehouse, P.O. Box M33 Missenden Road, NSW 2050, Australia. Email: Po.Yip@lh.org.au.

Abstract: Non-small cell lung cancer (NSCLC) is a devastating disease with poor prognosis. Systemic chemotherapy has been the mainstay of treatment in advanced disease for many decades. Personalized targeted therapy such as epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) and crizotinib has significantly changed the treatment paradigm in NSCLC. The future success of development of molecular targeted therapy relies on the understanding of signal transduction pathways. The PI3K-Akt-mTOR pathway is commonly deregulated in human malignancy including NSCLC. Therefore, this pathway is a target for many therapeutic developments. This review will provide an overview of PI3K-Akt-mTOR signaling pathway, genetic alterations activating the pathway and clinical therapeutic development of pathway inhibitors.

Keywords: PI3K-Akt-mTOR pathway; non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer is the most common cancer and leading cause of cancer death worldwide, with an incidence of 1.6 million new cases annually and 1.38 million deaths in 2008 (1). It is the fifth most common cancer and the leading cause of cancer death in Australia. There are approximately 9,700 new cases of lung cancer diagnosed each year. In 2007, there were 7,626 deaths from lung cancer in Australia, accounting for 19% of all cancer deaths (2,3).

For the past two decades, decisions regarding lung cancer treatment have been based largely on the histological distinction between non-small cell lung cancer (NSCLC) and small cell lung carcinoma. In recent years, more definitive histological classifications as well as identification of somatic mutations have become an essential component in determining the management of NSCLC. Molecular driven therapeutic targets such as *epidermal growth factor receptor* (*EGFR*) gene mutations (4) and abnormal fusion of *echinoderm microtubule-associated protein-like 4* and *anaplastic lymphoma kinase* (*EML4-ALK*) genes (5) have resulted in a paradigm shift in the treatment of advanced lung adenocarcinoma. However, they only account for a small proportion of NSCLC. Therefore, there are ongoing efforts in identifying more molecular targets for potential targeted therapeutics. *EGFR* mutation and abnormal fusion of *EML4-ALK* activate two main downstream signaling pathways, RAS-RAF-MEK-ERK and PI3K-Akt-mTOR, resulting in uncontrolled growth and cell proliferation. PI3K-Akt-mTOR is one of the most commonly deregulated pathways (6), which has been implicated in the tumourigenesis of NSCLC. Therefore, there has been an increasing research interest in identifying novel therapies to target this signaling pathway.

This review is going to provide an overview of the biology of PI3K-Akt-mTOR signaling pathway in normal cells under physiological conditions, mechanisms of deregulation of the pathway in NSCLC, therapeutic implications and potential prognostic and predictive biomarkers of the PI3K-Akt-mTOR signaling pathway.

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Biology of PI3K-Akt-mTOR pathway

Key components of PI3K-Akt-mTOR pathway

PI3Ks are a family of lipid kinases that phosphorylate the 3'-hydroxol group in phosphatidylinositol and phosphoinositides (7). There are three classes of PI3K (I-III), which are classified according to their structure and substrate specificity (8). They are heterodimeric proteins with catalytic and regulatory subunits. Class I divides into Class IA and IB based on the types of receptors that they are activated by and they consist of different catalytic and regulatory subunits, each has different isoforms. Class IA PI3Ks consist of a p110 catalytic subunit and a p85 regulatory subunit. They are activated by growth factor receptor tyrosine kinases (RTKs). The p110 catalytic subunit has three isoforms (p110α, p110β and p110δ) that are encoded by *PIK3CA*, *PIK3CB* and *PIK3CD* genes respectively. The p85 regulatory subunit has five isoforms (p50α, p55α, p85α, p85β and p55γ). The p85α, p50α and p55α are encoded by *PIK3R1* gene; p85β encodes for *PIK3R2* gene and p55γ encodes for the *PIK3R3* gene. Class IB PI3Ks consist of a p110γ catalytic subunit and a p101 regulatory subunit. They are activated by G-protein coupled receptors (GPCRs) (7,9). The role of Class IA PI3Ks in carcinogenesis has been well demonstrated but Class IB PI3Ks are less clear(10). Class II PI3Ks has only a single p110-like catalytic subunit that catalyze the production of PtdIns[3]P and PtdIns[3,4]P2 and regulate clathrin mediated membrane trafficking (8,11). Class III consists of a single member, hVPS34 which produces PtdIns[3]P and is involved in the regulation of vesicle trafficking, activation of mTOR by amino acids and autophagy (12,13).

Akt, is a serine/threonine-specific protein kinase and also known as protein kinase B (PKB). It consists of an amino-terminal pleckstrin homology (PH) domain, a central catalytic domain and a short carboxy-terminal regulatory domain. There are three isoforms: Akt 1 (PKBα), Akt 2 (PKBβ) and Akt 3 (PKBγ) (10). The function of Akt is to phosphorylate and activate or inactivate numerous downstream cytoplasmic and nuclear substrates such as forkhead (FOXO) family of transcription factors, p-53 binding protein (MDM2), pro-apoptotic protein BCL2-antagonitst of cell death (BAD) and tuberous sclerosis 1 and 2 (TSC1 & 2) to regulate cell survival, proliferation and protein synthesis, and hence cell growth (7,10).

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that is a member of the phosphatidylinositol kinase-related kinase (Pikk) family of kinases (9). It presents in two multi-protein complexes (mTORC1 and mTORC2). The mTORC1 is an mTOR complex combining rgulatory-associated protein of mTOR (Raptor), PRAS40 (also known as Akt substrate 1) and mLST8 while mTORC2 combines with rapamycin-insensitive companion of mTOR (Rictor), mSIN1, Protor and mLST8. It carries out its function of control on cell growth and division as well as protein translation through pathways of ribosomal p70 S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) (14).

PI3K-Akt-mTOR signaling in normal cells under physiological condition

Physiologically, the PI3K-Akt-mTOR pathway is activated by the binding of the ligand to the tyrosine kinase receptors (RTKs) such as EGFR, ErbB3, MET, PDGFR, VEGFR, IGF-1R, HER2/neu resulting in recruitment of class IA PI3Ks to the cell membrane where they convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Simultaneously, the Ras-Raf-MAPK pathway may also be activated leading to cross-talk with PI3K. The PIP3 generated at the cell membrane acts as a second messenger, which binds to the PH domain of Akt. Akt is then phosphorylated at Thr308 in the catalytic domain by the phosphoinositide dependent kinase 1 (PDK1) and at Ser473 in the C-terminal hydrophobic motif by the mammalian target of rapamycin complex 2 (mTORC2). This results in full activation of Akt that lead to phosphorylation and inactivation of the complex of tuberous sclerosis 2 and 1 (TSC2-TSC1), which is a GTPase-activating protein (GAP) for Ras homologue enriched in brain (RHEB). ERK-Rsk also inactivates TSC2. Both results in accumulation of GTP-bound RHEB, leading to activation of the mammalian target of rapamycin complex 1 (mTORC1), which phosphorylates p70 S6 kinase and 4E-binding protein 1 (4EBP1), 4EBP2 and 4EBP3 resulting increased protein translation, ribosome biogenesis and inhibition of autophagy (15-19) (*Figure 1*).

Phosphorylation of Akt not only activates the mTOR signaling but it also activates minute double minute 2 (MDM2) and inactivates targets for pro-apoptotic proteins such as forkhead box O (FOXO) belong to forkhead family of transcription factors and BAD, which are involved in cell survival (*Figure 1*). Akt phosphorylates FOXO resulting in activation of pro-apoptotic proteins such as BIM and FAS

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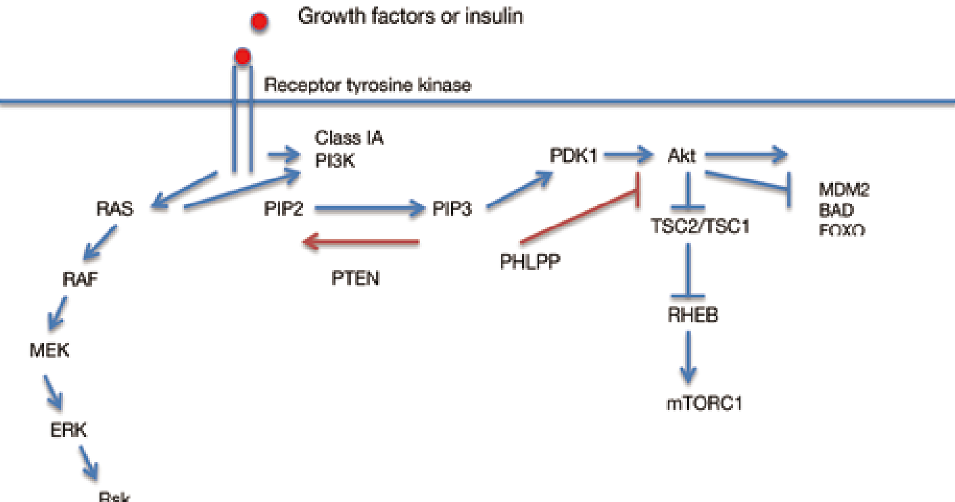

Figure 1 PI3K-Akt-mTOR signaling pathway.

ligand and expression of p27Kip1 and retinobloastoma-like 2 (RBL2) causing cell cycle arrest (20). There are a number of review articles that have elegantly illustrated and described this complex network of downstream substrates of Akt (7,8,10,14).

Feedback and regulation mechanisms of PI3K-Akt-mTOR signaling

In order to maintain cellular homeostasis, there are a number of different mechanisms that regulate the PI3K-Akt-mTOR signal transduction pathway preventing abnormal growth and cell division. Phosphatase and tensin homolog (PTEN) is the key component involved in deactivation of PI3K signaling by converting PIP3 back to PIP2 at the cell membrane (21) while the phosphatase PHLPP inactivates Akt signaling by dephosphorylating Akt at Ser 473 (22) (*Figure 1*). When it comes to the regulation of mTOR signaling, it is directly influenced by the activity of TSC2-TSC1 complex, which itself is controlled by the activity of TSC2-TSC1 complex, which itself is controlled by the cellular energy level via LKB1 (liver kinase b1)/STK11-AMPK (5'adenosine monophosphate-activated protein kinase) pathway (23), cellular oxygenation via hypoxia-inducible factor-α (HIFα) and DNA damage response 1 (REDD1) (24) and amino acid availability mediated by class III PI3K (25,26). Furthermore, there is an inhibitor protein, PRAS40 within the mTORC1 that exerts control on mTOR signaling. Carracedo *et al.* (27) illustrated that the feedback and regulation mechanisms of PI3K-Akt-mTOR are far more complex.

Deregulation of PI3K-Akt-mTOR signal transduction in NSCLC

Hanahan and Weinberg (28) described that tumourigenesis is the result of the ability of cancer cells to sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, enable replicative immortality, induce angiogenesis and resist cell death. Many studies have demonstrated that there is 50-70% overexpression of phosphorylated Akt in NSCLC indicating that abnormal activation of PI3K-Akt-mTOR pathway is a frequent event (29-31). Constitutive activation of PI3K-Akt-mTOR signaling pathway could be the result of genetic aberrations in any components of PI3K-Akt-mTOR pathway, its negative regulators, interconnected pathways and RTK signaling resulting in abnormal growth and cell proliferation. *EGFR* mutation and increased copy numbers, *MET* mutation and amplification and *EML4-ALK* rearrangement are examples of genetic changes that could result in abnormal RTK signaling. EGFR mutations are more prevalent in East Asian (up to 60%) than Caucasian patients (15-20%) (32-36). *EML4-ALK* rearrangement is much less prevalent (2-7%) and does not have an ethnicity predilection (37,38). However, both driver mutations are more common in patients who are non-smokers than smokers. At the PI3K-Akt-mTOR pathway, *PIK3CA* mutation and amplification, *PTEN* loss, *Akt1* and *LKB1* mutation are examples of genetic abnormalities involved in deregulation of signal transduction (6-8). Furthermore, Ras-Raf-MEK-ERK-Rsk pathway cross talks with PI3K-

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Akt-mTOR, therefore, *KRAS* mutations also play a role (39). Some of these genetic changes are more prevalent in certain histological subtypes of NSCLC than others. *EGFR*, *KRAS* mutations and *EML4-ALK* rearrangement are more common in adenocarcinoma than squamous cell carcinoma (32,33,35-38,40,41) while *PIK3CA* amplification is more common in squamous cell carcinoma than non-squamous cell carcinoma (42-48). *PTEN* loss is equally common in both NSCLC with squamous and non-squamous histology (33,49,50). In adenocarcinoma, *EGFR*, *KRAS* mutations and *EML4-ALK* rearrangement are mutually exclusive while *KRAS* and *PIK3CA* co-mutations are common (51). *LKB1* mutations are more common in NSCLC with non-squamous cell histology than squamous and often present with *EGFR* and/or *KRAS* mutations (52-56).

Therapeutic implications

From the RTKs signaling to PI3K-Akt-mTOR pathway, these are targets of major therapeutics development that are either already in clinical practice such as epidermal growth factor tyrosine kinase inhibitors, EGFR TKIs (gefitinib, erlotinib and afatinib) and mTOR inhibitors (everolimus and temsirolimus) or in development such as dual PI3K/mTOR, PI3K and Akt inhibitors.

Targeted therapy—approved for clinical use

EGFR TKIs are now the first line treatment for *EGFR* mutated advanced NSCLC of non-squamous histology. In 2009, Mok *et al*. (36) demonstrated in the Iressa Pan-Asia Study (IPASS) that patients treated with efitinib whose pulmonary adenocarcinoma harbored *EGFR* mutation have a longer progression-free survival than those who were treated with carboplatin and paclitaxel (HR 0.48; 95% CI: 0.36-0.64; P<0.001) in East Asia. Subsequent clinical trials with the same design using erlotinib or afatinib including Asian or western population reached the same conclusion (57-59). Crizotinib, an oral tyrosine kinase inhibitor targeting *ALK* (60), *MET* (61) and *ROS1* (62) has also been approved in advanced non-squamous cell lung carcinoma with *ALK* translocation. Shaw *et al*. (63) demonstrated that crizotinib improved both progression-free survival (PFS) (median 7.7 *vs*. 3 months; HR 0.49; P<0.001) and overall response rate (65% *vs*. 20%; P<0.001) in patients with ALK positive non-squamous cell lung carcinoma who progressed after platinum-doublet chemotherapy. Similar benefit when use in the first line setting among patients with ALK positive non-squamous cell lung carcinoma (64). mTOR inhibitors have been approved for clinical use as monotherapy in advanced renal cell carcinoma, pancreatic neuroendocrine tumour and in combination with examestane in estrogen receptor positive, HER2-negative breast cancer but not in advanced NSCLC because of poor response rate with increased toxicities. Soria *et al*. (65) showed that the overall response rate of everolimus monotherapy was less than 5% in patients with advanced NSCLC who progressed from chemotherapy. Ramalingam *et al*. (66) demonstrated that only 2 out of 28 (7%) patients had a partial response with the combination of everolimus with docetaxel while Besse *et al*. (67) failed to meet the predefined clinically meaningful treatment benefit threshold of 15% or greater with the combination of everolimus with erlotinib over erlotinib alone in the 3-month disease control rate but increased toxicities.

Targeted therapy—under development

Inhibitors targeting the key components of PI3K-Akt-mTOR pathway involving NSCLC are all undergoing either phase I or II therapeutic development. All these novel agents are administered orally. Their toxicities are unique to the components of the pathway that they are blocking but the common side effects include fatigue, anorexia, rash, hyperglycaemia, nausea and diarrhea (68).

PI3K inhibitors

Wortmannin and its derivative LY294002 were the first generation pan-PI3K inhibitors but they have not been moved into clinical use because of their toxicities. Pan-PI3K and isoform specific PI3K inhibitors are the two types of PI3K inhibitors that are in clinical development. Pan-PI3K inhibitors inhibit all isoform of class IA PI3K while isoform-specific PI3K inhibitors inhibit the specific isoforms of catalytic subunit of class IA PI3K (p110α, p110β and p110δ). BKM120 (Buparlisib), GDC-0941 (Pictilisib), PX-866 and XL-147 (SAR 245408) are examples of pan-PI3K inhibitors. BYI719 is an example of isoform-specific PI3K inhibitor.

Buparlisib (BKM120) is an oral pyrimidine-derived pan-PI3K inhibitor against both wild-type and mutant class I PI3Ks isoforms. It does not inhibit the class III PI3K or Mtor (69). The maximal tolerated dose has been established in phase I clinical trials with most common adverse events including rash, hyperglycaemia, anorexia, nausea and diarrhea and there was no ethnic difference in pharmacokinetics properties (70-72). It has advanced the

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furthest in the therapeutic development among all PI3K inhibitors as it is being evaluated in a randomised placebo-controlled phase III clinical trial in receptor positive but HER2 negative breast cancer (BKM120 + placebo versus BKM120 + fulvestrant) (73). However, buparlisib is still undergoing early phases of development in NSCLC.

Akt inhibitors

Their mode of action is to block the serine/threonine kinase Akt but their disturbance to the metabolic homeostasis causing severe hyperglycaemia and other potential metabolic abnormalities could hamper the development of this group of agents (74-76). MK-2206 is an example of a pan-Akt kinase inhibitor, which has been shown to potentiate the pathway inhibition when combined with systemic chemotherapy or molecularly targeted agents in preclinical study (77). Therefore, it is being evaluated in a phase II study in combination with erlotinib for patients who have advanced NSCLC after progression from erlotinib (NCT01294306).

Dual PI3K-mTOR inhibitors

SAR245409 and BEZ235 are two examples of dual PI3K-mTOR inhibitors. SAR245409 is a selective inhibitor of Class I PI3Ks, TORC1 and TORC2 (78) and BEZ235 is an imidazo-quinoline derivative blocking the activity of both PI3K and mTOR simultaneously (69,79). Both have been demonstrated to have anti-tumour effect in preclinical studies (80,81) and are being evaluated in early phases of clinical trials in combination with another targeted agents (69,78).

Trials in progress

All the novel agents targeting the PI3K-Akt-mTOR pathway mentioned above are still undergoing early phases of development in NSCLC. Hypotheses generated from the preclinical models, suggested that PI3K pathway inhibitors have the ability to overcome RTK resistance (77,82,83) and they have synergistic effect when combined with cytotoxic chemotherapy (42) or other targeted agents of interconnected pathways such as MEK inhibitors (69,84). Therefore, combination therapeutics approach would yield more success than monotherapy (85). Lockwood *et al.* (86) demonstrated in their genomic study that the genetic pathways involved in squamous cell lung carcinoma are different from those in non-squamous. NOTCH3 and FOXM1 are overexpressed in squamous cell lung carcinoma and may be involved in cross-talk with PI3K pathway (86-88). This highlighted the importance of histology. Therefore, not only do we need to evaluate markers by genetic alternations but also by histology subtype. Clinical trials in progress that evaluate the efficacy of PI3K pathway inhibitors in advanced NSCLC below include biomarker analysis by histology subtype (squamous cell carcinoma versus non-squamous cell carcinoma) and/or enriched with *PIK3CA*, *PTEN* gene mutated tumours.

PI3K pathway inhibitors + EGFR TKI

Ultimately, all patients with *EGFR* mutated lung adenocarcinoma who are on first generation EGFR TKIs (gefitinib and erlotinib), will eventually develop resistance and cancer progression. The mechanisms of resistance include acquired resistance mutation *T790M*, *MET* amplification or *PIK3CA* mutation resulting in activation of PI3K signaling. *PIK3CA* mutation account for up to 5% of EGFR-mutated NSCLC acquired resistance to EGFR TKI (89). Studies have demonstrated that adding PI3K inhibitors to EGFR TKIs can overcome the EGFR TKI resistant NSCLC cell lines (82,83,90,91). Tan *et al.* (92) demonstrated in a phase Ib study of 15 patients with *EGFR* mutant NSCLC who progressed during or after gefitinib that it was safe to combine buparlisib 80 mg with gefitinib 250 mg and there was antitumour activity but significant toxicities with 40% of patients experiencing delayed grade 3 rash and diarrhea. The median progression free survival was 2.8 (range, 2.3-8.1) months. Four out of nine patients in the group that included patients who progressed while on gefitinib had clinical responses including slight tumor shrinkage and reduced pleural effusion. Molecular analysis showed 6 (50%) of 12 patients whose tumors harbored *T790M* mutation, 2 (40%) of 5 had *MET* amplification while no patients whose tumor had *PIK3CA* mutation or *PTEN* loss. The dose expansion phase and alternate dose schedule are ongoing. Another similar phase Ib/II trial but in combination with erlotinib is currently recruiting (NCT01487265).

PI3K pathway inhibitors + chemotherapy

BASALT-1 (NCT01297491) is a two-stage phase II trial that included patients with metastatic NSCLC and PI3K pathway activation defined by *PIK3CA* mutation, *PTEN* mutation or loss of PTEN expression by immunohistochemistry. All patients in the first stage of the trial received buparlisib as a single agent and were stratified into squamous and non-squamous NSCLC subgroups. In the second stage, it was

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planned to randomise patients with squamous NSCLC to either buparlisib (100 mg/day) or docetaxel (75 mg/m^2 every 3 weeks) while patients with non-squamous NSCLC randomise to either buparlisib (100 mg/day) or docetaxel (75 mg/m^2) or pemetrexed (500 mg/m^2) every three weeks. However, Soria *et al*. (93) demonstrated the progression free survival rate at 12 weeks was less than 50% for both the squamous and non-squamous NSCLC groups. Therefore, the second stage was not initiated because of futility. BASALT-2 (NCT01820325) is a phase Ib/II trial to determine the safety and efficacy of buparlisib in combination with carboplatin (area under the curve, AUC 6) and paclitaxel (200 mg/m^2) every 3 weeks as first line treatment for patients with squamous NSCLC. NCT01911325 is another phase Ib/II trial that includes only squamous NSCLC.

There are other phase I or II trials using different chemotherapy regimens with buparlisib. Carboplatin with pemetrexed (NCT01723800 is recruiting) and cisplatin with gemcitabine are the two regimens used in two phase I trials and one has not opened yet (NCT01971489). Besse *et al*. (94) demonstrated in a phase Ib study that included patients with stage IIIB/IV NSCLC who failed first or second line chemotherapy that the overall response rate for squamous NSCLC patients was 75% (one complete responder and 2 partial responders) while non-squamous NSCLC eligible for bevacizumab was 66% (6 partial responders). There were no dose-limiting toxicities at 250 mg and the maximum dose for evaluation was at 330 mg. In the study, patients were stratified into two groups based on histology. For patients with squamous histology and the lesions centrally located or those with non-squamous histology and had contraindications to bevacizumab such as hypertension, haemoptysis or haematuria, pictilisib and carboplatin with paclitaxel were given for six cycles followed by maintenance pictilisib until progression (Arm A). For patients with non-squamous histology or squamous histology of peripherally locating lesions and no contraindications to bevacizumab, six cycles of carboplatin with paclitaxel and bevacizumab and pictilisib was given followed by maintenance bevacizumab with pictilisib (Arm B).

Prognostic and predictive biomarkers in NSCLC

Prognostic factor refers to patient or cancer characteristics that identify patients who are going to have a better or poorer survival regardless of treatment. They are validated in retrospective studies and are more important in early stage NSCLC because patients identified as high-risk may benefit from additional adjuvant therapies. Predictive factor refers to patient or cancer characteristics that identify patients who are more likely to respond to a particular treatment. They are validated in randomized controlled trials that can examine the predictive effect of the marker in both the placebo and treatment groups. They are more important in the advanced stage NSCLC because this allows delivery of personalised medicine to patients by maximizing benefit while minimizing potential harm. Genes, proteins, mRNAs and miRNAs are potential biomarkers, which can be discovered and evaluated using many techniques including immunohistochemistry, multi-gene profiling or next generations sequencing. With the advancement of molecular technology, we are increasingly more likely to classify and treat NSCLC according to their molecular genetic aberrations such as using EGFR TKIs and crizotinib in *EGFR* and *ALK* mutant NSCLC respectively. However, they only account for and benefit a small proportion of patients with advanced NSCLC. Furthermore, the clinical activity of novel targeted inhibitors targeting the PI3K-Akt-mTOR pathway, have been modest. Therefore, it would likely be cost ineffective to treat all patients with them. It is important to identify predictive biomarkers that could select patients who are more likely to respond to the novel targeted therapies than those who do not. To date, *EGFR* and *ALK* are the only predictive biomarkers in advanced NSCLC. There have been no predictive markers identified that correlate with clinical activity of novel targeted PI3K, Akt or dual PI3K/mTOR agents yet despite the effort of evaluating tumour molecular status in early phases of therapeutic clinical trials. In a preliminary analysis of a phase I buparlisib dose-escalation trial of 35 patients, over 70% of patients had adequate tumor to evaluate for *PIK3CA* status, PTEN expression and *KRAS* status (70). The study included only 2 patients (5.7%) with lung cancer and the majority of patients had colorectal (43%) or breast cancers (26%). There was one patient with triple-negative breast cancer who showed partial response while seven patients (20%) had stable disease of eight months or more. Five of them had tumors with PI3K pathway dependence. However, there were no correlation between tumor molecular alterations and clinical activity. Rodon *et al*. (71) reached the same conclusion at the final analysis of the full cohort of 83 patients and also among the subgroup of patients with breast or colorectal cancers. There were 43 patients in the expansion arm of the trial whose tumors harbored PIK3CA and/or PTEN mutations.

PIK3CA mutations or amplification and loss of PTEN are potential predictive markers of response because a

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Table 1 Immunohistochemical studies of prognostic significance on pAkt

Studies	Sample size	Stage of disease	Types of lung cancer	PAkt antibody	Methods	Compartments IHC stained	Prognostic factor (Negative/Positive)
Yip *et al*. (103) [2014]	471	IB	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	Yes (Negative)
Vincent *et al*. (104) [2011]	29	I & IIA	NSCLC	Ser 473 Thr 308	Western blotting	N/A	Not assessed
Al-Saad *et al*. (102) [2009]	335	I-IIIA	NSCLC	Ser 473 Thr 308	IHC	Cytoplasm Nucleus	Yes (Negative)
Tsurutani *et al*. (31) [2006]	230	I-IV	NSCLC	Ser 473 Thr 308	IHC	Not specified	Yes (Negative)
Lim *et al*. (101) [2007]	59	I, IV	NSCLC	Ser 473	IHC	Not specified	Yes (Negative)
Tang *et al*. (100) [2006]	102	I-IV	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	Yes (Negative)
Shah *et al*. (105) [2005]	82	I-IIIA	NSCLC	Ser 473	IHC Western blotting, WB	Cytoplasm Nucleus Membranous	No in pAkt expression by IHC Yes in PAkt/α-actin by WB (Positive)
Balsara *et al*. (29) [2004]	110	I-IV	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	No
Hirami *et al*. (106) [2004]	80	Not specified	NSCLC	Ser 473	IHC	Cytoplasm	Yes (Negative)
David *et al*. (99) [2004]	61	I-IV	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	Yes (Negative)
Massion *et al*. (46) [2004]	242 (NSCLC 215)	I-IV	NSCLC & limited- extensive stage SCLC	Ser 473	IHC	Cytoplasm Nucleus	No
Mukohara *et al*. (98) [2004]	91	I	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	No
Mukohara *et al*. (97) [2003]	60	I-III	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	No
Tsao *et al*. (96) [2003]	76	I-IV	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	No
Lee *et al*. (95) [2002]	43	Node +	NSCLC	Ser 473	IHC	Cytoplasm Nucleus	No

preclinical study has demonstrated that squamous cell lung cancer cells lines with PIK3CA mutations or amplifications and loss of PTEN protein expression are more sensitive to pictilisib (42).

As for prognostic biomarkers, there have been many evaluated but none have been used in daily clinical practice. The overexpression of phosphorylated Akt (pAkt) using immunohistochemistry is a promising potential prognostic biomarker, which is expressed in both squamous and non-squamous cell lung carcinoma (95). Earlier studies (29,46,95-98) demonstrated that pAkt has no prognostic value while later studies suggested the contrary. David *et al.* (99) was the first to demonstrate that high level of pAkt expression correlated with shortened survival and was an independent prognostic factor. Subsequently, more studies further confirmed that overexpression of pAkt (Ser 473) assessed by immunohistochemistry is a negative prognostic factor in NSCLC (31,100-103) (*Table 1*).

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Interestingly, Shah *et al*. (105) demonstrated that expression of pAkt assessed by immunohistochemistry did not correlate with patient prognosis. However, high levels of pAkt protein assessed by Western blotting and semiquantitative densitometry correlated with a good prognosis in both univariate and multivariate analysis. This highlighted the difficulties in identifying validated prognostic markers that are applicable to clinical use because studies included patients with heterogeneous stages of disease, used different immunohistochemical protocols and pAkt antibodies against different phosphorylation sites (Ser 473 *vs*. Thr 308), while others had small sample sizes, which were underpowered to demonstrate prognostic significance.

Conclusions

PI3K-Akt-mTOR signaling pathway is the target of many novel inhibitors. In order for patients to enjoy the maximal benefits and minimize side effects of these targeted therapies, identification of predictive markers are paramount. Incorporating biomarker analysis and patient enrichment strategies in clinical trials are essential in translational lung cancer research.

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Liquid biopsy for cancer screening, patient stratification and monitoring

Graham Brock, Elena Castellanos-Rizaldos, Lan Hu, Christine Coticchia, Johan Skog

Exosome Diagnostics, Cambridge, MA 02139, USA

Correspondence to: Johan Skog. Exosome Diagnostics, 840 Memorial Drive, Cambridge, MA 02139, USA. Email: Johan@exosomedx.com.

Abstract: Molecular characterization of a patient's tumor to guide treatment decisions is increasingly being applied in clinical care and can have a significant impact on disease outcome. These molecular analyses, including mutation characterization, are typically performed on tissue acquired through a biopsy at diagnosis. However, tumors are highly heterogeneous and sampling in its entirety is challenging. Furthermore, tumors evolve over time and can alter their molecular genotype, making clinical decisions based on historical biopsy data suboptimal. Personalized medicine for cancer patients aims to tailor the best treatment options for the individual at diagnosis and during treatment. To fully enable personalized medicine it is desirable to have an easily accessible, minimally invasive way to determine and follow the molecular makeup of a patient's tumor longitudinally. One such approach is through a liquid biopsy, where the genetic makeup of the tumor can be assessed through a biofluid sample. Liquid biopsies have the potential to help clinicians screen for disease, stratify patients to the best treatment and monitor treatment response and resistance mechanisms in the tumor. A liquid biopsy can be used for molecular characterization of the tumor and its non-invasive nature allows repeat sampling to monitor genetic changes over time without the need for a tissue biopsy. This review will summarize three approaches in the liquid biopsy field: circulating tumor cells (CTCs), cell free DNA (cfDNA) and exosomes. We also outline some of the analytical challenges encountered using liquid biopsy techniques to detect rare mutations in a background of wild-type sequences.

Keywords: Liquid biopsy; exosome; circulating tumor cell (CTC); cell free DNA (cfDNA); nucleic acids

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Introduction

The science of noninvasive disease monitoring has advanced greatly since circulating cell free DNA (cfDNA) was first reported in body fluids by Mandel and Metais (1). Since then, the evolution of sensitive cfDNA detection technologies has enabled the development of liquid biopsies with many clinical applications. For example, in oncology, the use of liquid biopsy allows for patient stratification (companion diagnostics), screening, monitoring treatment response and detection of minimal residual disease after surgery/recurrence.

Liquid biopsies have grown in importance because, the genetic profile of tumors can affect how well they respond to a certain treatment. However, this characterization is currently achieved through a biopsy despite the inherent problems in procurement of tissue samples and the limitations of tumor analyses. For example, the invasive nature of a biopsy poses a risk to patients and can have a significant cost. Tumor sampling from some cancer types also remains difficult resulting in inadequate amount of tissue for genetic testing. In the case of advanced or metastatic non-small cell lung cancers (NSCLC) as many as 31% of cases do not have accessible tissue (2). Even when tissue can be collected, preservation methods such as formalin fixation can cause C > T transitions through deamination of cytosine, potentially leading to false positive results for genetic tests (3). Finally, due to tumor heterogeneity, biopsies often suffer from sample bias (4).

More concerning with respect to guiding treatment decisions; biopsies will only inform of the genotype at that

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time-point. However, it is known that tumors are very dynamic and can change their dominant mutation pattern or acquire new mutations, especially after the selective pressure of drug treatment. This could be particularly unfavorable when stratifying patients to a specific targeted therapy based on historical mutation profiles of past tumor biopsies. In another example, approximately 50% of NSCLC patients become resistant to tyrosine kinase inhibitor therapy through an epidermal growth factor receptor (*EGFR*) T790M mutation (5,6), significantly only <5% of NSCLC patient have this mutation detectable in the primary biopsy (7). Another study showed that 38% of colorectal cancers with wild-type Kirsten rat sarcoma viral oncogene homolog (*KRAS*) developed mutations in this gene after anti-*EGFR* therapy as rapidly as 6 months after treatment (8).

Several reports have indicated there are difficulties in detecting tumor derived mutations in plasma, while others have been able to efficiently isolate circulating tumor derived nucleic acid in both metastatic and non-metastatic disease (9-11). This discrepancy is likely due to the methodologies used for detection of the mutation, as the allelic fraction of tumor derived circulating DNA varies from less than 0.01% (or undetectable) to over 90% (12,13). In addition, the amount of recoverable DNA varies significantly (over 3 logs) between patients with an average of about 17 ng of DNA per mL of plasma from advanced-stage cancers (14), corresponding to roughly 5,000 haploid genome equivalents.

Recent technological developments and the downstream analytics being applied to liquid biopsies are now capable of reproducibly detecting mutations at very low allelic frequencies. Advances have also been made in droplet digital PCR (ddPCR) (15), next-generation sequencing (NGS) (16), beads, emulsion, amplification and magnetics (BEAMing) (13), amplification of refractory mutation system (ARMS) (17), co-amplification at lower denaturation temperature-PCR (COLD-PCR) and its derivatives (18,19) and PointMan™ DNA enrichment technology (20), to name but a few.

Ultimately the choice of platforms and required detection limit will depend on the clinical sample being analyzed, as the most sensitive methods are reported to detect allelic frequencies of as little as 0.01%, providing a theoretical lower limit to detect one mutated copy in a background of 10,000 wild-type alleles (13). Thus, this level of sensitivity requires samples/patients where at least 10,000 target alleles enter the downstream analytical assay.

Although technically challenging, an inherent advantage of liquid biopsies over other traditional tissue-based methodologies is the enablement of longitudinal monitoring which could help clinical oncologists gain a broader molecular understanding of the disease. This review will focus on the application of genetic profiling of tumor associated RNA and DNA derived from biofluids.

Approaches to liquid biopsy analysis

Circulating tumor cells (CTCs)

CTCs are cells shed into the vasculature from a primary tumor and may constitute seeds for subsequent growth of additional tumors (metastasis) in distant organs. They have been detected in various metastatic carcinomas for example breast, prostate, lung, and colorectal cancer (21,22) but are extremely rare in healthy subjects and patients with nonmalignant diseases (23). Clinical evidence indicates that patients with metastatic lesions are more likely to have CTCs amenable to isolation but their frequency is low, often ~1-10 CTCs per mL of whole blood (24). As 1 mL of blood contains ~$7\times10e^6$ white blood cells and ~$5\times10e^9$ red blood cells (25), technologies capable of reproducibly isolating a single CTC from the background of all other blood components are fundamental. While such levels of sensitivity are challenging, there are several novel developments in this area. These include positive selection, negative selection, physical properties or even enrichment-free assays to efficiently isolate these rare CTCs (26,27).

Typically, CTCs are defined as cells with an intact viable nucleus, cytokeratin positive, epithelial cell adhesion molecule (EpCAM) positive and with the absence of CD45. Unfortunately EpCAM and other markers are not always expressed on CTCs and are down-regulated by processes such as epithelial to mesenchymal transition (28). In addition, non-tumor epithelial cells are known to circulate in the blood of patients with prostatitis (29) or patients undergoing surgery (30). From a technical standpoint, the heterogeneity of CTCs is a major challenge and this has led to alternative strategies of CTC enrichment, such as the CTC-iChip (31), which do not rely on tumor antigen expression.

Sequencing the genetic material from CTCs has demonstrated that, even when the isolated cell(s) fit the phenotypic criteria of being a CTC, the majority are not cancer cells. One study developed a protocol to recover the CTC enriched samples from the cartridge of the Veridex platform and found that from 37 NSCLC patients, the

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mutation allele abundance ranged between 0.02% and 24.79% with a mean of 6.34% (32). The number of CTCs found in the blood is therefore highly dependent on how the platform defines a cell as a CTC.

Currently, most CTC isolation platforms require that the whole blood is processed soon after collection, negating the option of long-term bio-banking. In addition, CTCs are fragile and tend to degrade when collected in standard evacuated blood collection tubes. The CellSearch CTC test, a Food and Drug Administration (FDA) approved actionable CTC test, requires that samples are processed within 96 hours of collection after being drawn into the *Cellsave* preservative tube. This test does not analyze the molecular genetics of the tumor; rather *Cellsave* is a platform for CTC numeration. A positive test (more than five detected CTCs for metastatic breast and prostate cancer and more than three CTCs for metastatic colorectal cancer per 7.5 mL of blood) is associated with decreased progression-free survival and decreased overall survival in these patients (33-37).

Cell free DNA (cfDNA)

There is currently an intensive research effort to understand the utility of cfDNA in various clinical fields such as cancer research (38,39), non-invasive prenatal testing (40) and transplant rejection diagnostics (41). Initial studies in cancer patients reported that cfDNA concentration in serum was significantly increased in comparison to healthy individuals (42), and it was suggested that this correlated with malignancy (43).

Most cfDNA in plasma is reportedly fragmented, around 150-180 bp in length (44) with a higher prevalence of tumor associated mutations in the shorter fragments (9). In fact, when analyzing the mutation abundance with massively parallel sequencing a significant correlation was found between mutations and fragments less than 150 bp (44). Notably, the size of the majority of cfDNA fragments overlaps well with the size of histone DNA (45).

The entry of cfDNA into the bloodstream is thought to originate from a cell following apoptosis or necrosis. Late stage cancer patients also have an increased level of cfDNA in plasma, however, most of this DNA is wild-type and believed to be from non-malignant cells and tumor stroma (9). It has also been suggested that the mutant fraction of cfDNA is derived from necrotic neoplastic cells phagocytized by macrophages, which then release digested DNA, a phenomena not seen in macrophages that engulf apoptotic cells (14). The extensive background of wild-type DNA limits the ability of downstream analytical platforms to detect tumor-derived mutation, presenting technical challenges for the use of cfDNA in liquid biopsies. While cell-free tumor DNA analyses are capable of examining the genetic or epigenetic changes that originate in tumor DNA (such as mutations, translocations, amplifications, indels and methylation abnormalities), they cannot analyze the tumor RNA transcriptome or proteome.

However, an advantage of cfDNA is that it can be analyzed from bio-banked biofluids, such as frozen plasma. In addition, a direct comparison of mutation detection on cfDNA *vs.* CTCs showed a higher abundance of the mutation on the cfDNA from the same patient (39). Finally, recent large studies comparing the effectiveness of cfDNA analysis to tissue biopsy in NSCLC showed the clinical value of the liquid biopsy approach (46). This positive result led to an approval to use cfDNA analysis for *EGFR* mutation analysis for IRESSA® in Europe (in patients where a tumor sample was not evaluable), making it the first *EGFR* tyrosine kinase inhibitor for which cfDNA testing is included in the label.

Although promising, challenges remain when using cfDNA to characterize the mutation status of a tumor. In addition to the low copy number of mutant alleles, the median half-life of cfDNA in circulation ranges from 15 minutes to a few hours (47). Also, the total concentration of cfDNA in the blood of cancer patients varies considerably (48) with tumor specific mutations ranging from undetectable (less than 1 copy per 5 mL of plasma) to patients with over hundred thousand copies of the mutation per ml of plasma (39). Thus, the challenge of how to maximize the yield of the cfDNA and pair this with a platform sensitive enough to detect rare variants in the background of wild-type DNA remains. Optimally, the ability to detect mutations in plasma should not be limited to a subpopulation of patients with very high mutant copy numbers in circulation. While many analytical platforms report the mutation load with an allelic frequency compared to the wild-type DNA, platforms relying solely on the allelic frequency without recording the number of mutations have limitations. The allelic frequency is affected by the amount of wild-type DNA not related to the tumor. Therefore, it is important to consider the processes that affect the amount of wild-type DNA in circulation. For example, exercise increases cfDNA levels 10-fold (49) and other pre-analytical variables such as blood collection and extraction protocols affect the amount and size range of cfDNA fragments in a sample (50). Delays in blood processing, blood storage

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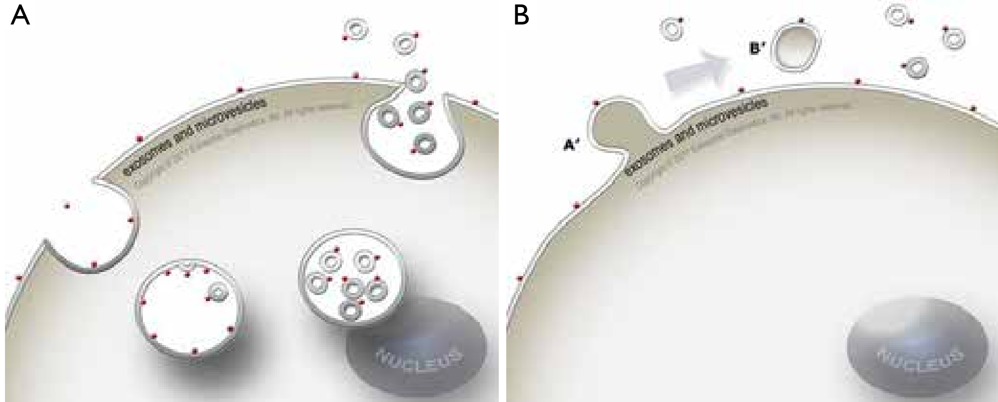

Figure 1 Exosome/microvesicle biogenesis. The classical exosome biogenesis pathway begins with the formation of an endosome, followed by inward budding of the endosome resulting in MVB with ILV. These ILV contain a sample of the cell's cytoplasm, including nucleic acids. (A) The ILV are then liberated by fusion of the MVB to the plasma membrane; (B) the second way of exosome/microvesicle biogenesis is through direct budding at the plasma membrane. MVB, multivesicular bodies; ILV, intraluminal vesicles.

temperature, agitation of the sample and shipment can all cause wild-type cfDNA release from lysed nucleated blood cells and effect the allelic frequency (51). For the same reason, plasma is often preferred over serum because of the potential for cell lysis during blood coagulation (52).

Exosomes

The exosome field has grown exponentially the last few years impacting various areas of research. Studies demonstrating that exosomes are actively released vesicles (carrying RNA, DNA and protein) and can function as inter-cellular messengers, have contributed to their elevated recognition in the scientific community (53-64). A recent review outlining the biological properties of exosomes and other extracellular vesicles (EV's) highlights these developments (65). However, with respect to nomenclature, the exosome field still lags behind as the definition and characterization of EV types are not yet firmly established (66). The majority of exosomes range in size from 30-200 nanometer in diameter and are isolated from all biofluids, including serum (60), plasma, saliva, urine and cerebrospinal fluid (67).

Exosomes and other EVs are particularly interesting as cancer biomarkers since they are stable carriers of genetic material and proteins from their cell of origin. They are also thought to be part of the disease process, for example, tumor exosomes have been shown to stimulate tumor cells growth, suppress the immune response and induce angiogenesis (60,68) and even be part of the metastatic process (63,69). Exosome release is also an active process and tumor cells can shed tens of thousands of vesicles per day resulting in hundreds of billions of vesicles per mL of plasma (55). The two mechanisms by which exosomes are released, either involve the formation of multivesicular bodies (MVB) and direct budding at the plasma membrane, or a process more akin to a retrovirus particle leaving the cell (*Figure 1*) (70).

In the early decades of exosome research, it was thought that they contained only protein and lipids. However, it has since been shown that exosomes are highly stable packages of RNA from the cell of origin (61). The finding that exosomes contain RNA with tumor specific mutations, can be isolated from biofluid samples and stored for many years in the freezer has opened up new opportunities in the field of diagnostics (60,71). Recent publications have also examined the DNA associated with exosomes and shown its utility for detection of gene amplifications as well as mutations (55,64,72).

Due to the size of an exosome, on average just over 100 nanometers, the entire transcriptome cannot be packaged inside every vesicle. By way of comparison, retrovirus particles with a similar size can package only around 10 kb (73), so it is likely that a single vesicle of that size carries only a limited number of transcripts. However, exosomes are extremely abundant ($10e^{11}$ per mL of plasma) and when isolating the vesicle fraction, most of the transcriptome can be detected (74). Exosomal RNA can be used for mutation detection (55,60,71,72) as well as global profiling of most types of RNA (74), and the profile alone (without mutation characterization) can be utilized for diagnostics (58,75,76).

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Table 1 Comparison of the analysis capability of CTC's, cfDNA and exosomes

Analysis capability	Examples	CTCs	cfDNA	Exosomes
Mutations	Point mutations, InDels, amplifications, deletions, translocations	Yes	Yes	Yes
Epigenetic modifications	Methylation patterns	Yes	Yes	Yes
RNA transcription profiles	Levels/activity of mRNA, microRNA, long non codingRNA, RNA splice variants	Yes	No	Yes
Phenotypic studies of cells from the tumor	Cell morphology, protein localization, *in vivo* studies	Yes	No	No
Inflammatory response, stromal and other systemic changes	Inflammatory RNA and protein markers	No	No	Yes
Analysis of RNA as well as DNA and protein profiles from tumor cells	Separate or in combination	Yes	No	Yes
Can utilize biobanked samples	Frozen plasma, urine and other biofluids	No	Yes	Yes

CTCs, circulating tumor cells; cfDNA, cell free DNA; InDels, insertions/deletions.

The precipitous release of exosomes by cancer cells seems to correspond to activation of the mitogen-activated protein kinases (*MAPK*) pathway frequently upregulated in tumor cells (77). Tumor derived mutations can be detected in exosomes from cerebrospinal fluid (67), serum (60), plasma (64) as well as in urine (71). However, as exosomes are released by all cells, they are particularly useful to profile not only mutations in cancer but also RNA profiles in inflammatory (78), metabolic (79), cardiovascular (80), neurodegenerative (81) and other disease processes.

Exosomes also carry surface markers from the cell of origin, which can be used for enrichment strategies, similar to CTCs (75). For example, characterization and analysis of exosome surface proteins hold great promise for the ability to identify, separate, sort and enrich exosomes originating from diverse cell sources. While the development of methods that allow for the routine analysis of exosome surface proteins has been a challenge, a number of recent advances have demonstrated potential. Immunoaffinity-bead based capture methods, microfluidic chip methods and antibody-based exosome arrays using both label and label-free detection platforms have all successfully exploited specific exosome surface proteins. This has enabled the capture, enrichment and characterization of unique populations of exosomes in the blood of healthy donors and of patients with pancreatic cancer (82), ovarian cancer (83), lung cancer (84,85). Surface protein-based exosome isolation methods combined with exosomal RNA extraction and qPCR detection assays have proven to be rapid and sensitive enough to monitor therapeutic response and resistance using exosomes from the blood of patients with glioblastoma (86,87).

In addition, the rapid advancement of a novel method of nanoscale fluorescence activated cell sorting call nanoFACS has further advanced methods of exosome isolation and sorting and allowed for the study of discrete, free, individual exosomes from body fluids (88). This technique and variants thereof hold great promise for future diagnostic applications where isolation and examination of individual exosomes is paramount. Finally, in addition to proteins, analysis of exosome protein-to-lipid ratios can be used to further isolate and characterize subpopulations of exosomes in body fluids (89).

Exosome investigations have focused on the important physiologic and pathophysiologic functions of these vesicles in micro-metastasis, angiogenesis and immune modulation (63,90) and as a means for detection of tumor specific mutations in biofluids. Consequently, in 2012, interest in this new field increased when the National Institute of Health (NIH) dedicated the large strategic Common Fund to study these new entities of extracellular RNA. The goal of this effort is to better understand how exosomes can be utilized for biomarkers and therapeutics as well as understanding this new mechanism of intercellular communication (http://commonfund.nih.gov/Exrna/index).

Mutation detection and RNA profiling

Analysis of nucleic acids present in bodily fluids can provide a better understanding of the disease, as summarized in *Table 1*.

Mutation detection in biofluids is a challenging task and requires highly sensitive analytical platforms. As this field

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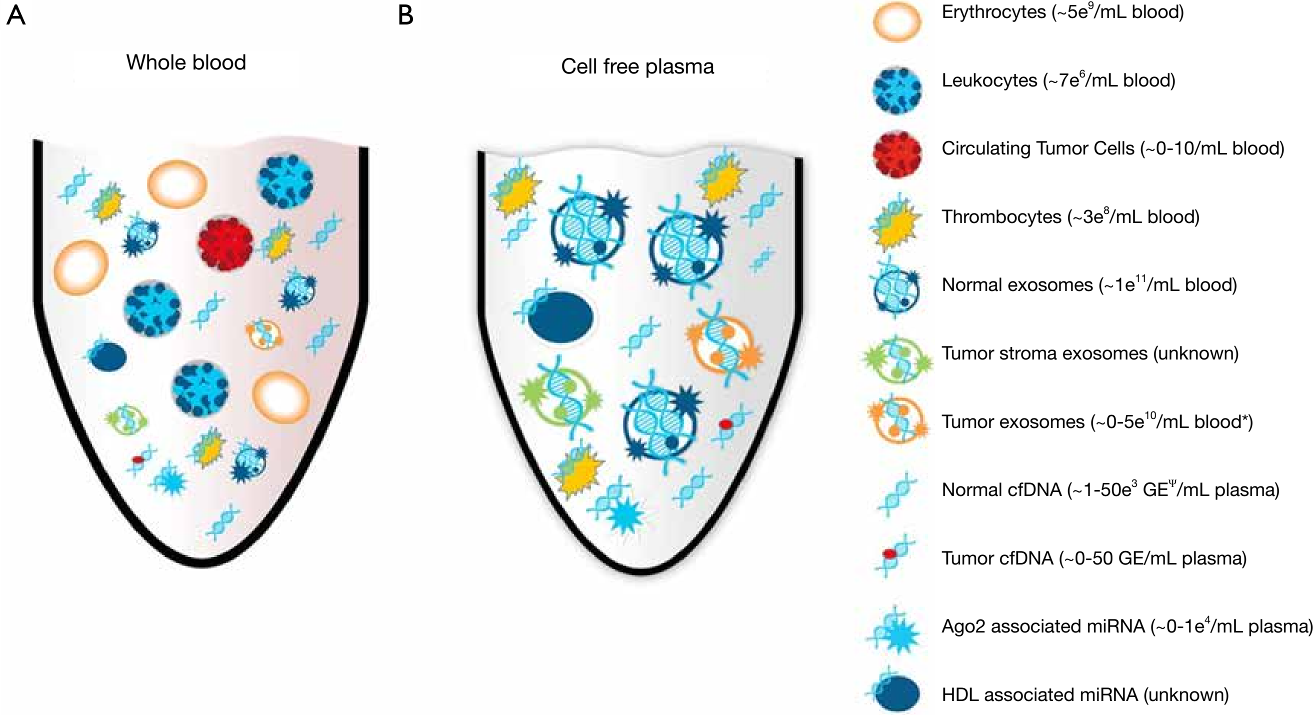

Figure 2 Circulating nucleic acids are coming from a wide range of cellular processes. It is important to optimize the sample processing for the particular target and understand where the RNA and DNA are coming from as well as their abundance. Whole blood as well as cell free plasma has multiple sources containing nucleic acids (shown in A and B respectively). Even components that lack a nucleus (like erythrocytes and thrombocytes) have been shown to carry RNA and can have cfDNA co-isolating in the preparations. *, based on a range of 0-50% of exosome RNA containing the tumor specific mutant allele (67); Ψ, GE, Genomic equivalents. (and Exosome Diagnostics unpublished data).

has evolved, the clinical applications of liquid biopsies have improved significantly. Examples of these analytical platforms include BEAMing (13), ARMS (17), and ddPCR (15). These platforms were developed specifically for the detection of extremely rare alleles and are used when the mutation type and position is known. Other platforms such as ice-COLD PCR and targeted resequencing using NGS platforms can detect rare allelic frequencies even when the type and location of the mutation in the gene is undefined. Targeted resequencing is becoming increasingly popular since it can easily accommodate larger panels of genes to cover the actionable mutations in cancer that have significant diagnostic, prognostic or therapeutic implications for a specific therapy. Initially, the inherent error rate of NGS platforms made it difficult to identify very rare alleles (<1%), but strategies using paired-end sequencing and background correction have enabled detection of allelic frequencies at or below 0.1% (91). Incorporation of unique identifiers to each target enables highly sensitive digital sequencing capable of quantifying the number of mutated reads as well as their allelic frequency (92,93).

RNA profiling from biofluids also poses numerous challenges. However, the discovery that exosomes contained RNA made it possible to separate the fragile RNA from the large amounts of RNases and PCR inhibitors that are present in most biofluids. As cell-free RNA in blood is immediately degraded, RNAs in serum and plasma are either protected inside vesicles like an exosome, in protein complexes with the Ago2 protein (94) or associated with HDL particles (95) as outlined in *Figure 2*. Most of the early studies were limited to the more abundant short (~22 nt) regulatory microRNAs. The levels of these microRNAs are tightly regulated in normal cells and dysregulation has been implicated in a number of human diseases e.g., cardiovascular (96) neurological and is strongly linked to cancer development and progression as reviewed by Croce (97). However, although robust and readily detectable, microRNAs represent only a minor fraction of the transcriptome. By contrast, if the appropriate methods are used, the nucleic acids in exosomes can be isolated and the entire transcriptome interrogated

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for effective molecular profiling and mutation detection. Successful RNA profiling from biofluids requires that the contaminants, which could inhibit downstream analysis are removed. The effective purification of the exosomes can remove these contaminants making the exosome isolation platform scalable, where the sample volume input is linear to the RNA output and not affected by the increased amount of RNases that can co-purify (98). This feature is important, since scaling the volume appropriately will enable profiling also of low copy number RNAs.

Finally, special precautions need to be taken to prevent degradation during the RNA extraction procedure, as the RNA purified from exosomes and the microRNAs from Ago2 complexes will now be exposed to RNases. Measuring integrity using an exogenous spiked-in sequence of similar size and structure as the RNAs in the exosomes is recommended. Ideally, the 'spike' should itself be protected from RNases, for example using a synthetic vesicle added directly into the biofluid as opposed to the lysed sample.

Discussion

The most obvious hurdle for all forms of liquid biopsy remains the relative rarity of nucleic acid derived from a tumor against the background of normal material found in most patient samples. In fact, the majority of cell, cell free nucleic acids, microRNAs and exosomes in a liquid biopsy will have originated from normal cells with numbers fluctuating as a consequence of biological variations. Such challenges are addressed using the strategies highlighted in the methods described above. These methods are currently sensitive enough to detect very rare mutation events. However, it is critical that laboratories undertaking such methods must be scrupulous in their methodologies to avoid erroneous results. Although clichéd, the analogy of a needle in a haystack applies and is appropriate for each of these approaches.

The analysis of CTCs and exosome has benefited from developments in the field of enrichment prior to the analytical readout. While still at an early stage, a number of studies have demonstrated that protein-based isolation and enrichment methods will be an important tool both in enhancing nucleic acid based assays and as stand-alone diagnostics in the future.

Clearly, exosomes have a number of advantages for diagnostics. They enable high quality RNA to be extracted from fresh or frozen biofluids, thus increasing the scope of detectable mutations to include mutations, splice variants, fusions as well as expression based assays for mRNA, microRNA, lncRNA and other non-coding RNAs. They are also released from living cells as an active process, whereas cfDNA is released through the process of apoptosis and necrosis. On cfDNA, all genes are present at an equal level, whereas RNA originating from a highly expressed gene could occur in thousands of copies/cell. However, as mutations exist on both exosome RNA (living process) and cfDNA (dying process), utilizing a platform that can use both will have obvious advantages for detecting rare mutations. This is especially true in the case of patients who do not have an abundant amount of mutated nucleic acid in circulation.

Improvements to analytical sensitivity and specificity will address some of the current hurdles, for example, cancer patients who have very few mutations in their biofluids, likely due to biology rather than analytical sensitivity. In many cases, the mutated alleles can occur at less than 1 copy per mL of plasma. So, combining exosome RNA and cfDNA has the advantage of increasing the detection sensitivity for low frequency mutations.

For the patient there is an obvious and clear advantage to a liquid biopsy in comparison to conventional surgical methods. However, most of the studies to date have focused on detection of actionable mutations in biofluids, and this is arguably only a fraction of the capability of liquid biopsies in enabling personalized medicine. As DNA mutations will only inform of some aspects of the disease, looking at RNA expression in biofluids can help further understand processes within the cancer patient.

Cancer is a complex and dynamic disease that can change quickly. To fully deliver on the promise of personalized medicine, development of reliable and robust non-invasive platforms for the diagnosis, patient stratification and to monitor treatment response are paramount. The various liquid biopsy platforms described in this review have the potential to add tremendous value to the care of cancer patients.

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Potential biomarkers for lung cancer screening

Gabriella Sozzi, Mattia Boeri

Tumor Genomics Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Istituto Nazionale dei Tumori, via Venezian 1, 20133, Milan, Italy

Correspondence to: Gabriella Sozzi. Tumor Genomics Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico (IRCCS), Istituto Nazionale dei Tumori, via Venezian 1, 20133, Milan, Italy. Email: gabriella.sozzi@istitutonazionaletumori.mi.it.

Abstract: Notwithstanding the encouraging results of the National Lung Screening Trial (NLST) the scientific community still debates on the cost-benefit profile of low dose computed tomography (LDCT) lung cancer screening. Several major concerns regard how to identify subjects at high risk of developing lung cancer, the optimal diagnostic algorithm, the management of lung nodules and the high false positive rates. The use of complementary biomarkers would be a useful strategy for dealing with most of these issues. This short review will focus on candidates' biomarkers circulating in serum or plasma that already reached an advanced validation phase also in LDCT lung cancer screening series. The biomarkers presented below are examples of the value of searching candidates by looking not only to the tumor itself but also to the interplay between the tumor and the host in order to identify early changes related to the biological reactivity of the host to a developing cancer.

Keywords: Lung cancer; risk prediction; screening; diagnosis; prognosis; biomarkers

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Background

Lung cancer is the second most frequent cancer both in man and women and continues to be the leading cause of death from cancer, accounting for over 20% of all cancer deaths in 2012 in Europe (http://globocan.iarc.fr).

The overall 5 years survival rate for lung cancer has risen from only 12% to 16% in the past 4 decades, due largely to the late stage at which most patients are diagnosed. This rate is very small if compared to that observed for the other big killers, colon and breast cancer, where survival exceeds 70% and 50%, respectively. In contrast survival of patients undergoing lung resection for small intrapulmonary cancers is greater than 80%. Thus in lung cancer, more than in any other cancer, early detection is essential to improve survivability through identification and therefore treatment of patients before their cancers become inoperable and lethal.

Imaging modalities and biomarkers

Great enthusiasm was raised by the publication in 2011 of the results of the National Lung Screening Trial (NLST), a randomized clinical screening trial enrolling 53,454 persons with three rounds of low dose computed tomography (LDCT) annual screening versus chest radiographs (1). It demonstrated a 20% reduction of lung cancer mortality and 7% reduction of all cause mortality in favor of LDCT. However, after three rounds of screening, 24.2% of subjects were classified as positive with 96.4% of these being a false positive with the need to screen 320 subjects to prevent 1 lung cancer death.

In a recent paper from the same team the issue of overdiagnosis in the trial was estimated (2). The authors reported an overdiagnosis global rate of >18% and that the number of cases of overdiagnosis in the 320 subjects needed to be screened to prevent 1 lung cancer death is 1.38. Thus reduction of false positive rate after initial screen, as well as reduction of overdiagnosis by more efficient prediction of tumor aggressiveness, represents critical and still unmet clinical needs.

Recently the results of three smaller European LDCT

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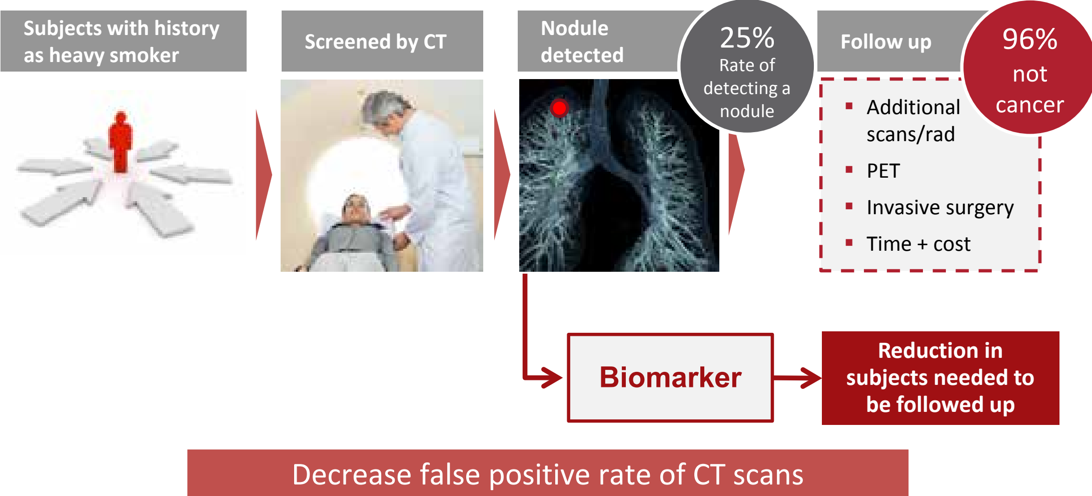

Figure 1 Clinical utility of biomarkers.

screening randomized trials were published and have reported non-significant mortality reductions (3-5). Two studies, the Multicentric Italian Lung Detection (MILD) (3) and the Danish Lung Cancer Screening Trial (DLCST) (5) showed a higher mortality in the screened LDCT arm and a meta-analysis of the four published studies demonstrated a small benefit in lung cancer mortality reduction (3).

In a systematic review of all randomized clinical trials that examined the benefits and harms of LDCT screening, the average nodule detection rate was around 25%, with 96% of nodules being benign. These high false positive rates of LDCT lead to multiple screening rounds and related radiation exposure, the use of unnecessary and sometimes harmful diagnostic follow-up and increased time and costs. The development of non-invasive complementary biomarkers could thus be very helpful for the reduction of subjects needed to be followed up and potentially to decrease false positive rate of CT scans and the over-diagnosis rate (*Figure 1*).

Biomarkers circulating in plasma or serum, if properly validated, could constitute the gold standard for a non invasive cancer diagnostics. In fact blood thanks to its rich content of different cellular and molecular elements that provide information on the health status of an individual, constitutes the ideal compartment to be tested for developing biomarkers. Moreover, blood samples can be easily and inexpensively collected by non invasive procedures throughout large clinical trials.

Several authors have based their biomarkers discovery strategy starting from the assumption that novel promising biomarkers are generated not only by cancer cells but also from the tumor microenvironment, the host response and their dynamic interaction. The cross talk among these components can be reflected in peripheral circulation and generate diagnostic and prognostic biomarkers and potentially, also biomarkers predicting the risk of disease development.

Table 1 reports the most promising candidate biomarkers for early lung cancer diagnostics detected in blood and their respective development phases according to the guidelines published in JNCI (6) and taking also into account the workflow for biomarkers validation described by other authors (7,8).

Several biomarkers have reached phase 3 which evaluates, as a function of time before clinical diagnosis, the capacity of the biomarker to detect preclinical disease. However, only few of them reached phase 4, prospective screening, which studies screen people and lead to diagnosis and treatment. None of them has reached so far phase 5, the final phase that will address whether screening with selected biomarkers will result in an overall benefit for the screened population by impacting on survival. A good biomarker should reduce the burden of cancer and would be not useful if it does not lead to change in treatments or outcomes and if it is only efficient in picking up indolent cancers.

However, concerning biomarkers, it must be recognized that there is a disconnection between promise and product and several reasons could be evoked:

- Discovery methods are often neither reliable nor efficient. This is in part related to the rapidly changing technology;

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Table 1 Circulating biomarkers for early lung cancer

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Candidates	Discovery, prediction	Assay validation	Retro-longitudinal	Prospective screening	Cancer Control
Autoantibodies (earlyCDT-test)	×	×	×	×	
C4d protein	×	×	×		
Serum microRNA	×	×		×	
Plasma microRNA (MSC test)	×	×	×	×	

- Selection of candidates: the choice of tumor-specific or high-throughput approaches. In particular genetic heterogeneity of tumors has limited the success of these initiatives;
- Reproducibility of the laboratory assays: several studies have to deal with over fitting, and lack of cross-validation and external validation;
- Most studies have poor design, just rely on case-control comparison and are not in the clinical context;
- The low concentration of analytes to be measured influences the reproducibility of the results;
- The availability of very few prospective collections of biological samples and in particular of bio-repositories related to screening trials.

Blood-based biomarkers

This review will focus on candidates' biomarkers circulating in serum or plasma since they are so far those that reached the more advanced validation phase.

All the studies selected in this review have validated their biomarkers in the context of LDCT lung cancer screening trials, by studying high risk subjects, and showed to be of value to predict the risk of lung cancer in asymptomatic individuals.

The biomarkers presented below are also examples of the value of searching candidates by looking not only to the tumor but also to the interplay between the tumor and the host in order to identify early changes related to the biological reactivity of the host to an incipient cancer.

Immune response biomarkers

C4d complement split product (9)—Phase of development: phase 2

These authors used an alternative approach not looking for cancer but for the immune response to cancer. In fact, immune activation may generate host-derived markers that are more homogeneous than cancer-derived markers. Immune responses against intracellular and surface tumor antigens are well documented in patients with lung cancer (10). In particular, the complement system is activated in lung tumor cells (11-14). Complement is a central component of innate immunity that plays an essential role in immune surveillance and homeostasis (15).

In their study these authors showed that lung tumors activate the classical complement pathway and generate C4d, a degradation product of this pathway and they evaluated if C4d may be of value for the diagnosis and prognosis of lung cancer.

They first examined plasma samples from 50 patients with early (stage I-II), clinically detected lung cancer and showed statistically significantly higher levels of C4d than those from 50 matched control subjects. The area under the ROC curve was 0.782 ($P<0.001$). Patients with higher levels of C4d (>3 µg/mL) had a statistically significantly shorter overall survival than those with low C4d levels ($P=0.002$). They also measured the levels of C4d in paired plasma samples (pre- and post-surgery) from 25 lung cancer patients with high (>2 µg/mL) C4d levels in the pre-surgery plasma. In all but one case, C4d levels were reduced after surgical removal of the tumor ($P<0.001$). As expected, in 19 patients with low plasma C4d levels (<2 µg/mL), the concentration of the marker did not change after resection of the tumor. These results provided evidence that plasma C4d levels depend on the presence of the tumor.

Plasma C4d levels were further evaluated in plasma samples from 190 asymptomatic individuals enrolled in a LDCT screening program. Thirty-two of them were diagnosed with lung cancer in the context of the program while the remaining 158 individuals had no evidence of cancer after LDCT screening. Both groups were matched

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Table 2 Performances of the autoantibody EarlyCDT®-Lung test

	Cases	Controls	Sensitivity	Specificity
Case-control studies	235	266	41%	91%
Clinical audit dataset	61	1,538	41%	87%
CT-detected lung nodules	43	146	44%	88%

by sex, age, and smoking history. Plasma C4d levels were statistically significantly higher in individuals with lung cancer than in individuals without the disease.

This result suggests that C4d levels may be of value to predict the risk of lung cancer in asymptomatic individuals. Additional validation sets are required to establish reliable cutoff values of this biomarker and it would be also critical to evaluate the performance of the test in specific clinical applications (e.g., in the context of a screening program) or in a cohort of prospectively collected patients presenting with one or more lung nodules discovered by chest LDCT.

Autoantibody signature (16)—Phase of development: phase 4

A more advanced and validated biomarker is the Autoantibody (AAB) signature developed by the group of Richardson JF in United Kingdom and now released by Oncimmune USA LLC.

It is well established that cancer patients produce autoantibodies to tumor proteins that are mutated, misfolded, ectopically presented, over-expressed, aberrantly degraded or anomalously glycosylated.

These authors discovered a 7 AAB signatures, previously 6 AAB, against oncogenes and TSG involved in lung cancer and also in other tumors: CAGE, GBU 4–5, HER2, p53, c-myc, NY-ES0-1 and MUC1. The strength of this AAB signature, called EarlyCDT-Lung test, is that it was validated in large series of patients and controls including either early and late stages tumors, NSCLC and SCLC. Across the various series, the signature showed high specificity, around 93%, but quite low sensitivity ranging around 40% in NSCLC and 55% in SCLC (*Table 2*) (16-20). However the test has the advantage to rely in an Elisa assay that is easily accomplished in a clinical laboratory.

In a recent paper (21) the test's performance characteristics in routine clinical practice were evaluated by auditing clinical outcomes of 1,600 US patients deemed at high risk for lung cancer by their physician, who ordered the EarlyCDT-Lung test for their patient.

The results obtained mirrored that of the extensive case–control training and validation studies previously reported (17-19,22). This audit has confirmed that EarlyCDT-Lung detects all types of lung cancer, all stages of the disease, and performs in clinical practice with the same sensitivity and specificity measured in the case–control studies. This is, therefore, the first autoantibody test that detects early stage lung cancer as shown with prospective validation data on a large number of individuals from a routine clinical practice setting (*Table 2*).

Recently Massion *et al*. evaluated the performance of the 7 AAB test in 189 lung nodules detected by LDCT, of which 43 malignant and 146 benign, and reported that EarlyCDT- Lung Oncimmune can provide significant discrimination between malignant and non-malignant lung nodules with sensitivity 44.2%, specificity 88.4%, PPV 52.8%, NPV 84.3%, with even better performance for nodules between 8-20 mm of diameter (*Table 2*) (unpublished data).

A prospective study is ongoing in Scotland (ECLS study) with the purpose to assess the value of the EarlyCDT-Lung test as a pre-CT screening tool. The study will enroll 10,000 people (50-75 yrs, smokers or ex-smokers) from Glasgow and the surrounding areas. Half of those taking part will be offered the EarlyCDT-Lung test (lung cancer test group). The other half (non-test group) will also have their blood taken, but it will not be tested as part of this study. People who have a positive lung cancer blood test will get a chest X-ray and a lung scan and 6 monthly scans for 2 years. However, only 1 in 9 people with a positive test is expected to develop LC within 2 years. People with a negative lung cancer blood test and those in the non-test group will not get any X-rays or scans will be monitored by their GP as normal: 98-99/100 people with a negative test are expected to not have LC at that time.

This study will potentially give insights on the utility of this biomarker as a first-line test to select subjects at increased risk for lung cancer development who need to undertake regular LDCT , potentially avoiding radiological exposure to low risk individuals with a negative test.

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Blood circulating miRNAs

Circulating microRNA in plasma and serum are promising biomarkers for a non invasive cancer diagnostics. After being transcribed in the nucleus, pre-miRNA molecules can be processed further by Dicer in the cytoplasm. In addition, based on recent findings there are at least two ways that pre-miRNAs can be packaged and transported using exosomes and MVBs or other (not fully explored) pathways together with RNA-binding proteins. After fusion with the plasma membrane, MVBs release exosomes into the circulating compartments and bloodstream. Likewise, pre-miRNA inside the donor cell can be stably exported in conjunction with RNA-binding proteins, such as NPM1 and Ago2, or by HDL (23). Circulating miRNAs enter the bloodstream and are taken up by the recipient cells by endocytosis or, hypothetically, binding to receptors present at the recipient cellular membrane capable of recognizing RNA-binding proteins. More studies are necessary to elucidate how miRNAs are loaded into exosomes and how they can be internalized by recipient cells. Exosomal miRNAs are processed by the same machinery used in miRNA biogenesis and thus have widespread consequences within the cell by inhibiting the expression of target protein-coding genes.

Thus, for their nature and biogenesis, miRNAs seem to remain rather intact and stable in biological fluids and, importantly, they are detectable quantitatively with simple assays (i.e., RT-qPCR) that are suitable also in a clinical context.

Serum-based 34 miRNA signature (24)—Phase of development: phase 4

The group of F. Bianchi at European Institution of Oncology (Milan, Italy) has developed a blood test for lung cancer diagnosis in asymptomatic high-risk individuals (heavy smokers, aged over 50) based on the detection of miRNAs from serum. Sera were collected from high-risk subjects enrolled in a large prospective early detection trial (the COSMOS study) for lung cancer by annual LD-CT. Starting from a total of 365 miRNA assay (microfluidic cards) the authors selected a pool of 147 miRNAs that were informative in a total of serum 253 samples from lung cancer screening patients and controls (COSMOS), symptomatic lung cancer patients and as a control group, a breast cancer and benign nodules series (*Figure 2*).

They used the training set to derive a diagnostic 34-miRNA signature capable of separating tumor from normal sera. As discriminant predictor a risk index was calculated based on the inner sum of the weights (*wi*) and expression (*xi*) of the 34 miRNAs greater than the threshold determined in the training set (*S wi xi*>3.235).

The performance of the IEO test in the validation set was 71% sensitivity, 90% specificity and 80% accuracy with better performance in stage II-IV only (30 normal/12 tumors) with 82% sensitivity, 90% specificity and 90% accuracy.

An analysis of the 34-miRNA model prediction strength in the testing set (all, 30 normal and 34 tumors) stratified by available clinical-pathological parameters showed odds ratio higher in Stage II-IV disease, in squamous carcinoma and in women.

When the 34-miRNA predictor was applied to evaluate the risk in a symptomatic set of 36 NSCLC patients and in 15 pulmonary hamartomas, it performed remarkably well.

By comparing the performance of the predictor in the normal sera of the testing set and in the sera of patients with the LDCT-detected benign nodules no significant differences in the average risk of the normal and nodule categories were found.

The authors also analyzed a group of sera collected before the onset of NSCLC (i.e., from patients who were negative at the screening round but who developed lung cancer >1 year after). For 13 of such cases, both the sera harvested before disease onset (BDO) and the tumor sera that were already included in the training or testing sets were available. When the risk predictor algorithm was applied, it indicated a significantly increased average risk index for sera collected after the onset of the disease (average risk BDO, 7.1; tumor, 10.4; P<0.001, paired *t*-test). Thus, at least in the cases analyzed, the 34-miRNA model was capable of detecting the conversion from a normal to a malignant state.

Finally, they tackled the question of the specificity of the 34-miRNA predictor for NSCLC detection, as opposed to other types of cancer, by screening sera from a cohort of 18 patients with invasive ductal breast carcinoma and 10 with breast benign nodules. When the 34-miRNA risk predictor algorithm was applied, it could not discriminate between breast tumors and benign breast nodules.

Plasma-based miRNA signature (25)—Phase of development: phase 4

In our first exploratory study we investigated miRNA profiles in plasma samples collected before and at time of disease detection in subjects enrolled in the first

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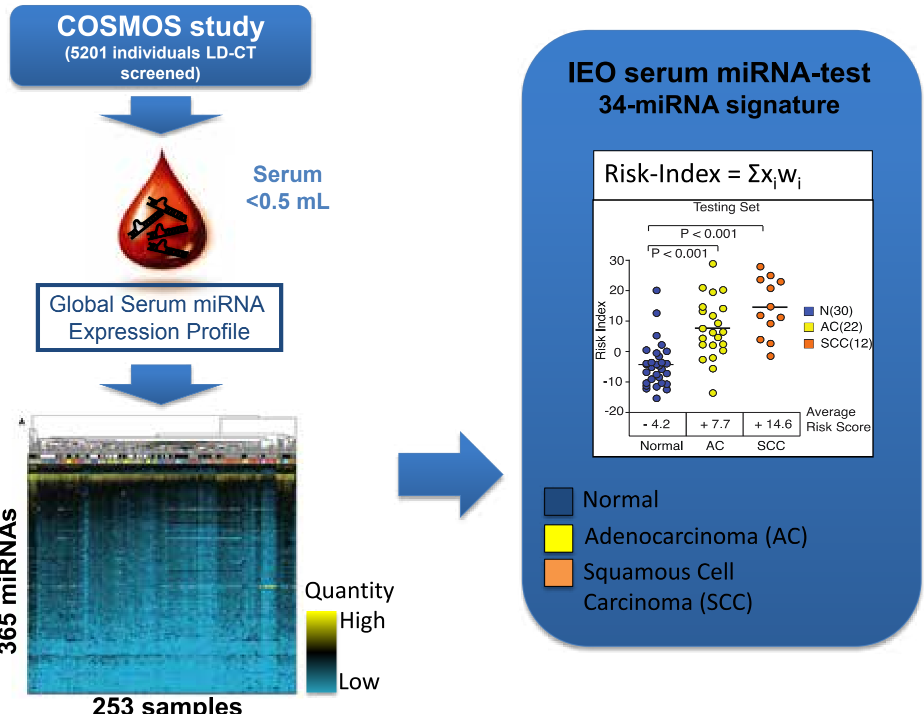

Figure 2 Flowchart of the COSMOS study.

observational trial and we validated selected miRNAs signatures in an independent series of subjects belonging to the randomized MILD trial (25). High-throughput miRNA expression profiles of plasma samples using TaqMan microfluidic cards and single assays for validation studies were performed and, importantly, we generated an original method to analyze data by looking at reciprocal miRNA ratios, an approach that allowed us to bypass the controversial issue of data normalization of miRNA in plasma. In this way, we identified 24 miRNAs whose reciprocal ratios were able to discriminate patients at risk of developing lung cancer and at risk for aggressive disease development in samples collected before disease detection, as well as diagnostic and prognostic signatures in plasma collected at the time of disease detection (*Figure 3*).

In order to have a more friendly and useful tool to classify plasma samples in clinical trials we recently generated a three-level risk categorization for disease: low, intermediate and high miRNA signature classifier (MSC) by combining the different signatures (*Figure 4*) and we used this pre-specified classifier to test diagnostic and prognostic performance in a Clinical Validation Study using the Multicentric Italian Lung Detection (MILD) Trial [2005-2012] cohort.

For this study, 1,000 consecutive plasma samples collected from June 2009 to July 2010 among lung cancer-free individuals enrolled in the trial were used to determine the specificity of the MSC. Plasma samples were first assayed for hemolysis to remove samples from patients that were potentially contaminated by red blood cells miRNAs (26,27).

Of the 1,000 samples, 130 were not evaluable because of hemolysis. Of the remaining 870 subjects, 594 (68%) belonged to the LDCT arms and 276 (32%) to the observational arm. To obtain a cohort for determining the sensitivity performance of MSC, plasma samples from almost all patients with lung cancer diagnosed by September 2012 were obtained (N=85). For 69 of these 85 patients, at least one evaluable sample was collected. For all patients we considered the sample closest to LDCT examination resulting in cancer diagnosis. Specifically, a sample at-diagnosis was available for 50 patients and a pre-disease sample for 19 patients. The pre-disease samples were collected from 8 to 35 months before lung cancer detection with a median lag time of 18 months.

Diagnostic and prognostic performance of MSC

MSC risk groups were examined for all 939 subjects according to lung cancer occurrence, lung cancer death,

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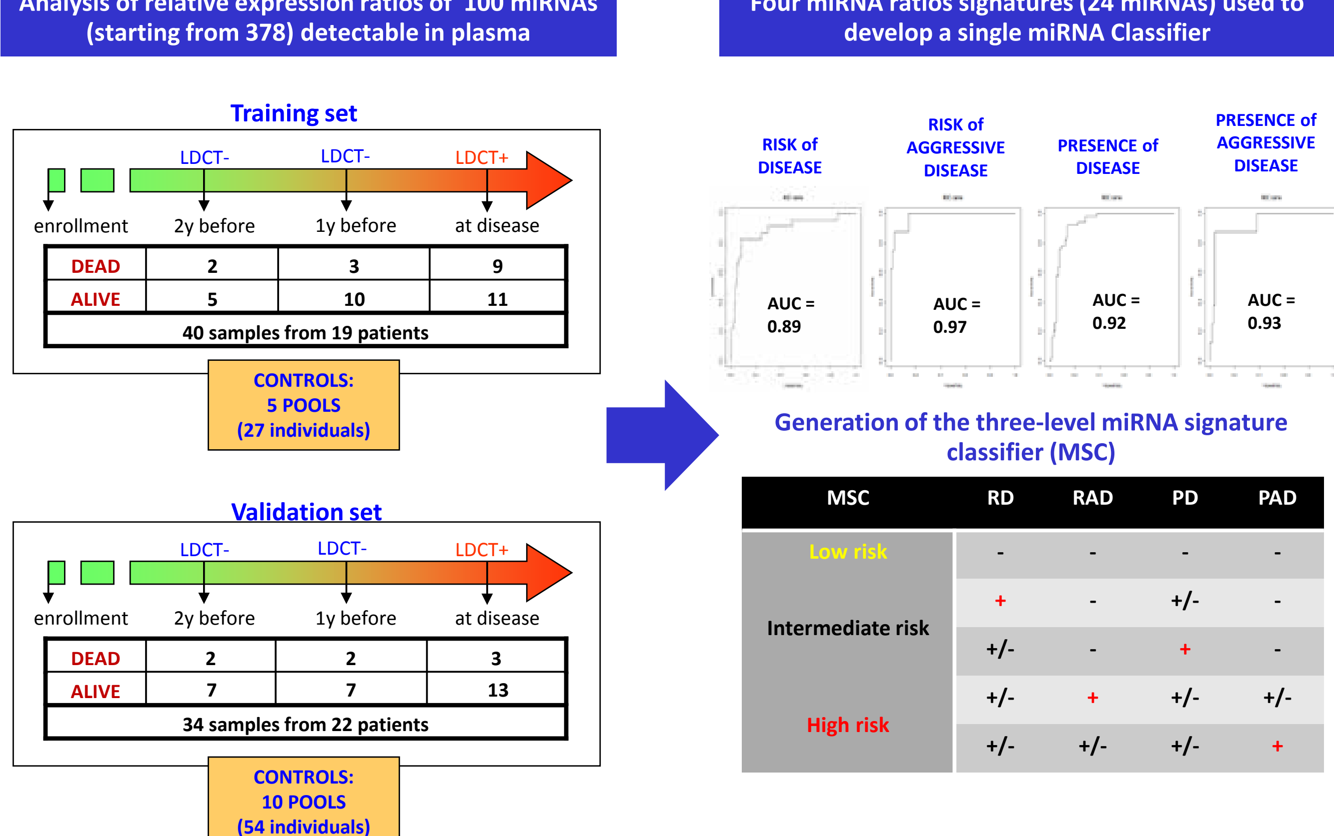

Figure 3 miRNA signatures discovery and initial validation.

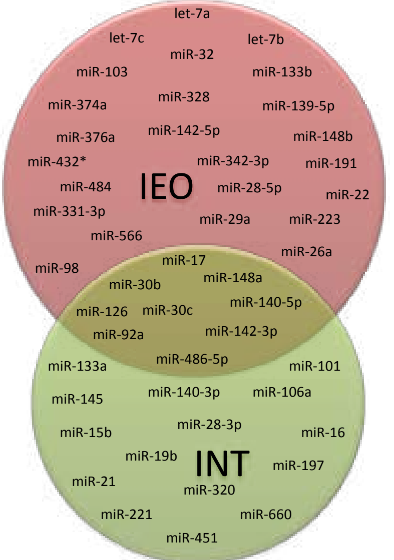

Figure 4 Comparison between serum- (IEO) and plasma-based (INT) miRNA tests.

and tumor stage. MSC Intermediate and High correctly classified 60 of 69 lung cancer patients with 87% SE, 81% SP, 27% PPV and 99% NPV (*Table 3*). MSC risk groups were not significantly associated (P=0.40) with varying tumor stage (I, II-III or IV). No significant differences were observed between MSC risk groups and histological subtypes (χ_1^2=1.60, p=0.4485), and between adenocarcinoma and squamous cell carcinoma (χ_1^2=0.55, P=0.759).

Time dependency analysis of diagnostic performance of MSC, showed similar values of SE, SP, PPV and NPV at 6-, 12-, 18- and 24-month intervals between blood sampling and lung cancer diagnosis supporting a strong diagnostic performance of MSC to predict LC development up to 24 months before disease detection.

Complementary diagnostic performance of LDCT and MSC

Restricting the analysis to the total of 652 subjects in the

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Table 3 Overall diagnostic performance of MSC

	Total	MSC (risk of lung cancer)		
		High (%)	Intermediate (%)	Low (%)
All subjects	939	63 (6.7)	159 (16.9)	717 (76.4)
No lung cancer	870	32 (3.7)	130 (14.9)	708 (81.4)
Lung cancer	69	31 (44.9)	29 (42.0)	9 (13.0)

MSC, miRNA signature classifier.

LDCT arm, LDCT identified 46 of 58 lung cancer subjects missing three patients with no pulmonary nodule detected and nine patients because of an interval cancer for a SE of 79%. Pre-specified binary risk groups of MSC (considering High and Intermediate versus Low) identified 40 of 46 LDCT-detected cancers, 8 of 9 interval cancers and all three subjects with "no pulmonary nodule".

LDCT had a SP of 81% for the clinically actionable subgroup of non-calcified nodules >5 mm and an associated false positive rate of 19.4% (115/594). When double-positive (LDCT and MSC) subjects were considered, the false positive rate decreased to 3.7% (22/594), with a decrease in SE (40/58, 69%). On the other hand, MSC detected 9 of 11 (82%) lung cancers that occurred in the observational arm.

The 5-fold reduction in false positives obtained by combining the MSC Lung Cancer assay to the results of the LDCT scan is of great clinical relevance in the context of reducing the false positive rate and the potential side effects associated with repeated LDCT scans or other unnecessary invasive diagnostic follow-ups.

Association of MSC risk groups with survival

The prognostic performance of the three pre-defined MSC risk groups to predict overall survival from plasma samples collected for all subjects with 3-year follow-up (N=939) was also evaluated. Three-year survival was 100%, 97% and 77% for Low, Intermediate and High respectively. The difference in survival between High/Intermediate and Low MSC was statistically significant (χ_1^2=49.53, P<0.0001) also after adjustment for age and gender (χ_1^2=12.57, P=0.0004).

This correlative study in lung cancer is the first of its kind, validating a biomarker using prospectively collected blood samples from a large randomized lung cancer screening trial. In addition to a significant reduction in the rate of false positive results, the performance of the MSC Lung Cancer assay was independent of the stage of lung cancer, as well as the time prior to detection of cancer with LDCT. This suggests additional potential utility for diagnosis and early detection with the MSC Lung Cancer assay.

Comparison between serum and plasma-based miRNA tests

Between the two miRNA signatures developed in serum and plasma, only nine miRNAs were overlapping, suggesting the relevance of this core of miRNAs for early lung cancer diagnosis (*Figure 4*).

The differences in the remaining miRNAs composing the signatures may be likely related to the type of biological samples used (i.e., serum *vs*. plasma) and the study design. In fact, our findings and those reported in literature suggest that miRNAs not released in physiological process, as during the cell lysis that occur during clot formation in serum samples, have a different physical state than miRNAs physiologically released and protected by lipoproteic complex or microvesicles (28,29). Moreover, the plasma signature was trained in samples of patients collected also before (and at the time of) disease detection, thus reflecting earlier, microenvironment-related changes whereas the serum-based signature was trained in serum samples of patients at the time of lung cancer diagnosis likely detecting more advanced tumor-specific changes.

A large validation phases in two different prospective screening trials in ongoing for both miRNA tests.

Conclusions

Early detection candidate biomarkers exist but only few of them are validated or tested in screening settings. The priority is now to validate existing candidates.

Biomarkers should provide knowledge about added value and therefore should be integrated to clinical, laboratory and imaging (LDCT) routine data.

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To demonstrate clinical utility requires significant investment in effort and resources towards prospective biomarkers driven clinical trial.

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Development of cancer diagnostics—from biomarkers to clinical tests

Yixin Wang

Ventana Medical Systems, Inc., Tucson, Arizona 85755, USA

Correspondence to: Yixin Wang, PhD. Ventana Medical Systems, Inc., A Member of the Roche Group, 1910 E. Innovation Park Drive, Tucson, Arizona 85755, USA. Email: ywang44@gmail.com.

Abstract: New biomarkers and methods have been emerging to improve cancer diagnosis, classification of cancer subtypes, prognosis and prediction of response to therapy. Insights gained from the role and significance of the biomarkers in tumor tissues and cells will aid in understanding tumorigenesis, metastasis and other disease processes. Diagnostic tests based on such information should enable more precise and objective decision-making about cancer staging, progression and response to therapy. On the other hand, many of the diagnostic techniques that are employed today in medicine have not changed over several decades. The fact highlights the challenges faced by new molecular and cellular technologies in having a real impact on patient management in clinic. One of the key challenges is to demonstrate the clinical value of a diagnostic test. In addition to clinical value, a routine test in clinic needs to be optimized so that the assay can fit into the clinical laboratory workflow and the assay result can be generated timely and reproducibly. The review will focus on development of molecular and cellular diagnostic assays that have the potential to aid clinical decision-making and patient management in oncology. The process described here demonstrates the steps to translate and develop novel biomarkers into quality diagnostic tests that can be readily deployed into clinical laboratories. The examples referenced here illustrate how tissue- and cancer-specific biomarkers, coupled with new molecular technologies, can add value to conventional diagnostic methods by providing standardized, objective and highly informative diagnostic tests.

Keywords: Analytical; biomarker; clinical; molecular diagnostics; translational research

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Introduction

The advent of molecular technologies has revealed a wealth of information about signaling pathways and gene regulation in cancer. New biomarkers and methods for classification of cancer subtypes, diagnosis, prognosis and prediction of response to therapy have been emerging. Advancements in analytical methods in molecular biology, such as polymerase chain reaction (PCR), deoxyribonucleic acid (DNA) arrays and next-generation sequencing have allowed researchers to interrogate a vast type of biological and clinical materials such as formalin-fixed, paraffin-embedded (FFPE) tissue, biopsies and cells present in blood, bone marrow or urine (1-8). Insights gained from the role and significance of the biomarkers in tumor tissues and cells will aid in understanding tumorigenesis and metastasis processes. In addition, the recent finding that circulating tumor cells (CTCs) and circulating DNA in blood can also have diagnostic value in metastatic cancers allowing clinicians to use them as surrogate endpoints (9,10). Diagnostic tests based on such information should enable "real time" biopsies of cancer progression and response to therapy. These new molecular and cellular technologies will enable more precise and objective decision-making.

On the other hand, many of the techniques that are employed today by pathologists and oncologists to generate a diagnosis, prognosis or prediction of therapy response have not changed over several decades. The fact highlights

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the challenges faced by new molecular and cellular technologies in having a real impact on patient management in clinic. One of the key challenges is to demonstrate the clinical value of a diagnostic test. For example, in the area of susceptibility/risk assessment, companies have commercialized molecular tests on the *BRCA1* and *BRCA2* genes for breast cancer (11). In the area of prognosis and prediction for therapy response, reverse transcription polymerase chain reaction (RT-PCR) based Oncotype Dx assay have also been adopted for breast cancer in predicting patients' benefit with chemotherapy (12). In addition, *in situ* hybridization (ISH) assays based human epidermal growth factor receptor 2 (Her-2) test and anaplastic lymphoma kinase (ALK) test have been used to predict responses to targeted therapies such as Herceptin and Xalkori in breast cancer and lung cancer, respectively (13,14). In addition to clinical value, a routine test in clinic needs to be optimized so that the assay can fit into the clinical laboratory workflow and the assay result can be generated timely and reproducibly.

The review will focus on development of molecular and cellular diagnostic assays that have the potential to aid clinical decision-making and patient management in oncology. The process described here demonstrates the steps to translate and develop novel biomarkers into quality diagnostic tests that can be readily deployed into clinical laboratories. The examples referenced here illustrate how tissue- and cancer-specific biomarkers, coupled with new molecular technologies, can add value to conventional diagnostic methods by providing standardized, objective and highly informative diagnostic tests. These new tests will impact not only the business of diagnostics from a low margin, single measurement science to a high value, information intensive science, but also, with acceptance by clinicians, the way medicine is practiced in the future.

Assay development process

Thousands of papers published every year reveal new genes as potential biomarkers for cancer diagnosis. However, a few of these biomarkers are really used as cancer diagnostics in clinic. *Figure 1* highlights the process to establish a specific biomarker as a diagnostic assay, which will require discovery of biomarkers, translational research, develop the biomarkers into diagnostic assays, incorporation of the assays in clinical trials to correlate the biomarkers with therapeutic responses and patient outcomes (*Figure 1*). One need to go through the entire development of a diagnostic assay with the new marker(s), which ensures that the assay used in the clinical trials are highly robust and reproducible to detect the intended disease state.

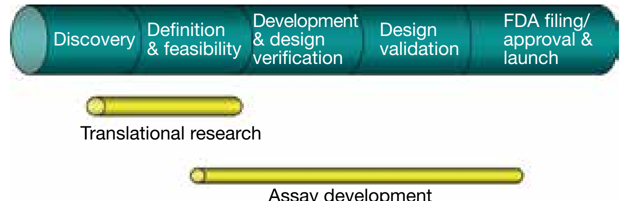

Figure 1 Process for assay development. Discovery is directed towards fundamental understanding of biology and disease processes. It provides the foundation for *translational research* and assay development. Translational research moves discovery results from concept into clinical evaluation and is often focused on specific clinical unmet need. *Assay Development* is directed towards improving the assay performance itself and validating the pre-determined assay format using relevant clinical specimens.

Biomarker discovery

Studies have shown that a wide variety of genomic changes, such as amplifications, translocations, deletions and point mutations may be present in a given type of cancer. Analysis of these genomic alterations led to the identification of oncogenes and tumor-suppressor genes involved in cancer development. Cancer development on the other hand is not restricted to genetic alterations. It can also be traced to epigenetic changes and changes in gene and protein expression levels. Studies of alterations in genetic, epigenetic and expression processes can help establish diagnostic biomarkers of tumors and classification of tumors based on recognition of complex molecular profiles or unique molecular alterations that occur in specific tumor types. However, it's very difficult to achieve such objectives in practice for several reasons: the cross talk of different cancer-related pathways complicates the understanding of cancer biology; there is considerable heterogeneity in the tumor and functions of the genes among individuals with same types of cancers; the treatment targets are not absolutely specific to cancer cells; the effectiveness of the treatments is limited because the targets are affected by other factors and the functions of the targets may change over time and produce resistance to the treatment.

Cancer diagnosis is mainly carried out by examining morphology and antibody staining in biopsy or resected

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tissue samples. The recent development of diagnostic methods based on analyses of CTC and circulating DNA (ctDNA) in blood opened new avenues for cancer detection and prognosis (9,10). CTCs and ctDNA from cancer patients are now analyzed to detect tumor markers such as mutations, microsatellite instability, hypermethylation and gene expression. It is also possible to detect cancer cells from other body fluids such as saliva, urine, broncho alveolar lavage, sputum and ductal lavage because epithelial tumors grow and cancer cells can be sloughed off the tumors into body fluids. This makes it possible to detect molecular markers using these samples.

In situations where not much is known about a particular disease state, there is a need for discovery of biomarkers that determine the cause(s) of the disease or the genetic basis of susceptibility of the disease. Examples of high-throughput molecular discovery tools include genomics and next-generation sequencing. Clearly this discovery step of biomarkers is required for developing diagnostic assays, but there is sometimes a tendency to jump to the conclusion that the technology used for biomarker discovery can automatically be used as a diagnostic tool in clinic. It is worth noting that when considering the use of the biomarker and the technology platform in a clinical laboratory, additional development needs to be carried out in order to meet specific requirements in clinical practice, including facility and infrastructure requirements, labor and ancillary laboratory equipment needs and the cost structure. These can, in theory, all be overcome, but this is a reality that most biomarkers and discovery technologies can't be directly used as diagnostics. The question remains as to whether or not the biomarker and the technology can become a clinically and economically feasible clinical tool. To answer the question requires a time- and resource-intensive development process.

Sample preparation

Sample preparation is a key pre-analytical step in diagnostic assay development. It ensures that the appropriate type of specimen is collected with a standard method and the handled in order to preserve specimen integrity. As to development of molecular assays, once the sample is obtained, it is important to confirm that DNA, RNA and proteins of the sample is stable and the specimen integrity is maintained during collection and transport. Numerous factors such as storage and transport time can affect quality of the specimen. Poorly handled samples may produce false negative test results. It's necessary to verify the sample collection and handling conditions. Similarly, the handling and storage conditions of test reagents must be tracked and monitored to ensure that their composition, concentration, and function are well maintained. Other important factors include standard documentation, ensuing that personnel have been adequately trained and that laboratory equipment is correctly calibrated and functions properly.

Biospecimen repositories and biobanks will play an increasingly important role in development of diagnostic assays. The integrity of the samples and the availability of associated clinical data are vital to analytical verification and clinical validation of the diagnostic assay. In some cases, prospective studies will need to be undertaken. Throughout diagnostic assay development, access to patient specimens and detailed clinical data is a key requirement for all the stages of the development process. In certain cases such as prognostic assay development, patient outcome data will be required, dictating either that prospective trials be conducted or that retrospective studies on archived material be performed. The latter choice is attractive because commercialization of new assays can be accomplished sooner. There are millions of specimens in biobanks throughout the USA managed by clinical trials cooperative groups, academic institutions and individual investigators (15), and the National Cancer Institute has been working to unify these biobanks through a National Biospecimen Network (NBN). Other repositories also exist. For example, the Breast Cancer Family Registry has enrolled nearly 12,000 families containing individuals with a wide range of familial risks of breast cancer (16). It is an excellent source of tissue and data for studies that require large numbers of samples with epidemiological, clinical and molecular data. On the other hand, one should note that using banked samples collected from different clinical institutes has its own set of risks. First, a lack of standardization in tissue acquisition and annotation across laboratories should be dealt with. Secondly, the integrity of samples and isolated nucleic acids may vary widely across sites, depending on age of sample, fixation method, storage method, and so on. Lastly, the clinical data must be available, properly annotated and sorted through very carefully to ensure its proper association with the corresponding sample. Despite these limitations, archived samples remain a rich source of tissue and clinical data and will become a fixture of diagnostic assay development in the molecular medicine era.

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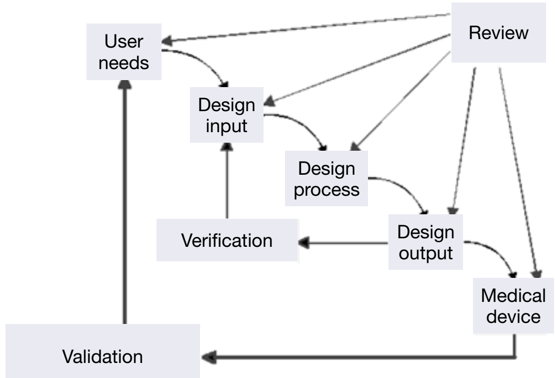

Figure 2 Application of design controls to assay development process (Adapted from FDA: Center for Devices and Radiological Health. Design Control Guidance for Medical Device Manufacturers).

Analytical assay development

Analytical verification and clinical validation of candidate biomarkers from discovery involve the identification of contributing factors that affect test accuracy, reproducibility and interpretation (*Figure 2*). *Figure 2* illustrates the definitions of each step in diagnostic assay development, especially verification and validation under design control. In developing a diagnostic test, one should recognize that the amount of process control required depends on the nature of the assay, the degree to which test reagents have been validated, the technology platform used for testing, and the assurance that specific regulatory approvals have been addressed, and that evolving knowledge regarding assay utility is incorporated into practice. For analytical and clinical assays to be considered reliable, it also requires statistically verifiable and reproducible results and a quality assurance process capable of aligning the various process elements with emerging knowledge. Accurate detection and quantification of the biomarker requires an assay capable of generating a reproducible result between the amount of input target in the sample and the output signal of the assay. Design and development of reproducible assays depend on the use of standards, reference materials and calibrators that contain known amount of the target. Samples containing concentration below the limit of detection of the assay will yield signals similar to the background noise of the assay. As the amount of input target in the sample is increased, a liner or near-linear signal response occurs over the assay's dynamic range or range of quantification. The analytical lower limit of quantification is defined by the concentration at which the target can be detected with acceptable precision and accuracy. When the upper limit of quantification is reached, the signal saturates. Most conventional assays have a dynamic range of about or less than 3-4 logs (e.g., ELISA assays). Molecular assays may have dynamic ranges of 5-7 logs or more. Many analytical factors are important for relating the input concentration to the output signal of an assay. For example, the analytical accuracy of the assay assesses the degree of agreement between the measured target and the true value of that target. Analytical accuracy is typically assessed through comparison of a new method to an established test or a different type of test. Precision refers to the agreement of independent test results under defined conditions. Intra-assay precision refers to test reproducibility in the same analytical run. Inter-assay precision concerns test reproducibility among different runs. Precision among days, sites, lots and batches can also be assessed. Protocol standardization and documentation are required to facilitate the comparison, validation and integration of the assay and avoid variation of the test results from different laboratories.

Data interpretation

How test results are interpreted and acted upon represents an integral part of diagnostic assay development. The more accurate information conveyed to the doctor regarding the analytical and clinical performance of a test, the more likely that the appropriate clinical decisions will be made. Unfortunately many publications do not clearly describe or articulate the difference between analytical and clinical sensitivity and specificity. As the result, the implications of positive or negative test results for diagnosis and management are not sometimes clearly conveyed. For example, the analytical sensitivity is the smallest amount of target that can be reproducibly detected by a test. This is distinctly different from clinical or diagnostic sensitivity that is generally considered to reflect the ability of a test to correctly identify individuals who have an illness or specified clinical disorder. Analytical specificity is the ability of a test to accurately distinguish the target of interest from other substances in the sample. The clinical or diagnostic specificity is the ability of a test to identify people who do not have the illness or specified clinical disorder.

Quality assurance of the assay reports can also be challenging. If multiple analytical tests are used to assess the clinical status of patients, clinicians may not be aware of the appropriate tests to request or be knowledgeable about

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the precise interpretation of the results. Another reality is that clinicians often deal with multiple laboratories, each of which may be involved in performing different tests. As a result, no comprehensive summary of the test results may be available. Individuals may also be seen by multiple clinicians who may not have the results of previous tests. Similarly, a laboratory may not have access to a previous result that might help it ensure that the appropriate testing algorithm is performed. In the long term and especially when test complexity is high, it is likely that an electronic longitudinal patient record of test results would be an effective way of ensuring that test results are available to support best clinical practices.

Because of the rapid evolution of both diagnostics techniques and therapeutic interventions, a need clearly exists for greater cooperation between clinicians, laboratories, researchers, and regulatory authorities to better define the analytical and clinical performance characteristics of tests. Proper validation of complex assays with sufficient statistical rigor must be thought of as a requirement rather than an optional step in the commercialization of the assays (17-19). For example, the validation studies should be based on patient cohorts that are sufficiently homogeneous for the test to be developed. The patient cohort in the validation set should be independent of the training set. Both training and testing sets should be large enough to enable the investigator to employ either cross-validation or split sample validation. Regulatory agencies such as Food and Drug Administration (FDA) may have suggested criteria on sample size determination as well (CFR - Code of Federal Regulations Title 21). FDA classifies *in vitro* diagnostic device (IVD) products into Class I, II, or III according to the level of regulatory control that is necessary to assure safety and effectiveness. The classification of an IVD (or other medical device) determines the appropriate premarket process. Independent validation is a prerequisite for adoption into clinical practice. Such validation studies should employ a 'locked' version of the assay, algorithm and cutoffs and should be of sufficient size to permit determination of the accuracy of the assay result, with confidence intervals.

Examples of cancer diagnostic assays

Biopsy and diagnosis of carcinoma of unknown primary (CUP)

CUP refers to wherein metastatic disease is present without an identifiable primary tumor site. It represents approximately 3-5% of all cancers (20). The prognosis and therapeutic regimen of cancer patients are dependent on the origin of the primary tumor, underscoring the need to identify the site of the primary tumor.

A variety of methods are currently used to resolve this problem. Immunohistochemical (IHC) markers, using panels of 4-14 tissue specific markers to improve sensitivity and specificity and identify tumor of origin, have demonstrated accuracies of 66-88% (21). More expensive diagnostic workups include imaging methods, such as chest X-ray, computed tomographic (CT) scans, and positron emission tomographic (PET) scans. Despite these sophisticated technologies, the ability to resolve CUP cases is only 20-30% ante mortem.

A promising new approach lies in the ability of gene expression or microRNA profiling to identify the origin of tumors (22-25). The technologies are able to utilize FFPE tissue of the metastatic tumor, since fixed tissue samples are the standard material in current practice. qRT-PCR has been shown to generate reliable results from FFPE tissue but, from a practical point of view, requires a smaller set of tissue specific gene markers. The assays are currently provided as CAP/CLIA laboratory service.

Diagnosis of prostate cancer

Prostate cancer is the second leading cause of male cancer-related death in the US, and its prevalence increases with age. In men with elevated prostate-specific antigen (PSA) levels or abnormal digital rectal examination (DRE) findings, the standard for prostate cancer detection has been trans-rectal ultrasound-guided sextant needle biopsy, a method introduced in 1989 by Hodge (26). However, the sensitivity of biopsy may be suboptimal, especially for larger and eccentrically shaped prostates, with false-negative rates as high as about 20% (26).

Insights into the molecular pathogenesis of prostate cancer have identified new markers. For example, *glutathione-S-transferase P1 (GSTP1)* gene encodes the glutathione-*S*-transferase π enzyme, which is a member of a large family of glutathione transferases that function to protect cells from oxidative insult. *GSTP1* has been extensively studied in prostate cancer, and its reduced expression, predominantly due to promoter hypermethylation, represents the most common epigenetic alteration associated with prostate cancer (27). Several studies have shown a high sensitivity for *GSTP1* to detect the presence of both prostatic intraepithelial

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Table 1 Comparison of commercial prostate cancer assays

Commercial	Product	Description
GenProbe	PROGENSA® PCA3 Urinary Assay	Primarily identifies those at higher risk for Prostate cancer (head-to-head versus PSA)
Myriad genetics	PROLARIS™	Determines the risk of recurrence in patients who have undergone RP surgery
Genomic health	Oncotype Dx	Identifies patients who are at low risk of disease progression
GenProbe	T2:ERG Urinary Assay	A new urinary assay that can detect prostate cancer and differentiate aggressive from less aggressive disease.

Table 2 Comparison of the discovery data of two key breast prognostic assays

Variables	MammaPrint	Oncotype Dx
Global gene expression	Yes	No
Signature	70 genes	21 genes
Assay	Microarray	RT-PCR
Other independent factors	T stage and N	Grade
ER+/ER– patients	Both	ER+ only
T sizes	T <5 cm	All
Pre/postmenopausal	<53 years	Both
Independent validation	Yes	Yes
Tissue	Frozen	Paraffin

RT-PCR, reverse transcription polymerase chain reaction.

neoplasia and prostate cancer, an ability to distinguish these from benign prostatic hyperplasia (BPH), and a prevalence of methylation in the range of 70-90% in prostate cancer (28-30).

A second dilemma exists in prostate cancer screening. Currently, screening is accomplished using DRE and measurement of PSA levels in serum, which is sufficiently sensitive but not specific to render a diagnosis of cancer (31). Confirmatory diagnosis via a trans-rectal biopsy is required. Prostate cancer screening could benefit from a test that demonstrated a high specificity and that could be used in conjunction with PSA testing, in order to determine which patients should actually undergo a biopsy. In fact, the methylation detection of several molecular markers could also have clinical utility in the screening setting. In addition, a different assay proposed for use in this setting is based on detection of the mRNA for two genes, *prostate cancer antigen 3 (PCA3)* and *PSA* (31-33) (*Table 1*).

Prognosis of breast cancer

Breast cancer is a heterogeneous disease that exhibits a wide variety of clinical presentations, histological types and growth rates. As a result of these variations, determining prognosis for an individual patient at the time of initial diagnosis requires careful assessment of multiple clinical and pathological parameters; however, traditional prognostic factors are not always sufficient to predict patient outcomes accurately (34,35). In primary breast cancer, metastasis to axillary lymph nodes is the most important clinical prognostic factor. Approximately 60-70% of lymph-node-negative (LNN) patients are cured by local–regional treatment alone (35), while most patients who relapse will eventually die from their disease. Therefore, identification of those patients that are at high risk for relapse would enable a physician to prescribe adjuvant systemic therapy selectively to those patients without giving adjuvant therapy to all LNN patients.

Genomic Health (CA, USA) has commercialized the Oncotype Dx assay, a set of 16 signature genes in their RNA expression and five control genes (11,36,37). As the signature was developed using data from patients who were treated with tamoxifen, it is not purely prognostic and is valid for estrogen-receptor-positive (ER+) patients initially and subsequently validated in other subtypes of breast cancer. Agendia (Amsterdam, The Netherlands) is commercializing another product for use in this setting referred to as the Mammaprint assay. It is a 70-gene signature based on the early work by Van't Veer and colleagues (38-41). The signature is valid for women under the age of 55. These two assays have been offered as reference laboratory services commercially (*Table 2*). Other assays in this area use a 76-gene signature or a ratio of the expression of two genes and is proposed to predict recurrence in patients treated with adjuvant tamoxifen (42,43).

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Circulating tumor cells (CTCs)

CTCs are rare, occurring at a frequency of one tumor cell for every million peripheral blood mononuclear cells (10). The number of patients exhibiting CTCs, and their absolute numbers of CTCs per patient increase as clinical stage rises (38). A 10,000-fold enrichment of CTCs in blood can be achieved by the use of ferrofluids linked to antibodies to the transmembrane glycoprotein epithelial cellular adhesion molecule (EpCAM) (44-47). For example, CellSearch could detect, enumerate and characterize CTCs, defined as nucleic acid-positive/CD45-negative/cytokeratin-positive, in the blood. Using the technology platform, studies can be designed to assess the clinical significance of CTCs in metastatic breast, colorectal and prostate cancer. Allard and colleagues (48) have demonstrated that the enumeration is linear over two logs (5-1,142 cells), that only one of 344 (0.3%) of healthy subjects had two or more CTCs per 7.5 mL of blood, and that, in 2,183 blood samples from 964 metastatic carcinoma patients, CTCs ranged from 0 to 23,618 per 7.5 mL of blood, with 36% exhibiting two or more CTCs. Of the major cancers, a larger percentage of prostate (57%) and breast (37%) cancer patients exhibited two or more CTCs. In a prospective, multicenter study, 177 patients with metastatic breast cancer were tested for levels of CTCs before treatment and at the first follow-up visit (43,49,50). Multivariate Cox proportional-hazards regression demonstrated that the levels of CTCs at baseline and at the first follow-up visit were the most significant prognostic factor of progression-free survival (PFS) and overall survival (OS).

Circulating endothelial cells (CECs) have also generated interest as a surrogate marker as CEC levels correlate with disease progression and reflect changes in the VEGF pathway (51,52). Angiogenesis plays an essential role in the growth and metastasis of tumors (51). Therefore, various anti-angiogenic agents are under development, targeting the vascular endothelial growth factor (VEGF) pathway (51). Reduction in the number of CECs accompanied a reduction in peripheral blasts in patients with refractory hematological malignancies who were treated with a microtubule inhibitor (53,54).

Enrichment of circulating cells can also enable a number of downstream applications. O'Hara and colleagues coupled *in vitro* transcription with multigene RT-PCR to analyze expression of 37 genes in CTCs (55). Smirnov and colleagues have amplified the RNA extracted from the CTC-enriched and CTC-depleted portions and applied this material to DNA arrays and have analyzed RNA extracted from enriched CTCs using qRT-PCR (56). Fehm and colleagues performed fluorescence *in situ* hybridization on CECs and demonstrated that patients had CECs that showed abnormal copy numbers (57).

Future directions

Novel diagnostics offers high sensitivity and high specificity in detection of cancer disease. In addition to the high sensitivity and specificity, these assays become accepted in clinical diagnostics owing to the ease with which they can be configured to detect almost any target, their requirement for minimal quantities of sample and their ability to be automated. Recent advances also allow such assays to be configured in a multiplex format, enabling simultaneous detection of multiple markers, which can be used to facilitate treatment of the disease. In addition, molecular markers of disease are stable, and the same assay chemistries can be used to develop diagnostic tests regardless of the type of disease being tested for.

There is a vast array of new technologies available, and they all have specific trade-offs with respect to speed, ease of use, throughput, multiplex level, ability to quantify, cost, availability of platform and resolution. It is important to determine the particular application for the test and specificity and sensitivity required for the application. Careful evaluation of specific needs will allow assay developers to choose solutions that are optimal for their specific needs. Failure to consider the strengths and weaknesses of each option will result in unnecessary costs and may limit effectiveness.

The discovery, validation, commercialization and clinical adoption of novel cancer diagnostic assays will change the paradigm of medical practice from single measurement, pathology- and clinical exam-driven decisions to more of an integrative approach in cancer patient management. Combining new medical content with emerging technologies and informatics will enable personalized medicine to reach its full potential. However, before these new technologies can reach the clinicians, issues in marker validation, sample acquisition and assay and platform development will have to be addressed. The focus of effort will have to shift from purely biomarker discovery to a more comprehensive approach that combines marker discovery, translational research, assay development and clinical validation.

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Molecular testing in lung cancer in the era of precision medicine

Helmut H. Popper[1], Ales Ryska[2], József Tímár[3], Wlodzimierz Olszewski[4]

[1]Research Unit for Molecular Lung & Pleura Pathology, Institute of Pathology, Medical University of Graz, Austria; [2]The Fingerland Department of Pathology, Charles University Faculty of Medicine and Faculty Hospital in Hradec Kralove, Czech Republic; [3]2nd Department of Pathology, Semmelweis University, Budapest, Molecular Oncology Research Group, HAS-SU, Budapest, Hungary; [4]Department of Pathology, Cancer Center, 5 Roentgen Str. 02-781 Warsaw, Poland

Correspondence to: Univ. Prof. Dr. Helmut H. Popper. Research Unit for Molecular Lung & Pleura Pathology, Institute of Pathology, Medical University of Graz, Auenbruggerplatz 25, Graz, 8036, Austria. Email: helmut.popper@medunigraz.at.

Abstract: The clinical expectations how pathologists should submit lung cancer diagnosis have changed dramatically. Until mid 90-ties a clear separation between small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) was mostly sufficient. With the invention of antiangiogenic treatment a differentiation between squamous and non-squamous NSCLC was requested. When epidermal growth factor receptor (EGFR) mutation was detected in patients with pulmonary adenocarcinomas and subsequent specific treatment with tyrosine kinase inhibitors (TKIs) was invented, sub-classification of NSCLC and molecular analysis of the tumor tissue for mutations was asked for. Pathologists no longer submit just a diagnosis, but instead are involved in a multidisciplinary team for lung cancer patient management. After EGFR several other driver genes such as echinoderm microtubule associated protein like 4-AL-Kinase 1 (EML4-ALK1), c-ros oncogene 1 , receptor tyrosine kinase (ROS1), discoidin domain receptor tyrosine kinase 2 (DDR2), fibroblast growth factor receptor 1 (FGFR1) were discovered, and more to come. Due to new developments in bronchology (EUS, EBUS) the amount of tissue submitted for diagnosis and molecular analysis is decreasing, however, the genes to be analyzed are increasing. Many of these driver gene aberrations are inversions or translocations and thus require FISH analysis. Each of these analyses requires a certain amount of tumor cells or one to two tissue sections from an already limited amount of tissues or cells. In this respect new genetic test systems have been introduced such as next generation sequencing, which enables not only to detect multiple mutations in different genes, but also amplifications and fusion genes. As soon as these methods have been validated for routine molecular analysis this will enable the analysis of multiple genetic changes simultaneously. In this review we will focus on genetic aberrations in NSCLC, resistance to new target therapies, and also to methodological requirements for a meaningful evaluation of lung cancer tissue and cells.

Keywords: Non-small cell lung carcinoma (NSCLC); molecular pathology; target (driver) genes; tissue based assessment

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Introduction

Within the last decade many important discoveries were made in the regulation of growth, differentiation, apoptosis, and metastasis of lung cancers. These findings have dramatically changed the view of the oncology community about the importance of the classification of lung carcinomas. With the findings of different responses for cisplatin treatment in adenocarcinomas versus squamous cell carcinomas (SCCs) this simple clinical lung carcinoma classification schema small cell lung carcinoma (SCLC) versus non-small cell lung carcinoma (NSCLC) was abolished. In addition, results of recent research show even the category of adenocarcinoma is in fact a heterogeneous group of different tumors with a broad spectrum of molecular changes. The chance of targeting at least some of

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the mutations by currently available treatment thus requires much more precise classification of lung tumors based not solely on morphology, but including even detection of various molecular predictive markers.

Therapy relevant molecular changes in pulmonary carcinomas

NSCLC and angiogenesis

In the last decade humanized antibodies have been developed to interfere with the neoangiogenesis in primary as well as metastatic carcinomas (1,2). However, anti-angiogenetic drugs can cause severe bleeding, especially when administered in patients with centrally located NSCLC. However, it is still not clear, if the reported bleeding episodes in these patients are due to the squamous histology or more logically to the central located tumors, which are usually supported by arteries and veins arising from large branches. In addition, it was reported that cavitation within the tumor is prone to hemorrhage, again something more common in central tumors located close to large blood vessels (3). The erroneous perception of oncologists about SCCs most probably is due to the fact that SCCs arise predominantly in central bronchi.

Angiogenesis, better neoangiogenesis is a process by which primary tumors get access to nutrients and oxygen and is characterized by the sprouting of endothelial cells from the preexisting vessels (in contrast to vasculogenesis, which is the process of growth of the vessels de novo—e.g., during embryonic development). The process of neoangiogenesis is still not fully understood. Under normal circumstances endothelial cells are virtually quiescent, therefore a crucial requirement for neoangiogenesis is their stimulation to proliferation by angiogenic factors, such as vascular endothelial growth factors (VEGFs). In some cases are these factors produced by the tumor cells themselves, in other cases are these growth factors produced by elements of the immune system, such as macrophages present in the tumor microenvironment (4). However, once new blood vessels (capillaries, small arteries, veins) are formed, this provides advantage for the tumor cells over their normal neighbor cells in getting better oxygen and nutrient supply. Nutrients and oxygen are not the only important factor for rapid growth, also purine and pyrimidine bases are essential for a dividing tumor cell (5,6). Increased angiogenesis itself in invasive adenocarcinomas has a negative impact on survival and progression of disease in these patients (7).

Angiogenesis is essential for the primary tumor as well as for metastasis. The secretion of VEGFs facilitates most often neoangiogenesis. Tumor blood vessels are immature, with incomplete basement membrane, fragile, and are therefore prone to rupture. Using antibodies against VEGF (bevacizumab) the angiogenesis can be inhibited and regression of the tumor is induced. However, in some cases, mostly in centrally located tumors can this therapy result in severe hemorrhage.

New developments are focusing on the inhibition of the VEGF receptors (VEGFRs) and also on the role of hypoxia inducible factor (HIF) and hypoxia in tumor development and metastasis. In several studies the importance of VEGF and VEGFR axis was stated for vascular invasion and metastasis, mainly involving VEGF-C and VEGFR3 (7-10). Studies aiming to target this axis showed positive results in experimental settings (11-13). Bringing these targeted therapies into clinical trials is still in its infancy (14). A major problem in targeting VEGF-VEGFR is the fact that its regulation is under the major influence of the hypoxia pathway. Hypoxia is an important factor in invasion and angioinvasion, and HIF1-signaling will result in the upregulation of VEGF (15,16). So the hypoxia pathway might constantly overrule a blockade of VEGF-VEGFR unless also HIF1 production is inhibited (17). In addition, several other independent pathways regulate the angiogenesis and thus blocking of just one of them is sooner or later bypassed by another one resulting in resistance and failure of the anti-angiogenic treatment.

NSCLC and cisplatin drugs, the effect of anti-apoptotic signaling

In a large multi-institutional study the effect of cisplatin chemotherapy was investigated. High expression of deoxyribonucleic acid (DNA) repair enzymes, especially excision repair cross complementation group 1 (ERCC1) was found to be responsible for failure of cisplatin chemotherapy and this expression correlated predominantly with squamous cell histology (18). ERCC1 is part of the excision repair machinery involved in the repair of damaged DNA. In NSCLC showing a high expression of this enzyme, the action of cisplatin-based chemotherapeutics is inefficient, most probably because DNA damage induced by the drug is immediately repaired. In a subsequent report the usefulness of ERCC1 immunohistochemistry failed, probably because the antibody clone did not pick up the relevant splice variant of ERCC1. Therefore the authors suggested using messenger ribonucleic acid (mRNA)

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quantification instead.

Thymidilate synthase (TS) blocker

Pemetrexed is an inhibitor of TS less for the other enzymes in the thymidine cycle. Thymidine uptake is essential for rapidly dividing carcinoma cells. In tumors with low expression of TS pemetrexed can block the enzyme resulting in growth inhibition. TS expression most often is low in adenocarcinomas, but is highly expressed in many SCCs. Thus pemetrexed is efficient in most adenocarcinomas and not in SCCs (19). However, the action of pemetrexed is still not entirely clear: thymidylate metabolism does not only rely on enzymes of the thymidylate cycle, but also needs active and passive uptake mechanisms; and thymidine uptake might also be influenced by pemetrexed (20).

Receptor tyrosine kinases (RTKs) in lung carcinomas

RTKs are membrane-bound protein receptor composed of an extracellular receptor domain, a transmembrane spanning portion, and an internal (intracellular) domain, which at its C-terminal end contains the kinase domain. The external receptor domain has a specific configuration for the binding of growth factors. Such stimulation results in dimerization of the receptor, where two molecules form either homo- or heterodimer. This specific binding changes the configuration of the whole receptor and leads to the phosphorylation and activation of the kinase domain. There are two ways of activation of RTKs in lung cancer: overproduction of ligands either by the tumor cell or by cells within the microenvironment, such as macrophages; or activation by a mutation of the receptor gene, most often within the kinase domain. The receptor kinase itself can act also in two different ways: one is transfer of phosphorylation to transfer molecules (21,22), like GAB1 or Grb2; or the kinase splits into fragments, where one activated protein fragment translocates into the nucleus and binds to specific DNA elements and induces transcription of downstream proteins (23). In lung cancer RTKs can be constantly activated by different mechanisms: amplification of the RTK gene, mutations of the RTK gene, gene rearrangements (translocation/inversion) with constant activation or inactivation of regulatory proteins. Another mechanism is downregulation of regulatory proteins by microRNAs (miRNAs), so a tumor suppressor or a negative feedback protein is not synthesized because of mRNA inactivation by miRNA (24-29).

Adenocarcinomas

Adenocarcinomas in highly industrialized countries are the most common lung carcinoma, representing up to 40% of all lung carcinomas. In addition what was previously regarded as a single entity has become a huge diversity of carcinomas. Adenocarcinomas in never-smokers most probably represent a separate entity with different etiology, pathogenesis, and gene signatures and a slower progression rate compared to adenocarcinomas in smokers. Also recent studies of gene signatures have contributed to a more heterogeneous picture of these neoplasms. Morphologically adenocarcinomas can show a variety of patterns, which in part correlate with gene signatures, although our knowledge in this respect is still in its infancy.

Adenocarcinoma is defined by the formation of papillary, micropapillary, cribriform, acinar, and solid structures, the latter with mucin synthesis-mucin-containing vacuoles in at least 10% of the tumor cells. Adenocarcinomas can be either mucinous or non-mucinous. Both will show the above-mentioned patterns. Some rare variants are fetal, colloid, and enteric adenocarcinomas. Most often a mixed pattern is seen with a predominance of at least one component.

Tumor cells in adenocarcinomas can show differentiations along well-known cell types as Clara cells, pneumocytes type II, columnar cells, and goblet cells. Due to the importance of targeted therapy the exact classification of adenocarcinomas and their differentiation from other NSCLC has become a major task in pulmonary pathology. Differentiation factors are used to prove the nature of the carcinoma especially in poorly differentiated tumors. A variety of useful markers have been tested, the most important ones are thyroid transcription factor-1 (TTF1), cytokeratin 7 and Napsin A.

Epidermal growth factor receptor (EGFR)

In 2004, an EGFR mutation was detected in a patient with lung adenocarcinoma and responded to tyrosine kinase inhibitor (TKI) treatment—a new era of targeted therapy in NSCLC has started (30,31).

Mutation of EGFR has been detected in a small percentage of lung cancer patients in the Caucasian population. These are activating mutations found in exons 18, 19, 20, and 21 of the EGFR gene (kinase domain) (32). Mutations are most often found in never smokers, females, and in patients with adenocarcinoma histology. Mutations change the configuration of the kinase, which does not need anymore the ligand-based activation from the receptor domain. The receptor stays in an activated stage and constantly signals

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downstream. Proliferation of neoplastic cells in carcinomas with this activating mutation can be inhibited by small receptor TKIs such as gefitinib, erlotinib, and afatinib. These TKIs bind either reversibly or irreversibly into the adenosine triphosphate (ATP) pocket of the mutated EGFR kinase domain and thus inhibit phosphor-transfer to downstream molecules, thus blocking the signaling cascade (33). The most common mutations are deletions within exon 19 with a variation of 9-18 nucleotides, and a point mutation at exon 21 (L858R). Other less common mutations are point mutations in exon 18, and insertions in exon 20.

However, mainly within exon 20 there are also resistance mutations, the best known is T790M. This type of mutation inhibits or reverses the binding of the TKIs gefitinib and erlotinib and prevents the receptor blockade. The occurrence of T790M is most frequently associated with previous TKI treatment. This mutation can be present in the tumor cells already before the treatment initiation and becomes detectable as a result of clonal selection (overgrowth of resistant cell population) or it originates de novo. The irreversible TKI afatinib might overrule some of these resistance mutations, but more data are needed to prove this (34).

Treatment response with TKIs is best in exon19 deletions, followed by exon21 point mutation. Mutations within exon 18 and 20 are less responsive (35).

For targeted therapy with TKIs tissue samples of NSCLC have to be analyzed for these mutations. Within the different subtypes of adenocarcinomas some will show a higher percentage of EGFR mutations, whereas others not. In Caucasian population adenocarcinomas with acinar or papillary pattern are mutated in up to 27%, whereas mucinous adenocarcinomas are constantly negative for EGFR mutations (and show KRAS mutation instead). Carcinomas with biphasic morphology such as adenosquamous carcinomas and mixed small cell and adenocarcinomas can show mutations but usually in a very small percentage of cases.

Another therapy approach was tested with humanized monoclonal antibodies for EGF. By competitive binding to the receptor, this antibody replaces EGF and thus inhibits transactivation of the kinase. This type of therapy seems to be especially promising in EGFR-naïve (wild-type) adenocarcinomas and in addition also in SCCs (36,37).

Echinoderm microtubule associated protein like 4-AL-Kinase 1 (EML4-ALK1) and additional fusion partners

Inversion of the ALK1 kinase gene and fusion with the EML4 gene has been recently shown in patients with NSCLC, especially in solid adenocarcinomas with focal differentiation into signet ring cells. Subsequently other patterns have been associated with this type of gene rearrangement, such as micropapillary. Both genes are on chromosome 2; the chromosomal break is inversely rearranged whereby the kinase domain of ALK and EML4 are fused together. The ALK kinase thus is under the control of EML4, which results in a constant activation of the kinase. ALK similarly to EGFR stimulates proliferation and inhibits apoptosis. Patients with this inversion respond excellently to crizotinib treatment, which is now the second example of targeted therapy in NSCLC (38). Proof of EML4ALK1 inversion can be done with different methods: the most common is FISH where two probes (3' and 5') detecting the ALK gene on both sides of the breakpoint are used. In the normal situation these probes will detect the two portions close together or overlapping within the tumor nucleus (resulting in fused FISH signal). In cases of rearrangement, the probes will highlight each of the splitted portions of the ALK1 gene, so instead of two overlapping signals the signals split apart. In the Caucasian population EML4ALK1 rearrangement is usually found in 4-6% of NSCLC; in adenocarcinomas this might be increased to 8%.

Other genes joining the ALK1 gene in the same way can replace the EML4 gene. If kinesin family member 5B (KIF5B) joins to ALK1, the overexpression of KIF5B-ALK (27) in mammalian cells led to the activation of signal transducer and activator of transcription 3 (STAT3) and protein kinase B and enhanced cell proliferation, migration, and invasion (27). Another fusion partner recently described is ALK-KLC1 (39). These other ALK1 fusions are rare; the incidence is about 1%.

C-ros oncogene 1, receptor tyrosine kinase (ROS1)

ROS1 is another kinase involved as a driver gene in adenocarcinomas of the lung (40). Usually the rearrangement of ROS1 is evaluated by two FISH probes for the 3'- and the 5'- ends. Only few fusion partners have been identified so far, CD74, SLC34A2, EZR, and GOPC/FIG (41,42). This gene rearrangement has no influence on outcome, but similar to ALK1 this is usually a younger population of cancer patients (43). The incidence of ROS1 rearrangement is in the range of 1%. The function of one of the fusion genes EZR-ROS was studied in a mouse model and showed that in this experimental setting the fusion gene acted as an oncogene inducing multiple tumor nodules in mice (44). Most important patients with this type of gene aberrations

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responded well to the ALK1 inhibitor crizotinib (45-47).

KIF5B and ret proto-oncogene , receptor tyrosine kinase (RET)

KIF5B is one of the fusion partners for either ALK1 or RET. The KIF5B-RET fusion gene is caused by a pericentric inversion of 10p11.22-q11.21. This fusion gene overexpresses chimeric RET RTK, which can spontaneously induce cellular transformation (48). Besides KIF5B, CCDC6, and NCOA4 can form fusion genes with RET. Patients with lung adenocarcinomas with RET fusion gene have more poorly differentiated tumors, are younger, and more often never-smokers. Solid adenocarcinomas predominate, tumors are smaller but lymph node involvement is higher. The incidence of RET fusion is about in 1% of NSCLCs and almost 2% of adenocarcinomas (48-50).

Met proto-oncogene, receptor tyrosine kinase (MET)

MET is another RTK bound to cell membranes in NSCLC. The ligand for MET is hepatocyte growth factor (HGF), originally found in hepatic carcinomas. This receptor came into consideration in NSCLC because amplification of MET or alternatively upregulation of HGF was identified as a mechanism of the resistance in EGFR mutated adenocarcinomas treated by TKI (25,51). A search for the role of MET in other NSCLC excluding EGFR mutated adenocarcinomas showed, that MET amplification was a rare event, but upregulation of MET is relatively common: approximately 20% of NSCLC including adenocarcinomas and SCCs showed high protein expression, but only 2% MET amplification (Popper *et al.* in preparation). Clinical studies are in progress to evaluate the possibility to interfere with MET signaling using monoclonal antibodies. Other studies use small molecule inhibitors for MET. Since MET expression is common in EGFR mutated adenocarcinomas some studies aim to inhibit both EGFR and MET signaling pathways (52). In a phase III trial the combination of EGFR TKI and MET inhibition failed, most probably because the cut-off levels were not properly set (personal experience and Popper *et al.* in preparation).

Squamous cell carcinomas (SCCs)

SCC is defined by a plate-like layering of cells, keratinization of at least single cells, intercellular gaps and bridges (represented by desmosomes and hemidesmosomes), and expression of high molecular weight cytokeratins (CK 3/5, 13/14). There are some morphologic variants as small cell and baseloid SCC, but these have not been associated with specific gene signatures and therefore are only important in diagnostics.

The incidence of SCC has dropped in the last three decades from a major entity representing 35% of lung carcinomas to around 17%. One of the major reasons is the shift from filter-less to filter cigarettes. This has resulted in the reduction of particle-bound carcinogens and increase of vaporized carcinogens, which more easily reach the bronchioloalveolar terminal unit, inducing mainly adenocarcinomas.

In the past, SCC was mainly a diagnosis required to exclude several therapeutic options in the clinic: no pemetrexed therapy, no antiangiogenic drugs, less responsiveness to cisplatin treatment. However, this has changed within the last 3 years, as there are several emerging new targets for treatment of SCC.

Fibroblast growth factor receptor 1 (FGFR1)

FGFR1 was identified being amplified in about 20% of SCCs (53) [M. Sharp *et al.*, Poster presentation, American Association for Cancer Research (AACR) meeting 2011]. In experimental studies as well as in ongoing clinical trials it was found that only amplification, proven by *in-situ* hybridization methods identified patients, who respond to small molecule inhibitor treatment (54). In subsequent trials the FGFR1-TKI therapy failed despite amplification: it became clear recently that there are additional genetic changes in some of these patients, specifically CA-PI3K mutations or amplifications. So in future the tumor in these patients will require analysis for several genes.

Discoidin domain receptor tyrosine kinase 2 (DDR2) and FGFR2

DDR2 and FGFR2 mutations are found exclusively in SCCs, however, only in a small percentage, 4% and 2%, respectively (55). In DDR2 mutated SCC patients some TKIs were successfully applied (56,57). For FGFR2 multikinase inhibitors might be an option for specific treatment (58,59).

Large cell carcinoma (LCC)

LCC is defined by large cells (nuclei >25 µm) devoid of any cytoplasmic differentiation, and large vesicular nuclei. They have a well-ordered solid structure. By electron microscopy differentiation structures can be seen such

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as hemidesmosomes, tight junctions, intracytoplasmic vacuoles with microvilli, and ill-formed cilia. This fits clearly into the concept of a carcinoma, at the doorstep of adenocarcinoma and SCC differentiation. LCC numbers have dramatically decreased due to the routine use of immunohistochemistry for more precise sub-classification of NSCLC. Using TTF1, low-molecular cytokeratins, as well as p63 and cytokeratin 5/6 most cases of LCC were either reclassified into adenocarcinoma or SCC, respectively (60). These recent changes make an evaluation of genetic aberrations in LCC quite difficult, since genetic studies were based on previous classifications.

Not surprisingly EGFR mutations, MET amplifications, and EML4ALK1 fusions have been reported in LCC (61). LKB1, a gene mutated in a small percentage of adenocarcinomas was also shown in squamous and large cell carcinomas (62). LKB1, also known as serine/threonine kinase 11 (STK11), is involved in the negative regulation of mechanistic target of rapamycin (mTOR) and closely cooperates with tuberous sclerosis gene (TSC) 1 and 2 genes (63).

Resistance mechanisms

There are general classes of resistance mechanisms to TKI therapy. The target can be altered by a secondary inhibitory mutation or by amplification. The second class is a bypass track, by which the blocked TK is circumvented. Finally the tumor may undergo phenotypic and genotypic changes, which makes TKI-therapy inefficient.

The most frequent resistance mechanisms for EGFR are inhibitory mutations on exons 20 and 19. The most common ones on exon 20 are D770_N771 insertions (up to 3%) and the mutations T790M, V769L, N771T, and the D761Y mutation on exon 19 (64-66). Several of these mutations might be targeted by second and third generation TKIs (67). A common bypass track in EGFR mutated adenocarcinomas is amplification of the MET receptor (64,68,69). A third mechanism is a phenotypic change of the tumor. A transition from adenocarcinoma to small cell carcinoma has been reported. Also re-biopsies have shown a transition from a well-differentiated adenocarcinoma to an undifferentiated carcinoma (57,70-72). Concomitant to this phenotypic change also genotypic changes are seen: a SCLC no longer presents with EGFR mutation but will respond to classical chemotherapy. In transgenic mice an upregulation of pS6 might explain some of these phenomena. Two new resistance mechanisms have been reported on a recent poster session: methylation of PTEN promoter region caused a deactivation of PTEN (similar to PTEN loss) and subsequent upregulation of PI3K-AKT pathway. The second resistance mechanism was an aberrant signaling of EGFR into SRC kinases, thus circumventing the effect of EGFR blockade by TKI (Izumi *et al.*, ERS Congress Munich, Sep. 6th, 2014).

Resistance mechanisms in EML4ALK rearranged lung adenocarcinomas do exist, however, the exact mechanisms are still under investigation (73,74). Most common are secondary mutations in the ALK domain. Most common are L1196M and G1269A, less common are 1151Tins, L1152R, C1156Y, F1174L, G1202R, and S1206Y (75-77). Again bypass mechanisms do occur such as MET activation, but also ALK amplification. Interestingly second and third generation ALK inhibitors can target most of the secondary mutations. However, also these new generation ALK inhibitors will induce secondary resistance mutations, for which new drugs have to be designed (78,79).

Similar to EGFR and EML4ALK also for ROS1, KIF5B, and RET secondary mutations have been reported (80,81). For MET this can be expected, but so far treatment has just started with MET inhibitors.

Resistance mechanisms for FGFR1 inhibition are still not exactly known. The major problem in this setting of SCCs is complicated, because response to treatment might be dictated by the mode of FGFR1 modification in the carcinoma: mutation, amplification, deletion, and/or multiple alterations. In lung SCCs the prevalent alterations are amplification and mutation (53,82). This has largely been ignored, therefore the outcome and response has to be reevaluated. Using TKIs for FGFR1 some carcinomas responded quite well, whereas others not. Another problem in FGFR1 amplified pulmonary SCCs is the coincidence of FGFR1 amplification with PI3K mutations and amplifications (82). These new findings have to taken into account, before resistance mechanisms can be further explored.

Treatment for DDR2 and FGFR2 mutations has been applied in few patients. A resistance mutation has already been shown in cell culture studies using cell lines with DDR2 mutation (83). So far this has not been seen in patients.

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Molecular methods for somatic mutation testing in lung adenocarcinoma: *EGFR* and beyond

Christine Khoo[1], Toni-Maree Rogers[1], Andrew Fellowes[1], Anthony Bell[1], Stephen Fox[1,2]

[1]Department of Pathology, Peter MacCallum Cancer Centre, St Andrews Place, Victoria 3002, Australia; [2]Department of Pathology, University of Melbourne, Victoria 3002, Australia

Correspondence to: Dr. Christine Khoo. Department of Pathology, Peter MacCallum Cancer Centre, 7 St Andrews Place, East Melbourne, Locked Bag 1, A'Beckett Street, Victoria 3002, Australia. Email: Christine.Khoo@petermac.org.

Abstract: Somatic mutational profiling in cancer has revolutionized the practice of clinical oncology. The discovery of driver mutations in non-small cell lung cancer (NSCLC) is an example of this. Molecular testing of lung adenocarcinoma is now considered standard of care and part of the diagnostic algorithm. This article provides an overview of the workflow of molecular testing in a clinical diagnostic laboratory discussing in particular novel assays that are currently in use for somatic mutation detection in NSCLC focussing on epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*), *ROS1* and *RET* rearrangements.

Keywords: Epidermal growth factor receptor (*EGFR*); anaplastic lymphoma kinase (*ALK*); *ROS1*; *RET*

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Introduction

There has been a recent and significant paradigm shift in the diagnosis and management of lung cancer, with the discovery of driver mutations that can be targeted by specific therapeutic inhibitors (1). This translates into clinical outcomes for patients whose cancer harbour these mutations or rearrangements. Personalized treatment is driving the demand for somatic mutation testing in cancer not only in absolute patient numbers for which worldwide lung cancer affected approximately 1.8 million patients in 2012 and caused an estimated 1.6 million deaths (2), but also in the number of genes. Molecular testing of lung adenocarcinoma for the epidermal growth factor receptor epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase anaplastic lymphoma kinase (*ALK*) is now considered standard of care with other "driver mutations" in oncogenes such as *KRAS*, *ROS1*, *RET*, *HER2*, *BRAF*, *PIK3CA*, *NRAS*, *AKT1*, *MET* and *MEK* (3) also being part of the diagnostic algorithm and work-up of these patients. The results of the base biomarker findings are now incorporated into the standardized structured reporting by the College of American Pathologist (CAP) (4) and the Royal College of Pathologists Australasia (RCPA) (5). Recently, the CAP, International Association for the Study of Lung Cancer (IASLC) and Association for Molecular Pathology (AMP) published a joint guideline communicating the recommendations for molecular testing in lung cancer (6). In these guidelines the pathologist plays a crucial role in this endeavour optimizing tissue handling and triaging of tumor material for appropriate testing downstream. This article provides a brief overview of the workflow of molecular testing in a clinical laboratory and also discusses the various assays that are currently in use for somatic mutation testing specifically focussing on *EGFR*, *ALK*, *ROS1* and *RET* mutations.

Molecular genetics of non-small cell lung cancer

Background

Adenocarcinoma

Recently The Cancer Genome Atlas (TCGA) Research Network published results from their work on the

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comprehensive molecular profiling of lung adenocarcinoma (using messenger RNA, microRNA, DNA sequencing, copy number analysis, methylation and proteomic analyzes) (7). In this study, aberrations in eighteen genes were found to be statistically significant, with the genes identified being: -*TP53* (46%), *KRAS* (33%), *EGFR* (14%), *BRAF* (10%), *PIK3CA* (7%), *MET* (7%), *RIT1* (2%), *STK11* (17%), *KEAP1* (17%), NF1 (11%), *RB1* (4%), *CDKN2A* (4%), *SETD2* (9%), *ARID1A* (7%), *MARCA4* (6%), *RBM10* (8%), *U2AF1* (3%) and *MGA* (8%). The key pathways affected in lung adenocarcinoma are the *RTK/RAS/RAF* pathway activation, the *PI(3)K-mTOR* pathway, p53 pathway, cell cycle regulator pathway, oxidative stress pathways and mutations in chromatin and RNA splicing factors. The analysis identified that amplification in *MET*, *ERBB2* and mutations in *NF1*, *RIT1*, *TP53*, *KEAP1* were enriched in oncogene negative tumors (i.e., tumors that lack receptor tyrosine kinase activation and that do not harbour *H/N/KRAS*, *EGFR*, *ERBB2*, *BRAF* mutations and *ALK*, *RET*, *ROS1* rearrangements) (7). The list of mutations are ever increasing, highlighting the drive to identify potential therapeutic targets. In the following discussion, we will be highlighting the recent updates pertaining to *EGFR*, *ALK*, *ROS1* and *RET*.

Epidermal growth factor receptor (*EGFR*)

In 2004, the discovery of *EGFR* gene (also known as *HER1* or *ERBB1*) mutations linked to clinical response with EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib in patients harbouring mutations, transformed the management of lung cancer and fuelled the drive for the discovery of other oncogenic drivers (8-10). Subsequently second generation *EGFR* TKIs are being trialled to improve efficacy in first line treatment of *EGFR* mutated non-small cell lung cancer (NSCLC) and to provide an alternative strategy for treating cases of acquired resistance (10,11). The proposed mechanism by which these second generation TKIs circumvent the issue of acquired resistance is said to occur via three methods: (I) by intensifying *EGFR* inhibition (through binding with/inhibition of other members of the *ERBB* family); (II) by specific inhibition of the *EGFR* downstream signalling pathway; (III) by dual targeting of parallel signalling pathways combining *EGFR* with another pathway inhibitor (i.e., vascular endothelial growth factor *VEGF* pathway) (10). Second generation *EGFR* TKIs (neratinib, dacomitinib, afatinib) are pan *HER* inhibitors aiming to intensify *EGFR* inhibition by forming irreversible covalent binding to *EGFR* kinase domain and other members of the *ERBB* family (*HER2*, *HER4*) (10,11). The most common form of acquired resistance is the T790M mutation, and specific *EGFR* T790M inhibitors (CO-1686) have been developed and investigated to address this issue (10,11). In preclinical studies, AP26113, a dual *EGFR/ALK* inhibitor has shown selective activity against mutated *EGFR* tumors including those harbouring the T790M mutation (10,11). Dual *EGFR/VEGF* inhibitors such as XL647 (vandetanib) and BMS-6905214 aim to inhibit the cross talk between the *VEGFR* and *EGFR* signalling pathway, as *VEGFR* expression is said to be associated with *EGFR* resistance (10,11).

EGFR gene mutations occur more commonly (but not exclusively) in light/never smokers, females and Asians as compared to other ethnic groups, however demographics alone should not be the sole criteria to exclude patients for mutational testing (6). *EGFR* mutations have been described in association with lepidic predominant adenocarcinoma, papillary, micropapillary adenocarcinoma subtypes and adenocarcinoma in situ (AIS) and are less common in adenocarcinoma with mucinous differentiation or with a solid growth pattern (12). *KRAS* mutations on the other hand, are commonly associated with invasive mucinous adenocarcinoma (formerly mucinous BAC pattern) and extracellular mucin (13).

EGFR mutations are present in approximately 15% of primary lung adenocarcinomas and are mutually exclusive of *KRAS* and *BRAF* mutations. *EGFR* is a member of the *ERBB* family of receptor tyrosine kinases and the gene is located at 7p12. It encodes a transmembrane receptor protein with cytoplasmic tyrosine kinase involved in downstream signalling transduction pathways. The most common activating somatic mutations in the cytoplasmic tyrosine kinase domain of *EGFR* occur in exons 18-24. Of these, the two most common mutations are the short inframe deletion in exon 19, clustered around amino acid residues 747-750 and the L858R missense mutation in exon 21, together accounting for approximately 80-90% of all *EGFR* mutations (14). Nevertheless, a significant number of mutations that may respond to TKIs have been identified outside of these "hot spots" and this has a bearing on the methodology of mutation detection (see below). Acquired secondary resistance to *EGFR* TKI can occur during the course of treatment, with the most common mechanism identified as the T790M mutation in exon 20 (10,11,15). This can sometimes be present below the assay limit of detection if retesting for this mutation is performed on the original biopsy, suggesting in some patients clonal outgrowth occurs under selective therapeutic pressure. Other pathways conferring resistance includes reactivation of

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downstream signalling pathways (*MET* amplification, *HER2* amplification, mutation in *PI3K* gene), phenotypic alteration (transformation of original NSCLC histology to small cell histology) and epithelial mesenchymal transition (15).

Anaplastic lymphoma kinase (*ALK*)

In 2007, a rearrangement in the *ALK* gene on 2p23 resulting in a fusion oncogene was discovered as an oncogenic driver mutation in a subset of lung adenocarcinomas (2-5%). It is commonly found in younger, light/never smokers (14). The histological features said to be associated with *ALK* rearranged tumors range from those with a solid growth pattern, signet ring cells with mucin production to those with well differentiated tubulopapillary and cribriform patterns (16). Treatment response in the early clinical trials in patients with such a rearrangement led to the accelerated U.S. Food and Drug Administration (FDA) approval of crizotinib in 2011. Crizotinib is an oral selective *ALK/MET* TKI for the treatment of NSCLC patients harbouring such an *ALK* rearrangement. In lung cancer, the most common *ALK* rearrangement is an inversion on chromosome 2, inv[2] (p21 p23) resulting in fusion of the 3'kinase domain of *ALK* with the (echinoderm microtubule-associated protein-like 4) *EML4* gene and its promoter region. The *EML4-ALK* gene fusion results in constitutive activation of the *ALK* kinase domain. This leads to activation of the three major downstream signalling pathways: *MAPK/MEK/ERK*, *PI3K/AKT*, and *RAS/STAT3*. The breakpoints in *EML4* are variable, whilst the *ALK* breakpoint is mostly in exon 20. This results in multiple variant of *EML4-ALK* due to the different truncations in *EML4* (16). There are at least 11 known *EML4-ALK* reported variants. The most common variants are variant 1 (E13, A20) with this nomenclature representing breakpoint in exon 13 of *EML4* juxtaposed to exon 20 of *ALK* (33%) and variant 3a/b (E6a/b, A20) representing breakpoint in exon 6 of *EML4* juxtaposed to exon 20 of *ALK* (29%). The other *EML4* variants are known as variant 2 (E20, A20) (9%), variant 7 (E14, A20) (3%), variant 5' (E18, A20) (2%), variant 4 (E15, A20) (2%), variant 5a/b (E2, A20) (2%) and E17, A20 (1%). Besides *EML4*, other less common translocation partners exist (*KIF5B-ALK*, *TFG-ALK*) (14). To date, further novel rearrangements have been identified including *HIP1-ALK* (17), *KLC1-ALK* (18) and *STRN-ALK* (19). A recently discovered variant *PTPN3-ALK* results from translocation of part of the *ALK* gene to the third intron of *PTPN3*, which does not result in a protein with enzymatic activity but instead results in a loss of one allele of *PTPN3* and is hypothesized to contribute to tumorigenesis through loss of the tumor suppressive functions of the *PTPN3* gene. The *PTPN3-ALK* will not respond to crizotinib as the *ALK* kinase domain is absent (20). The significance of these diverse *ALK* fusion variants is unknown. As in *EGFR*, resistance to crizotinib may arise from secondary "gate keeper" mutations in the *ALK* tyrosine kinase domain, activation of alternative signalling pathway or outgrowth of clones that contain a different driver mutation (21). The most common "gatekeeper" mutation identified in the *ALK* tyrosine kinase domain is the L1196M which results in structural alteration of the adenosine triphosphate (ATP) binding pocket of the receptor, which in turn obstructs crizotinib from binding to its target (21). Other secondary mutations are distributed over *ALK* kinase domain. Activation of alternative downstream signalling pathways via the *PI3K/AKT/mTOR* pathways, heat shock protein 90 (HSP90) and activation of *EGFR* through increased phosphorylation and upregulation of *EGFR* ligands (rather than by *EGFR* gene mutations) have been shown to contribute to crizotinib resistance. Novel new generation *ALK* inhibitors (Ceritinib, Alectinib, AP26113) show activity against the L1196M gatekeeper mutation and other mutations (*ROS1* and *EGFR*). HSP-90 Inhibitors (retaspimycin, ganetespib) are also currently in clinical trial (21).

ROS1

ROS1 is a receptor tyrosine kinase of the insulin receptor family and is located on chromosome 6q22 (22). *ROS1* kinase alterations lead to activated downstream signalling of several oncogenic pathways controlling cell proliferation, survival and cell cycling (*STAT3*, *PI3K/AKT/mTOR*, *RAS-MAPK/ERK* pathways). As compared to *ALK* and *RET* rearrangements, whereby coiled-coil domains in the 5'fusion partners lead to ligand independent homodimerization, many of the *ROS1* fusion proteins do not have dimerization domains and the mechanism of constitutive activation of *ROS1* fusion proteins is unknown (22). *ROS1* rearrangements have been identified in 2% of lung adenocarcinoma, with patients sharing similar clinical profiles (younger age at diagnosis, non-smoking history) to those harbouring ALK rearrangements. The different *ROS1* fusion partners identified to date include *EZR*, *CD74*, *SLC34A2*, *LRIG3*, *SDC4*, *TPM3*, *FIG* or *GOPC*, *CCDC6*, *KDELR2* (22-30). Two novel translocation partners *LIMA1* and *MSN* were detected recently (31). With all different translocation partners, the breakpoint in *ROS1* occurs at the 5'end of exons 32, 34, 35 or 36 and the *ROS1* kinase domain is retained (22). Cell lines harbouring *ROS1* fusions

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and case reports have shown that *ROS1* mutated lung adenocarcinoma show response to crizotinib therapy (25). The structural homology of crizotinib binding sites in the *ROS1* and the *ALK* tyrosine kinase domains is said to account for this (28). A phase 1 study using crizotinib in 50 patients with *ROS1* rearranged advanced NSCLC showed marked clinical response (in terms of duration of response and progression free survival, with no difference between type of *ROS1* translocation partners). In this study, the objective response rate was 72%, with 3 patients showing compete responses and 33 patients showing partial responses in their tumor with crizotinib treatment (31). This highlights the importance of including *ROS1* in the current testing algorithm.

RET

RET (rearranged during transfection) is a receptor tyrosine kinase mapped to chromosome 10q11.2 (14). *RET* rearrangements have been identified in thyroid carcinoma whereby germline gain of function mutation leads to multiple endocrine neoplasia (MEN) type 2 and somatic gain of function mutation to sporadic medullary thyroid carcinoma. In lung adenocarcinoma, *RET* rearrangements were discovered in 2011, with the investigators using whole genome/transcriptome sequencing, multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) and Sanger sequencing, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) as identification and verification methods (1). *RET* rearrangements have been identified in 1-2% of lung adenocarcinomas (24,32) but the prevalence is higher (quoted up to 16%) when preselected and enriched for tumors which are pan negative for other known driver mutations (i.e., *EGFR*, *KRAS*, *NRAS*, *BRAF*, *HER2*, *PIK3CA*, *MEK1*, *AKT*, *ALK*, *ROS1*) (33). Patients with *RET* translocated NSCLC tend to be younger and never smokers (23). The most common fusion is the *RET-KIF5B*, formed from the intrachromosomal rearrangement/somatic inversion of chromosome 10 in the pericentric region, resulting in ligand independent dimerization and constitutive activation of the *RET* tyrosine kinase. Seven different *KIF5B-RET* variants have been recognized; each differs with respect to *KIF5B* (1). *CCDC6*, *NCOA4*, *TRIM33*, *CUX1* account for the remainder of fusion partners (23,24,32,34,35). The coiled-coil domain of the translocation partner functions to promote ligand independent dimerization, inducing homodimerization leading to constitutive activation of *RET* and downstream growth signalling. The oncogenic mechanism is similar to that seen *ALK* rearrangements (34). Histologic features of lung adenocarcinoma with *RET* rearrangement include those with solid growth pattern containing signet ring cells, mucinous cribriform pattern with abundant extracellular mucin. Lung adenocarcinomas with *ALK*, *ROS1* and *RET* rearrangements share similar histological features (solid signet-ring cell pattern and mucinous cribriform patterns) and it has been proposed that these features could be a marker of an underlying rearrangement associated adenocarcinoma (23). Commercially available multikinase inhibitors such as vandetanib have been shown to inhibit the proliferation of cell lines with *KIF5B-RET* and *CCDC6-RET* fusion (24). Preliminary data from a phase II trial using multitargeted kinase inhibitor cabozantinib showed three *RET* positive patients experienced partial response and disease control (33). This data highlights that *RET* rearrangements are an oncogenic driver in a subset of lung adenocarcinoma and is a potential druggable target, hence the importance of incorporating this into diagnostic assays.

Case selection for testing

The new IASLC/American Thoracic Society (ATS)/European Respiratory Society (ERS) international multidisciplinary lung adenocarcinoma classification guideline highlights the role of the pathologist in reporting lung cancer in resection specimens, small biopsies/cytology specimens and provides guidelines for the management of tumor tissue in patients with advanced lung cancer. The histologic distinction into NSCLC subtypes (adenocarcinoma versus squamous cell carcinoma) is still based on tumour morphology. The use of a limited panel of immunohistochemical markers (TTF1/Napsin A, p63 or p40) is employed when this distinction is not possible, i.e., when dealing with small biopsy/cytology samples (NSCLC-NOS) with the ultimate aim of conserving tissue for further molecular testing (12). Currently all lung adenocarcinoma, mixed tumors with an adenocarcinoma component or a small sample where an adenocarcinoma component cannot be excluded should be forwarded for molecular testing. Cytology specimens are suitable for molecular testing with cell block preparations preferred over smears (6). Samples for metastatic lesions to bone are an issue as acidic decalcifying solutions cause extensive DNA fragmentation but fixatives such as EDTA preserve DNA integrity to some extent. The choice of testing of the primary lesion versus metastatic lesion is dictated by the quality of the specimen (tumor content and preservation) (36), although the most recent site of metastatic disease should be tested in a case of

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a previously treated TKI sensitive tumor which progresses on treatment. There are many potential algorithms for the sequence of molecular testing that are usually dictated by local requirements and availability of testing. It is suggested that *EGFR* and *ALK* should be reflexly tested at the time of diagnosis to ensure results are available at the time when therapy needs to be instituted as DNA degrades even with optimal storage and block retrieval can take significant time and can delay instigation of treatment. Nevertheless, the choice of reflex testing versus clinician requested testing may best be decided at a multidisciplinary team setting (6).

Workflow in a laboratory

The routine work flow for analysis of somatic mutation starts with histologic assessment, review and confirmation of the diagnosis on a representative haematoxylin and eosin (H&E) stained slide of the tumor. The proportion of tumor content is documented and the area containing the highest proportion of tumor is demarcated on the slide. The aim of this initial step is to enrich and prepare a high concentration of tumor cells that can be isolated using tissue macrodissection. The assessment should also document the presence of mucinous material, necrotic tissue, pigment and haemoglobin as these can inhibit the polymerase chain reaction (PCR). Nevertheless, in the authors' experience, depending on the assay selected, a result can be obtained on as few as 50 well fixed cells. The Illumina Truseq Custom Amplicon Cancer Panel recommends 250 ng of input DNA, however results can be obtained with as little as 150 ng. Although limited tissue availability is one issue, preanalytical factors such as fixation, tissue processing, long term and poor storage conditions have a far greater impact on the nucleic acid integrity of the tumor. During tissue processing, inadequate fixation/low pH formalin can induce DNA degradation and fragmentation. 10% neutral buffered formalin is an important and widely used fixative in diagnostic pathology to preserve tissue architecture, prevent enzymatic degradation/tissue autolysis and to support high quality and consistent staining with H&E. The two common forms of DNA changes caused by formalin fixation is fragmentation of DNA and sequencing artefacts (37-39). Formalin by its nature of fixation via cross linking of DNA also causes fragmentation of DNA, resulting in template DNA of short and variable fragment lengths. Other factors affecting the quality of the template DNA is the type of fixative used, time in fixation and temperature during tissue storage which can significantly alter/modify the DNA fragment. After DNA is extracted from formalin fixed paraffin embedded (FFPE) material, a quick quality control measure is introduced to quantify the amount of DNA/RNA material. The aim of this step is to identify and select samples that would be suitable for further sequencing. The DNA can be quantified by spectrophotometry (the NanoDrop Spectophotometer is an example of this) or by fluorometry using dyes that bind to double stranded DNA (the Qubit® assay is an example of this. Whilst these methods determine the bulk or concentration of DNA, they do not provide information regarding the quality of the template DNA (in terms of the underlying potential molecular damage and fragmentation) (39). The information regarding the DNA quality and template fragment lengths from FFPE material may be determined by using a multiplex PCR assay. This quality control measure uses amplicons of known varying lengths (e.g., 100, 200, 300, 400 and 600 bp) to assess the tumor DNA template for fragment size and to ensure that there are enough templates of suitable lengths for further molecular processing/next generation sequencing (38). Formalin also causes chemical modification of DNA, and cytosine deamination resulting in C > T sequence artefact post PCR amplification, which is particularly evident when using very fragmented template DNA (37-39). These will need to be taken into account when interpreting downstream results. In general, pre-analytical factors are difficult to control, but play a vital role in the quality of the DNA material for further molecular testing. It is imperative that tumour tissue be managed properly to ensure accurate and reliable data output as molecular assays are highly dependent on the quality of input DNA.

Molecular method/assays used in lung adenocarcinoma

There are a wide variety of commercially available molecular assays used to detect mutations in lung adenocarcinoma. An ideal assay should be sensitive and specific enough to comprehensively cover all clinically relevant targets using limited samples, while being cost effective and efficient. In NSCLC the main types of somatic mutations in cancer include single nucleotide variants (SNV)/point mutations, small duplications/insertions or deletions (indels), exon/gene copy number changes and structural variants (from translocations/inversions) (3).

The methods used will depend on the type of mutation that is being detected. The techniques used to identify

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EGFR mutations can be divided into "screening (or scanning)" or "targeted" (or specific mutation) genotyping methods (40). "Screening" technologies such as Sanger sequencing, Next Generation Sequencing (NGS), High Resolution Melt Analysis (HRMA) and Pyrosequencing have the potential to detect all *EGFR* mutations in the region of interest including novel mutations. In contrast, "targeted" assays such as the Agena MassARRAY Oncocarta panel, the Cobas EGFR Mutation Test (Roche Molecular Systems), the Therascreen *EGFR* Kit (Qiagen) and SNaPShot (by Life Technologies/Applied Biosystem) are usually highly sensitive to detect a preselected/ specific known mutations or "hot spot" mutations but by their design are unable to identify novel mutations. The consensus opinion of the CAP/IASLC/AMP is that any routine *EGFR* assay used in clinical practice should be able to detect the common *EGFR* TKI sensitizing mutations (exon 19 deletions and L858R) and mutations that confer decreased sensitivity to *EGFR* TKI (T790M, exon 20 insertions). Assays used should also be able to detect the following common and less common mutations in the *EGFR* gene: - exon 19 (15-bp, 18-bp, 9-bp, 12-bp, 24-bp, 27-bp deletions and 15-bp, 18-bp insertions), exon 18 (E709, G719 mutations), exon 20 (S768, T790M, insertions), exon 21 (L858R, T854, L861Q mutations) (6).

The techniques used for clinical detection of the underlying gene rearrangement as occurs with *ALK*, *ROS1* and *RET* include FISH, reverse transcription-PCR (RT-PCR) and IHC to detect the overexpressed protein caused by the underlying fusion transcript. Target specific break-apart FISH probes can detect a rearrangement regardless of the fusion partner but this technique is highly technical and expensive, and not feasible for screening of large samples for rearrangements of *ALK*, *ROS1* and *RET* that occur at low frequency. IHC offers an alternative option for screening, and is widely available in diagnostic pathology laboratories. Fusion specific RT-PCR combined with Sanger or next generation sequencing of the PCR products allows specific identification of the fusion partners, however the predesigned fusion specific primer/probes used may miss novel or unknown translocation partners that may not be detected by the preselected probes. The results of RT-PCR are also affected by the often degraded and poor RNA quality obtained from the FFPE material. A novel multiplexed expression gene expression/ transcript based assay known as the Nanostring nCounter assay works on the premise that a rearrangement causes mRNA overexpression of the 3'end of the gene compared to the 5'end of the gene. Novel next generation sequencing assays based on either the relative expression of 5' versus 3'amplicons derived from the cDNA of the oncogenic partner of known fusions, or specific fusion targeted amplicons, have recently become available. The Archer™ *ALK*, *RET*, *ROS1* Fusion Detection Kit is a targeted sequencing assay based on Anchored Multiplex PCR (AMP) to simultaneously detect and identify fusions of human *ALK*, *RET* and *ROS1* genes (41).

Molecular methods/assays for EGFR mutations: screening assays and targeted assays

Screening assays

Sanger sequencing

Traditional Sanger sequencing or direct DNA sequencing is considered the gold standard for characterizing all mutations. Sanger sequencing is performed on PCR products and requires sequencing primers spanning the region of interest, DNA polymerase for primer extension, labelled nucleotides/ bases and a low concentration of modified nucleotide/bases (also known as dideoxyNTP). All four nucleotide bases (adenosine, thymine, guanine and cytosine) are each labelled with a different flourophore. Sanger sequencing is also known as "sequencing by termination" or "chain terminator sequencing" as it uses the ddNTP (modified nucleotides/bases) to stop primer extension. This creates DNA fragments of different lengths, which are then separated out with capillary gel electrophoresis. Sanger sequencing is often the orthogonal method used to confirm results due to its ability to characterize a wide variety of mutations (SNVs, small insertions/duplications/deletions/indels), however it is limited in detecting gene copy number changes. It is not scalable (as compared to massively parallel sequencing/ next generation sequencing). Sanger sequencing works on a small amount of input DNA (5-10 ng) however has low sensitivity. It requires that the mutant variant, which may be a minor component of the mixture be present at least 20% of the total tumour DNA to be detected (3,42).

High resolution melt analysis

High-resolution melt (HRM) analysis is a cheap, rapid and sensitive mutation screening (or scanning) method. It is used to identify samples that contain mutations for further characterization by sequencing. The starting DNA material is amplified in a real-time PCR reaction and a melt analysis is subsequently performed in the presence of a DNA binding dye (the dye fluoresces brightly only when bound to double stranded DNA). The process of HRM begins with increments in temperature to a point (melting temperature,

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Tm) where the double stranded DNA (with high fluorescence) will "melt apart" to become single stranded DNA fragments (low fluorescence). The DNA containing the mutation will "melt" at a different temperature compared to the wild type DNA. This difference in melt curve signature is used to detect the presence or absence of a mutation. As HRM is a screening tool, a more specific method like DNA sequencing is needed to identify the precise mutation (42).

Pyrosequencing

Pyrosequencing is also known as "sequencing by synthesis" and uses chemiluminescent detection of inorganic pyrophosphate to detect specific base additions. This is a quick, sensitive method to detect mutant DNA that utilises the template containing the region of interest, primers, DNA polymerase and a set of enzymes/substrates (ATP sulfurylase, luciferase, apyrase, adenosine 5'phosphosulfate and luciferin). During primer extension, pyrophosphate is released each time a nucleotide is sequentially incorporated onto the 3'end of a DNA which through an enzymatic reaction results in light emission. The resultant sequence is determined from the pyrogram generated. Compared with Sanger sequencing, pyrosequencing is a sensitive method that allows detection of mutations in tumor samples as low as 5% (as is often the case when tumor material is heterogeneous and admixed with adjacent normal tissue) compared with 10-20% tumor material needed for Sanger sequencing. Pyrosequencing is best used to detect SNVs and is limited in its ability to detect gene copy number changes/structural chromosomal changes (3,42). Pyrosequencing, and the related next generation sequencing systems utilizing this technology (Roche 454, Ion Torrent Personal Genome Machine (PGM) (Life Technologies/Thermo Fisher Scientific) next suffer from insensitivity in homopolymer repeats greater than 7-8 nucleotides in length.

Next generation sequencing (NGS)

Massively parallel sequencing or next generation sequencing (NGS) is a mutation screening method. NGS technology has the ability for high throughput sequencing of a large number (up to millions) of DNA templates in a single reaction with multiple patient samples. NGS platforms can detect somatic mutations as low as 5% of tumor material (43). The many applications of NGS include sequencing of the whole genome, exome (protein-coding regions of the genome), or transcriptome (all expressed sequences). There are many available NGS platforms available that differ in their sequencing chemistries and methods of sequence detection but all share the same fundamental principles and steps (44,45). Firstly a library is constructed followed by PCR amplification and sequencing. The initial library preparation may be created via random fragmentation of the starting DNA of interest and ligation/annealing of the DNA fragments to an adapter sequence/linker to create a "library". The library is then amplified by repeated cycles of PCR reaction (on a solid surface) and then sequenced. The presence of specific adapter/linker sequences allows selective amplification by PCR reaction. Amplicon libraries may also be generated directly from unfragmented target DNA. The clonal amplification of templates can be performed by emulsion PCR (e.g., Ion Torrent PGM, Ion Proton, Roche 454 platform and ABI SOLiD) or with bridge PCR amplification to form clusters on a flow cell surface (e.g., Illumina platform) (44,45).

In massively parallel sequencing, the repeated cycles of nucleotide addition and detection of the incorporated bases (i.e., sequencing and detection) occur simultaneously (44,45). The platforms utilize different sequencing chemistries (44,45). In the Illumina platform, sequencing is by synthesis with reversible dye terminators. The identity of the incorporated nucleotide is determined by the specific fluorescence it emits (each nucleotide carries a specific fluorescent label, hence emits a specific wavelength) and this signal is detected. After the detection step, the 3'OH group is deblocked such that the fragment continues to be extended in each cycle. The Ion PGM instruments use a chemistry related to pyrosequencing, however the base addition is detected by the release of hydrogen ions during native nucleotide incorporation rather than inorganic pyrophosphate. This is a variation of pyrosequencing which monitors the pH change rather than pyrophosphate/light to detect the incorporation of nucleotide. Pacific Biosciences uses single molecule real time (SMRT) DNA sequencing whereby the fluorescently labelled nucleotide is added to the growing strand by DNA polymerase. The fluorescence which is attached to the terminal phosphate end of the nucleotide is cleaved by the DNA polymerase and the diffusion of emitted light is detected by zero-mode-waveguide (ZMW) (44). The sequenced "reads" are then aligned to a reference genome and analyzed with bioinformatics software (45). While whole genome sequencing provides extensive data on SNV, indels, complex structural arrangements and copy number changes, it is relatively expensive and the huge amount of data generated requires complex bioinformatics analysis and storage. Due to its high sensitivity, often incidentally discovered novel variants may pose challenges in interpretation as these are

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of unknown clinical significance.

Compared with whole genome sequencing, targeted NGS/exome sequencing offers a more affordable, efficient and clinically applicable method for somatic mutational profiling in cancer as it focuses on clinically relevant genes. Targeted NGS/exome sequencing enriches the target of interest and focusses higher coverage or read depths over genomic regions of interest (46). In this method, the target of interest is enriched (either by PCR amplicon method or hybridization capture) and the application of deep sequencing focuses a high number of reads targeted to a region known to contain variants of clinical significance. A variety of bench top sequencers are now being used in diagnostic laboratories for targeted mutational profiling, as these have the ability to generate clinically important data at a lower cost and with a faster turnaround time.

A significant advantage of NGS that is particularly valuable for NCSLC is its ability to test multiple targets/genes of interest (as compared to sequential testing) on limited material from small biopsies and cytological samples. It also, unlike targeted genotyping assays (discussed below), is able to detect any type of mutation in the region of interest as compared to an assay used to detect only the specific mutations. Nevertheless, NGS technology uses PCR for amplifying target DNA and as such, is susceptible to issues inherent to PCR enzymatic amplification such as preferential amplification of certain library fragments. False artefacts/false variants may also occur due to substitution errors by PCR polymerase. Due to its inherent sensitivity, application of NGS in the diagnostic setting raises issues pertaining to the discovery of low frequency variants and their clinical validation and how these should be reported and applied to patient care. There are currently no standardized model or guidelines for the application of NGS in clinical practice, highlighting the need for validation of NGS technologies mainly in terms of the NGS analytical process (minimum coverage/depth of coverage) and standardization of bioinformatics packages (47).

Targeted assays

Commercially available targeted assays for *EGFR* mutations include those from Agena Bioscience MassARRAY, SNaPShot by Life Technologies/Applied Biosystems, cobas® (Roche Molecular Systems) and therascreen® Mutation Kits (Qiagen). Targeted assays are also available for *KRAS* and *BRAF* mutations. The therascreen® KRAS kit (Qiagen) covering 7 mutations in *codons 12*, *13* was approved by the U.S. FDA in June 2012 as a companion diagnostic device for cetuximab for patients with metastatic colorectal carcinoma. The cobas® KRAS (Roche Molecular Systems) is designed to detect 19 KRAS mutations in *codons 12*, *13* and *61*. In 2011, the U.S. FDA approved the cobas® 4800 BRAF V600 Mutation Test (Roche Molecular Systems) as a companion diagnostic test in conjunction with the approval of vemurafenib for patients with metastatic melanoma with the BRAF V600E mutation. The therascreen® BRAF kit is also available. These targeted assays allow for multiplex genotyping of known validated, “hotspot mutations” or genetic alterations simultaneously within a single assay, although the Agena assay looks at multiple genes depending on the particular assay. These multiplex testing platforms detect specific alterations/mutations that are known to be present in specific genes however are limited in their abilities to detect new or additional mutations outside the targeted region. Targeted assays are highly sensitive and can be performed with a lower amount of starting DNA material (5-10%) depending on the mutation compared with traditional Sanger sequencing (48-52).

Agena bioscience massarray® system

Agena MassARRAY® system utilizes PCR amplification and allele specific single-base primer extension. Each nucleotide/base added to the primer contains a defined molecular mass and the primer extension products are analyzed using the principle of MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight). The time of flight is proportional to the mass/charge which is translated into specific genotype calls (43,53). There are multiplexed somatic mutation panels (reagent sets) that allow detection of known oncogenes. These customised panels with selected candidate genes are selected and distilled from large scale sequencing studies, to target clinically actionable mutations. Currently there is a multi-gene panel OncoCarta™ Panel v1.0. covering key “actionable” mutations in the *EGFR*, *BRAF*, *KRAS*, *NRAS*, *c-Kit* genes and a LungCarta panel which comprises 214 somatic mutations in 26 tumor suppressor and oncogenes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *ALK*, *AKT1*, *DDE2*, *EPHA3*, *EPHA5*, *ERBB2*, *FGFR4*, *JAK2*, *MAP2K1*, *STK11*, *MET*, *NOTCH1*, *NRF2*, *NTRK1*, *NTRK2*, *NTRK3*, *PIK3CA*, *PTCH1*, *PTEN*, *PTPRD* and *TP53* (48,49).

Snapshot® multiplex kit (applied biosystems®)

The SNaPshot multiplex kit/platform from Applied Biosystems uses multiplex PCR and single base primer extension using fluorescent labelled probes. The fluorescently labelled primer extension products are then detected by conventional capillary electrophoresis.

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The SNaPshot panel tests for a smaller panels of genes and mutations (8 to 14 genes, >50 hotspot mutations) compared to the Agena MassARRAY® system (43). It allows multiplexing and rapid identification of single nucleotide polymorphism (SNP)/point mutations at specific sites of the PCR generated templates. This can be then be combined with a further sizing assay to detect deletions (e.g., in exon 19) and insertions (e.g., in exon 20). Although this is a commercially available platform, it allows users the flexibility to customize the kit and design the assay to meet the needs of the individual laboratories as an in-house assay. The workflow is simple and easily incorporated into diagnostic laboratories. The capillary electrophoresis automated DNA sequencer is a familiar and available equipment present in most clinical laboratories, avoiding further overhead costs. SNaPshot assays require less input DNA compared to Sanger sequencing. The main disadvantage of the SNaPshot platform is the limit to the number of assays/reactions that can be multiplexed (optimally below 10). It is not designed to detect amplifications, insertions or deletions.

cobas® EGFR mutation test

The cobas® EGFR Mutation Test (Roche Molecular Systems) is another allele specific real time PCR assay. In 2013, the cobas EGFR Mutation Test was approved by the U.S. FDA as a companion diagnostic test to select patients with EGFR exon 19 deletions or L858R substitution in exon 21 for treatment with erlotinib, concurrently as it was approved for use as first line treatment of metastatic NSCLC (50). The pivotal trial leading to the approval of erlotinib as new first line treatment was the based on the results of the phase 3 European Randomized Trial of Tarceva Versus Chemotherapy (EURTAC) trial assessing the safety and efficacy of erlotinib compared to standard platinum based chemotherapy (54). The Cobas EGFR mutation test was used in this study to determine the EGFR mutation status of the trial patients. This assay uses Taqman probes in a qPCR reaction to simultaneously amplify and detect the mutations using specific probes (each with their own fluorescence). TaqMan probe based assays use two target specific primers flanking the region of interest and a third sequence specific probe to hybridize with the area of interest. The sequence specific probe contains a reporter molecule at the 5'end and a quencher molecule on the 3'end of the probe. When these two molecules are in close proximity, the interaction between the quencher molecule and reporter molecule prevents emission of fluorescent signals. The TaqMan probe relies on the exonuclease activity of Taq polymerase to cleave the dual labelled sequence specific probe upon encounter during the PCR amplification phase. The cleaving process separates the reporter molecule from the quencher, resulting in a signal that can be detected. For the EGFR gene, it is able to detect 41 mutations in Exons 18, 19, 20 and 21 of the EGFR gene. The mutations covered by the cobas® system includes G719X (G719S/G719A/G719C) in exon 18, 29 deletions and mutations in exon 19, T790M, S7681, 5 insertions in exon 20 and L858R in exon 21 (2 variants) (51).

therascreen® EGFR kit (qiagen)

The therascreen® EGFR kit (Qiagen) is also another allele specific real time PCR assay. In 2013, afatinib was approved by the FDA as first line treatment of patients with metastatic NSCLC with *EGFR* exon 19 deletions or L858R mutations. This approval was based on the results of the LUX-Lung 3 trial. The therascreen® EGFR kit, used in the study was approved as a companion diagnostic test at the same time (50,55). For the EGFR gene it has been designed to detect 29 mutations in exons 18, 19, 20 and 21 of the gene. The mutations detected include G719X (G719S/G719A/G719C) in exon 18, 19 deletions in exon 19, T790M in exon 20, S7681 in exon 20, 3 insertions in exon 20, L858R in exon 21 and L861Q in exon 21. The therascreen® kit uses ARMS (amplification-refractory mutation system) and Scorpions for the detection of these mutations. ARMS is an allele specific amplification process using Taq DNA polymerase to selectively amplify specific mutated sequences. Scorpions are used to detect the ARMS amplicon, hence detect the presence of mutations. Scorpions are molecules that contain a PCR primer linked to a probe (which contain both a fluorophore and quencher). When the Scorpion primer binds to the ARMS amplicon, it starts primer extension resulting separation of the fluorophore and quencher, with release of fluorescence (52).

Currently there is no consensus regarding the best method to conduct *EGFR* mutational testing (6). The two early pivotal trials in 2004 that showed an association with *EGFR* activating mutations in the tyrosine kinase domain being strong predictors to response to *EGFR* TKIs used traditional direct Sanger sequencing (8,9). The Iressa Pan-Asia Study (IPASS), a phase III randomized study of gefitinib versus carboplatin/paclitaxel in previously untreated never/light smokers with advanced NSCLC tested the clinically enriched population for *EGFR* for mutation status (using PCR ARMS *EGFR* mutation detection kit), *EGFR* gene copy number (with FISH) and *EGFR* protein expression (with IHC). The presence of *EGFR* mutation, rather than gene copy number and protein expression correlated with

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better outcome with gefitinib (56). There are a number of commercially available PCR based targeted *EGFR* mutation detection kits (as listed above) which have high analytical sensitivity but may not cover all possible spectrum/variables outside the scope of their detection. Diagnostic laboratories providing this service will need to report all findings and integrate the findings into a clinically usable report for the oncologist to aid therapeutic decision making. All findings should be reported, with a comment if the mutation is: (I) one of the commonest mutation known to show sensitivity to EGFR TKIs; (II) uncommon, but has been reported in the literature to confer EGFR TKI sensitivity; (III) uncommon with unknown clinical significance; (IV) known to confer EGFR TKI resistance; (V) uncommon mutation of unknown clinical significance but the mutation is occurring in an exon where mutations are usually related to EGFR TKI resistance.

Molecular methods/assays for ALK, ROS1 and RET mutations

Rearrangements and inversions characterize the mutations within the *ALK*, *ROS1* and *RET* gene in lung adenocarcinoma. As opposed to the above methods which are geared towards detecting SNVs and indels, FISH is the technique used to identify exon/gene copy number changes and structural variations from rearrangements and inversions in clinical practice. An alternate approach to the detection of *ALK*, *ROS1* and *RET* rearrangement is IHC. In NSCLC, IHC can be used to either detect either mutant specific product (e.g., specific *EGFR* L858R, *EGFR* exon 21 deletion, BRAF V600E) or in the case of *ROS1*, *RET* and *ALK*, *IHC* can detect overexpression of protein (resulting from underlying translocation) that does not occur in non-rearranged tumours.

In general, FISH and IHC testing methods detects *ALK* rearrangements without prior knowledge of the translocation partner. In the Australian experience, testing for ALK rearrangements vary depending on the individual testing laboratory. In general, centralized laboratories perform *ALK* testing either in parallel with or in a sequential manner after a negative result from *EGFR/KRAS* mutational testing. Simultaneous testing reduces turnaround times (TAT) but sequential testing is more cost effective. Many laboratories perform ALK IHC as a rapid and cheap triage, with equivocal or positive results being sent for confirmatory FISH testing at a reference laboratory (57). However, this often uses more of the limited material available for testing and it is recommended that the two are performed in parallel. The other issue with IHC is the relatively poor quality assurance that occurs in laboratories without an orthogonal method that ensures that the IHC is accurate and reproducible. *ROS1* testing has also been implemented in some laboratories using both FISH and IHC.

Fluorescence in situ hybridization (FISH)

FISH is the current gold standard for the detection of *ALK* rearrangements although it cannot identify the fusion partner. FISH technology utilizes dual probes containing specific sequences of DNA to bind specifically to the nucleotide sequence on the target DNA. The probes are conjugated to a fluorescent molecule allowing detection. In NSCLC, FISH testing using the Vysis *ALK* Break Apart probe Kit (Abbott Molecular) was approved as a companion diagnostic test concurrently with crizotinib based on the clinical response seen in patients with *ALK* rearranged tumors using this method (58). There are other ALK FISH probes that are commercially available but not yet FDA approved (59) (e.g., ZytoLight® SPEC *ALK/EML4* TriCheck™ Probe, Cytocell ALK Breakapart probe, Cytocell Aquarius *EML4* breakapart probe). In ALK wild type, the close proximity of the probes result in closely opposed or a fused (yellow) signal. Additional copies of the fused signal indicate polysomy, which can occur in both wild type and A*LK* rearranged tumours. A tumor is considered to have a rearrangement when (I) there is separation of the red and green signal by more than 2 signal widths or (II) when there is a single red signal without a corresponding green signal in addition to fused (normal) signals although the translocation partner will be unknown. Interpretation of *ALK* break apart FISH differs from other FISH probes as the translocation and inversion occurs on the same chromosome arm. False positive break apart signals may be due to the slight separation of the probes in some wild type cells and truncation artefact which may result in artificial signal separation (59). FISH is relatively expensive compared with IHC, requires technical expertise for interpretation and is usually only available in larger reference centres.

FISH is also used to detect *RET* and *ROS1* rearrangements using *ROS1* and *RET* Dual Colour Break Apart Probes (23).

Immunohistochemistry (IHC)

The use of IHC for ALK protein expression is based on the premise that ALK protein is normally absent in the lung

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and the overexpression of ALK protein infers an underlying rearrangement of the *ALK* gene leading to constitutive activation and subsequent overexpression of the protein (59). There have been many studies comparing IHC with gold standard FISH testing using a variety of different antibodies (60,61). A recent study used five different ALK antibody clones 5A4 (Novocastra), D5F3 (Cell Signaling), ALK1 (Dako), ALKO1 (Ventana) and SP8 (Abcam), and comparing the results to ALK FISH showed that the D5F3 and 5A4 ALK clones stained all ALK FISH rearranged cases with weak/moderate/strong intensity with some false positive cases (61). The 5A4 and D5F3 clones have generally been shown to have higher staining intensity compared with the ALK1 clone (61,62). In studies using ALK IHC, two scoring systems are used for evaluation. One of these is a four tiered scoring systems with 0 (negative), 1+ (weak intensity cytoplasmic staining), 2+ (moderate intensity cytoplasmic staining) and 3+ (strong intensity cytoplasmic staining). Samples have been evaluated by the presence or absence of staining, or using several semi-quantitative methods including a histoscore (H score) of 1+ to 3+ by assessing the percentage of cells showing expression together with the intensity of staining. Cases are considered positive is there is 1+, 2+ or 3+ staining. The other scoring algorithm is a binary system from Ventana. In 2011, Ventana/Roche collaborated with Pfizer Inc. and Cell Signaling Technology to develop an automated and standardized IHC companion diagnostic test for *ALK* rearrangements to identify patients who would be eligible for treatment with Pfizer's Xalkori® (crizotinib). As such, the binary scoring system can also be applied when using the Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody, as the assay has been developed to maximize concordance with ALK status as determined by FISH. A positive ALK IHC is determined by the presence of strong granular cytoplasmic staining in tumor cells, regardless of the percentage of positive tumor cells. The specimen is considered negative for ALK when there is an absence of strong granular staining in tumour cells. Staining may be seen in non-tumour elements (alveolar macrophages, nerve and ganglion cells, normal mucosal glandular epithelium, scattered lymphocytes, mucin, and necrotic tumour areas) and this is not regarded as a positive result. Some 1-2% of ALK negative cases may demonstrate a weak, diffuse granular cytoplasmic staining but these cases are considered negative for ALK due to the lack of strong intensity staining (62).

It is critical that IHC for ALK testing in NSCLC is optimized and modified for this specific use in lung tissue, as the ALK expression in NSCLC is lower than it is in anaplastic large cell lymphoma. In NSCLC, *ALK*-rearranged staining is noted to be less intense, more granular, with staining within the cytoplasmic compartment as compared to in lymphoma (whereby the staining is more intense and with nuclear and cytoplasmic expression) (60). Although the low prevalence of *ALK* rearrangements would support IHC as a feasible pre-screening triage test with selected cases to be confirmed using FISH, IHC is subject to pre-analytical factors (technical aspects pertaining to tissue fixation), analytical factors (type of antibody clone used, endogenous peroxidase activity, necrosis/crush artefact) and post analytical factors (interobserver variation in evaluating scoring, different cut offs used for a positive/negative result). The observation that even the presence, absence or semi-quantitive analysis of protein expression by IHC in general community laboratories that do not have an orthogonal method to ensure accuracy and reproducibility is poor suggests that IHC use should be performed only where FISH is available. The European Society of Pathology (ESP) provides an external quality assurance assessment (EQA) scheme for testing of biomarker mutations in NSCLC. In 2012, a pilot EQA programme was conducted for *ALK* testing (IHC or FISH) and a second pilot was conducted for *EGFR*, *KRAS*, *ALK* (IHC, FISH or RT-PCR). *ROS1* testing was included in the 2014 scheme. Participation in such a scheme provides laboratories with an opportunity to verify and standardize their current practices, and to also improve the reliability of their testing platforms (63).

IHC has also been used to detect *ROS1* and *RET* rearrangements in NSCLC, with comparable results to FISH and RT-PCR (23) .In this study, the novel *ROS1* rabbit monoclonal antibody antibody D4D6 from Cell Signaling Technology showed differences between *ROS1* rearranged tumors and those without a *ROS1* rearrangement. The optimal immunostaining interpretive criteria to predict underlying rearrangements is not yet clearly defined. In a study by Yoshida (29), adenocarcinomas containing the *ROS1* rearrangement showed a range of staining pattern from diffuse to focal cytoplasmic staining, with some tumors showing cytoplasmic membrane accentuation at the apical or lateral surfaces. They suggest that H-score of more than 150, diffuse staining extent of more than 75% and moderate-strong intensity staining was felt to discriminate between *ROS1* rearranged tumors and those without the rearrangement. In rare cases, there was occasional staining of non-neoplastic type II pneumocytes and macrophages (29). As these rearrangements are

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rare, IHC can be used as a screening tool for further confirmatory test.

EGFR IHC

In terms of using IHC for *EGFR* testing, three main types of EGFR IHC tests exists: (I) IHC for total *EGFR*; (II) IHC for phosphorylated *EGFR*; (III) mutant specific EGFR IHC. Experience with the former two IHC types are limited and currently not recommended as standalone tests for patient selection for *EGFR* TKI therapy (6). The mutation specific EGFR IHCs that are commercially available target the two most common *EGFR* mutations (the L858R mutation in exon 21 and the common 15 bp/5AA deletion (E746_A750del) in exon 19. The L858R antibody has shown high sensitivity and specificity for detecting the specific mutation compared to the accepted orthogonal methods. The other *EGFR* E746_A750 exon 19 deletion antibody is limited at identifying other rarer variant exon 19 deletions other than 15 bp (64,65). As such, mutant specific EGFR IHC testing should be used in conjunction with orthogonal molecular methods in cases negative for mutant specific EGFR IHC tests. Mutant specific antibodies may play an important role in situations whereby molecular testing is limited by the amount of available tumor tissue, however mutant specific IHC are limited in identifying other less common *EGFR* mutations that account for up to 10% of cases. They also suffer from the vagaries of ALK IHC and thus it is not recommended as a first line test.

Reverse transcriptase polymerase reaction (RT-PCR) to detect translocations/gene fusions in *ALK*, *ROS1*, *RET*

Besides FISH and IHC, multiplex RT-PCR is another method used to detect the different translocation in *ALK*, *ROS1* and *RET*. This method of detection is popular with Japanese investigators as highlighted in their work (24). RT-PCR combined with DNA sequencing allows precise and specific variant detection of the translocation partner, however this requires prior knowledge of the possible fusions/translocation partner in order to design multiple primer sets to detect this. For example, in *EML4-ALK* rearrangements whereby there are many breakpoints for *EML4*, the RT-PCR method would require multiple primer sets to discriminate between all known variants (18,23,29,66,67). Other rare non *EML4* fusion partners for *ALK* also exist (KIF5B, TFG, KLC1, STRN and *HIP1* as mentioned earlier) and this limitation needs to be taken into account when using the RT-PCR method for clinical detection of *ALK* rearranged NSCLC. FISH and IHC methods can detect all fusions regardless of the fusion partner, and are useful for screening but specific identification of the (potentially novel) translocation partner will require multiplex RT-PCR.

The future

The ability to multiplex and simultaneously detect many mutations at once is advantageous and important especially when dealing with small tumor samples as with NSCLC that are often procured during advanced disease. The patient may have metastatic disease to sites hampering access to adequate tumor material. The clinical condition of the patient may also limit the options of an invasive procedure to obtain tumor material. Archival FFPE tumor tissue hold a wealth of material for research however FFPE material is often degraded and of poor quality. As such, the need to adapt to these conditions is highly important as there is an increasing demand for more information from the often small amount of material received.

A recently described automated digital multiplexed gene expression/transcript based assay to simultaneously test for *ALK*, *ROS1* and *RET* fusions in NSCLC holds exciting promise as a practical modality for high throughput detection of fusion transcripts (66,68). Known as the nCounter gene expression analysis system (by Nanostring Technologies), this platform combines the advantages of FISH and IHC methods to determine the mutational/expression status of many genes simultaneously in a single test. The novel Nanostring nCounter system is capable of multiplexing up to 800 genes in a single test using a small amount of tumor material (100 ng of total RNA). The technology can be used on RNA/DNA samples and is compatible with RNA of variable quality, in particular FFPE material. As the targets are directly quantified, the nCounter system does not require a polymerase reaction (no conversion step to cDNA by RT-PCR or an amplification PCR step, hence avoiding errors that may potentially be introduced when using short/fragmented DNA material from FFPE). The low yields of RNA/DNA extracted from FFPE material are often degraded or may contain modifications that can inhibit the polymerase reaction, hence this may introduce possible bias to the results. Lira et.al used the nCounter transcript based assay to simultaneously detect *ALK*, *ROS1* and *RET* fusions in NSCLC samples, showing concordance with FISH and IHC methods (68). The benefit of the nCounter system is its ability to directly detect and quantify many targets in a single reaction using a limited sample. Whilst it

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can detect the presence/absence of a fusion/translocation, the 3' overexpression detection method depends on only the higher expression levels of probes distal to the known fusion junctions. As such, it is limited in its ability to discriminate between the specific variant types/translocation partners (68).

The coupling of NGS technologies in conjunction with detection of circulating tumor cells (CTCs) and cell-free circulating tumor DNA (ctDNA) from lysed CTCs in plasma or serum provides a non-invasive method to monitor treatment and track disease progression (69,70). CTCs are thought to shed into the blood stream from the primary or the metastatic tumor deposits, while ctDNA are fragments of DNA that have been released from cells during cell turnover, cell lysis or cell death. The relative levels of CTCs and ctDNA in a patient can be used as a marker of tumor burden and treatment response. Molecular genotyping of the CTCs and ctDNA can be a proxy of the underlying mutations in the tumor from which they derive. CTCs can be characterized by their morphology (the whole cell can be analyzed), by IHC or FISH and genotyped with DNA/ RNA based assays. ctDNA are easier to isolate and extract as compared to CTCs and can be genotyped (for point mutations point mutations, copy number variations, chromosomal rearrangements and structural variations and methylation patterns). These "liquid biopsies" provide a surrogate and additional method of sampling tumor material (compared to more invasive biopsies and resection specimen). CTCs are thought to be mechanism by which tumour cells spread to its distal sites, and this methodology enables real time study of tumor *in vivo* complementing traditional radiologic imaging which is used for follow-up of these patients, to monitor treatment response. It also has the potential for early diagnosis of malignancy and intervention. The application of NGS technology for mutational analysis of CTCs enables detection of treatment resistance and guide clinical decision making (69,70).

Conclusions

Molecular testing to detect oncogenic drivers for targeted treatment is now part and parcel of oncology practice in the era of personalized medicine. There are a multitude of platforms available for somatic mutational testing and the selection of platform is based on the type of mutation to be detected and local clinical and laboratory circumstances. It highlights the importance in using the right test and to select the right patient for the right drug. Screening assays offer the ability to detect all *EGFR* mutations and have the potential to detect novel mutations, while targeted assays offer higher specificity and sensitivity to detect specific known mutations that are clinically actionable. FISH is used to detect fusions characteristic of *ALK*, *ROS1* and *RET* in lung cancer. IHC for *ALK* can be used as an effective screening strategy to select out cases for FISH testing. Novel technologies with the ability to simultaneously detect *ALK*, *ROS1* and *RET* fusions in a single assay show promise for use in the clinical setting as do liquid biopsies. The challenges of genomic testing lie in the complexity of cancer pathways, their heterogeneous nature with an evolving tumor genome that has potential to develop resistance. Rather than sequential testing of specimens for single mutations at the time of treatment, there is an increasing demand for multiplexing and simultaneous detection of many targets at once at the time of diagnosis.

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Overview of clinicopathologic features of *ALK*-rearranged lung adenocarcinoma and current diagnostic testing for *ALK* rearrangement

Hyojin Kim[1], Jin-Haeng Chung[1,2]

[1]Department of Pathology, Seoul National University Hospital, College of Medicine, Seoul, Korea; [2]Department of Pathology, Seoul National University Bundang Hospital, Seongnam, Korea

Correspondence to: Jin-Haeng Chung, MD, PhD. Professor, Department of Pathology, Seoul National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam 463-707, Korea. Email: chungjh@snu.ac.kr.

Abstract: Patients with non-small cell lung cancer (NSCLC) who harbor anaplastic lymphoma kinase (*ALK*) gene rearrangements can derive significant clinical benefit from ALK tyrosine kinase inhibitor. Accurate patient identification is absolutely crucial for successful using ALK inhibitor treatment. However, lung cancer patients with *ALK* gene rearrangement after ALK inhibitor therapy eventually develop acquired resistance to treatment. In this review, the authors summarize the clinicopathologic features of *ALK*-rearranged NSCLC and the pros and cons of current diagnostic testing. In addition, we discuss the current diagnostic flow of ALK testing and consideration of rebiopsy sample during disease progression in patients treated by ALK inhibitors.

Keywords: Anaplastic lymphoma kinase (*ALK*) gene rearrangement; histology; fluorescent in situ hybridization (FISH); immunohistochemistry (IHC); non-small cell lung cancer (NSCLC)

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Introduction

The echinoderm microtubule-associated protein-like 4 and the anaplastic lymphoma kinase (*EML4-ALK*) fusion genes were identified in non-small cell lung cancer (NSCLC) in 2007 (1). The presence of *ALK* fusion in NSCLC is the best predictor of response to crizotinib, an ALK tyrosine kinase inhibitor (2,3), and these data led to the accelerated approval of crizotinib by the U.S. Food and Drug Administration (FDA). The incidence of the *ALK* rearrangement in NSCLC has been reported to be approximately 5% in various studies (1,4-6). Several studies showed particular clinical characteristics of patients with *ALK*-rearranged NSCLC (7-9). In addition, *ALK*-rearranged tumors were associated with histomorphologic features and positive correlation with histologic subtypes using the new International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) lung adenocarcinoma (ADC) classification (4,10-13).

Currently, the three primary methods of detecting *ALK* rearrangements are fluorescent in situ hybridization (FISH), immunohistochemistry (IHC), and the reverse transcriptase polymerase chain reaction (RT-PCR). Each of these individual methods has both advantages and disadvantages. There are many efforts to improve the sensitivity of identifying *ALK* rearrangement and recently, the Ventana ALK assay is a new method of detecting *ALK* rearrangements with high sensitivity (14).

This review is focused on clinicopathologic features of *ALK*-rearranged lung ADC and current diagnostic testing for *ALK* rearrangement.

ALK gene rearrangement in NSCLC

The *EML4-ALK* fusion in NSCLC results from an inversion in the short arm of chromosome two, fusing the

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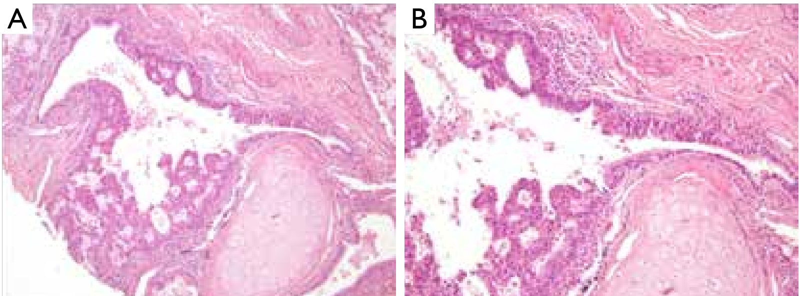

Figure 1 Relationship of *ALK*-rearranged tumors with the bronchiole. (A) Tumor cells invaded the adjacent bronchiolar epithelium (magnification, 10×); (B) at higher magnification, dysplastic epithelial lesions that resembled adjacent tumor cells continued the non-neoplastic bronchial epithelium (magnification, 20×). *ALK*, anaplastic lymphoma kinase.

N-terminal domain of *EML4* to the intracellular kinase domain of *ALK* (3' gene region), resulting in a constitutively active *ALK* tyrosine kinase (1). *EML4-ALK* fusion gene, by itself, is a potent oncogenic driver, reported in about 3-7% of all NSCLC patients. Other fusion partners for ALK have been discovered in NSCLC, such as *TFG-ALK* (15), *KIF5B-ALK* (16), and *KCL1-ALK* (17), and multiple *EML4-ALK* isoforms (18-20) have been identified, but their clinical significance still remains unknown.

Clinicopathologic characteristics of *ALK*-rearranged NSCLC

ALK rearrangements are more often found in never or light ex-smokers, in younger age patients and in lung ADC. Published studies have consistently reported that young age and history of never smoking are statistically different between patients with *ALK*-rearranged and *ALK*-negative lung ADCs (6,8,21,22). Although approximately 70-80% of *ALK*-rearranged patients are nonsmokers, the remaining 20-30% includes ex- or current smokers. Some previous studies, however, found that the *ALK* rearrangements were not associated with non-smoking (23,24). The age range of *ALK*-rearranged patients is commonly lower than NSCLC patients' and even younger than the *EGFR*-mutated population (25). A major challenge is that a younger age at presentation and a lack of smoking history of patients with tumors harboring *ALK* rearrangement are overlapped characteristic of those who harbor *EGFR* mutations.

In our previous study, *ALK*-rearranged tumors exhibited aggressive behavior such as nodal metastasis and advanced disease stage at diagnosis (25,26). In another study, they also observed a strong association of *ALK* rearrangement with advanced stage in NSCLC patients (27), which strengthened the importance of ALK testing in advanced stage disease.

Distinct histomorphologic features of *ALK*-rearranged lung ADC

Several studies have investigated the predictive value of pathological and morphological features in detecting *ALK*-rearranged tumors. Although the results of these studies have been inconsistent because of the limited number of *ALK*-rearranged tumors, solid signet-ring cell and cribriform pattern has been known to be associated with *ALK* rearrangement in lung ADC (7,9-12,28). In our previous study, *ALK*-rearranged lung ADC exhibited several histological characteristics that differentiated it from other genotypes: cribriform formation, presence of mucin-containing cells and presence of psammoma bodies (25). We also identified a close relationship to the adjacent bronchial epithelium is a unique feature of *ALK*-rearranged tumors. In some *ALK*-rearranged cases, tumor cells invaded the adjacent bronchiolar epithelium and showed the appearance of "budding off" of small epithelial cell clusters into the lumen. Furthermore, flat atypical lesions that resembled adjacent tumor cells infiltrated the non-neoplastic bronchial epithelium (*Figure 1*). *ALK*-rearranged tumors were more likely to be centrally located and easily obtained from the bronchoscopic biopsy procedure. Our findings suggest that *ALK*-rearranged tumors might be originated from different cell type, in contrast to *EGFR*-mutated tumors that is originated terminal respiratory unit (TRU) (29-31). In addition, frequent immunoexpression of p63 as well as TTF-1 in *ALK*-rearranged tumors has been described in a few studies (10,25) (*Figure 2*). Although the frequent reactivity to TTF-1 in *ALK*-rearranged tumors indicates

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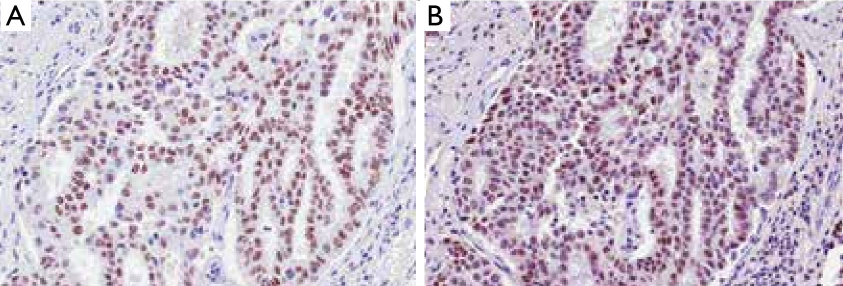

Figure 2 (A) Dual nuclear expression of TTF-1 (magnification, 40×) and (B) p63 in *ALK*-rearranged tumors (magnification, 40×). *ALK*, anaplastic lymphoma kinase.

derivation from TRU (31), type II pneumocytes or Clara cells native to that unit are typically negative for p63 (32). We proposed that a cell type that dually expresses TTF-1 and p63, as a cell of origin of *ALK*-rearranged tumors and overexpression of p63 might have functional roles related with carcinogenesis or tumor differentiation in specific subset of lung ADCs, however, a specific cell type of *ALK*-rearranged tumors has not yet been elucidated.

A few studies have reported a positive histological correlation with *ALK* rearrangement in lung ADC using the new IASLC/ATS/ERS classification that was published in 2011 (4,10-13). The solid subtype was significantly more frequent in the *ALK*-rearranged cancers, however, an *ALK*-positive rate is about 8% among the solid subtype ADCs that is similar with 9% in acinar subtype (SNUBH unpublished data). In our study, *ALK*-rearranged lung ADCs were also significantly associated with solid predominant subtype and not with acinar or papillary predominant subtypes (33). Another study showed that the existence of a minor mucinous component was independently associated with a relatively high prevalence of *ALK* rearrangement (34). However, no morphological characteristics could identify a specific genetic subtype, suggesting that genetic alterations are associated with a spectrum of morphological features.

Diagnostic methods for detecting *ALK* gene rearrangement

Currently three main methods of detecting *ALK* rearrangement are FISH, IHC, and the RT-PCR.

FISH has been considered the gold standard method for detecting *ALK* rearrangement. The FDA in the USA approved the Abbot Vysis *ALK* Break Apart FISH Probe Kit for companion diagnostic testing for *ALK*-rearranged NSCLC. Although FISH can detect rearrangements regardless of the fusion partners, it is expensive, generally requires specialized technical resources and expertise and thus cannot be applied in all pathological laboratories. In clinical practice, it is important to determine the presence of an *ALK* rearrangement in small biopsy samples with advanced stage NSCLC patients. Therefore, FISH analysis may not be available for screening all NSCLC patients.

Alternatively, IHC is less expensive and less time-consuming than *ALK* FISH, and is a well-established method in the routine work of every pathology department. IHC is less sensitive than FISH analysis to variations in handling or pretreatment of specimens, and a diagnosis can be established with a smaller number of tumor cells than required for FISH analysis. Several antibodies and detection systems have been investigated for overcoming the low expression level of the ALK fusion protein (35-37). 5A4 and D5F3 are known to be high affinity antibody clones (*Figure 3*) (35-38). Recently, the novel fully automated ALK IHC assay developed by Ventana company has been introduced that uses D5F3 antibody and relies on the tyramide amplification technique bound to the Ventana automated BenchMark XT for high sensitivity (*Figure 3B*). Several studies have demonstrated that there is a high concordance between the Ventana IHC and FISH (14,39,40). In September 2013, this automated IHC of Ventana Company has received China's FDA approval as a companion diagnostic identifying ALK protein expression in lung cancer patients.

The RT-PCR is a more sensitive and rapid method that can identify specific variants of the *ALK* rearrangements. However, RT-PCR requires *ALK* fusion variants to be known so that primers to all variants are included in the reaction. Although with an ever-expanding list of *ALK* fusion variants, all the reported variants require skillful application. In addition, majority of current *ALK* fusion variants were detected by RT-PCR in fresh frozen tumor tissue. However, in daily clinical practice, most of the tumor tissue available for molecular profiling is from

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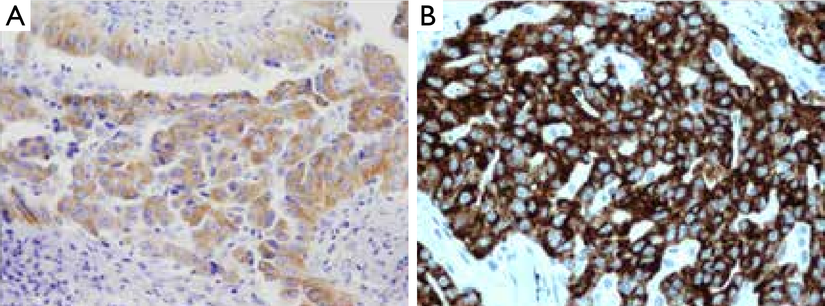

Figure 3 (A) ALK protein expression on immunohistochemistry using 5A4 antibody (magnification, 40×) and (B) D5F3 antibody (magnification, 40×). ALK, anaplastic lymphoma kinase.

FFPE tissue, where the integrity of RNAs is likely to be greatly compromised compared with fresh frozen tissue. Although FISH and IHC can be performed on a single FFPE slide, RT-PCR requires multiple slides in order to extract sufficient RNA for a successful reaction. Therefore, detecting *ALK* rearrangements using RT-PCR continues to be challenging in routine practice.

ALK testing in routine practice

Currently, crizotinib therapy is indicated only in *ALK*-rearranged NSCLC patients who are either inoperable or have residual or recurrent disease after surgery. However, the majority of lung cancer patients present with advanced stage and at the time of initial biopsy many patients have not been fully-staged or assessed for surgery. In this context, the guidelines for molecular testing in lung cancer recently published by the College of American Pathologists (CAP), IASLC, and Association for Molecular Pathology (AMP) has recommended performing *ALK* FISH testing at the time of diagnosis for patients presenting with advanced-stage NSCLC who are suitable for medical therapy or at a time of recurrence (41). That is, reflex ALK testing in all lung cancer patients would be encouraged but is only possible if it can be performed in a cost effective and timely manner.

As a companion diagnostic test, reflex *ALK* FISH in all lung cancer patients would be desirable, however, this strategy in the routine practice is difficult due to several limitations such as cost, resource and time constraints. Recently, many retrospective studies have suggested that ALK IHC can be used as a screening test for *ALK* gene rearrangements in lung cancer (6,35,38,42-45). Thus, reflex ALK IHC followed by confirmatory FISH testing can be readily integrated into the routine clinical setting and represents a cost effective and practical approach to screening for this druggable gene rearrangement. For the successful reflex test of ALK, we caution that ALK IHC should be fully validated in individual laboratories, performed with appropriate lung specific protocols when applied in clinical setting and controlled based on the results of the test. Even if IHC result is negative, FISH studies can still be performed on patients with a high clinical suspicion of *ALK* gene rearrangement.

ALK testing of rebiopsy samples during disease progression in patients treated by ALK inhibitor

Many of advanced NSCLC harboring *ALK* gene rearrangement treated with ALK inhibitors eventually relapse due to acquired resistance. Identifying the various mechanisms of resistance is critical to developing new treatment strategies in the acquired resistance setting. Several studies have identified several resistance mechanisms to crizotinib in rearranged *EML4-ALK* NSCLC and more studies are needed to fully understand the resistance mechanisms and to define new targeted strategies (46-48). This resistance has been associated with various tumoral genetic changes, such as other mutations in the *ALK* gene, *ALK* gene amplification or activating mutations of other genes (49). These changes may guide the selection of further treatments in these patients with resistant tumors. Therefore, it is widely accepted that rebiopsy is useful at the time of progression. However, this depends on the feasibility of rebiopsy at this time. Bosc *et al.* evaluated the percentage of patients who underwent rebiopsy with mutant *EGFR* or *ALK*-rearranged NSCLC and acquired resistance to tyrosine kinase inhibitors (50). A rebiopsy was considered as feasible in 72% while a biopsy was in fact performed in 46%. When rebiopsy was performed, there was sufficient

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tumor material in the vast majority of cases (more than 85%) in several studies (50,51).

There were few contraindications to biopsy, reflecting the fact that patients with activating mutations are often nonsmokers or former light smokers and therefore less prone to tobacco related comorbid conditions such as COPD and heart disease. The most frequent constraint was poor physical condition, probably associated with cancer progression.

It should be considered that some degree of heterogeneity may occur between the primary tumor and its metastases. We previously found ALK protein expression in 11.9% (8/67) of primary NSCLCs and 25.4% (17/67) of their matched metastatic lesions, indicating that metastatic progression can be associated changes in ALK expression (52). Regarding the biopsy site, some authors consider that the highest failure rates are observed when the tissue is obtained from bone samples (53). These high failure rates are mostly observed when a decalcification process is needed. Despite significant improvements using EDTA (54), we have to recognize that bone biopsies are still not recommended for molecular testing. Patients and physicians may be reluctant to accept a surgical brain biopsy, even a minimally invasive stereotactic biopsy.

Understanding the molecular mechanisms of resistance and personalizing the treatment accordingly justify the need for rebiopsy. Although a vast majority of patients may undergo a second biopsy procedure, in one third of cases a biopsy was either not feasible, contraindicated or not suitable for molecular analysis. This emphasizes the need for the development of less invasive techniques.

Clinical impact and conclusions

The codevelopment of drug with a companion diagnostic assay has accelerated rapid development in the area of diagnostic assays in lung cancer. This led to the most sensitive, specific, and cost-effective assay for the screening of *ALK* rearrangement. As well as diagnostic testing, understanding distinct clinical and histomorphological characteristics of *ALK*-rearranged lung cancer may improve diagnostic accuracy and help us to detect all patients with *ALK*-rearranged lung cancer.

With the advances in acquired resistance after crizotinib therapy, the importance of repeat tissue acquisition and molecular testing during disease progression and the need for close collaboration between pathologists and clinicians are increasing.

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Circulating DNA in diagnosis and monitoring *EGFR* gene mutations in advanced non-small cell lung cancer

Paola Bordi[1], Marzia Del Re[2], Romano Danesi[2], Marcello Tiseo[1]

[1]Medical Oncology Unit, University Hospital of Parma, Parma, Italy; [2]Department of Clinical and Experimental Medicine, Clinical Pharmacology Unit, University of Pisa, Italy

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Correspondence to: Paola Bordi. Medical Oncology Unit, University Hospital of Parma, Via Gramsci 14, 43126 Parma, Italy. Email: paolabordi@yahoo.it.

Abstract: Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are current treatments for advanced non-small cell lung cancer (NSCLC) harboring activating *EGFR* gene mutations. Histological or cytological samples are the standard tumor materials for *EGFR* mutation analysis. However, the accessibility of tumor samples is not always possible and satisfactory in advanced NSCLC patients. Moreover, totality of EGFR mutated NSCLC patients will develop resistance to EGFR-TKIs. Repeat biopsies to study genetic evolution as a result of therapy are difficult, invasive and may be confounded by intra-tumor heterogeneity. Thus, exploring accurate and less invasive techniques to (I) diagnosis *EGFR* mutation if tissue is not available or not appropriate for molecular analysis and to (II) monitor EGFR-TKI treatment are needed. Circulating DNA fragments carrying tumor specific sequence alterations [circulating cell-free tumor DNA (cftDNA)] are found in the cell-free fraction of blood, representing a variable and generally small fraction of the total circulating DNA. cftDNA has a high degree of specificity to detect *EGFR* gene mutations in NSCLC. Studies have shown the feasibility of using cftDNA to diagnosis of EGFR activating gene mutations and also to monitor tumor dynamics in NSCLC patients treated with EGFR-TKIs. These evidences suggested that non-invasive techniques based on blood samples had a great potential in EGFR mutated NSCLC patients. In this review, we summarized these non-invasive approaches and relative scientific data now available, considering their possible applications in clinical practice of NSCLC treatment.

Keywords: Circulating DNA; epidermal growth factor receptor (EGFR); non-small cell lung cancer (NSCLC); tyrosine kinase inhibitor (TKI)-resistance

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Serum biomarkers for non-small cell lung cancer (NSCLC)

NSCLC is still the main cause of cancer related death in males and females across Western countries. It is commonly known that about 50% of NSCLC is diagnosed in advanced stage and for the majority of these patients, even if encouraging data regarding immunotherapy have been published, to date chemotherapy still represents the mainstay of treatment and prognosis remains poor (1,2). However, approximately 15-20% of advanced NSCLC presents a targetable driver mutation, a condition that dramatically changes therapeutic perspectives and patient outcome (3-6).

Mutations in the gene encoding for the epidermal growth factor receptor (EGFR) represent the first driver mutations identified in NSCLC. The presence of the mutation implicates a receptor constitutively activated that continuously gives the cell input favoring proliferation

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(7,8). In 90% of cases EGFR activating mutations are represented by exon 19 deletions and exon 21 L858R point mutations (9). It has been established that EGFR activating gene alterations are more common in patients with specific clinico-pathological characteristics, such as female, never smoker, Asiatic origin and adenocarcinoma histological subtype. *EGFR* mutations represent the most important factor for prediction of response to EGFR tyrosine kinase inhibitors (TKIs). In fact, they are associate with significant increase in response rate (approximately 70%) and improvement in progression free and overall survival (OS) (4,5,10). To date, TKI registered as first line therapy for patients with EGFR mutated NSCLC are gefitinib, erlotinib and afatinib and their toxicity profile is certainly more tolerable than standard chemotherapy. However, for drug prescription purpose, the presence of *EGFR* mutation needs to be demonstrated and therefore neoplastic tissue sample is always required.

Unfortunately, biopsies in lung cancer represent a criticism. Bronchoscopy and trans-thoracic biopsies are not well accepted by patients and the event that tumoral material is not sufficient or adequate for molecular analyses is not so infrequent (11). Bone biopsies are a critical issue because decalcification procedures interfere with molecular testing and results (12). Moreover, a single biopsy cannot reflect the clonal heterogeneity of the tumor, which could be present in a single tumor lesion (intratumoral heterogeneity) or between different sites of the tumor (intermetastatic heterogeneity) (13-15). Finally, bioptic procedures are not free from related risks (16). Recent advances in therapeutic management of patient with EGFR mutated NSCLC demonstrated the importance of identifying, after the progression to TKI, the molecular mechanisms of acquired resistance in order to continue, as long as possible, a tailored therapy based on the developed resistance alteration (17,18). This approach entails the repetition of a biopsy theoretically every time a patient experiences a progression of disease with a consequent increased discomfort for the patient who undergoes re-biopsy. Moreover, the re-biopsy after progression is not feasible when disease progression involves a body site that can be reached only with complicated surgical procedures (i.e., brain). All this considerations have given the research the incentive for the identification of more accessible and tolerated methodologies for molecular alteration identification.

Several attempts were done in order to identify reliable serum biomarkers for cancer. In the past, serum proteins, such as for example carcinoma carcinoembryonic antigen (CEA), have been commonly used for diagnosis of different cancer but due to low specificity and sensibility their routinely use is not recommended (19). Subsequently, the identification of circulating tumor cells (CTCs) in serum of patient with cancer seemed to represent the solution for cancer serum diagnosis and monitoring. However, several problems emerged regarding the best method for their isolation as different available devices, basing the selection on cells dimension or antigen expression, presented a moderate risk of false negatives (20). Recently, the attention moved to the possibility of isolation and analysis of cell-free tumor DNA (cftDNA) that, to date, represents the best candidate for identification and monitoring of molecular tumor-related alterations in blood of patients with cancer (21).

Fragments of circulating DNA were isolated in plasma many years ago (22). In particular, patients with cancers present higher levels of circulating DNA comparing to healthy volunteers because of the presence of tumoral counterpart, which express the same molecular abnormalities expressed by DNA of primitive mass (13). The elevate cellular turnover and consequent cellular necrosis and apoptosis cause a massive release of tumoral DNA into the bloodstream were it can be isolated and analyzed. Therefore, tumor size, localization and vascularity may influence cftDNA plasmatic levels. It is also possible that part of cftDNA comes from CTCs lysis (13). The analysis of cftDNA, defined as liquid biopsy, could be repeated every time needed and without any discomfort for patients. Moreover, the mutational analysis of cftDNA demonstrated a significantly better sensitivity if compared with CTCs one, establishing cftDNA as the best circulating source for molecular analysis (23). Information derived from liquid biopsy could be used in future for early cancer diagnosis, assessment of genetic determinants for targeted therapies, monitoring of tumor dynamics and early evaluation of tumor response, identification of resistance mechanisms (13).

In the last years, techniques for cftDNA analysis have been largely employed for identification of activating and resistance mutations in NSCLC EGFR mutated patients and the aim of this review is discuss principal findings.

Circulating free tumor DNA and technologies for its detection

cftDNA could be a relevant biomarker to molecular

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diagnosis and monitor treatment resistance, because of its sensitivity and specificity, but it really needs reproducible and standardized methods, both for the extraction and for its analyses.

Most of the published papers used conventional methods for the cftDNA extraction with commercially available kits for routine use, based on selective binding to a silica-based membrane for improved recovery of fragmented nucleic acids (i.e., Qiagen, Norgen). While the amount and the quality of cftDNA can deeply vary, high-analytical sensitivity and specificity techniques are required for its detection; moreover, a critical issue is to make a distinction and a choice between the importance and the clinical role of cftDNA quantification and mutation analysis. Because of it, many published studies applied a combined quantitative and qualitative analysis of cftDNA starting from surgery and during follow-up, founding that during follow-up, cftDNA levels decrease progressively, but rapidly increased when a relapse occurred, whereas specific mutations were detected only in relapsed patients (24). Dawson and colleagues analyzed the cftDNA of 30 metastatic breast cancer patients to monitor response to treatment. cftDNA was detected in 29/30 patients, showing that cftDNA levels have a dynamic range and the correlation with variations in tumor burden were better than did CA 15.3 serum biomarker or CTCs (25).

Regarding the mutation analysis of cftDNA, a large number of technologies is now available to analyze mutations in cftDNA, including automatic sequencing, real-time polymerase chain reaction (PCR) platforms, mass spectrometry (MS) genotyping, amplification protocols with magnetic beads in oil emulsions [beads, emulsion, amplification and magnetics (BEAMing)] and next-generation sequencing (NGS), digital PCR platforms (26-30). The sensitivity range of the available techniques varies from 15% to 0.01%, but one of the major gaps in this field is the lack of standardization of techniques, in order to understand how those techniques are cost-effective and reliable to fit clinical needs.

Among techniques most of them are able to detect mutant allele frequencies with a sensitivity of at least 2%, other, like cold-PCR, can reach somatic mutations at very low frequencies of 0.1-0.5%, and many genotyping approaches can be combined with it to analyze known mutations [i.e., MS-based matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) genotyping technologies] (31).

Real-time PCR

One of the widely used methods to detect known mutations is the real-time PCR. The real-time PCR works with either TaqMan probes or TaqMan Detection Mutation Assay. TaqMan probes have a sensitivity detection limit of approximately 10% (32), otherwise, TaqMan Detection Mutation Assay is a competitive allele-specific TaqMan PCR technology, with high sensitivity and specificity because the mutant allele detection is based on an allele-specific primer, while an MGB blocker oligonucleotide suppresses the wild-type background and high sensitivity. Thanks to this mechanism of action, the TaqMan Detection Mutation Assay is able to detect as low as 0.1% mutant molecules in a background of wild type genomic DNA (Cancer Biomarker Research using castPCR™ Technology, AACR 2012). Real-time PCR can also work with Scorpion primers, a kind of bi-functional molecule in which a primer is covalently linked to the probe, with a fluorophore and a quencher. In the absence of the mutation, the quencher close to the fluorophore absorbs its fluorescence. During the Scorpion PCR reaction, the presence of a mutation separates the fluorophore and the quencher increasing the emitted fluorescence (http://www.premierbiosoft.com/tech_notes/Scorpion.html).

Most of the published studies adopted this technology for the analysis of cftDNA in lung cancer. In particular, results coming from analyses focused on the detection of the *EGFR* mutations in cftDNA of patients with EGFR mutated tumors showed a wide variability: the concordance ranges from 43% to 100% (23,33). Unfortunately, in some cases, mutations can be missed using Real Time technology, and therefore the results are inferior compared to more sensitive approaches.

Digital PCR

The digital PCR approach is based on the same principle of the real-time PCR, but while the real-time PCR works as a unique solution, the digital PCR is able to divide the amplification mix in several thousand of replicates. This partition permits the amplification and the analysis considering single spots, which means that the system is able to decrease the ration of cftDNA/germinal DNA, increasing sensitivity. Digital PCR can works on various principles, for example, silicon chips (Quant studio 3D, Life technologies, Carlsbad, CA, USA) or micro droplets (Bio-Rad Qx100, BioRad, Hercules, CA, USA). This kind of technology can theoretically increase the sensitivity to 1:100,000 molecules of cftDNA in a germinal DNA background (34). Disadvantage of this technology is the not

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standardized threshold to establish the presence and the amount of mutations.

Beads, emulsion, amplification and magnetics (BEAMing)

Many other approaches, like BEAMing technology, are able to detect a very small amount of mutant DNA sequences in a larger pool of fragments containing wild-type DNA, in order of a single mutant allele in a background of 10,000 wild-type alleles, and it is able to enabling copy-number quantification (35). BEAMing is a sensitive method to detect known genetic mutations, even when at very low copy numbers. The technique is based on a combination of emulsion digital PCR and flow cytometry, with beads, emulsification, amplification and magnetics to achieve the necessary level of sensitivity. DNA sequences are amplified via emulsion PCR covalently bound to magnetic microbeads via streptavidin-biotin interactions; the PCR products generated in each emulsion droplet will remain physically affixed to the microbeads at the end of the reaction, allowing them to be easily separated and purified using a magnet, to determine the presence and number of known mutant variations. The wild-type or mutant DNA can be easily differentiated using flow cytometry. Unfortunately, the BEAMing workflow results complex limiting the feasibility and reproducibility of the technology.

Next-generation sequencing (NGS)

All the mentioned techniques are able to find only known mutations in samples, and this means that a patient need to have a tumor biopsy screened in advance to capture the mutational status, consequently, in terms of costs and standardization of the analysis, it is need to personalized a panel test for each patient. The analysis of cftDNA using NGS technology has recently demonstrated to offer increase detection sensitivity, showing also a good specificity in patients with advanced cancers (27). Published studies demonstrate that deeper sequencing of plasma DNA may allow the problem of clonal heterogeneity and selection (36).

Many NGS technologies are available to date, all of them produce short sequences from single molecules of DNA and it is compared to a reference sequence, allowing the sequencing of large portion of the genome. Selecting only a limited number of sequences of frequently mutated genes, it is easy to reach very deep cover- age of sequencing for candidate mutation loci. This allows the identification of mutated alleles even if highly diluted. Moreover, one of the advantages of the NGS is that whole-genome sequencing of cftDNA can allow the identification of rearrangement and region of copy number aberrations, not detectable with other techniques (27).

Unfortunately, in terms of daily application into the clinic, the use of a NGS technique is still so far, the management of the data requires expert biologists in library preparation, a dedicated bioinformatics support is recommended to solve computational problems that occur during the project and it is an expensive technique.

Genotyping MS

A considerable number of technologies are available for the detection of mutations using MS, but nowadays, the MALDI-TOF MS has become the most used method. The genotyping method is able to distinguishing different alleles by the different masses of primer extension products. The experimental procedure is divided into three steps: amplification, primers extension reaction, transfer of the reaction product into a chip that contains a specific matrix, with two intermediate cleaning reactions, before detection of the extension products. At the end of the analysis, the peak spectrum resulting from MALDI-TOF MS analysis can be analyzed with software that traces back primer masses to assayed alleles. MALDI-TOF MS is relatively more expensive and time consuming than RT-PCR-based methods, but it is more suitable for the simultaneous analysis of multiple mutations. Sequenom is nowadays into clinical routine for the analysis of somatic mutations from FFPE tissue; one of the limitations of this method, common to other similar genotyping techniques, is that it only returns genotypic data. For this reason, analyses with more than one single nucleotide polymorphism (SNP), such as linkage disequilibrium or haplotype diversity, require the most likely haplotypes to be inferred.

cftDNA for identification of *EGFR* mutations in patients with NSCLC

To validate cftDNA analysis for *EGFR* mutations detection, results obtained in serum have been compared with the actual gold standard that is analysis on tissue from tumor biopsy. To our knowledge, the first authors that compared results from serum and paired tissue samples were Kimura and colleagues in 2006 (37). Even if paired samples were just 11, authors reported a 72.7% of concordance between serum and tissue. One year later, the same author published

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another casistic of 42 patients were EGFR mutational status was consistent with tissue one in 92.9% of cases (38). In 2009, Yung *et al*. detected EGFR 19del and L858R in 17% and 26%, respectively, of 35 pre-therapy plasma samples by using digital PCR; when data were compared with results from tumor samples, overall serum analysis demonstrated very high sensitivity and specificity (92% and 100%, respectively) (26).

Other studies published and conducted on Asiatic populations, revealed high grade of specificity and moderate grade of sensitivity (39,40). Furthermore, authors observed a significant increase in sensitivity when only patients with advanced stage or poorly differentiated adenocarcinoma were evaluated. These data can be explained considering that overall tumor mass and aggressivity can influence levels of cftDNA and therefore the possibility of *EGFR* mutation detection.

The principal data regarding Caucasian patients were published by Weber and Douillard (41,42). Weber *et al*. analyzed pairs of diagnostic biopsy and plasma sample of 199 patients obtained prior commencing therapy with EGFR-TKI (41). The overall concordance between plasma and tissue was 179/199 (90%) and six mutations were present only in plasma sample but not in bioptic specimens suggesting a possible role of tumoral heterogeneity. Douillard and colleagues published data regarding patients enrolled in the phase IV study of gefitinib in Caucasian patients with advanced stage IV EGFR mutated NSCLC (42). All patients were centrally screened for *EGFR* mutation in tissue sample and matched baseline plasma samples were mandatory. Authors matched 652 tumor and plasma samples and concordance resulted 94.3%, sensitivity 65.7% and specificity 99.8%, concluding that, even if tumor remains the preferred source, plasma testing could be appropriate in patients without available tissue. This statement is based on the evidence that patients with EGFR mutated cftDNA presented a response rate similar to patient with EGFR mutated tissue.

Recently, Mok published results of analysis conducted on data from the FASTACT-2 study where patients were randomized to receive platinum-based chemotherapy plus sequential erlotinib or placebo (43). Authors matched 238 plasma and tissue samples and concordance was 88%, sensitivity 75% and specificity 96%. Similar to previous study, patients with EGFR-positive cftDNA treated with erlotinib presented a significantly better outcome than patients treated with placebo [progression-free survival (PFS) 13.1 *vs*. 6.0 months; P<0.0001], while no difference emerged between EGFR-negative cftDNA patients treated with erlotinib or placebo. These results enforce the role of cftDNA *EGFR* mutations as predictive factor for response to EGFR-TKI confirming they could represent a reliable surrogate of tissue determination.

Considering the high number of reports present in literature, two meta-analysis investigating the diagnostic value of cftDNA for *EGFR* mutations identification have been published and both included studies with paired tissue and plasma samples (44,45). Characteristics of the studies included in the two meta-analyses are summarized in *Table 1*. The first one considered results from 20 published studies of which all were conducted in Asia but one conducted in USA (44). Results showed a pooled sensitivity of 0.674 (95% CI: 0.517-0.800) and a pooled specificity of 0.935 (95% CI: 0.888-0.963). Positive and negative likelihood ratios were 10.307 (95% CI: 6.167-17.227) and 0.348 (95% CI: 0.226-0.537), respectively. The summary receiver operating characteristic (SROC) curve was generated and area under the curve (AUC) resulted 0.93 [0.90-0.95] indicating high diagnostic accuracy. The other meta-analysis considered 27 studies of which a consistent part already included in the previous one, five studies regarding Caucasian populations and five studies published in 2014 including ones by Douillard and Weber. Pooled sensitivity and specificity were 0.620 (95% CI: 0.513-0.716) and 0.959 (95% CI: 0.929-0.977), respectively and AUC was 0.91 (95% CI: 0.89-0.94). As previously reported, accuracy increased in patients with advanced stage disease (AUC 0.96, 95% CI: 0.94-0.97). The authors of both meta-analyses conclude in favor of the high diagnostic accuracy showed by cftDNA underlying the high specificity and non-invasivity that make it a useful tool for screening. However, some limitations have been described including the presence of heterogeneity between studies and the absence of a unique and specified time of blood collection that could have a significant impact as chemotherapy could influence EGFR status (66).

After publication of these meta-analysis, results of two relevant studies (ASSESS and IGNITE trials) investigating the utility of ctDNA in plasma for the detection of *EGFR* mutation were presented at European Lung Cancer Conference 2015 (67,68). Both are multicenter diagnostic studies evaluated the utility of ctDNA for *EGFR* mutation testing in a real-world setting (Europe and Japan in ASSESS and Asia-Pacific and Russia in IGNITE, respectively), having as primary objective the concordance between *EGFR* mutation status obtained via tissue or cytology and plasma-based testing (*Table 2*). Both studies have

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Table 1 Characteristics of studies included in the two meta-analyses evaluating cftDNA in *EGFR* mutation detection

First author	Country	Year	Detection methods	Female (%)	Adenocarcinoma (%)	Ever smokers (%)	No. of samples	Sensitivity (%)	Specificity (%)
Kimura H (37)	Japan	2006	ARMS	37.3	85.2	NA	11	75	40
Kimura H (38)	Japan	2007	ARMS	33.3	73.8	66.7	42	75	97
He C (46)	China	2009	ME-PCR	36.6	75.4	53	18	89	100
Yung TK (26)	China	2009	Digital PCR	NA	NA	NA	29	100	94
Kuang Y (47)	USA	2009	ARMS	81.5	NA	NA	43	70	85
Bai H (48)	China	2009	DHPLC	46.5	74.3	44.8	230	97	92
Sriram KB (49)	Australia	2011	ME-PCR	33.9	56.3	93.7	64	50	100
Jiang B (50)	China	2011	ME sequencing	31	72.4	62.1	58	78	100
Taniguchi K (51)	Japan	2011	BEAMing	65.9	95.5	NA	44	73	0
Brevet M (52)	USA	2011	Sequenom	51.6	96.8	54.8	31	44	85
Goto K (33)	Japan	2012	AS-APEX	87.6	NA	9	86	43	100
Nakamura T (53)	Japan	2012	I-PCR-QPM	51.3	100	46.2	70	45	100
Hu C (54)	China	2012	HRM	50	58.3	45.8	24	100	0
Huang Z (55)	China	2012	DHPLC	46.7	78	41.4	822	64	85
Xu F (56)	China	2012	ARMS	39.2	84.3	NA	34	50	100
Yam I (57)	China	2012	AS-APEX	60	94.3	14.3	35	100	80
Jing CW (58)	China	2014	HRM	42.5	58.3	NA	120	64	97
Liu X (59)	China	2013	ARMS	34.9	98.8	54.7	86	68	100
Lv C (60)	China	2013	DHPLC	54.5	NA	45.5	6	0	100
Zhang H (61)	China	2013	MEL	43	75.6	51.2	86	68	100
Kim ST (62)	Korea	2013	PNA-LNA PCR clamp	38.6	70.2	56.1	57	66	93
Zhao X (39)	China	2013	ME-PCR	31.5	65.8	51.4	111	35	98
Kim HR (63)	Korea	2013	PNAClamp	NA	NA	NA	40	17	100
Li X (plasma) (64)	China	2014	ARMS	42.5	78	46.8	141	48	95
Li X (serum) (64)	China	2014	ARMS	44	79.6	43.5	108	40	96
Weber B (41)	Denmark	2014	Cobas EGFR blood test	49	95	91	196	61	96
Douillard JY (42)	Europe	2014	ARMS	NA	NA	NA	652	66	99
Wang S (65)	China	2014	ARMS	48.5	80.6	46.3	74	22	97

ARMS, amplification refractory mutation system; ME-PCR, mutant-enriched-PCR; DHPLC, denaturing high-performance liquid chromatography; ME-sequencing, Mutant-enriched sequencing; BEAMing, beads, emulsion, amplification and magnetics; AS-APEX, allele-specific arrayed primer extension; I-PCR-QPM, inhibiting-PCR-sequencing probe method; HRM, high-resolution melting; MEL, mutant-enriched liquid chip; PNA-LNA, peptide nucleic acid-locked nucleic acid; NA, not available

controversial results, probably in relation to heterogeneous methodologies used; in fact, if plasma samples were processed in central designated laboratories, nevertheless *EGFR* mutation testings on tissue were performed according to local practices and, sometimes, with low sensitive techniques. In ASSESS trial, 1,311 patients were enrolled with data available on both tissue and plasma samples of 1,162. Considering overall results, the concordance obtained was 89.1%, with a sensitivity of 46%, specificity of 97.4%, positive predictive value (PPV) of 77.7% and negative predictive value (NPV) of 90.3%. Considering a subgroup with same methodology used in tissue and plasma,

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Table 2 ASSESS and IGNITE trials

Parameter	ASSESS trial				IGNITE trial			
	Overall (n=1,162)		Same method (n=254)		Asian pacific patients (n=1,687)		Russian patients (n=894)	
	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI
Concordance	1,035/1,162 (89.1)	87.1-90.8	221/254 (87.0)	82.2-90.9	1,310/1,687 (77.7)	75.6-79.6	767/894 (85.8)	83.3-88.0
Sensitivity	87/189 (46.0)	38.8-53.4	25/56 (44.6)	31.3-58.5	343/692 (49.6)	45.8-53.4	33/109 (30.3)	21.8-39.8
Specificity	948/973 (97.4)	96.2-98.3	196/198 (99.0)	96.4-99.9	967/995 (97.2)	96.0-98.1	734/785 (93.5)	91.5-95.1
PPV	87/112 (77.7)	68.8-85.0	25/27 (92.6)	75.7-99.1	343/371 (92.5)	89.3-94.9	33/84 (39.3)	28.8-50.5
NPV	948/1,050 (90.3)	88.3-92.0	196/227 (86.3)	81.2-90.5	967/1,316 (73.5)	71.0-75.8	734/810 (90.6)	88.4-92.5

n, numerator value for each parameter; N, denominator value for each parameter; PPV, positive predictive value; NPV, negative predictive value.

in particular as Therascreen®, results improve (concordance 94.9%, sensitivity of 72.7%, specificity 99.1%, PPV 94.1% and NPV 95%) and are similar those obtained in previous small experiences (42). In IGNITE trial, 3,382 patients were enrolled with data available on both tissue and plasma samples of 2,581. Results obtained (see *Table 2*) showed findings that need some clarification, in particular in Russian patients; in fact, PPV is low, the percentage of mutations in non-adenocarcinoma is higher than expected (about 10% of cases overall, with higher percentage in plasma than in tissue samples in Russian patients, 7.1% *vs*. 3.7%, respectively), as well as the percentage of rare mutations (15.5% and 26.7% in Russian adenocarcinoma and non-adenocarcinoma samples, respectively).

The role of *KRAS* mutations in patients with EGFR mutated NSCLC is still controversial. In fact, *EGFR* and *KRAS* mutations have always been considered mutually exclusive in lung cancer and *KRAS* mutations demonstrated a negative predicting effect for response to EGFR-TKI. However, recently studies demonstrating the coexistence of these molecular alterations on tissue samples were published (69). Authors observed that KRAS mutation did not preclude response to EGFR-TKI suggesting that the interaction between the two pathways may be more complex (69). Coexisting *EGFR* and *KRAS* mutations have been isolated also in plasma in some studies (62,70,71). Wang *et al*. reported EGFR/KRAS co-presence in five out of 120 patients who presented PFS and OS significantly inferior to patients harboring only *EGFR* mutation (70). The presence of both mutations at diagnosis was reported also by Kim *et al*. in five out of 57 patients. However, in their experience, *KRAS* serum mutation did not influence prognosis (62). It is worth noting that advances in technologies for DNA molecular analysis could open new scenarios and the role of different mutations may be re-assessed.

Acquired resistance to EGFR-TKI and role of cftDNA

Unfortunately, acquired resistance is an inevitable process during therapy with EGFR-TKI and usually it develops after a median treatment period of 10-12 months (72). Molecular mechanisms underlying acquired resistance have been largely investigated and the occurrence of a second *EGFR* mutation in exon 20 (T790M) resulted the most frequent resistance-associated molecular alteration with a prevalence ranging from 49% to 63% (72,73). Other less frequent mechanisms of resistance are represented by HER2 amplification (12-13% of cases), MET amplification (5-11%), *PIK3CA* mutations (about 5%) or *BRAF* mutations (1%) (73,74). A particular situation is represented by the emergence of a neoplastic clone with clinical and histological features consistent with small cell lung cancer (SCLC) that is reported in 3-14% of cases and implies a more aggressive behavior (72-74).

T790M was reported for the first time in 2005 and its presence increases receptor affinity for ATP that reduces TKI capability to bind EGFR translating in drug

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inefficacy (75-77). The presence of a clone harboring T790M resistance mutation has been associated with indolent progression and favorable prognosis (78). In fact, Oxnard and colleagues evaluated T790M expression in patients with EGFR-TKI acquired resistance and found out that T790M was significantly more frequent in loco-regional sites of disease than in distant ones and associated with longer post-progression survival. On the contrary, patients without T790M were more likely to progress with new sites of disease in previously uninvolved organs and presented poorer performance status. Similar results have been reported by Oya and colleagues (79); 48% of patients presented T790M in the re-biopsy specimen that was significantly associated with more local than systemic disease progression. Different results were recently reported by Zheng *et al*. in a Chinese cohort of 117 patients; in fact, even if T790M prevalence (47%) in resistant patients and early onset are confirmed, authors showed that T790M patients presented significantly shorter OS (80).

The importance of the identification of the mechanism involved in acquired resistance is not only theoretical since the efficacy of next generation EGFR-TKI has been demonstrated. Recently, results from trials testing two new molecules AZD9291 and rociletinib have been published and show an impressive efficacy especially in T790M-positive patients, with response rate ranging between 59% and 61% and a median PFS ranging from 9.6 to 13.1 months after progression to first-line TKI (17,18). Similarly to what stated above, the T790M presence need to be demonstrated with re-biopsy after progression and frequently this could represent a limit in lung cancer patients. However, the feasibility of resistance monitoring by plasma DNA sequencing has been proved in several cancers, including EGFR mutated NSCLC [(36), *Table 3*]. In this study, authors evaluated the variation of mutant allele fractions associated with resistance to oncological treatment in patients with different cancers. Principal findings included the increase of mutations in *PIK3CA* after therapy with paclitaxel in breast cancer, increase of RB1 mutations after cisplatin in ovarian cancer and increase of T790M in patient with NSCLC EGFR positive treated with gefitinib. T790M was not detectable in plasma at the start of treatment and increased along with *NFkB1* and *p53* mutations.

Oxnard *et al*. reported on a series of nine EGFR mutated patients treated with first-line erlotinib and six of them exhibited T790M in plasma during treatment (81). Sorensen *et al*. described a group of 23 EGFR mutated patients treated with erlotinib as second-line therapy and the presence of T790M was documented in nine patients as acquired resistance mechanism (82). In particular, authors identified a new response parameter, represented by the plasmatic response, a condition defined by the reduction or disappearance of EGFR activating mutation in plasma during TKI treatment. Reduction in *EGFR* mutations plasmatic levels can be demonstrated very early, as recently also reported by Marchetti *et al*., that observed decreased levels starting from the 4th day of therapy with TKI (83). Several authors demonstrated that in patients that developed T790M-mediated acquired resistance, the level of plasmatic EGFR activating mutations started to increase along with the appearance of T790M (81,82,84). Interestingly, in all reports authors demonstrated that T790M was detectable in plasma several days (range: 15-344) before the evidence of disease progression per RECIST criteria. This observation is consistent with the hypothesis of the selection of a resistant neoplastic clone operated by EGFR-TKI, that growths until becomes clinically relevant. However, it should be note that the presence of T790M in association with EGFR sensitizing mutations has been documented in pre-treatment tissue and plasmatic samples, suggesting that the resistance clone could be present since the beginning and reach the blood stream after the clonal expansion (85,91,92). The identification of T790M in patients TKI-naïve could have a significant impact as double-positive patients presented shorter PFS than patients positive only for activating mutations.

Dynamic evolution of *EGFR* mutation plasmatic levels has been confirmed form others authors. Nakamura *et al*. reported on a series of 49 patients diagnosed with adenocarcinoma of whom 19 with acquired resistance (86). They found that 53% of resistant patients were positive for T790M and observed that T790M was not detectable in non-responders since T790M appeared in plasma only in responsive patients supporting the theory of a clone selection. Marcq and colleagues described two cases of patients treated with EGFR-TKI (87). In one case activating mutation decreased in plasma and the subsequent increase at progression was associated with T790M appearance; in the other case the patient experienced a complete plasmatic response, with only EGFR activating mutation re-appearing at progression. Wang *et al*. retrospectively analyzed a series of 135 patients treated with EGFR-TKI and found out that patients with pre-TKI plasma sample positive for T790M had significantly inferior PFS and OS comparing with pre-TKI negative patients (85). Moreover, among

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Table 3 List of studies evaluating *EGFR* gene activating and resistance mutations and their level modification

First author	Year	Methodic	No. of patients	EGFR determination	EGFR variation levels	T790M determination (timing)	T790M variation levels	Others
Murtaza M (36)	2013	Digital PCR	1	√	√	√ (R)	√	p53, NFKB1
Oxnard GR (81)	2014	dd-PCR	9	√	√	√ (R)	√	–
Sorensen BS (82)	2014	Cobas EGFR blood test	23	√	√	√ (R)	√	–
Marchetti A (83)	2015	Cobas EGFR blood test	57	√	√	–	–	–
Ahn MJ (84)	2015	dd-PCR	60	√	√	√ (R)	–	–
Wang Z (85)	2014	Digital PCR, ARMS	135	–	–	√ (D)	√	–
Nakamura T (86)	2011	MBP-PQ	49	–	–	√ (R)	√	–
Marcq M (87)	2014	ARMS	2	√	√	√ (R)	√	–
Piotrowska Z (88)	2015	BEAMing	12	√	√	√ (R)	√	–
Sequist LV (89)	2015	BEAMIing	113	–	–	√ (R)	√	–
Thress KS (90)	2015	NGS, dd-PCR	19	√	√	√ (R)	√	EGFR C797S

dd-PCR, digital droplet-PCR; ARMS, amplification refractory mutation system; MBP-PQ, mutation-biased PCR quenching probe; BEAMing, beads, emulsion, amplification and magnetics; (R), at resistance; (D), at first diagnosis.

patients with pre-TKI positive sample, higher levels were associated with significantly shorter PFS. On the contrary, patients with increased quantity of T790M during TKI therapy presented better PFS and OS than patients with decreasing T790M levels. Interestingly, authors observed high plasmatic levels of MET amplification in patients with decreasing T790M suggesting that TKI pressure could select a MET-amplified tumoral clone responsible of earlier resistance. Similarly to what reported for EGFR activating mutations, also reduction in T790M plasmatic levels can be considered as early parameter of response. In fact, Sequist reported that plasmatic T790M positivity is a predictor of durable response in patients treated with rociletinib, a third generation EGFR-TKI, and that responding patients show decrease of circulating T790M during treatment. However, authors have noted that about 33% of patient with T790M negative plasma responded and that also non-responding patients' present level reduction during treatment, concluding that probably T790M is not always the dominant resistance driver (89).

Finally, as new third-generation TKI with high affinity for T790M positive receptor have been developed, mechanisms of acquired resistance to new TKI have been studied and identified (88). In a group of 12 re-biopsied patients resistant to rociletinib, Piotrowska and colleagues reported the disappearance of T790M in six patients (of whom two presented transformation to small cell histology) and EGFR amplification in three T790M-positive patients. Regarding plasma analysis, they observed an increased in EGFR activating mutation during TKI therapy that was associated in some patients with T790M increase and in other patients with persistent T790M suppression. Similarly, Thress *et al.* analyzed plasmatic modifications of patients treated with AZD9291. Together with fluctuations of T790M circulating levels, the appearance of a new mutation C797S was documented as mechanisms of acquired resistance. In vitro studies have documented that this mutation impairs binding of TKI to EGFR thus inducing resistance (90).

Conclusions

Despite tissue biopsy still represents the gold standard for diagnosis, sophisticated technologies have permitted the isolation and identification of lung cancer related mutations in plasma opening new scenarios with a major impact in cancer patients management. Mutational analysis of cftDNA represents one of the most important recent breakthroughs in thoracic oncology. In fact, in certain situations, liquid biopsy could be an essential tool for clinicians because it gives the chance of a targeted therapy also in patients who cannot undergo invasive diagnostic procedures, due to comorbidities or the absence of biopsable tumor lesions. Moreover, liquid biopsy presents the advantages of a non-invasive technique that, without any discomfort, can be repeated every time needed during a patient therapeutic history. In particular, cftDNA analysis assumes a crucial

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role for patients with EGFR mutated lung cancer, since they represent a group of patients receiving a huge benefit from targeted mutation identification, not only at diagnosis but also at the onset of acquired resistance, but for whom obtaining tissue sample is sometimes not feasible.

Several issues remain outstanding regarding the routine employment of cftDNA. First, many devices for cftDNA detection and analysis have been developed, characterized by a slight different spectrum of sensitivity and specificity. Data in literature are extremely heterogeneous from this point of view as different authors tested the reliability of different devices. Therefore, univocal conclusions cannot still be formulated and two meta-analyses were conducted to clarify the feasibility of plasmatic *EGFR* mutation detection. Many studies were included, even though conducted with different methods, and globally emerged that plasmatic molecular analysis of EGFR presents a high accuracy suggesting its possible employment when tissue is not available. The evidence that the predictive role of plasmatic *EGFR* mutation has been confirmed and is consistent with data obtain from tissue enforces the utility of plasmatic analysis for *EGFR* mutations detection lung cancer. However, diagnostic sensitivity and specificity are influenced also by plasmatic cftDNA levels that depend on cftDNA mechanisms of release and clearance. Moreover, it has been demonstrated that the levels of cftDNA are also determined by several tumor-related factor including tumor mass, stage of disease, vascularization, aggressivity and certainly other are unknown. These issues need to be clarified before cftDNA enter in current clinical practice.

In a minority of patients, the analysis on cftDNA permitted the isolation of *KRAS* mutation along with the presence of EGFR activating mutation. This is an element of particular interest, as these two alterations have been always considered mutually exclusive and only one report signaled their co-existence in tissue. This finding could be explained considering that plasmatic molecular characterization overcome the limit of tumoral heterogeneity and theoretically permit to identify mutations expressed by clones situated in different body sites. However, it should be considered that new technologies present higher sensitivity than previous ones and therefore could be able to detect molecular alterations expressed by limited number of tumoral cells opening new perspectives on tumor biology.

Finally, the application of cftDNA analysis in the field of acquired resistance to EGFR-TKI is of particular interest. In general, the profile of acquired resistance mechanisms expressed in plasma is consistent to what revealed in tissue samples and T790M, which represent a predicting factor of response to third-generation TKI, emerged as the most frequent resistance mutation. The opportunity of obtaining molecular information avoiding serial re-biopsies permitted to explore the dynamic process leading to resistance. Different authors demonstrated that levels of EGFR activating mutation promptly decreased in plasma after the initiation of EGFR-TKI and that the occurrence of T790M is an early phenomenon that anticipates of several weeks the radiological progression. Again, modifications of T790M levels in response to third-generation EGFR-TKI have been described, even if predictive and prognostic impact is unclear. To date, these findings have not any clinical consequences. However, the efficacy of TKI-therapy modulation basing on fluctuations of plasmatic activating and resistance mutations levels deserved to be valuated prospectively in the future and represent a promising research topic.

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Footnote

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Lung cancer diagnosis and staging in the minimally invasive age with increasing demands for tissue analysis

Erik Folch[1], Daniel B. Costa[2], Jeffrey Wright[3], Paul A. VanderLaan[4]

[1]Division of Thoracic Surgery and Interventional Pulmonology, [2]Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA; [3]Baptist Memorial Hospital, Memphis, TN, USA; [4]Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

Contributions: (I) Conception and design: E Folch, DB Costa, PA VanderLaan; (II) Administrative support: All authors; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Erik Folch, MD, MSc. Division of Thoracic Surgery and Interventional Pulmonology, Beth Israel Deaconess Medical Center and Harvard Medical School, 185 Pilgrim Road, Deaconess 201, Boston, MA 02215, USA. Email: efolch@bidmc.harvard.edu.

Abstract: The diagnosis and staging of patients with lung cancer in recent decades has increasingly relied on minimally invasive tissue sampling techniques, such as endobronchial ultrasound (EBUS) or endoscopic ultrasound (EUS) needle aspiration, transbronchial biopsy, and transthoracic image guided core needle biopsy. These modalities have been shown to have low complication rates, and provide adequate cellular material for pathologic diagnosis and necessary ancillary molecular testing. As an important component to a multidisciplinary team approach in the care of patients with lung cancer, these minimally invasive modalities have proven invaluable for the rapid and safe acquisition of tissue used for the diagnosis, staging, and molecular testing of tumors to identify the best evidence-based treatment plan. The continuous evolution of the field of lung cancer staging and treatment has translated into improvements in survival and quality of life for patients. Although differences in clinical practice between academic and community hospital settings still exist, improvements in physician education and training as well as adoption of technological advancements should help narrow this gap going forward.

Keywords: Lung cancer; staging; molecular testing; minimally invasive; endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA); interventional pulmonology

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide. The most frequently encountered primary lung cancers include epithelial-derived non-small-cell lung cancer (NSCLC), with adenocarcinoma and squamous cell carcinoma as the main histologic subtypes; and neuroendocrine carcinomas, with small cell lung cancer (SCLC) as the major high-grade neuroendocrine carcinoma. Most NSCLCs are diagnosed at advanced stages, and historically (up to the early 2000s), palliative therapeutic decisions were based solely on the differentiation between NSCLC and SCLC. Hence, the main diagnostic modalities and focus on tissue acquisition were geared towards obtaining small samples for simple histopathological characterization that would be added to non-invasive imaging studies to complete tumor, node, metastasis (TNM) staging. The paradigm of NSCLC histology not otherwise specified (NOS) with advanced TNM staging drove the development of anti-cancer therapies for NSCLCs in the 1980s, 1990s, and early 2000s; with the evidence-based introduction of platinum-doublets as the main palliative modality for stage IV NSCLC (1).

A need to better define NSCLC subtypes occurred in the early 2000s with the introduction of novel cytotoxic chemotherapies (pemetrexed) and biological agents

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(bevacizumab) that had enhanced efficacy or worsened toxicity, respectively, based on histology (2,3). To this end, a diagnosis of NSCLC NOS was no longer sufficient, and the more widespread use of both histochemical and immunohistochemical ancillary studies helped to more consistently distinguish adenocarcinoma from squamous cell carcinoma in small biopsy/cytology specimens. The 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) lung adenocarcinoma classification was developed by an international core panel of expert medical oncologists, pulmonologists, pathologists, and thoracic surgeons, to address minimum requirements in immunohistochemical testing markers to differentiate between adenocarcinoma and squamous cell in small samples that were previously classified as NSCLC NOS (4). This shift in tumor acquisition goals and requirements, continues to reverberate in clinical lung cancer care and drug development, with, for example, the initial approval by the US Food and Drug Administration (FDA) of the immune-checkpoint, anti-programmed death-ligand 1 (PD-1) inhibitor, nivolumab, for advanced squamous cell lung cancer (5).

The need for adequate tissue for the diagnosis and management of NSCLC has increased substantially over the last decade, as new anti-cancer therapies have begun to explore vulnerabilities in the genomic underpinnings of cancer. Cancer is a heterogeneous group of diseases that lead to invasion and metastasis, induction angiogenesis, replicative immortality, resistance to cell death, reprogramming of energy metabolism, evasion of immune surveillance, circumvention of growth suppressors, and sustained proliferative signaling (6). The latter is especially prevalent in subgroups of NSCLC, since sustained proliferative signaling is usually derived from genomic mutations in key oncogenes that encode for activated tyrosine kinases.

Three main genomic events lead to the direct activation of tyrosine kinases in NSCLC: overexpression or amplification (due to increased copy numbers of a certain oncogene), mutation (due to point mutations or insertions/deletions), and rearrangement with partner genes (by preserving or activating the kinase domain of oncogenes). The most prevalent oncogenes that are amplified, mutated or rearranged in NSCLCs are listed in *Table 1* (7-9).

Tyrosine kinase inhibitors (TKIs), small molecules that can block the function of kinases, have been developed as precision therapies in NSCLC. As of mid-2015, EGFR and ALK mutations are the most prevalent, clinically relevant driver oncogenes in NSCLC care. First generation reversible EGFR TKIs (gefitinib and erlotinib) and second generation irreversible EGFR TKIs (afatinib) have been shown in multiple randomized phase III trials to be superior to standard platinum-doublet chemotherapies in the first line treatment of advanced EGFR mutant lung adenocarcinomas and are FDA approved for use in this setting (10-13). In addition, novel third generation covalent EGFR TKIs that are more specific to the most common first/second generation TKI resistance mutation (EGFR-T790M) are active and have FDA 'breakthrough' review designation.

ALK mutations in lung adenocarcinomas occur through gene rearrangements (the most common partner is EML4) that lead to constitutive activation of the tyrosine kinase domain of ALK. The multitargeted ALK/MET/ROS1 TKI crizotinib led to significant responses in phase I and II trials of ALK rearranged lung adenocarcinoma, and phase III randomized trials in the second line (crizotinib versus docetaxel or pemetrexed) and first line (crizotinib versus platinum-pemetrexed) setting have confirmed that crizotinib is more effective than chemotherapy for these tumors (14-17). The FDA label of crizotinib requires tumor identification of ALK rearrangement status. In addition, the second generation ALK TKI ceritinib is FDA approved for the therapy of crizotinib-resistant ALK rearranged lung adenocarcinoma and the related compound alectinib has a FDA breakthrough designation (18,19). Other TKIs have differing levels of evidence for off-label use in lung adenocarcinomas with other genotypes (*Table 1*).

To standardize the use of tissue for the ever-changing needs of molecular diagnostics in lung cancer, in 2013, IASLC, Association for Molecular Pathology (AMP), and College of American Pathologists (CAP) published minimum molecular testing guidelines for selection of lung cancer patients for EGFR and ALK TKIs that are now widely used for day-to-day medical oncology care (20). The current guidelines prioritize use of rapid single gene assays for these two driver oncogenes. However, it is becoming evident that technological advances have reached a point where comprehensive molecular profiling using a variety of next generation sequencing (NGS) platforms is feasible in routine clinical practice; with a multitude of commercial or academic vendors providing Clinical Laboratory Improvement Amendments (CLIA)-certified NGS assays that use formalin-fixed paraffin-embedded (FFPE) specimens or cytology specimens to isolate DNA and/or RNA for analyses of a targeted panel of genes to select for the most readily targetable alterations (*Table 1*) (21,22).

Therefore, the need for sufficient, high-quality tissue

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Table 1 Known driver mutations in NSCLC with associated targeted therapeutics

Molecular target/driver oncogene	Prevalence (%)	US FDA-approved TKIs in 2015	US FDA-breakthrough designation TKIs in 2015	Off label use of TKIs with significant level of evidence (NCCN category 2A)	Off label use of TKIs with lesser levels of evidence
Adenocarcinoma					
KRAS mutations	25-30	None	None	None	None
EGFR mutations	15-20	Erlotinib, afatinib	AZD9291, rociletinib	N/A	N/A
ALK rearrangements	3-7	Crizotinib, ceritinib	Alectinib	N/A	N/A
ROS1 rearrangements	2-4	None	Crizotinib	Crizotinib	Cabozantinib
MET exon 14 skipping mutation	2-4	None	None	None	Crizotinib
ERBB2 mutations	1-3	None	None	None	Afatinib
BRAF mutations (V600E)	1-3	None	Dafrafenib, dafrafenib + trametinib	Dafrafenib, vemurafenib	N/A
RET rearrangements	1-2	None	None	None	Cabozantinib
MET amplification	1-2	None	None	Crizotinib	N/A
MAP2K1 mutations	1	None	None	None	None
NTRK1 rearrangements	<1	None	None	None	None
FGFR2/3/4 rearrangements	<1	None	None	None	None
Squamous cell carcinoma					
FGFR1 amplifications	15-20	None	None	None	None
FGFR2/3/4 mutations/ rearrangements	5-10	None	None	None	None
PI3KCA mutations	5-10	None	None	None	None
DDR2 mutations	1-5	None	None	None	Dasatinib

NSCLC, non-small-cell lung cancer; FDA, Food and Drug Administration; TKIs, tyrosine kinase inhibitors; N/A, non-applicable.

material for diagnosis, staging, and treatment selection has grown significantly, concurrently with the expansion of minimally-invasive tissue acquisition methods. We will address current minimally invasive methods for tissue acquisition in the diagnosis and management of patients with lung cancer, their performance characteristics, and consider current gaps in patient care in different practice environments.

Minimally invasive techniques for tissue acquisition

Prompt and accurate diagnosis and staging of patients with lung cancer should be sought through an efficient process: one that minimizes the number of procedures before initiating treatment. Ideally, the preferred initial procedure would be able to simultaneously provide tissue for diagnosis, tumor classification, molecular testing, as well as provide staging information. However, this may or may not be possible depending on the individual patient and the need for sufficient and appropriate tissue for current and future cytological, immunohistochemical, and molecular studies. The available techniques are: mediastinoscopy, endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA), endoscopic ultrasound (EUS) with fine needle aspiration (FNA), traditional bronchoscopic TBNA and computed-tomography guided core needle biopsy (CT-CNB) or CT-FNA. The overall performance measures of these different techniques are summarized in *Table 2*.

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Table 2 Non-invasive and minimally-invasive staging modalities for non-small cell lung carcinoma*

Procedure	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Number of studies	Number of specimens	Cancer prevalence (%)
CT	55	81	58	83	43	7,368	30
Integrated PET-CT	62	90	63	90	19	2,014	22
Mediastinoscopy^	81	100	100	91	35	10,648	34
TBNA	78	100	100	77	27	2,408	81
EUS-FNA	89	100	100	86	26	2,443	58
EBUS-TBNA	89	100	100	91	26	2,756	58
EBUS-TBNA + EUS-FNA	91	100	100	96	7	811	33

*, median data values, compiled from the most recent 3rd edition ACCP Guidelines for the Diagnosis and Management of Lung Cancer [Silvestri *et al.* (23)]. PPV, positive predictive value; NPV, negative predictive value; PET-CT, positron emission tomography-computed tomography; TBNA, transbronchial needle aspiration; EUS-FNA, endoscopic ultrasound guided fine needle aspiration; EBUS-TBNA, endobronchial ultrasound with transbronchial needle aspiration; ^, includes traditional mediastinoscopy and video-assisted mediastinoscopy.

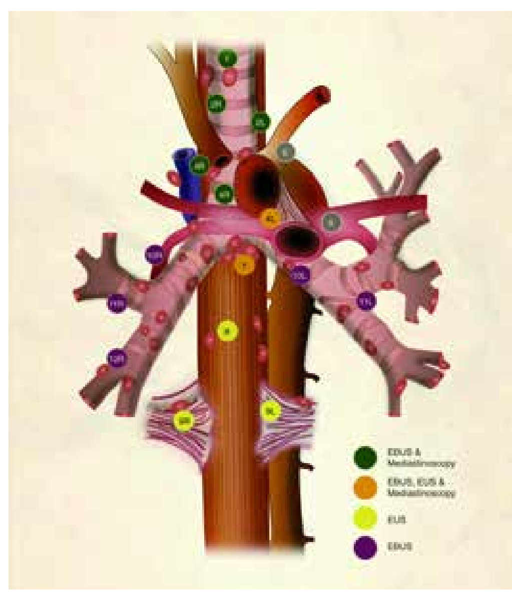

Figure 1 Lymph node map adapted from the 2009 IASLC lung cancer staging project. The lymph node stations are color coded to indicate the minimally-invasive staging techniques that can readily access each lymph node station. The close proximity to vascular structures highlights the importance of direct visualization or ultrasound guidance to avoid bleeding complications. EBUS, endobronchial ultrasound; EUS, endoscopic ultrasound; IASLC, International Association for the Study of Lung Cancer.

Mediastinoscopy

Mediastinoscopy is a surgical procedure that allows for the exploration of the superior mediastinum from the sternal notch to the subcarinal space and sometimes can reach the main bronchi (*Figure 1*). It is done under general anesthesia, with the neck maximally extended and through a 2-3 cm collar incision at the sternal notch carried out through the platysma. The strap muscles are separated to expose the trachea and after incising the pretracheal fascia, the pretracheal plane is developed. Finger dissection is initially used as caudally as possible while palpating key structures such as the innominate artery and the aortic arch. This space is then used to advance the video-mediastinoscope. This process is continued by using suction/coagulation device sweeps to advance caudally. Before carrying out biopsies, the surgeon identifies the innominate artery, aortic arch, pulmonary artery and the azygos vein. Occasionally, the appearance of a lymph node and a vascular structure are similar, and a fine needle is used to gently penetrate the structure and identify if there is blood flow or not (24).

In a similar fashion to EBUS or EUS, exploration of the lymph nodes starts on the contralateral side of the tumor to rule out N3 disease and then proceeds in a systematic way. The subcarinal lymph nodes are usually sampled last because bronchial artery and perinodal bleeding can be more difficult to control. It is important to mention that by convention the specificity and positive predictive values of cervical mediastinoscopy are considered 100%, as entire lymph nodes are excised for histologic evaluation. However, positive results are not confirmed by other tests. The median sensitivity of conventional mediastinoscopy is reported to be 78% with a median negative predictive value of 91% (23). Video-mediastinoscopy has a median

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sensitivity of 89% with a negative predictive value of 92%. Although rare, complications occur in 3% of cases with serious bleeding in 0.4% occasionally requiring mediastinotomy (25,26). Mortality is under 0.5% (27,28).

There are two technical variations of mediastinoscopy intended for systematic removal of mediastinal lymph nodes: video-assisted mediastinoscopic lymphadenectomy (VAMLA) and transcervical extended mediastinal lymphadenectomy (TEMLA). These two procedures, also called "supermediastinoscopies", are not widely used but their exceptional operating characteristics warrant a comment. Both are done through an incision similar to the one used for mediastinoscopy but with systematic removal of the lymph nodes. In VAMLA, the removal of subcarinal and right inferior paratracheal lymph nodes en block followed by the left inferior paratracheal lymph nodes is done through a 2-blade spreadable mediastinoscope (29).

In TEMLA, a sternal retractor elevates the sternum allowing for complete mediastinal lymphadenectomy from the supraclavicular to the paraesophageal lymph nodes. A thoracoscope is also used to remove the subaortic and para-aortic lymph nodes (30).

Although both are rarely used, the sensitivity of VAMLA was close to 100%, while TEMLA has shown to be superior to mediastinoscopy and EBUS (31,32).

Interestingly, some experts and authors of the prior research studies, conclude that VAMLA and TEMLA have no current role in the routine mediastinal staging of lung cancer. In part due to their invasiveness and high risk of complications when compared to equally accurate but less invasive options including EBUS and EUS (33). Furthermore, VAMLA and TEMLA are not mentioned (23) or recommended only within clinical trials (34) in the most recent guidelines for staging of lung cancer.

Endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA)

Endoscopic techniques have emerged as the procedure of choice for diagnosis and staging of lung cancer (23). These techniques have also been associated with lower morbidity and mortality, and have been suggested to be more cost effective than mediastinoscopy (35,36). Complications are very rare, with the rate of pneumothorax between 0.07% and 0.2% (37). The procedure is usually done in the outpatient setting by pulmonologists, interventional pulmonologists, or thoracic surgeons in a procedure suite or in the operating room. Anesthesia largely depends on local practices, but may involve moderate sedation or general anesthesia. A dedicated flexible bronchoscope with an ultrasound (5, 7.5, 10 and 12 MHz) at the distal end is inserted through the mouth, an endotracheal tube, or a laryngeal mask and advanced to the distal trachea where apposition of the ultrasound probe to the airway wall reveals adjacent structures in high detail. After identifying the lymph node station based on anatomic landmarks, a 21 or 22 gauge needle is advanced under direct visualization on ultrasound.

Although there is no consensus on the number of times each lymph node is punctured (passes), in our experience, three passes with 15 needle excursions per pass provides diagnostic material in over 95% of cases (38). After each pass, the needle is withdrawn and a small amount of material can be either placed on a slide for immediate preparation or the entire sample can be placed in a preservative solution for cytologic analysis and cellblock preparation. As shown in *Figure 1*, EBUS can access the following stations: 2R and 2L (upper paratracheal), 4R and 4L (lower paratracheal), 7 (subcarinal), 10R and 10L (hilar), 11R and 11L (interlobar), on occasion 12R and 12L (lobar) as well as paratracheal and parabronchial masses that occur close to the airway. At least one case series that encompasses multiple institutions described access to station 5 (subaortic) through a transpulmonary artery route (39).

Endoscopic ultrasound guided fine needle aspiration (EUS-FNA)

EUS is also a real-time ultrasound procedure guiding trans-esophageal needle aspiration. It allows posterior mediastinal sampling through the esophageal wall. The lymph nodes preferentially accessible to EUS are the inferior pulmonary ligament (level 9), paraesophageal (level 8), subcarinal (level 7), and left paratracheal (level 4L) (*Figure 1*). However, anterolateral paratracheal (levels 2R, 2L, and 4R) are difficult to sample with EUS. EUS also has a high safety profile, similar to EBUS (40,41). The main feature that sets apart EUS from other techniques is the access to locations outside of the mediastinum, such as the left lobe of the liver, a significant part of the right lobe of the liver, and the left adrenal gland (42). Given its relative strengths and weaknesses, it is best to think of EUS as a complement to EBUS for the diagnosis and staging of lung cancer patients. When used in combination, the yield is higher than with either technique used alone. Pooled analyses have shown sensitivity of 91% and specificity of 100% (23,43).

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CT-guided biopsy

Computed tomography provides details on the anatomic location, shape, margins, attenuation of the primary lesion as well as the extent of invasion of the chest wall, presence of suspicious mediastinal, hilar, segmental lymph nodes, and proximity to surrounding structures (44). However, this radiologic evaluation is not entirely specific and should not be used as the single source of staging. The median sensitivity and specificity of CT for identification of mediastinal lymph node involvement were 55% and 81% respectively (23). Other studies have shown similar low sensitivity when pooled in meta-analysis demonstrating sensitivity of 51-64% for NSCLC (45,46). Whenever CT guidance is used to obtain tissue by core needle biopsy or fine needle aspiration, the pooled sensitivity and specificity are 90% and 97% respectively (47). However, the complications include a 15% risk of pneumothorax and 1% risk of major hemorrhage (48). The risk factors for major complications during trans-thoracic needle aspiration include emphysema, small lesion, greater depth of needle penetration, and multiple needle passes. For these reasons, it is not common to use trans-thoracic needle aspiration to sample mediastinal lymph nodes.

In summary, the different minimally invasive techniques are designed to help clinicians identify lung cancer patients who are likely to benefit from primary resection, neo-adjuvant chemotherapy and/or radiation, or palliative chemotherapy. However, recent studies suggest that the strategic combination of staging techniques (such as EBUS, followed, when negative, by mediastinoscopy) provides better outcomes and may be more cost-effective (49). A study by Farjah and colleagues reported severe underuse of multimodality staging; with the use of multimodality staging increasing over time from 1998 to 2005 resulting in an association between use of multimodality staging and improved survival, irrespective of the stage of disease (50).

If only imaging studies are used for staging, 15-40% of patients will be denied curative intent therapy (51). For these reasons, radiologic images that are concerning for lung cancer or metastatic disease should be confirmed with cytology or histopathology. Inadequate lymph node evaluation is unfortunately common and its consequences are hard to estimate, but likely translates into reduced lung cancer survival if nodal disease is not identified and treated (52-54).

Lymph node mapping

Regardless of how thoracic lymph nodes are sampled for staging purposes, it is important to use a common vocabulary when describing the location of these lymph node stations as well as to state what specific lymph node stations were sampled. The Japanese (Naruke) and US/European (Mountain and Dresler) lymph node maps were reconciled into a single universal map by the IASLC in 2009 (55). This provides a uniform, specific anatomic definition of the lymph node stations, and facilitates the identification of the exact location during surgery, radiologic interpretation and minimally-invasive biopsy techniques (see Rami-Porta *et al.* in this special issue). It is recommended that we abandon loose anatomic descriptions such as "lower paratracheal" or "parahilar" as these terms are not specific to a lymph node station and can easily be misinterpreted.

Definitions for mediastinal lymph node evaluation

Using standard definitions for the thoroughness of mediastinal nodal staging is as important as using a uniform mediastinal lymph node map (56). The following categories have been used for surgical staging, but they can easily be extrapolated to minimally invasive techniques such as EBUS TBNA. The extent of lymph node assessment can be broadly categorized into the following groups (57):

(I) Random sampling: the sampling of lymph nodes by convenience or by preoperative or intraoperative findings. The most common situation is the sampling of a single enlarged lymph node. Unfortunately, this practice has been found to be very common in the mediastinoscopy literature (52).

(II) Systematic sampling: the sampling of predetermined lymph node stations, such as 2L, 4L, 7, and 10L for a left sided lung tumor, and 2R, 4R, 7 and 10R for a right sided tumor.

(III) Mediastinal lymph node dissection: the complete surgical removal of all identifiable mediastinal lymph node tissue based on anatomic landmarks.

(IV) Extended lymph node dissection: the removal of bilateral paratracheal and cervical lymph nodes by formal dissection.

(V) Lobe-specific systematic node dissection: the removal of ipsilateral mediastinal lymph node tissue based on the location of the tumor.

Guidelines on tissue acquisition and processing for diagnosis, staging, and genotyping

The American College of Chest Physicians (ACCP) evidence-

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based clinical practice guidelines, the European Society of Thoracic Surgeons (ESTS) guidelines, and Cancer Care Ontario (CCO) Program in Evidence-Based Care Practice Guidelines are in agreement on their recommendations for indications and techniques for invasive staging (23,34,58). It is important to emphasize that random sampling or sampling of a single enlarged lymph node is considered inadequate surgical staging. Some authors have extrapolated this to minimally invasive techniques and have advocated against random sampling (59). It is recommended that appropriate staging include stations 2R, 2L, 4R, 4L, and 7. However, TBNA of lymph nodes that are smaller than 5 mm is very difficult and likely will result in sub-optimal amount of tissue for diagnosis. Clinically suspicious lymph nodes, such as enlarged (≥1 cm short axis diameter) or FDG-avid nodes, should also be sampled. Guidelines, such as those published by ESTS, the United Kingdom's National Institute for Health and Care Excellence, and CCO, recommend that appropriate lymph node assessment should be systematic and include a minimum of three mediastinal lymph node stations, one of which should be station 7 (subcarinal) (34,58,60).

Sample acquisition and processing differences: how does needle aspiration (cytology) differ from core biopsy (histology)?

It is important to have an appreciation for how small biopsies obtained by minimally invasive means are processed and evaluated by the pathologist/cytopathologist. In general, these small biopsy or cytology specimens must be sufficient to establish a diagnosis of malignancy, to make a reliable subclassification of disease (e.g., adenocarcinoma *vs*. squamous cell carcinoma) using immunochemical stains, and, increasingly, for molecular testing to identify targetable driver mutations. The amount of information to be gleaned from these small biopsy and cytologic specimens is great, and has increased dramatically over the past decade.

Minimally invasive biopsy specimens are small, with limited cellular material. Transbronchial/endobronchial biopsies and transthoracic core needle biopsies of lung lesions can provide some tissue architecture, helpful in delineating invasive carcinoma from in-situ/lepidic pattern of spread, though sampling limitations can be an issue for these specimens. Cytologic aspirates (EBUS-TBNA or EUS-FNA) oftentimes lack these architectural cues, though frequently larger tissue fragments that are almost biopsy-like can be aspirated and appreciated on direct smears or cell block preparations. Establishing a diagnosis of malignancy on cytologic specimens should rarely be a problem though, as the cytologic features of malignancy are generally easy to appreciate. In contrast to biopsy specimens, which are nearly always formalin-fixed and paraffin-embedded, cytologic specimens can be processed and evaluated in a number of ways, including by direct smears or touch-preparations of tissue biopsies (either air-dried or alcohol fixed), alcohol-fixed liquid based concentration methods (such as using cytospin, ThinPrep, or SurePath), as well as the creation of a tissue cell block. The latter captures the cellular material into a cell pellet that is formalin-fixed and paraffin-embedded, creating for all intents and purposes a tissue-biopsy-like specimen from which multiple serial slides can be cut from the paraffin block and used for immunohistochemical stains and molecular testing. In reality, the lines between small biopsy specimens and cytology specimens (especially with the creation of a good cell block) have become blurred, with both types of specimens capable of providing specific histopathologic diagnoses and serving as substrates for molecular testing.

In order to preserve cellular material for downstream molecular testing, the 2015 iteration of the WHO classification of lung tumors (61) and the 2011 IASLC/ATS/ERS classification of lung carcinomas on small biopsy/cytology specimens (62) recommends that a focused panel of immunostains be employed for the work-up of a suspected primary NSCLC when histology or cytomorphology alone is insufficient to distinguish adenocarcinoma from squamous cell carcinoma. Specifically, one lung adenocarcinoma marker (traditionally the transcription factor TTF-1) and one squamous cell marker (usually p63 or more recently p40—the N-terminal truncation isoform of p63 shown to be more specific for squamous cell carcinoma) (63). If these results are inconclusive, then second line lung adenocarcinoma markers (such as the aspartic proteinase Napsin-A) and squamous cell carcinoma markers (cytokeratin 5/6) can be employed. A mucicarmine histochemical stain can also be helpful to demonstrate glandular differentiation. Clinical and radiologic correlation are always helpful, to focus the immunohistochemical work-up of carcinoma metastatic to the lungs, especially when more lung-specific markers are negative.

Genotyping: yield of different techniques

The most current guidelines from the CAP, IASLC, and AMP call for testing all advanced stage lung

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adenocarcinomas (or mixed tumors with an adenocarcinoma component) for EGFR mutations, generally by PCR-based methods, and ALK gene rearrangements (via FISH assay or with screening immunohistochemistry) (20). Lung cancers are also commonly tested for KRAS mutations which are associated with resistance to tyrosine kinase inhibitors. In addition to these three main molecular targets, the list of less common driver mutations (*Table 1*) in lung adenocarcinoma is growing rapidly. With the growing number of actionable targets for lung cancer, relying on the current paradigm of one-off testing using these small biopsy or cytology specimens will inevitably deplete the cellular material despite the cytopathologist's best efforts to maximize cell block cellularity and minimize material loss during the initial diagnostic work-up. Therefore, a shift towards multiplexed panels seems inevitable in future (21).

Many groups have published very good molecular testing success rates using small biopsy and cytology specimens. In general, the success rates for small biopsy specimens (including transthoracic core needle biopsies or transbronchial biopsies) are comparable to those for cytology cell block specimens. Recent studies comparing these modalities report a molecular testing success rate for small biopsy specimens of 55-100%, and a success rate for FNA or EBUS-TBNA cell block specimens of 46-95%, depending on the study parameters (64-67). In general there is a higher molecular testing failure rate from small biopsy or cytology specimens as compared to larger surgical resection specimens, inferred from the limiting tumor cellularity present in the former (68).

A recent publication from the Lung Cancer Mutation Consortium, a multi-institutional program investigating selected oncogene drivers in lung adenocarcinoma, revealed that in an 8-gene panel testing approach, 35% of cytology specimens and 26% of small biopsies were insufficient for molecular testing (compared to only 5% of surgical resection specimens). Importantly however, the authors comment that once a specimen was deemed adequate for molecular testing (i.e., has sufficient tumor cellularity), the specimen type (cytology/small biopsy/surgical resection) had no influence on subsequent molecular testing performance and (69) that minor differences between completion rates were not felt to be clinically significant. Therefore, cytology and small biopsy specimens have been proven to be excellent substrates for molecular testing, as long as enough tumor cells are obtained and the preceding pathologic work-up is efficient and minimized tumor cell loss.

Advanced bronchoscopy techniques in non-academic settings

EBUS-TBNA has become increasingly commonplace outside of academic medical centers. However, appropriate training for thorough and systematic mediastinal staging is still lagging (59). Electromagnetic navigation bronchoscopy (ENB), and other advanced diagnostic techniques have also become increasingly commonplace in the community setting. Each of these procedures has an associated learning curve, requiring the development of a systematic approach to proper procedural techniques for biopsies and tissue handling. Increasing interest has led to implementation of training in advanced bronchoscopy techniques in pulmonary/critical care fellowships, as well as dedicated interventional pulmonary fellowships.

For physicians who did not have exposure to these techniques during their formal training, the training options include taking a sabbatical year, participating in an intense 1-7 day course, or direct proctoring by experienced colleagues. Current ACCP guidelines for procedural training are based on minimum number of procedures and not necessarily on the cognitive and technical skills required (70). In the United States, the need for the procedures at community and regional hospitals has led to the implementation of bronchoscopy services, including EBUS, or the creation of referral channels to tertiary care centers (71). Ultimately, the success of community programs depends on adequate investment of human and technological capital, ideally within multidisciplinary teams of pulmonologists, thoracic surgeons, radiologists, cytopathologists, radiation oncologists, and medical oncologists, who should collaborate to apply evidence-based guidelines while continuously evaluating their performance using mutually accepted yield and quality metrics.

A number of authors have advocated the utility of rapid onsite examination (ROSE) for the evaluation of EBUS samples. Although immediate feedback for the bronchoscopist as well as appropriate specimen collection and triage can be helpful in certain circumstances, the current guidelines from the World Association for Bronchology and Interventional Pulmonology state that use of ROSE is not recommended for every case if the operator is experienced (72), and certainly should not limit the implementation of a much needed service for lung cancer patients. In this setting, EBUS-TBNA samples for driver oncogene mutation analysis has been successful in close to 95% of the cases, even with use of a commercial laboratory

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and no sample enrichment (64). Appropriate tissue handling and preparation with methanol based fixatives and paraffin-embedded cell blocks have been used successfully by our group and others (68,73).

Conclusions

The diagnosis and treatment of lung cancer has undergone multiple dramatic changes in the last decade. We have a better understanding of the molecular biology of lung cancer and driver mutations that can be targeted through the use of specific tyrosine-kinase inhibitors. Significant technological advances allow interventional pulmonologists and surgeons to obtain diagnostic material in a safe and minimally invasive manner. Ongoing refinements in diagnostic and ancillary molecular testing by pathologists and cytopathologists has allowed small biopsy and cytology specimens to be used to accurately diagnose and characterize lung cancer, helping direct appropriate therapeutic decisions. Moving forward, a pressing task for the health care community at large will be to narrow existing practice gaps between high-performing (often academic) and lower performing (often community-based) care delivery settings.

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Footnote

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Computer modeling of lung cancer diagnosis-to-treatment process

Feng Ju[1], Hyo Kyung Lee[1], Raymond U. Osarogiagbon[2], Xinhua Yu[3], Nick Faris[2], Jingshan Li[1]

[1]Department of Industrial and Systems Engineering, University of Wisconsin, Madison, WI 53706, USA; [2]Thoracic Oncology Research Group, Baptist Memorial Health System, Memphis, TN, USA; [3]School of Public Health, University of Memphis, Memphis, TN, USA

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Correspondence to: Dr. Jingshan Li. Department of Industrial and Systems Engineering, University of Wisconsin, Madison, WI 53706, USA. Email: Jingshan.li@wisc.edu.

Abstract: We introduce an example of a rigorous, quantitative method for quality improvement in lung cancer care-delivery. Computer process modeling methods are introduced for lung cancer diagnosis, staging and treatment selection process. Two types of process modeling techniques, discrete event simulation (DES) and analytical models, are briefly reviewed. Recent developments in DES are outlined and the necessary data and procedures to develop a DES model for lung cancer diagnosis, leading up to surgical treatment process are summarized. The analytical models include both Markov chain model and closed formulas. The Markov chain models with its application in healthcare are introduced and the approach to derive a lung cancer diagnosis process model is presented. Similarly, the procedure to derive closed formulas evaluating the diagnosis process performance is outlined. Finally, the pros and cons of these methods are discussed.

Keywords: Lung cancer quality improvement; process modeling; discrete event simulation (DES); analytical model; Markov chain; closed formula

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Introduction

Lung cancer is the second most common cancer in both men and women, accounting for about 13% of all new cancers. The American Cancer Society estimates that for year 2015, there will be about 221,200 new cases of lung cancer (115,610 in men and 105,590 in women) in the United States, with an estimated 158,040 deaths (86,380 in men and 71,660 in women) from lung cancer (1). It is the leading cause of cancer death among both men and women, accounting for 27% of all US cancer mortality (1). Survival in lung cancer mainly depends on the extent of spread (stage) at the time of treatment. The 5-year survival rate ranges from more than 60% for stage I patients, to about 40% for stage II patients. It quickly drops to 20% for stage III patients, and only 4% for stage IV patients (2). Treatment selection is also stage-dependent. Therefore, early diagnosis and staging of lung cancer is of critical importance.

The lung cancer diagnosis process is long and complex, with substantial variations. It typically starts with an abnormal X-ray, followed by computed tomography (CT) scan and diagnostic biopsy. After radiologic (noninvasive) staging and/or invasive staging, depending on the stage, patients may be treated by surgery, chemotherapy, radiation therapy, or (as is increasingly the case) a combination of these modalities. For surgical patients, medical clearance is needed before surgery. However, different patients may follow different procedures. For instance, some patients may skip some tests, while other patients may need to go backward and repeat some tests. *Figure 1* illustrates the lung cancer diagnosis process with variations for surgical patients, where the dashed lines represent unusual practices. As one can see, these variations make the diagnosis process extremely difficult to be represented using simple routes.

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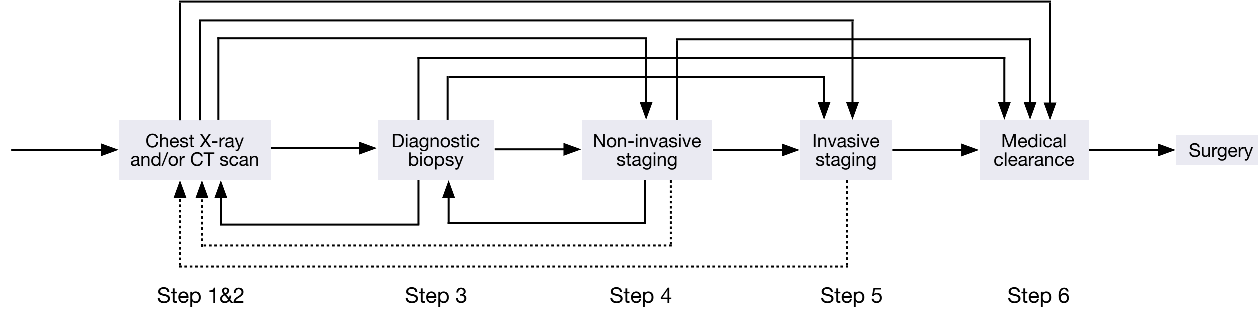

Figure 1 Lung cancer diagnosis process for surgical patients. CT, computed tomography.

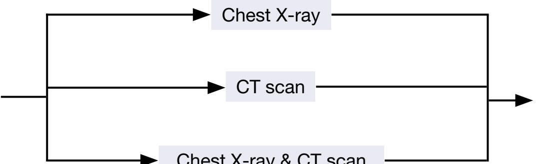

Figure 2 Chest X-ray and CT scan process. CT, computed tomography.

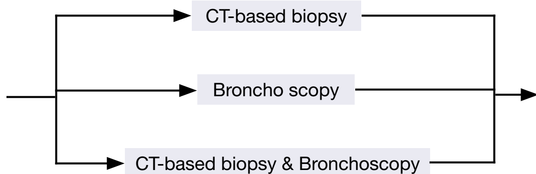

Figure 3 Diagnostic biopsy process. CT, computed tomography.

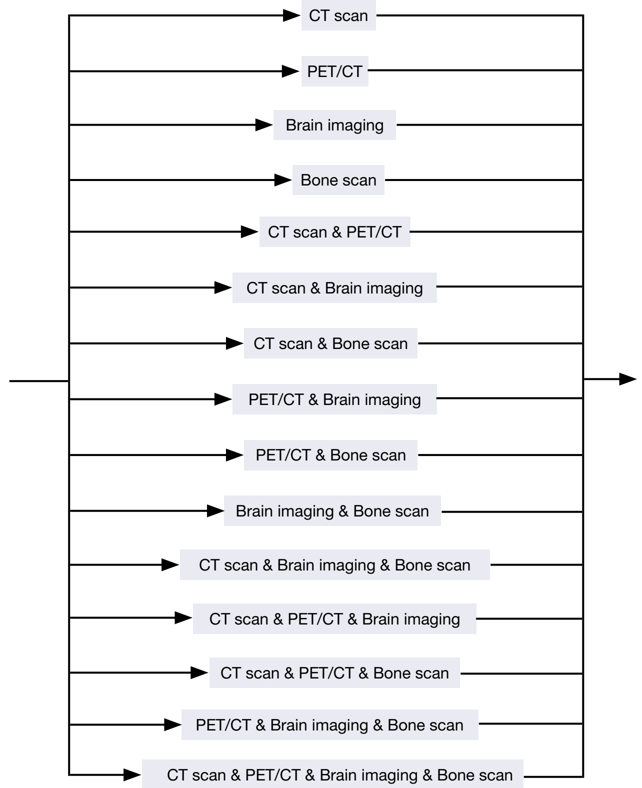

Figure 4 Non-invasive staging process. PET, positron emission tomography; CT, computed tomography.

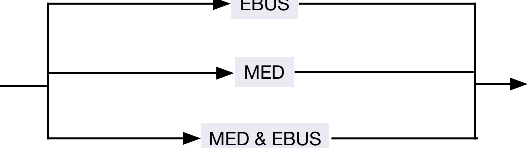

Figure 5 Invasive staging process. EBUS, endobronchial ultrasound; MED, mediastinoscopy.

The whole diagnosis process can be divided into six steps. Within each step, the process flow can still have many variations. For instance, during the abnormal chest X-ray and/or CT scan step (see *Figure 2*), a patient may go to either option or both. Similarly, for the diagnostic biopsy step, the biopsy process can be carried out by major procedures, such as CT guided biopsy, bronchoscopy, or both tests may be used (see *Figure 3*). The non-invasive staging step is much more complex. There are more than a dozen combinations of CT scan, positron emission tomography (PET)/CT, brain imaging, and bone scan. The patient may take only one of them, or two or three of them, or even all of them (see *Figure 4*).

The invasive staging step is much simpler compared to non-invasive staging. The major procedures such as endobronchial ultrasound (EBUS) staging, mediastinoscopy (MED) staging, or both may be used (see *Figure 5*). For

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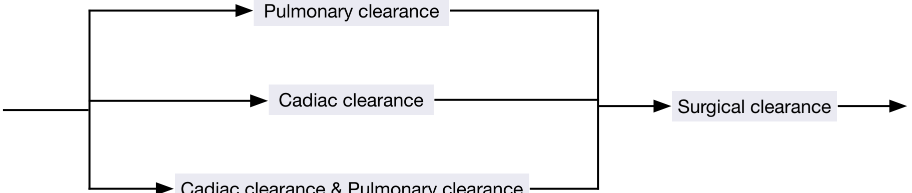

Figure 6 Medical clearance process.

simplicity, we have limited the schematic to the two invasive mediastinal nodal staging procedures most commonly used in community hospitals. Finally, the medical clearance step before surgery usually includes pulmonary and cardiac clearance, each of which presumably involves visits with providers from each of these specialties. A possible combination of them provides all the variations in this process (see *Figure 6*). Note that the pulmonary clearance can occur well before cardiac clearance, or even before staging tests.

There can be significant waiting time between each two steps, some of which may be substantial. Although each actual test may only take a few hours, the waiting time could last from days to weeks or even months. Because of the importance of accurate staging to treatment selection and prognosis, reducing the waiting times at various steps is of significant importance. Although the importance of waiting time reduction is intuitive, how to reduce it effectively is not. First, the relationship between the waiting times between various steps, and their contribution to the overall delay time from initial lesion detection to definitive treatment is not clear. Second, although ideally all waiting times should be reduced substantially, their reduction may have different impact on the total time for diagnosis process. How to identify the most critical waiting time so that its reduction has the largest reduction of overall process time is not known. Third, how to ensure that the waiting time can be controlled within a desired time limit has not been studied. Finally, even if the mean diagnosis time is short, large variability can still lead to a substantial number of patients waiting much longer than desired. Therefore, the variance of diagnosis time plays a significant role in reducing the possibility of treatment delay. How to address the above concern in terms of variance is unknown.

To answer these questions, a detailed analysis of the lung cancer diagnosis-to-treatment process is needed. Although clinical trials can be carried out, that strategy would take an inordinate and substantial amount of effort and time. The "small tests of change" or plan-do-check-act (PDCA) model may not be either appropriate or safe in many cases. Therefore, a model based approach is needed. Computer process models can provide significant guidance to system improvement efforts, before any potentially disruptive changes in process are implemented. It can present a fresh look at the whole process, offer an alternative method to "test" changes (virtually) in practice, and evaluate the impact of those changes. In this review, we focus on two types of computer process models that can be used for this purpose: discrete event simulation (DES), and analytical modeling.

Discrete event simulation (DES) models

Literature review

Computer or discrete-event simulation has been a prevailing tool in healthcare delivery research. It has been successfully implemented in emergency departments (EDs), hospital pharmacy units, critical care units, outpatient clinics and diagnostic centers. The rapid development in information technology and data analytics has substantially enhanced the functions and efficiency of simulation tools. Using the simulation model, the practitioners can vividly emulate the events randomly happening in healthcare delivery process, test sophisticated logics and schedules, evaluate design options, assess system efficacy, and carry out 'what-if' analyses to investigate the complex relationships among system variables, study the impact of potential changes, and finally to provide decision support for healthcare management. By testing different scenarios of patient arrivals, staffing level, workforce and equipment configuration, bed capacity, scheduling and team policies, lab turnaround time, etc., the simulation models can help find solutions to reduce patient length of stay, increase bed utilization, identify the most critical constraints (or bottlenecks), and improve efficiency and care quality.

Comprehensive reviews of computer simulation models used in health care systems have recently been presented (3-6).

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In these reviews, simulation studies in multiple healthcare organizations are introduced, such as outpatient clinics, EDs, surgical centers, orthopedic departments, and pharmacies. A substantial number of studies using simulations have focused on patient flow and crowding reduction in EDs. For instance, Storrow *et al.* (7) discovers that reducing lab turnaround time can help reduce ED length of stay and the need for ambulance diversion. Brenner *et al.* (8) and Zeng *et al.* (9) have identified diagnostic testing as the main bottleneck in the EDs under study. Integration of registration and triage is also studied in (10). Using DES, a decision support framework is introduced in (11), which shows that in-patient bed management is the key to unblock ED outflows. In addition, Konrad *et al.* (12) introduces a split flow approach to bring patients to resources and providers. By verifying through DES, it shows that such an approach has the advantage to typical fast-track practice in ED.

In addition to EDs, other hospital departments and clinics also received substantial amount of research attention. For example, for intensive care units (ICUs), a simulation model is developed in (13) to determine the number of supplementary nurses in an ICU that are required to minimize overall nursing staff costs. Griffiths *et al.* (14) intends to optimize the number of available ICU beds in order to maintain an acceptable level of bed occupancy. Zhu *et al.* (15) also studies bed capacity in ICU to estimate the proper number of beds needed to meet the target service level and the extra number of beds to respond to demand growth. Azari-Rad *et al.* (16) studies the perioperative process in a general surgery service using simulations to reduce the number of surgical cancellations. The results indicate that scheduling surgeons on a weekly basis, sequencing surgeries in order of increasing length and variance, and adding beds to the surgical ward help reduce the number of surgical cancellations.

In pharmacies, a simulation study introduced in (17) discovered that early preparation for the returning patients and dedicating an infusion staff member for medication delivery could substantially reduce patients' waiting time for antineoplastic medications, with up to 50% reduction achieved through such improvement efforts. Reynolds *et al.* (18) investigates the impact of changes in staffing levels and skill-mix on prescription workload and dispensing robot utilization in hospital pharmacy outpatient dispensing systems. Moreover, it is found in Zeng *et al.*'s study (19) that the pharmacist is not the main constraint in discharge process delay, but rather, early release of discharge orders by physicians is the key to speeding up the discharge process.

For outpatient clinics, an orthopedic outpatient clinic is studied in Rohleder *et al.*'s study (20) to optimize staffing levels and patient scheduling. Werker *et al.* (21) describe the model to reduce planning time and waiting time in radiation therapy process. Berg *et al.* (22) shows that the maximum number of patients served in an endoscopy suite is linearly related to the number of procedure rooms, whose turnaround time has a significant impact on the utilization of procedure rooms and endoscopist. Patient scheduling is analyzed through simulations in Ogulata *et al.*'s study (23) to determine appropriate scheduling policy under different environmental conditions. Outpatient radiology scheduling procedure is analyzed in Lu *et al.*'s study (24) to reduce the number of tests without pre-approvals so that financial losses can be minimized. In addition, Villamizar *et al.* (25) analyzes the impacts of changes in patient volume, arrivals, and clinic scheduling. Reynolds *et al.* (26) studies the staffing model design for a health clinic for homeless people. A complete model of patient flow analysis in (27) shows that implementation of "swing" rooms (flexible between antepartum and mother-baby rooms) could help to balance bed allocation in a women's health center. More DES models in various healthcare systems can be found in (28-34).

Discrete event simulation (DES) in lung cancer diagnosis process

To study lung cancer diagnosis process using DES, the simulation model can be constructed by following the paths in *Figures 1-6*. The following data are needed to define such a model.

Waiting time

This is the time between two consecutive steps or tests, i.e., the time a patient waits for the next test or diagnosis. Examples of the waiting time could be: the time between chest X-ray and CT scan in step 1&2; the time between CT-based biopsy and bronchoscopy in step 3; the time between step 1&2 and step 3, etc.

By checking the time stamps when the patients take each test, the waiting times for each patient can be collected. Then through statistical analysis, the collected waiting times are fitted into a distribution. The mean, the variance and other statistical parameters can be obtained. Such functions are included in most simulation software. These results are the time inputs to the simulation model.

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Routing probabilities

The probability a patient may take one specific route or test. Examples of routing probabilities include: the probability a patient may only take CT scan in step 1&2; the probability a patient may take CT scan and brain imaging in step 4; the probability a patient will go to step 5 directly after step 3, etc.

By counting the number of patients in each possible route from one step, and dividing the total number of patients leaving that step, such probabilities can be calculated and will be the routing inputs to the simulation model.

Using the simulation model, a validation study can be carried out by comparing the simulation model output with the results obtained through data collection. If the difference is small enough, the simulation model is validated and can be used for further analysis, such as 'what-if' analysis. For example, by reducing one waiting time by 10%, we can evaluate its impact on the overall diagnosis time. By carrying out such activity for all the waiting times, one can compare the results and discover the activity leading to the largest reduction in overall diagnosis time. Such a waiting time is viewed as a 'bottleneck waiting time' or the 'system constraint'. Then efforts can be focused on reducing the bottleneck waiting time. This effort can be repeated continuously until the overall diagnosis time reaches the desired value.

Analytical models

Markov chain model

To study the lung cancer diagnosis process, two types of analytical models could be useful. One is referred to as Markov chain, the other is closed formula.

Continuous time Markov chain (CTMC)

To briefly introduce the Markov chain model (35-37), consider a continuous time stochastic process $X(t)$, $t \geq 0$, taking non-negative integer values. If for all s, t, $u \geq 0$, and non-negative integers i, j, k, the following property holds:

$$P\left(X(t+s)=j \middle| X(s)=i, X(u)=k, 0 \leq u < s\right) = P\left(X(t+s)=j \middle| X(s)=i\right)$$

then such a process is a CTMC. In other words, in such a process, the conditional distribution of a future state at time $t+s$, given the current state at time t and all past states, only depends on the current state and is independent of the past states. Such a property is referred to as the Markovian property.

Introduce

$$P_{ij}(t) = P\left(X(t+s)=j \middle| X(s)=i\right)$$

to denote the probability that the process is in state j at time $t+s$, given that it is in state i at time s. Such a probability is referred to as the *transition probability* of the CTMC. If $P_{ij}(t)$ is independent of s, then the CTMC has stationary or homogeneous transition probabilities. When $t \to \infty$, the probability that a CTMC will be in state j often converges to a limiting value P_j, independent of the initial state, i.e.,

$$P_j = \lim_{t \to \infty} P_{ij}(t)$$

Such limiting probability exists if, given a process starts in state i, there exists a positive probability it is in state j and it takes a finite time returning to state i. Probability P_j represents the proportion of time the process is in state j.

For a CTMC, the amount of time the process stays in state i before transitioning to another state follows exponential distribution with rate v_i. Then the transition rate that the process will transit from state i into state j is denoted as q_{ij}, i.e.,

$$q_{ij} = v_i P_{ij}$$

Then the rate that the process transits into state j equals to the rate that the process transits out of state j, i.e.,

$$v_j P_j = \sum_{i \neq j} P_i q_{ij}, \quad \forall j,$$

where the left-hand side is the rate that the process leaves state j (flow-out), and the right-hand side is the rate that the process enters state j (flow-in). As one can see, such equations balance the flow-in and flow-out rates, so they are often referred to as *balance equations*. In addition, the sum of all the state probabilities equals to 1,

$$\sum_j P_j = 1$$

By solving these balance equations, P_j, the probability that the process is in state j can be obtained, which will lead to the performance measure of interest.

In addition to CTMC, discrete time Markov chain can be defined similarly. Consider a stochastic process $X(n)$ at time n, n=1,2,..., taking a finite or countable number of values and satisfying:

$$P\left\{X(n+1)=j \middle| X(n)=i, X(n-1)=r, \cdots, X(n-l)=s\right\} = P\left\{X(n+1)=j \middle| X(n)=i\right\} = p_{ij}$$

where $X(n)=i$ implies that the process is in state i at time n.

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Box 1 Balance equations

$P(0;0,0)(\mu_1+\mu_2) = P(0;1,0)\lambda_1 + P(0;0,1)\lambda_2,$	[1]
$P(0;0,1)(\mu_1+\lambda_2) = P(0;1,1)\lambda_1 + P(0;0,0)\mu_2 + P(1;0,1)c_2,$	[2]
$P(0;1,0)(c_1+\lambda_1+\mu_2) = P(0;0,0)\mu_1 + P(0;1,1)\lambda_2,$	[3]
$P(0;1,1)(c_1+\lambda_1+\lambda_2) = P(1;1,1)c_2 + P(0;0,1)\mu_1 + P(0;1,0)\mu_2,$	[4]
$P(1;0,0)(\mu_1+\mu_2) = P(1;1,0)\lambda_1 + P(1;0,1)\lambda_2,$	[5]
$P(1;0,1)(\mu_1+\lambda_2+c_2) = P(1;1,1)\lambda_1 + P(1;0,0)\mu_2,$	[6]
$P(1;1,0)(\lambda_1+\mu_2) = P(0;1,0)c_1 + P(1;0,0)\mu_1 + P(1;1,1)\lambda_2$	[7]
$P(1;1,1)(\lambda_1+\lambda_2+c_2) = P(0;1,1)c_1 + P(1;0,1)\mu_1 + P(1;1,0)\mu_2$	[8]

The transition probabilities and balance equations can be derived as well (35-37).

An illustrative example

To illustrate such a method, consider the two-service model introduced in (38), where a patient needs to go through nurse check and physician diagnosis within the patient room (or on patient bed). Denote the two services as s_1 (nurse check) and s_2 (physician diagnosis). Since both physician and nurse need to take care of multiple patients and they also have other duties in addition to meeting with patients, the status of their service is characterized by m_i=1, i=1,2, if they are available and m_i=0 otherwise. Let p_1 represent the number of patients waiting for or being served by service s_2. Since only one patient is allowed in the patient room, p_1 could be either 0 or 1. Then, the states of the system are defined as $\{p_1;m_1,m_2\}$. The probability the process stays in these states is denoted as $P(p_1;m_1,m_2)$.

Assume there is unlimited patient arrival. Each service has exponential service time with rate c_i, and the providers have exponential available time and non-available time with rates λ_i and μ_i, respectively. From the Markov property that the rate of the system leaving a state should be equal to the rate of the system entering that state, the following balance equations are obtained (*Box 1*):

In addition, we have

$$\sum_{k=0}^{1}\sum_{i=0}^{1}\sum_{j=0}^{1}P(k;i,j)=1$$

Solving these equations, all state probabilities $P(k;i,j)$ can be obtained. According to Little's law (39), flow time equals to number of patients divided by throughput. Since there is only one patient in the room, the patient length of stay T_s can be calculated as the inverse of throughput, i.e., the rate that the patient finishes physician diagnosis service.

$$T_s=\frac{1}{P(1;0,1)c_2+P(1;1,1)c_2}$$

Markov chain model in healthcare systems

Markov chain model has been used extensively in many engineering and science fields, such as informatics, manufacturing, finance, medicine, physics and chemistry. In recent years, application of Markov chain in healthcare delivery systems has attracted a lot of research efforts.

As illustrated above, Wang *et al.* (38) models the care delivery activities inside a patient room in ED to evaluate patient length of stay and provider utilization. For general emergency medical service systems, Wiler *et al.* (6) reviews the available models for ED, including Markov chain and DES models. A two-dimensional Markov chain model is introduced in (40) to characterize the number of busy ambulances and whether the system is in compliance or not. The model can provide accurate estimates of response time distribution and number of busy ambulance distribution. Similarly, Almehdawe *et al.* (41) derives the steady state probability distributions of queue lengths and waiting times for ambulance patients. A three-hospital EMS-ED model is presented to analyze the impact of system resources on offload delays.

Patient flow and care deliveries have been studied using Markov chain models. A care activity model with multiple patient rooms and limited number of care providers in primary care clinics is presented in (42). Wang *et al.* (43) study work flow and staffing level in a CT test center and identify the imaging formatting process as the main constraint in the system. The patient flow in a gastroenterology clinic is evaluated in (44) based on a Markov chain model. Using this model, various policies on check-out scheduling are investigated. In addition, using a single room Markov chain model as a building block, an iterative method is introduced in (45) for a mammography imaging center with multiple rooms to study the work flow with a shared Technologist Assistant. In home care, Lanzarone *et al.* (46) introduces a Markov chain model of patient care pathway to provide predictions on number of patients who are followed up, the duration of each care and the amount of required visits, which can provide support for human resource planning.

Using the Markov chain model, hospital admissions have been studied. For example, Tang *et al.* (47) evaluate patient length of stay and use it to admit acute myocardial infarction patients into the hospital. It shows that the phase-type distribution can help account for the heavy skewness and heterogeneity in the data. The phase-type distribution is a convolution of exponential distributions, resulting from one or more inter-related Poisson processes occurring in

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sequence or phases. A survey of phase-type distribution modeling in healthcare systems is presented in (48) and ideas for further utilization are proposed (49); also studies hospital admission control and proposes a new gateway to improve admission through adding an expedited patient care queue. Using a Markov chain model of patient flow (50), discusses admission scheduling, resource requirement forecasting and resource allocation to satisfy demand and resource constraints.

Patient safety has been studied using Markov chain in (51), where the state space includes normal and risk status of patients, nurse check, physician intervention, and rapid response team (RRT) diagnosis. Through a recursive procedure, the limited availability of providers is considered when multiple patients are present. In addition, to improve patient safety in surgeries, the disruptions in surgical work flow are also modeled by Markov chain in (52), and bottleneck analysis is carried out to identify the most impeding disruption, removing which can reduce the impact of surgical disruptions in the strongest manner.

In addition, Markov chain has been used to model biologic processes, such as lung cancer growth and metastasis. In paper (53), the metastatic progression for primary lung cancer is modeled based on a Markov chain, which offers a probabilistic description of the time history of the disease unfolding through the metastasis cascade. This enables evaluation of disease progression pathways and timescales of progression from the lung to other sites. In (54), the progression of the disease is divided into four phases and calculated using a Markov chain model for familial nasopharyngeal carcinoma. Then four screening policies [(A) annual screening; (B) biennial screening; (C) triennial screening; and (D) triennial screening for participants who tested Epstein-Barr virus (EBV) negative and annual screening for participants who test EBV positive] are compared. The results show that screening policy (D) has the highest efficacy. Additional Markov chain models in health care applications can be found in (55-60).

Markov chain model of lung cancer diagnosis process

Using the CTMC outlined above, the lung cancer diagnosis process can be modeled. The system states can be defined as follows: Let the patient's waiting for a test be a state of the process. For example, waiting for CT scan after chest X-ray in *Figure 2*, waiting for CT-based biopsy in *Figure 3*, and waiting for bone scan after brain imaging in *Figure 4*, can be defined as the states for the diagnosis process. Similarly, all other states can be defined.

From the collected data, the average waiting time can be calculated for each state. Reversing them we obtain parameter v_i. The transition probability from one state to another one, P_{ij}, will be the routing probability from one test to another. With these parameters, the balance equations can be obtained. Solving the equations, the overall diagnosis time is calculated.

Closed formulas

Due to the special feature in lung cancer diagnosis process, it is possible to develop closed formulas to evaluate the overall waiting time and the variability. It has been shown that for a serial process with multiple independent stages, the mean and variance of overall flow time will be equal to the sum of all process times and the associated variances, respectively. In other words, consider a serial process with M independent stages, if each stage i, i = 1,...,M, takes an average time τ_i and variance var_i to finish, then the mean T and variance Var of the overall diagnosis time will be:

$$T = \sum_{i=1}^{M} \tau_i, \quad \text{Var} = \sum_{i=1}^{M} \text{var}_i$$

Such an approach has been used in studying the 'Rapid Response' process to improve patient safety in acute care. In papers (61-63), when deterioration in a patient's clinical condition is detected, the primary nurse may call the intern, resident, or RRT for help. The provider can either make a decision or call for further help from the upper level physicians (e.g., intern to resident, RRT to resident, resident to fellow, fellow to attending). Thus, the response process can be modeled as a complex network with split, merge, and parallel structures. By considering the combination of possible routes (e.g., RN-intern-resident-fellow-attending), the closed formulas can be developed to evaluate the decision time and its variability.

As shown in section 1, similar to the rapid response process, the lung cancer diagnosis process is very complicated and can also be modeled by a complex network. However, for one specific patient, he/she can only take one possible route. Thus, from his/her point of view, a serial process will be taken during the whole diagnosis period. Thus, by assuming all testing steps are independent, the closed formula can be applied for his/her route. To consider many patients, by including the routing probabilities, the whole diagnosis process can be represented by a combination of a set of specific routes, each being weighted through its routing probability. *Figure* 7 illustrates such

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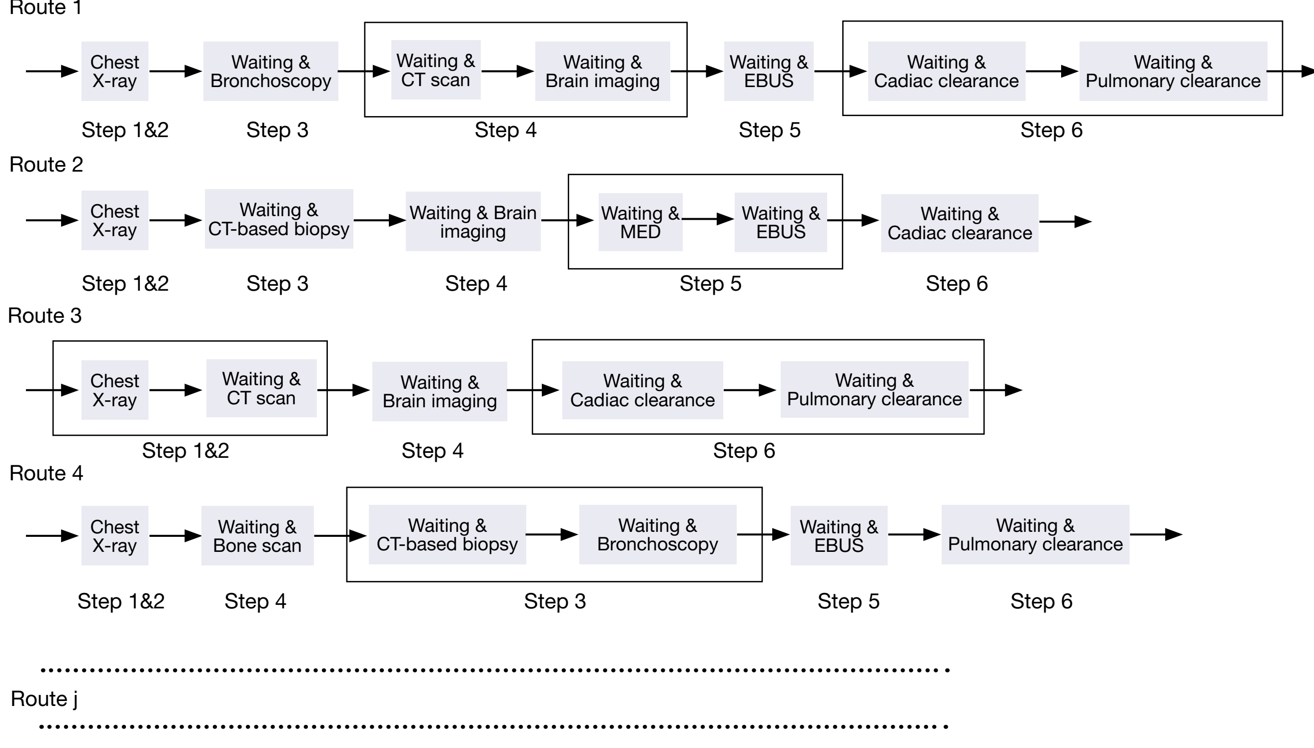

Figure 7 Illustration of possible routes. CT, computed tomography; MED, mediastinoscopy; EBUS, endobronchial ultrasound.

an approach. The complex diagnosis process can be decomposed into a set of possible routes that the patients may go through. Four examples are illustrated in the figure. Then for a particular route j, the mean diagnosis time T_j and the variance Var_j can be calculated. Calculating the product of all probabilities going to the next test after each one in this route, we obtain the probability α_j of a patient taking such a route, which will be the weight of route j. Then $\alpha_j T_j$ and $\alpha_j \mathrm{Var}_j$ provide the weighted mean and variance, respectively. Summing them up, we obtain the final mean and variance of the overall diagnosis process.

$$T = \sum_j \alpha_j T_j, \quad \mathrm{Var} = \sum_j \alpha_j \, \mathrm{Var}_j$$

Discussion

Both the DES and analytical models are useful in studying the lung cancer diagnosis-to-treatment process. The simulation model can provide more detailed and more vivid analysis as well as user friendly graphic interface and animation. There are many DES software programs available, such as Simul8, Arena, Flexsim, ProModel (or MedModel). However, it takes longer time to develop and execute the simulation model, needs more inputs, and relies on the software environment. For complex processes and extensive scenarios, computation intensity may become an issue. More importantly, most simulation models are case-study based, which makes it difficult to discover some common features of the system.

The analytical models, on the other hand, can provide quick analysis, which is extremely useful during what-if analyses. In addition, it is possible to derive system properties, such as monotonically increasing property with respect to process parameters and bottlenecks. Also it requires less data inputs and is not dependent on software. However, the results are less detailed and do not have animation capability. The assumptions in the models may also limit their applications.

Concerning the analytical model for lung cancer diagnosis process, the Markov chain model assumes exponential service time, and may need a large number of states, which make the analysis difficult to proceed. Typically, empirical formulas need to be developed to approximate the performance in non-exponential scenarios (42-45).

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For small variations, i.e., the coefficient of variation (CV), defined as standard deviation divided by the mean, is small or less than 1, the average performance usually only depends on the mean and CV. In addition, it will be difficult to evaluate the variance. The closed formulas can handle any service time distributions and evaluate variance. However, the number of possible routes can be very big so that it may need to ignore some routes which have very small probabilities and have almost no impact on system performance. In both approaches, an independent assumption is introduced. In practice, a patient's probability of receiving a certain test is usually conditioned on the previous test results. Thus the waiting time is also conditioned on the previous diagnostic results. Therefore, both the state dependent Markov chain and closed formula should be developed.

Conclusions

In this paper, computer process modeling methods are introduced for the lung cancer diagnosis-to-treatment process. Both DES and analytical models (including Markov chain model and closed formulas) can be used to estimate patients' diagnosis-to-treatment time. Using these models, the complex relationship between waiting times and overall process time can be investigated, 'what-if' analyses can be carried out to determine the most critical waiting time that impedes early detection and staging in the strongest manner. Such methods provide quantitative tools and an alternative way to improve care quality in the lung cancer management process.

The methods introduced here are not only applicable to the lung cancer diagnosis process, but also useful in many healthcare delivery processes, such as patient or work flow, care transition, information transfer, as well as clinical decision process. The developed models can be used for staffing analysis, resource management, scheduling and decision support, among other things.

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Footnote

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Techniques to define segmental anatomy during segmentectomy

Hiroyuki Oizumi, Hirohisa Kato, Makoto Endoh, Takashi Inoue, Hikaru Watarai, Mitsuaki Sadahiro

Second Department of Surgery, Yamagata University Faculty of Medicine, Yamagata, Japan

Correspondence to: Hiroyuki Oizumi. Second Department of Surgery, Yamagata University Faculty of Medicine, Yamagata, Japan. Email: hohizumi@med.id.yamagata-u.ac.jp.

Abstract: Pulmonary segmentectomy is generally acknowledged to be more technically complex than lobectomy. Three-dimensional computed tomography (3D CT) angiography is useful for understanding the pulmonary arterial and venous branching, as well as planning the surgery to secure adequate surgical margins. Comprehension of the intersegmental and intrasegmental veins makes the parenchymal dissection easier. To visualize the segmental border, creation of an inflation-deflation line by using a method of inflating the affected segment has become the standard in small-sized lung cancer surgery. Various modifications to create the segmental demarcation line have been devised to accurately perform the segmentectomy procedure.

Keywords: Segmentectomy; thoracoscopy; video-assisted thoracic surgery (VATS); three-dimensional computed tomography (3D CT); slip knot; subsegmentectomy

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Introduction

In recent years, the diagnosis of small lung nodules and non-solid lung cancers has been increasing due to developments in computed tomography (CT) technology. It is reported that the prognosis of such malignancies is good even with a sublobar resection (1-3). It is reasonable to perform a less invasive resection of a smaller volume of lung tissue, and the simple procedure of wedge resections may be sufficient if tumors are located in the peripheral sub-pleural parenchyma. However, wedge resection is inadequate for most primary lung cancers and for nodules located deep in the lung. Segmentectomy is preferred in such cases to secure an adequate surgical margin (4). In open thoracotomy surgery, a tumor is dissected bluntly by maintaining a sufficient margin while directly palpating the tumor. However, in thoracoscopic surgery, in which a hand cannot be passed directly into the thoracic cavity, it is important to proceed with the operation with a clear anatomical understanding.

Anatomical segmentectomy

In a lobectomy, demarcation of the lobar anatomy is usually relatively straightforward. In contrast, segmentectomy is more complex. In particular, the recognition of the subsegmental fissures within the pulmonary parenchyma may be difficult, with unclear boundaries between adjacent segments. In addition, when the target disease is a malignant tumor, it is necessary to secure enough surgical margin. In a thoracotomy, the tumor is dissected bluntly from the adjacent segments by maintaining a sufficient margin while directly palpating the tumor, and involved blood vessels are also treated. During thoracoscopic surgery, in which a hand cannot be passed directly into the thoracic cavity, it is important to proceed with the operation with a clear anatomical understanding.

The lung segments extend to the peripheries with the bronchus as the base. There are ten segments in the right lung (upper lobe, three; middle lobe, two; lower lobe, five) and eight segments in the left lung (upper lobe, four; lower lobe, four). Each segment has a different morphology, size and blood vessel branch, which depend on its site, and there are many variations among patients (5-7). The left upper lobe is divided into the upper and lingular divisions, while the bilateral lower lobes are generally divided into the superior and basal segment that is combined with the remaining area. As lobation is occasionally observed between

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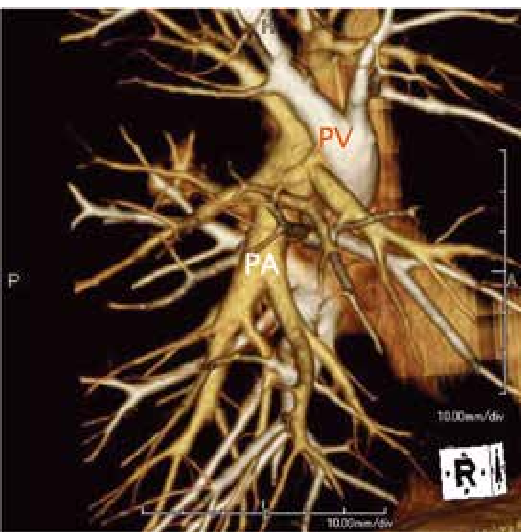

Figure 1 Three-dimensional computed tomography angiography. PA, pulmonary artery; PV, pulmonary vein.

these segments, the anatomy is relatively simple and easily understood. Therefore, video-assisted thoracic surgery (VATS) segmentectomy has often been performed along this plane (8,9). The problem lies with resections of other segments. It is important to plan and accurately perform the procedure (10-12). A variety of methods have been devised and used clinically, especially in thoracoscopic surgery, to solve the problem of the lack of tactile guidance (13-15).

With non-anatomical segmentectomy, the pulmonary parenchyma is roughly incised after treating the pulmonary artery and bronchus at the pulmonary hilum. However, it is not yet possible to cover resection of all segments with this method alone. The next branch of the segmental bronchus is called a subsegmental bronchus (16). Thoracoscopic resection of this subsegment has recently been performed (17). Thus, we describe herein the methods of understanding the dissection required for anatomical segmentectomy.

Understanding vascular structure

As the segmental artery is located at the pulmonary hilum in the superior segment of the lower lobe, identification and dissection are relatively easy. However, as arterial branches are embedded in the pulmonary parenchyma in some segments, it is sometimes necessary to preserve the proximal branch and divide the peripheral. Also, in many cases, more than one arterial branch is present even in a single segment. In such cases, it is useful to observe in detail and understand the morphology of the branch by employing contrast-enhanced CT, in order to carry out the surgery smoothly. A segmental artery normally accompanies the segmental bronchus. After completing division of the affected artery, the segmental bronchus can be easily traced as it is less flexible in the surrounding tissues.

With rapid advances in multi-detector CT (MDCT) in recent years, it has become possible to easily perform three-dimensional (3D) processing not only in a workstation but also on a personal computer (*Figure 1*). By using MDCT, we understand each patient's individual anatomy and can perform operations mainly by defining the course of arteries and veins (13-15). Usually, radiologists or technicians construct the 3D image using a workstation. The arteries and the veins are separately segmented and color-coded by CT value, and these volume-rendered images are then merged into the 3D-CT angiography. This image is ideal but it takes a long time to create. Thoracic surgeons know the basic anatomy of the lung, and therefore don't need complex images. When we use volume rendering methods, we prepare simple images that meet our needs in as little time as approximately seven minutes (http://www.youtube.com/watch?v=tSO58k9Lja8). By cutting out the area of interest, the image can be magnified, de-magnified or rotated during surgery (*Figure 2*). We previously reported that port-access thoracoscopic segmentectomy could be safely be performed in all segments using this approach, termed Segmentectomy Achieved by MDCT for Use in Respective Anatomical Interpretation (SAMURAI) (15). Since 2004, we have performed thoracoscopic segmentectomy in 160 patients including subsegmentectomy in 20 patients, and our completion rate is 98%. The surgical results for small lung cancer are still insufficient, with a mean follow-up period of only 3.5 years as yet. However, the 5-year survival rate is 100%, which is very favorable.

The venous branches within the segment become intersegmental veins as they converge, and return to the hilum. In segmentectomy, it is very important to understand these intersegmental and intra-segmental veins (*Figure 3*). The pulmonary parenchyma is dissected along the intersegmental vein, and intrasegmental vein thereby is identified. Division of the intrasegmental veins allows identification of the intersegmental border and facilitates the further parenchymal dissection (14,15). It is as if a clam can be opened when the adductor is cut.

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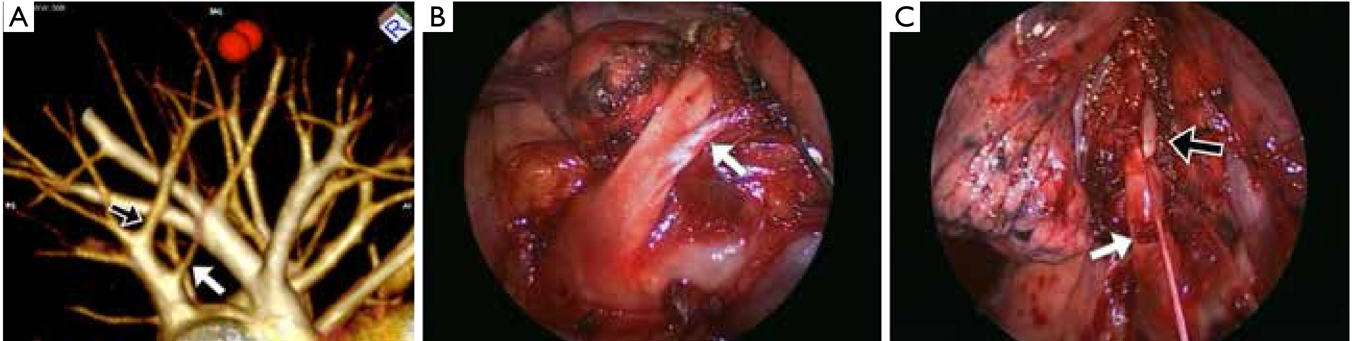

Figure 2 S1+2a (apical subsegment in left apical posterior segment) resection of the left upper lobe. (A) Three-dimensional computed tomography angiography with a marking of the tumor indicates two subsegmental arterial branches should be divided from the left apical posterior segmental artery. White arrow, first branch of the subsegment; Black arrow, second branch of the subsegment; (B) Operative view of the patient. The white arrow indicates the first arterial branch; (C) Operative view of the patient. The white arrow indicates the stump of the first arterial branch. The black arrow indicates the second arterial branch that was encircled in the deep parenchyma.

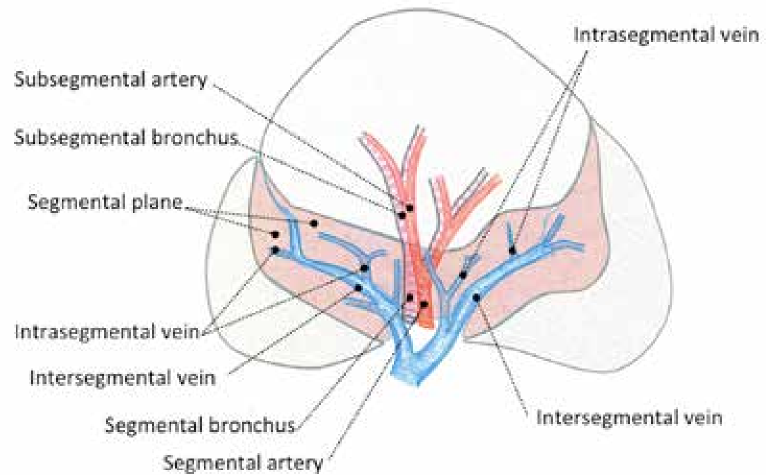

Figure 3 Schema of lung segmentectomy. The intersegmental plane is dissected preserving the intersegmental veins. Intrasegmental veins of the affected segment should be identified and divided.

Surgical margin

The SAMURAI method not only defines the running of blood vessels but also determines the extent of resection by virtually defining surgical margins. If it is difficult to preserve the margin in a single segment resection, we perform an extended resection of the parenchyma of adjacent segments.

Iwano and colleagues reported that radiologists propose the extent of resection to surgeons by superimposing a spherical safety margin on 3D images using a workstation for CT (18). While this method is ideal, preparing the images can be complex and time-consuming for surgeons. Although the SAMURAI method cannot create a perfect sphere in images, the surgeons themselves can evaluate resection margins intraoperatively using an appropriate scale in real time (15).

Identification of the intersegmental border

Inflation-deflation line

The basis of segmentectomy is to isolate and divide the bronchus and then dissect its peripheral pulmonary parenchyma. For conventional segmentectomy in open thoracotomy, division at the intersegmental border was

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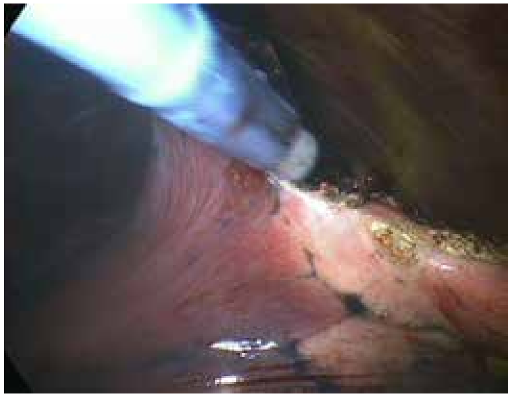

Figure 4 Inflation-deflation line created by slip knot method.

generally performed by dissecting the bronchus in the affected lung and collapsing the lung on the peripheral side. In lung cancer patients, the actual method involved securing a margin by directly palpating the tumor. Meanwhile, Tsubota reported a method of inflating the affected segment to be beneficial (19). Moreover, Okada and colleagues visualized the intersegmental plane by selectively inflating the segment using a jet ventilator and reported this approach to be effective in securing an operative field. Expansion of the affected segment allows not only visualization of intersegmental borders but also maintains the morphology and size of the resected lung in the same state as the actual systemic physiological state, thereby achieving more accurate evaluation of resection margins (11). Therefore, it is considered to be more advantageous oncologically and is becoming a standard method in Japan.

Thus, jet ventilation is useful as an inflation method for the affected segment in thoracoscopic surgery or small thoracotomy. However, this method requires equipment and another doctor to maneuver the bronchoscope. Some institutions experienced such difficulties and various modifications have been devised. Direct inflation into the bronchus using a butterfly needle from the operative field was reported to be useful (20). However, great care is essential as this approach can reportedly cause air embolism (21).

We were not able to effectively insert the bronchoscope into the smaller bronchi during resection at the subsegmental (third order) bronchial branches (16). Therefore, we attempted to block the bronchus by ligation with expansion of the affected segment, especially in segmentectomy of smaller bronchial calibers. We ligated a bronchus conventionally using a knot pusher after ventilation when the bronchus was narrow. However, this method cannot be performed quickly after inflation; therefore, the affected segment will be partly deflated. We found that the monofilament slip-knot, customized from the previously reported modified Roeder knot, was useful since it enabled the surgeon to ligate the bronchus during ventilation of the lung. The bronchus is closed by pulling the thread (http://www.youtube.com/watch?v=XH2jt7kL3mo), and was effective for creating the inflation—deflation line (*Figure 4*) (22). We believe that this method can be generalized because it doesn't need any special equipment and is applicable at any time.

Intersegmental veins

As described earlier, intersegmental pulmonary veins serve as important landmarks (15). The dissection of their branches, the intrasegmental pulmonary veins, facilitates intersegmental dissection. When it is difficult to reach the segmental artery and bronchus located in the deep areas of the pulmonary parenchyma, we can reach the target bronchus by dissecting the parenchyma along the intersegmental pulmonary vein. For example, in segmentectomy of S9+10 or S10 of the lower lobe, the bronchus is located in a very deep area far from the interlobar area. We have devised a posterior approach to dissecting the pulmonary parenchyma along the vein (V6) between the superior and the basal segment, initially, thereby reaching the bronchus posteriorly (http://www.youtube.com/watch?v=V2Rq92JB6vk) (23). Once the bronchus is reached, a line between the inflated and deflated areas is created using the aforementioned method. This facilitates dissection of S9 and S10, formerly classified as the most difficult segments, and reduces the operative time. As such, visualization of the line between the inflated and deflated areas and the intersegmental vein dissection are both important in performing intersegmental dissection.

Other techniques

There is a report describing a fluorescence method, wherein indocyanine green is injected into a blood vessel after treating the target segmental artery (24,25). It is based on the premise that the segmental bronchus is accompanied by the pulmonary artery. As the running vessels do not match in some cases, it is necessary to read CT images in detail to identify the pulmonary artery to ultimately be treated. A method of injecting dyes into the bronchus has also been reported (26). While this direct

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Figure 5 A solid model of pulmonary arteries and veins of right upper and middle lobe molded from computed tomography data using three-dimensional (3D) printer.

method is promising, it requires an additional procedure of injecting materials via bronchoscopy. Although both methods require special instruments and procedures, we anticipate that there will be further reports describing their general use in the future.

Future simulation: virtual to real

Computer technology is rapidly advancing. We are now able to visualize the surfaces of pulmonary blood vessels, output the dendritic structure as an STL file, and create a 3-dimensional solid model using a 3D printer. After sterilization, this device can be hand held and observed during surgery (*Figure 5*). As 3D printer equipment and consumable supplies are expensive, there is an issue of cost in creating the model. While it still cannot be regarded as an item for actual use as compared with virtual technology, there is potential for this approach to become a useful tool if the manufacturing cost can be brought down in the future.

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Open, thoracoscopic and robotic segmentectomy for lung cancer

Chi-Fu Jeffrey Yang, Thomas A. D'Amico

Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA

Correspondence to: Thomas A. D'Amico, MD, Professor and Vice Chairman. Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA. Email: damic001@mc.duke.edu.

Abstract: While lobectomy is the standard procedure for early stage lung cancer, the role of sublobar resection is currently under investigation for selected patients with small tumors. In this review, studies reporting outcomes on open, thoracoscopic and robotic segmentectomy were analyzed. In patients with stage I lung cancer, with tumors <2 cm in diameter and within segmental anatomic boundaries, segmentectomy appears to have equivalent rates of morbidity, recurrence and survival when compared to lobectomy. Segmentectomy also resulted in greater preservation of lung function and exercise capacity than lobectomy. It appears reasonable to consider segmentectomy for patients with stage I lung cancer (particularly in air-containing tumors with ground glass opacities) where tumors are <2 cm in diameter and acceptable segmental margins are obtainable, especially in patients with advanced age, poor performance status, or poor cardiopulmonary reserve. The results of two ongoing randomized controlled trials (CALGB 140503 and JCOG0802/WJOG4607L) and additional well-designed studies on open, thoracoscopic, and robotic segmentectomy will be important for clarifying the role of segmentectomy for lung cancer.

Keywords: Lung cancer surgery; minimally invasive surgery; thoracoscopy/video-assisted thoracoscopic surgery (VATS); segmentectomy

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Introduction

The first segmentectomy, a lingulectomy, was performed by Churchill and Belsey in 1939 for the treatment of bronchiectasis (1). Over the subsequent decades, segmentectomy was increasingly applied to small primary lung cancers (2,3). However in 1995, the Lung Cancer Study Group (LCSG) performed a randomized controlled trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer (NSCLC) and found that limited pulmonary resection for tumors <3 cm in size resulted in increased locoregional recurrence compared to lobectomy (4). Subsequently in North America, the use of segmentectomy for NSCLC was generally limited to patients with marginal cardiopulmonary function (5).

The LCSG trial is the only randomized controlled trial of lobectomy versus limited resection for lung cancer to date, and is indeed a landmark study. However, it enrolled patients from 1982-1988 (4) and the landscape of thoracic oncology has changed considerably. Since then, there have been new developments leading to renewed interest in segmentectomy for small primary lung cancer tumors (5). Firstly, there is now strong evidence that low-dose computed tomography (LDCT) screening in high-risk patients reduces lung cancer deaths. Importantly, the screening protocols have identified greater numbers of smaller lung tumors (<2 cm), which are more frequently operable and curable (6,7). Of note, the LCSG trial did not specifically assess the effect of lobectomy versus segmentectomy on smaller tumors, as 30% of patients in that study had tumors that were larger than 2 cm (4). Secondly, since 1995, newer staging modalities have emerged which will likely improve patient selection for anatomic lung resection (4). Thirdly, surgeons have advanced the fields of video-assisted thoracoscopic surgery (VATS) and robotic surgery, with increasing experience at applying those approaches to segmentectomy. These new developments have led to a growing number of studies

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investigating the use of open, minimally invasive and robotic segmentectomy for carefully selected patients with smaller tumors less than 2 cm in size, especially in patients with marginal cardiopulmonary function (5).

A previous review of these studies demonstrated that when compared to thoracoscopic lobectomy, thoracoscopic segmentectomy had equivalent rates of morbidity, recurrence and survival in selected patients (5). When compared to open segmentectomy, thoracoscopic segmentectomy was found to have equivalent oncologic results, with shorter length of stay, reduced rates of morbidity, and lower cost. There have since been additional studies on segmentectomy, including further reports on uniportal and robotic approaches. This review is an update on the current role of segmentectomy and will focus on the most relevant recent studies on open, minimally invasive and robotic segmentectomy for lung cancer.

Open segmentectomy *vs.* open lobectomy

Since the LCSG study, although there have been no new randomized trials, there have emerged several retrospective studies comparing open segmentectomy to open lobectomy (8). In contrast to the LCSG trial, which enrolled patients from 1982-1988 and included 30% of patients with tumors >2 cm, these studies reflected a more current medical and surgical practice, and focused on examining the role of segmentectomy for tumors >2 cm in diameter. These studies reported similar outcomes and have found no significant differences in morbidity, mortality, locoregional recurrence or survival between segmentectomy and the lobectomy (8).

Most of these studies had groups well-matched for pulmonary function, but an important limitation of these studies is that many did not include information on preoperative co-morbidities. Three recent retrospective studies on segmentectomy *vs.* lobectomy did however include preoperative comorbidities and pulmonary function tests in their analysis. In 2011, Schuchert and colleagues compared the results of 107 patients undergoing resection for stage IA NSCLC (≤1 cm) via lobectomy (n=32), segmentectomy (n=40) or wedge resection (n=35) (9). Preoperative forced expiratory volume in 1 second (FEV1) was significantly lower in the sublobar resection (segmentectomy, wedge) groups compared with the lobectomy group; but age, sex distribution, tumor size, histology and preoperative comorbidities were similar between groups. Mean follow-up was 42.5 months and there was no statistically significant difference in overall disease recurrence or estimated 5-year disease-free survival (lobectomy, 87%; segmentectomy, 89%; wedge, 89%; P>0.402). While the authors note that a VATS approach was used more often than an open approach (57% *vs.* 43%) they did not specifically study the effects of open *vs.* VATS approach on outcomes.

Carr and colleagues conducted a retrospective study comparing the outcomes of 429 patients undergoing resection of stage I NSCLC via lobectomy or anatomic segmentectomy (10). The segmentectomy group (n=178) was older and had more co-morbidities—more likely to have coronary artery disease (18.5% *vs.* 12.8%, P=0.036) or chronic obstructive pulmonary disease (26.4% *vs.* 14.4%, P=0.0001)—than the lobectomy group (n=251). The segmentectomy group also had worse pulmonary function than the lobectomy group (FEV1 81.1±17.6 *vs.* 71.8±25.6, P=0.02). The authors found no difference in 30-day mortality (1.1% *vs.* 1.2%), recurrence rates (14.0% *vs.* 14.7%, P=1.00), or 5-year cancer-specific survival (T1a: 90% *vs.* 91%, P=0.984; T1b: 82% *vs.* 78%, P=0.892) when comparing segmentectomy and lobectomy for pathologic stage IA non-small cell lung cancer, when stratified by T stage. Of note, this study included patients who underwent both open and VATS approaches, and an open approach was used less often with segmentectomy than with lobectomy (41% *vs.* 60.6%, P=0.0001). The authors did not specifically evaluate outcomes by type of approach.

With regard to the role of open segmentectomy in the elderly, Kilic and colleagues conducted a retrospective review of 78 patients >75 years of age who underwent segmentectomy *vs.* lobectomy for stage 1 NSCLC. The segmentectomy group included more patients with chronic obstructive pulmonary disease (COPD) and diabetes. The tumors were significantly larger in the lobectomy group (3.5 *vs.* 2.5 cm, P<0.0001). The authors found no significant difference in 5-year disease-free survival between segmentectomy and lobectomy (11). Outcomes associated with an open *vs.* VATS approach were not specifically evaluated.

In addition to the single-institution retrospective studies described above, there has been one population-based study of open segmentectomy and lobectomy for stage I NSCLC. In 2011, Whitson and colleagues analyzed 14,473 patients undergoing anatomic segmentectomy or lobectomy for stage I NSCLC derived from the Surveillance Epidemiology and End Results (SEER) database. The authors were unable to stratify by open or VATS approach, but presumably

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most of the operations were performed open. Lobectomy was demonstrated to be associated with improved overall ($P<0.0001$) and cancer-specific ($P=0.0053$) 5-year survival compared with segmentectomy. After adjusting for tumor size, this improvement in survival remained. However, it is difficult to draw specific conclusions from this study because, in addition to its retrospective nature, the study did not have data on patient preoperative co-morbidities and pulmonary function—important variables which may have significantly affected both procedure selection and postoperative outcomes.

Advantages of open segmentectomy *vs.* open lobectomy

Since the 1995 LCSG randomized trial, there have been numerous retrospective studies that have shown that there are no differences in recurrence and survival between open segmentectomy and open lobectomy, even in patients with greater co-morbidities and worse pulmonary function (10), patients older than 75 years of age (11), and patients with larger tumors between 2 and 3 cm in size (10). Furthermore, in one study, open segmentectomy was found to preserve postoperative pulmonary function at 90%±12% of preoperative levels (12). There is one recent population-based analysis which found that patients undergoing anatomic segmentectomy had a decreased survival rate when compared to those undergoing lobectomy for stage I NSCLC. However, this study did not include information about patient comorbidities or cardiopulmonary function; patients in segmentectomy could have had reduced cardiopulmonary function, greater co-morbidities or other factors that affected survival.

Advantages of segmentectomy *vs.* wedge resection

With regard to the outcomes of patients undergoing an open segmentectomy versus wedge resection for stage I NSCLC, multiple reports show a decreased risk of recurrence and equivalent or improved survival in patients undergoing open segmentectomy compared to those undergoing wedge resections (8). When compared with the wedge resection, segmentectomy has also been shown to be associated with a larger parenchymal margin (13,14), a higher yield of lymph nodes and rate of nodal upstaging (14), and reduced risk of locoregional recurrence (15). Based on these studies, segmentectomy would be the preferred procedure for patients considering sublobar resection.

Predictors for prognosis and recurrence

With regard to predictors for prognosis and recurrence for patients with NSCLC who underwent segmentectomy, Koike and colleagues found age >70 years, gender (male), >75% consolidation/tumor ratio on high-resolution CT, and lymphatic permeation to be independent poor prognostic factors, and lymphatic permeation to be an independent predictor for recurrence (16). Yamashita and colleagues found KI-67 proliferation index to be a predictor of early cancer death (17). Traibi and colleagues have also shown male gender, FEV1 ≤60% and open (as opposed to VATS) surgery to be risk factors for postoperative complications (18).

In 2013, Koike and colleagues reported risk factors for locoregional recurrence and survival in patients undergoing sublobar resection (patients who underwent segmentectomy or wedge resection in the analysis) (15). They found four independent predictors of locoregional recurrence: wedge resection, microscopic positive surgical margin, visceral pleural invasion, and lymphatic permeation. Independent predictors of poor disease-specific survival were smoking status, wedge resection, microscopic positive surgical margin, visceral pleural invasion, and lymphatic permeation.

Thoracoscopic segmentectomy *vs.* open segmentectomy

Since the 1995 LCSG randomized trial, there have been significant advancements in thoracoscopic surgical techniques, including a better understanding of the potential advantages of the thoracoscopic lobectomy and segmentectomy for anatomic pulmonary resection (5). The studies included in the present review will use the definition of thoracoscopic segmentectomy as the completion of sublobar anatomic pulmonary resection, with individual vessel ligation and without the use of a utility thoracotomy, retractors or rib-spreading (5). Studies using a "hybrid" segmentectomy with mini-thoracotomy fall into the category of open surgery and are not included in this section.

The first retrospective study comparing outcomes of thoracoscopic and open segmentectomy was performed by Shiraishi and colleagues in 2004 (19). The authors selected patients with clinical stage IA peripheral tumors (<2 cm) and reviewed the outcomes of 34 patients who underwent VATS segmentectomy versus 25 who underwent open segmentectomy. They found no significant differences

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in postoperative complications and perioperative deaths. Long-term survival was not evaluated in this study.

In 2007, Atkins and colleagues conducted a retrospective study comparing the results of 48 patients who underwent VATS versus 29 who underwent an open approach (20). The authors found no significant differences in preoperative co-morbidities, pulmonary function, operative time, estimated blood loss, nodal stations sampled and chest tube duration between the two groups. In addition, no significant differences were seen in locoregional recurrences between the open (8.3%) and the VATS (7.7%) approaches (P=1.0). However, there was a significantly decreased length of hospital stay for the VATS group when compared to the thoracotomy group (4.3±3 *vs.* 6.8±6 days; P=0.03). At approximately 30 months postoperatively, it was found that the VATS group had improved long-term survival when compared with the thoracotomy group (P=0.0007), although the groups were not matched oncologically.

Schuchert and colleagues performed a retrospective review of patients who underwent VATS segmentectomy (n=104) versus those who underwent thoracotomy (n=121) (21). There were no significant differences between the two groups in age, gender, histology, and pulmonary function as measured by FEV1 and DLCO. The VATS group had slightly smaller tumor sizes than the thoracotomy group (2.1±1.1 *vs.* 2.4±1.2 cm, P=0.05) and there were fewer lymph nodes harvested during VATS segmentectomy when compared with open segmentectomy (6.4. *vs.* 9.1, P=0.003). The VATS group also had a decreased length of hospital stay compared to the thoracotomy group (5 *vs.* 7 days, P<0.001). There were significantly fewer perioperative pulmonary complications in the VATS group as well (15.4% *vs.* 29.8%; P=0.012) but both groups, VATS and open, had similar rates of postoperative complications. Most importantly, regarding margins, it was demonstrated that a margin: tumor size ratio >1 was associated with a decrease in recurrence (14.7%) when compared to a ratio <1 (28.9%, P=0.037). In addition, the authors performed a propensity analysis that showed no significant difference in recurrence-free or overall survival. Interestingly, there were also no significant differences in locoregional or overall survival between groups with tumors >2 cm and tumors <2 cm.

In another analysis, Leshnower and colleagues conducted a retrospective review of 17 patients who underwent VATS segmentectomy versus 26 who underwent a thoracotomy approach for patients with primary lung cancer and metastatic disease (22). The two groups were similar with regards to age, tumor size, gender, body-mass index, co-morbidities and pulmonary function. An average of 3 lymph node stations were sampled in both groups and there were no significant differences in numbers of lymph nodes sampled (VATS 4.0±3 *vs.* open 6.1±5, P=0.40). There was also no significant difference between the groups in operative time. There were 2 (4.8%) deaths within 30 days after surgery in the thoracotomy group but none in the VATS group. Furthermore, the VATS group had decreased chest tube duration (VATS 2.8±1.3 *vs.* open 5.2±3 days, P=0.001) and reduced hospital length of stay (VATS 3.5±1.4 *vs.* open 8.3±6 days, P=0.01). In addition, the authors found that average hospital costs were approximately $1,700 less for the VATS group, although this finding was not statistically significant.

Advantages of thoracoscopic segmentectomy *vs.* open segmentectomy

In summary, the above studies comparing VATS segmentectomy with open segmentectomy show that VATS segmentectomy for stage I NSCLC is feasible and safe (19-22). VATS segmentectomy appears to be associated with an equivalent survival rate when compared to the open approach: all studies report 0% 30-day mortality for the VATS group, compared to 1.7-7.7% 30-day mortality for open segmentectomy, and there is no apparent difference in long-term survival. The VATS approach was also found to be associated with shorter length of stay, lower costs, reduced rates of overall complications, including fewer cardiopulmonary complications and reduced length of chest tube duration (5). At this time, it appears that there are no significant differences in operative times between the VATS *vs.* open approach: one study has shown a longer operative time (19), and the other three have shown similar operative times (20-22).

Thoracoscopic segmentectomy *vs.* lobectomy *vs.* wedge resection

Evaluation of thoracoscopic segmentectomy *vs.* thoracoscopic lobectomy or wedge resection for NSCLC is also under current investigation. Harada and colleagues conducted an analysis of pulmonary function for patients undergoing VATS segmentectomy (n=38) or VATS lobectomy (n=45) for stage I NSCLC (23). The authors found that 50% fewer segments were resected in the segmentectomy group and that the number of resected segments was associated with reduced forced vital capacity (FVC) and FEV1 at 2-

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and 6-month postoperatively (P<0.0001). Consequently, at six months after surgery, the segmentectomy group had regained exercise capacity while the lobectomy group continued to have a 10% loss in exercise capacity.

In 2004, Iwasaki and colleagues performed a retrospective review of patients who underwent VATS lobectomy (n=100) or VATS segmentectomy (n=40) for stage I and II NSCLC (24). The authors found no significant differences in 5-year survival between the segmentectomy and lobectomy groups (77.8% *vs*. 76.7%, P=0.47). Shapiro and colleagues also conducted a retrospective study of VATS segmentectomy (n=31) *vs*. VATS lobectomy (n=113) but solely for stage I NSCLC (25). The segmentectomy group was found to have a longer smoking history and reduced pre-operative pulmonary function when compared to the lobectomy group (FEV1 83% *vs*. 92%, P=0.04). Despite differences in baseline patient fitness between the segmentectomy and lobectomy groups, there were no significant differences in complication rates, perioperative mortality, hospital length of stay, local recurrence (3.5% *vs*. 3.6%) and total recurrence rate (17% *vs*. 20%). In terms of lymph nodes dissected, segmentectomy was equivalent to lobectomy, with both groups having approximately five nodal stations sampled and ten lymph nodes resected. Mean follow-up for the segmentectomy and lobectomy groups were 21 and 22 months respectively, and both groups had similar overall and disease-free survival rates (P>0.5).

In 2010, Sugi and colleagues conducted a retrospective study of 159 patients who underwent VATS wedge resection (n=21), VATS segmentectomy (n=43) or VATS lobectomy (n=95) for stage I NSCLC (26). The lobectomy group had a higher percentage of patients with pathological stage greater than pT1N0 when compared to the segmentectomy group (18% *vs*. 8%, P=0.07). Follow-up was five years and the groups had similar 5-year recurrence-free and overall surviva, although there were differences in tumor size between the groups—the VATS wedge group had tumors <1.5 cm, the segmentectomy group had tumors <2 cm and the lobectomy group had tumors >2 and <3 cm. Yamashita and colleagues compared the results of VATS segmentectomy (n=38) or VATS lobectomy (n=71) with systemic lymphadenectomy (27). Both groups had similar recurrence-free and overall survival, although there were differences in tumor size between the segmentectomy and lobectomy groups (1.5 *vs*. 2.5 cm, P<0.0001).

Nakamura and colleagues performed a retrospective review of patients undergoing VATS lobectomy (n=289), VATS segmentectomy (n=38) or VATS wedge resection (n=84) for stage I NSCLC (28). The authors found differences in the mean tumor size between the lobectomy (2.57 cm), segmentectomy (1.98 cm) and wedge resection groups (1.85 cm). In this study, 5-year survival was lower for the wedge resection group (71.2%), compared to the lobectomy (90%) and segmentectomy (100%) groups. However, compared to the other groups, the wedge resection group comprised sicker patients with more co-morbidities.

Yamashita and colleagues evaluated the results of patients undergoing VATS segmentectomy (n=90) or VATS lobectomy (n=124) for stage IA NSCLC (29). There was a higher percentage of T1a tumors in the segmentectomy group when compared with the lobectomy group (84% *vs*. 58%, P<0.001). The segmentectomy group had a smaller median tumor size (15 *vs*. 20 mm). However, both groups were similar with regards to operative time, intraoperative blood loss, chest tube duration, and hospital stay. There were fewer numbers of dissected lymph nodes in the segmentectomy group when compared to the lobectomy group (12.1 *vs*. 21, P<0.0001) but both groups were also similar with regards to morbidity, 30-day mortality, recurrence, disease-free and overall survival.

Zhong and colleagues conducted a retrospective review of patients undergoing VATS segmentectomy (n=81) or VATS lobectomy (n=120) for stage IA NSCLC (30). There were no significant differences between the groups in pre-operative co-morbidities, pulmonary function, tumor size or histology. Both groups had similar operative times, similar rates of postoperative complications and no perioperative deaths. There were no differences between VATS segmentectomy and lobectomy with regards to lymph nodes resected (11.2±6.5 *vs*. 14.5±8.1, P=0.18). Length of hospital stay was also similar between both groups. There were no significant differences in local recurrence rates and 5-year overall or disease-free survivals. Multivariate Cox regression analyses also showed that tumor size was the only independent prognostic factor for disease-free survival. Another study compared the results of 73 VATS trisegmentectomies for stage IA (n=45) and IB (n=11) lung cancer with 266 VATS left upper lobe lobectomies for stage IA (n=105) and IB (n=73) lung cancer (31). There were no significant differences in overall complication rates or survival between patients undergoing VATS trisegmentectomy and those undergoing lobectomy for either stage IA lung cancer or stage IB lung cancer.

A retrospective review of patients undergoing VATS segmentectomy (n=26) or VATS lobectomy (n=28) for stage

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IA NSCLC was also conducted by Zhang and colleagues (32). Again, there were no significant differences in operative time, estimated blood loss, number of lymph nodes resected and postoperative complications. Both groups had similar local recurrence rates and 3-year survival. Of note, the authors did find a significantly decreased length of hospital stay in the VATS segmentectomy group by approximately three days (P=0.03). Postoperative FEV1 was also decreased to a lesser degree in the VATS segmentectomy group. Tumor size, however, was not reported in this study.

Zhao and colleagues compared the results of patients undergoing VATS segmentectomy (n=36) or VATS lobectomy (n=138) for stage I NSCLC (33). There were no significant differences in blood loss, operative time, chest tube duration and length of hospital stay between the two groups. There was also no significant difference in local recurrence and in recurrence-free survival between the two groups, although the study was limited by a relatively short follow-up of less than one year and by not including tumor size data.

Advantage of thoracoscopic segmentectomy over thoracoscopic lobectomy and wedge resection?

These studies demonstrate that although thoracoscopic segmentectomy is a more complex procedure than the thoracoscopic lobectomy (5), the rates of morbidity, recurrence and survival are similar among patients with tumors >2 cm in diameter. Specifically, there were no significant differences in overall complication rates (25,26,29,30,32,33), local recurrence rates (25,26,29,30,32,33), 5-year recurrence-free survival (26,27,29,30) and 5-year survival rates (24,26,27,29,30). The studies also show no difference in operative time between the two groups (29,30,32,33). In addition, the segmentectomy groups had similar (25,29,30,33), or reduced lengths of hospital stay (32) when compared to the lobectomy groups. It appears that thoracoscopic segmentectomy is able to preserve more lung function (23,32) and exercise capacity (23) than thoracoscopic lobectomy, although long-term follow-up data is needed.

There are, however, important limitations to the abovementioned studies. Firstly, some studies did not report the tumor size data (31-33). Of the studies that did, most found that the lobectomy groups had significantly larger tumors than the segmentectomy groups (23-29). This difference in tumor size limits interpretation of results because tumor size is known to be a prognostic factor of survival for NSCLC (30,34). However, in one recent study where both thoracoscopic segmentectomy and lobectomy groups were well-matched in tumor size, histology, preoperative co-morbidities and pulmonary function (30), both groups had similar local recurrence rates, disease-free and overall survival. This is consistent with previous data from the open segmentectomy literature. For example, in 2006, Okada and colleagues conducted a multi-center study of 567 patients with tumor size <2 cm who underwent open segmentectomy or lobectomy (35). Mean tumor size for the segmentectomy and lobectomy groups were 1.57 cm and 1.62 cm (P=0.056), respectively. The segmentectomy was associated with equivalent 5-year survival when compared to the lobectomy (83.4% *vs.* 85.9%, respectively).

Another limitation of the above-referenced studies is that many of them, with the exception of four studies (27,29,30,33), did not report the percentage of patients with bronchoalveolar carcinoma or adenocarcinoma *in situ*. This is an important variable to account for (5), as demonstrated by a study performed by Nakayama and colleagues that examined the results of 63 patients with adenocarcinoma who underwent open sublobar resection of clinical stage IA NSCLC (36). The authors classified the patients' tumors as either "air-containing type" (n=46) or "solid-density type" (n=17) according to the tumor shadow disappearance rate on high-resolution CT. After resection, 38 of the 46 air-containing tumors were identified as bronchoalveolar carcinomas whereas all solid-density type tumors were non-bronchoalveolar carcinomas. Air-containing tumors were associated with better overall 5-year survival than solid-density tumors (95% *vs.* 69%, P<0.0001).

The VATS wedge resection procedure yields a smaller parenchymal margin, reduced number of resected lymph nodes and reduced sampling of nodal stations when compared to segmentectomy (14). There have also been two studies comparing the survival outcomes of this procedure with that of the VATS segmentectomy and lobectomy. However, in the wedge resection group, the tumors were smaller (26,28) or the patient population had greater co-morbidities, which limits interpretation of results (28); further studies with groups that are better matched will be needed prior to making any conclusions regarding the role of VATS wedge resection role in NSCLC.

Further study is also needed regarding selection criteria for the thoracoscopic segmentectomy. Based on the reviewed evidence, it appears reasonable to consider segmentectomy for patients with small, peripheral tumors (in particular air-containing tumors with ground glass opacities suggesting bronchoalveolar histology) that are

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less than 2 cm in diameter when an acceptable segmental margin is obtainable (margin ≥ tumor diameter), especially in patients with advanced age, poor performance status, or poor cardiopulmonary reserve. Future retrospective studies would benefit from controlling for tumor size, operative co-morbidities, type of cancer, tumor location (including distance from the margin to the edge of the tumor and resection margin) and propensity score matching. There are two ongoing randomized trials (discussed below) that will clarify the role of the thoracoscopic segmentectomy in lung cancer.

Feasibility of mediastinal lymph node dissection (MLND)

Mediastinal lymph node assessment is a critical component of segmentectomy for NSCLC. Mattioli and colleagues reported that open segmentectomy procures an adequate number of N1 and N2 nodes for pathologic examination (37). When comparing the thoracoscopic segmentectomy to the thoracoscopic lobectomy, two studies preliminarily demonstrate no significant differences in lymph nodes harvested or nodal stations sampled (25,30) while one reported fewer lymph nodes harvested with the segmentectomy (29). When comparing open *vs.* thoracoscopic segmentectomy, one study found no difference in lymph nodes harvested (22), while another reported fewer lymph nodes harvested with the VATS approach (21).

In addition, two studies compared the completeness of lymph node evaluation during anatomic resection of primary lung cancer by open and VATS approaches (38,39). Most of the analyses performed in these studies grouped segmentectomies together with lobectomies, thereby limiting the ability to draw any conclusions specifically regarding segmentectomy. However, in one of the studies which reported analyses of nodal upstaging from the Society of Thoracic Surgery national database, the authors did report one subset analysis that showed off the 170 VATS segmentectomies analyzed, upstaging from cN0 to pN1 was seen in 4% of patients compared with 5.3% among 280 open segmentectomies (38). The authors noted that the differences in upstaging between VATS and open approaches may have been the result of approach bias, and that equivalent nodal staging may be possible with increasing experience with VATS (38).

Preliminarily, based on the available evidence, it appears that it is possible to achieve adequate lymph node dissection with segmentectomy, but that surgeon experience does play an important role, particularly in the case of the thoracoscopic segmentectomy. More detailed investigation on lymph node evaluation in VATS versus open segmentectomy and VATS segmentectomy *vs.* VATS lobectomy is therefore needed.

Other types of thoracoscopic segmentectomy

Totally thoracoscopic segmentectomy

There have been a few small case series reported on the "totally thoracoscopic" or "complete VATS" technique for segmentectomy (39-46). In this technique, there is no access incision, and the specimen is retrieved through one of the port sites that is enlarged at the end of the procedure; only video-display and endoscopic instrumentation are used (47). There is no evidence that there are advantages associated with this approach, although it does allow the surgeon to use carbon dioxide insufflation. The largest series reported is from Gossot and colleagues, who performed totally thoracoscopic anatomic segmentectomy on 117 patients (48). The authors reported five conversions to thoracotomy with mean operative time of 181±52 minutes, mean intraoperative blood loss of 77±81 mL, and postoperative complication rate of 11.7%. The mediastinal lymph node harvested and nodal stations sampled were 21±7 and 3.5±1. The average length of hospital stay was 5.5±2.2 days. Preliminarily, it appears that totally thoracoscopic segmentectomy is feasible and safe, although further studies with longer follow-up that compare this technique with traditional open and VATS approaches are needed.

Uniportal segmentectomy

VATS segmentectomies are typically performed via two to three incisions, but Gonzalez-rivas and colleagues presented the first case report demonstrating that the procedure is feasible with one incision and through one port (49). Subsequently, they reported their initial results for 17 uniportal VATS anatomic segmentectomies. Mean operative time was 94.5±35 minutes, 4.1±1 nodal stations were sampled and 9.6±1.8 lymph nodes were resected. There were no conversions. Median tumor size was 2.3±1 cm, chest tube duration was 1.5 days (range, 1-4 days) and the median length of stay was 2 days (range, 1-6 days) (50). Wang and colleagues also demonstrated their experience, performing thoracoscopic lobectomy (n=14) and segmentectomy (n=5) with radical MLND through a single small (3- to 5-cm)

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incision (51). Mean operative time was 156±46 minutes, median number of lymph nodes harvested was 22.9±9.8, and blood loss was 38.4±25.9 mL. There were no conversions and 30-day mortality was 0%. The authors did not assess for differences by type of operation and there was no long-term follow-up. Preliminarily, it appears that single-incision segmentectomy is feasible and safe, although further studies comparing single-port to traditional open and VATS approaches are needed.

Robotic segmentectomy

A recent review of a national database demonstrated that robotic pulmonary resections have increased from 0.2% in 2008 to 3.4% in 2010 (52). The vast majority of robotic procedures are lobectomies, but there has been a small increase in robotic segmentectomies performed as well.

A retrospective study of 35 patients who underwent robotic thoracoscopic segmentectomy was performed, including 12 patients who had stage IA NSCLC (53). In this series, median age was 66.5 years, tumor size was 1.4 cm, operative time was 146 minutes and number of lymph node stations sampled was 5 (54). Four patients had perioperative complications, and 60-day mortality was 0%, while length of hospital stay was two days. Pardolesi and colleagues reported the initial results of 17 patients who underwent robotic segmentectomy at three institutions (55). The authors used a 3- or 4-incision strategy with a 3-cm utility incision in the anterior fourth or fifth intercostal space. Mean age was 68.2 years and mean duration of surgery was 189 minutes. There were no major intraoperative complications and no conversions were needed. Postoperative morbidity rate was 17.6%, median postoperative stay was five days and postoperative mortality was 0%.

Based on these reports, robotic segmentectomy appears to be a safe and feasible operation although additional studies comparing the outcomes of the robotic segmentectomy with the open and VATS approaches, as well as with the lobectomy, will be needed.

Limitations

There were several key limitations to the studies discussed above. Firstly, because the studies were retrospective in nature, there was the potential for surgeons' bias to affect the type of operation a patient received, which could have affected outcomes. In addition, often, the studies did not compare groups that were well-matched—which could have affected results. For example, in studies where patients in the VATS segmentectomy group were sicker than those in the comparison group (9-11,21,25), the benefits of VATS segmentectomy could have been underestimated. In studies where the VATS group had slightly smaller tumors than those in the comparison group (21,24,26-29), there may have been an overestimation of the benefits of VATS segmentectomy.

To reduce the impact of treatment-selection bias and confounding in estimating the effects of segmentectomy *vs.* lobectomy, randomized controlled trials should continually be performed (described below). Future retrospective studies should also aim to match variables that have confounding effects, use stratification or multivariate regression analysis where appropriate, and incorporate propensity score matching when possible (56,57).

Future research

In the studies reviewed above, there was no data reported on the tolerance of patients for resection of secondary cancers. This would be an important area for future research because up to 11.5% of patients who undergo pulmonary resection for stage I NSCLC develop additional primary lung cancers (25,58). By causing less trauma than open segmentectomy, and preserving more lung function than lobectomy, VATS segmentectomy theoretically would offer patients higher tolerance for resection of secondary cancers when compared to the open segmentectomy or open or VATS lobectomy (5).

In addition, future studies should aim to include data on the number and type of nodal stations sampled or lymph nodes dissected. Only four of the studies in this review (22,25,29,30) reported specific information on lymph node sampling with segmentectomy. The effect of surgeon experience on outcomes in segmentectomy also deserves attention, as there is currently no published data on the topic.

There are two ongoing large-scale randomized controlled trials that will improve our understanding of the outcomes of limited resection for NSCLC: CALGB 140503 and JCOG0802/WJOG4607L (59,60). CALGB 140503, sponsored by the Alliance for Clinical Trials in Oncology, will evaluate the outcomes of patients who are randomly assigned to undergo limited resection (segmentectomy or wedge resection) or lobectomy, with the VATS or thoracotomy approach determined by the surgeon (60). JCOG0802/WJOG4607L, sponsored by the Japan Clinical Oncology Group and the West Japan Oncology Group, will evaluate outcomes of patients who are randomly assigned

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to undergo segmentectomy (wedge resections are excluded) or lobectomy (59). Both studies will clarify the role of segmentectomy for NSCLC but will have some limitations as well. CALGB 140503 may be limited in its final analysis because the limited resection group includes not only patients undergoing segmentectomy, but also patients undergoing wedge resection. And in both CALGB 140503 and JCOG0802/WJOG4607L, the operative approach—VATS *vs*. open—will not be a primary outcome variable.

Conclusions

Based on the reviewed evidence, it appears reasonable to consider segmentectomy for patients with stage I NSCLC tumors (particularly in air-containing tumors with ground glass opacities) that are <2 cm in diameter when an acceptable segmental margin is obtainable (at least 2 cm), especially in patients with advanced age, poor performance status, or poor cardiopulmonary reserve. The outcomes of CALGB 140503 and JCOG0802/WJOG4607L and additional well-designed studies on open, thoracoscopic, and robotic segmentectomy will be important for further clarifying the role of segmentectomy for NSCLC.

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Segmentectomy versus lobectomy for clinical stage IA lung adenocarcinoma

Morihito Okada[1], Takahiro Mimae[1], Yasuhiro Tsutani[1], Haruhiko Nakayama[2], Sakae Okumura[3], Masahiro Yoshimura[4], Yoshihiro Miyata[1]

[1]Department of Surgical Oncology, Hiroshima University, Hiroshima, Japan; [2]Department of Thoracic Surgery, Kanagawa Cancer Center, Yokohama, Japan; [3]Department of Thoracic Surgery, Cancer Institute Hospital, Tokyo, Japan; [4]Department of Thoracic Surgery, Hyogo Cancer Center, Akashi, Japan

Correspondence to: Morihito Okada, MD, PhD. Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3-Kasumi, Minami-ku, Hiroshima City, Hiroshima 734-0037, Japan. Email: morihito@hiroshima-u.ac.jp.

Background: Despite the increasing prevalence of the early discovery of small-sized non-small cell lung cancers (NSCLCs), particularly adenocarcinoma, sublobar resection has not yet gained acceptance for patients who can tolerate lobectomy.

Methods: We compared the outcomes of segmentectomy (n=155) and lobectomy (n=479) in 634 consecutive patients with clinical stage IA lung adenocarcinoma and in propensity score-matched pairs. Those who had undergone wedge resection were excluded.

Results: The 30-day postoperative mortality rate in this population was zero. Patients with large or right-sided tumors, high maximum standardized uptake value (SUVmax), pathologically invasive tumors (with lymphatic, vascular, or pleural invasion), and lymph node metastasis underwent lobectomy significantly more often. Three-year recurrence-free survival (RFS) was significantly higher after segmentectomy compared to lobectomy (92.7% *vs*. 86.9%, P=0.0394), whereas three-year overall survival (OS) did not significantly differ (95.7% *vs*. 94.1%, P=0.162). Multivariate analyses of RFS and OS revealed age and SUVmax as significant independent prognostic factors, whereas gender, tumor size and procedure (segmentectomy *vs*. lobectomy) were not. In 100 propensity score-matched pairs with variables adjusted for age, gender, tumor size, SUVmax, tumor location, the three-year RFS (90.2% *vs*. 91.5%) and OS (94.8% *vs*. 93.3%) after segmentectomy and lobectomy respectively were comparable.

Conclusions: Segmentectomy with reference to SUVmax should be considered as an alternative for clinical stage IA adenocarcinoma, even for low-risk patients.

Keywords: Adenocarcinoma; segmentectomy; sublobar resection; lung cancer; lobectomy

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Introduction

Sublobar resection for intentionally treating patients with small non-small cell lung cancer (NSCLC) who are able to withstand lobectomy has remained highly controversial, although lobectomy is considered a standard procedure even for sub-centimeter lung cancers. The Lung Cancer Study Group (LCSG) revealed a three-fold increase in local recurrence rates and poorer survival in patients who had undergone sublobar resection rather than lobectomy in a singular randomized phase III study published in 1995 (1). The dogma that lobectomy is the standard of care for stage I NSCLC has been upheld until recently. However, several current investigations have found equivalent outcomes of sublobar resection and lobectomy when NSCLC are ≤2 cm (2-7).

Sublobar resection consists of segmentectomy and wedge resection, which are quite different from each other as

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curative surgery for lung cancer, since segmentectomy is more likely to provide sufficient margins and allows access to subsegmental and hilar lymph nodes. The present study retrospectively compared the outcomes of segmentectomy, not wedge resection and lobectomy among patients with clinical stage IA lung adenocarcinoma, and adjusted for clinical factors to minimize selection bias of patients. This analysis is an extended and updated version of our previous investigation (8).

Patients and methods

We analyzed data from 634 patients who had undergone lobectomy and segmentectomy for clinical T1N0M0 stage IA lung adenocarcinoma since October 2005. All patients were assessed using high-resolution computed tomography (HRCT) and F-18-fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT). Patients with incompletely resected (R1 or R2) or multiple tumors were excluded from the prospectively maintained database that was analyzed herein. All patients were staged according to the TNM Classification of Malignant Tumors, 7th edition (9). Platinum-based chemotherapy was administered to patients with pathological lymph node metastasis after surgery. The institutional review boards of the participating institutions approved the study and the requirement for informed consent from individual patients was waived because the study was a retrospective review of a database. Chest images were acquired by multi-detector HRCT independently of subsequent FDG-PET/CT examinations. Tumor sizes and maximum standardized uptake values (SUVmax) were determined by radiologists at each institution. Because of the heterogeneity of PET techniques and performance, we corrected inter-institutional errors in SUVmax resulting from PET/CT scanners of variable quality based on outcomes of a study using an anthropomorphic body phantom (NEMA NU2-2001, Data Spectrum Corp, Hillsborough, NC, USA) that conformed to National Electrical Manufacturers Association standards (10). A calibration factor was analyzed by dividing the actual SUV by the gauged mean SUV in the phantom background to decrease inter-institutional SUV inconsistencies. Postoperative follow-up of all patients from the day of surgery included physical examinations and chest X-rays every three months, as well as chest and abdominal CT and brain MRI assessments every six months for the first two years. Thereafter, the patients were assessed by physical examinations and chest X-rays every six months, and annual CT and MRI imaging.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences software version 10.5 (SPSS Inc., Chicago, IL, USA). Continuous variables were compared using t-tests and Mann-Whitney U tests in all cohorts and Wilcoxon tests for propensity-matched pairs. Frequencies of categorical variables were compared using the χ^2 test and propensity-matched pairs were analyzed using McNemar tests. Propensity score matching was applied to balance the assignments of the included patients and to correct for the operative procedures (lobectomy or segmentectomy) that confounded survival calculations. The variables of age, sex, tumor size, SUVmax, side and lobe were multiplied by a coefficient that was calculated from logistic regression analysis, and the sum of these values was taken as the propensity score for each patient. Lobectomy and segmentectomy pairs with equivalent propensity scores were selected by a 1-to-1 match.

We defined recurrence-free survival (RFS) as the time from the day of surgery until the first event (relapse or death from any cause) or last follow-up, and overall survival (OS) as the time from the day of surgery until death from any cause or the last follow-up. The durations of RFS and OS were analyzed using the Kaplan-Meier method, and differences in RFS and OS were assessed using the log-rank test. Both RFS and OS were assessed by multivariate analysis using the Cox proportional hazards model.

Results

Of the 634 patients analyzed in this study, 479 and 155 underwent lobectomy and segmentectomy, respectively (*Table 1*). Patients with large tumors, right-sided tumors, pathologically invasive tumors, (presence of lymphatic, vascular, or pleural invasion), high SUVmax, and lymph node involvement were significantly more often treated by lobectomy. However, age and gender did not differ significantly between the two procedures. *Table 2* shows the segments that were removed during segmentectomy.

None of the patients died within 30 days of surgery, and tumors recurred in 54 patients at a median postoperative follow-up period of 34.2 months. Twenty recurrences were local only and 34 were distant (with or without local recurrence). Local recurrence occurred in 17 patients after

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Table 1 Patient characteristics

Variables	Lobectomy (n=479)	Segmentectomy (n=155)	P value
Age	66 [30-89]	66 [31-89]	0.37
Gender			
Male	223 (46.6%)	74 (48.1%)	0.78
Tumor size (cm)	2.2 (0.7-3.0)	1.5 (0.6-3.0)	<0.001
SUVmax†	2.1 (0-16.9)	1.1 (0-9.8)	<0.001
Side			
Right	325 (67.8%)	81 (52.3%)	<0.001
Lobe			<0.001
Upper	254 (53.0%)	82 (52.9%)	
Middle	48 (10.0%)	0 (0%)	
Lower	177 (37.0%)	73 (47.1%)	
Lymphatic invasion	97 (20.3%)	10 (6.5%)	<0.001
Vascular invasion	111 (23.3%)	10 (6.5%)	<0.001
Pleural invasion	66 (13.9%)	8 (5.2%)	0.0024
Lymph node metastasis	50 (10.6%)	3 (1.9%)	<0.001

†, maximum standardized uptake value.

lobectomy (hilar lymph node, n=1; mediastinal lymph node, n=11; pleura, n=2; hilar and mediastinal lymph nodes, n=1; bronchial stump and mediastinal lymph node, n=1; mediastinal lymph node and pleura, n=1) and in three patients after segmentectomy (bronchial stump, n=1; pleura, n=1; residual lung and mediastinal lymph node, n=1).

The 3-year OS rates between patients who underwent lobectomy and segmentectomy were similar (94.1% *vs.* 95.7%, P=0.162), whereas three-year RFS rates significantly differed (86.9% *vs.* 92.7%, P=0.0394; *Figure 1*). *Table 3* shows that the multivariate analyses of RFS and OS selected age and SUVmax as significant independent prognostic factors, but not sex, tumor size, or procedure (lobectomy *vs.* segmentectomy).

Propensity score-matching based on clinical variables of age, gender, tumor size, SUVmax, side and lobe, allowed good matches of 100 lobectomy and segmentectomy pairs in terms of clinical and consequently pathological factors, except for more advanced age and higher SUVmax in the segmentectomy group (*Table 4*). Patients who underwent middle lobectomy were excluded from matching for a fair comparison, since tumors located in a middle lobe were never treated by segmentectomy. *Figure 1* shows that the three-year RFS and OS did not significantly differ between

Table 2 Details of segmentectomy (n=155)

Site	Number
Right (n=81)	
S1	11
S1+2	1
S2	13
S3	7
S6	31
S7	3
S8	8
S9	1
S10	1
S7+8	1
S8+9	2
S9+10	1
S7+8+9+10	1
Left (n=74)	
S1+2	17
S3	9
S1+2+3	10
S1+2+3c	1
S4	5
S5	1
S4+5	7
S6	15
S8	2
S9	5
S10	1
S8+9+10	1

propensity score-matched patients after lobectomy or segmentectomy (91.5% *vs.* 90.2% and 93.3% *vs.* 94.8%, respectively).

Discussion

The RFS and OS curves of patients with clinical stage IA lung adenocarcinoma seemed better after segmentectomy than lobectomy, although the clinical and pathological backgrounds significantly differed and would obviously affect their survival (11-16). Multivariate analyses of the clinical background for RFS and OS demonstrated that procedure (lobectomy *vs.* segmentectomy) was not a significant prognostic factor. The clinical features or

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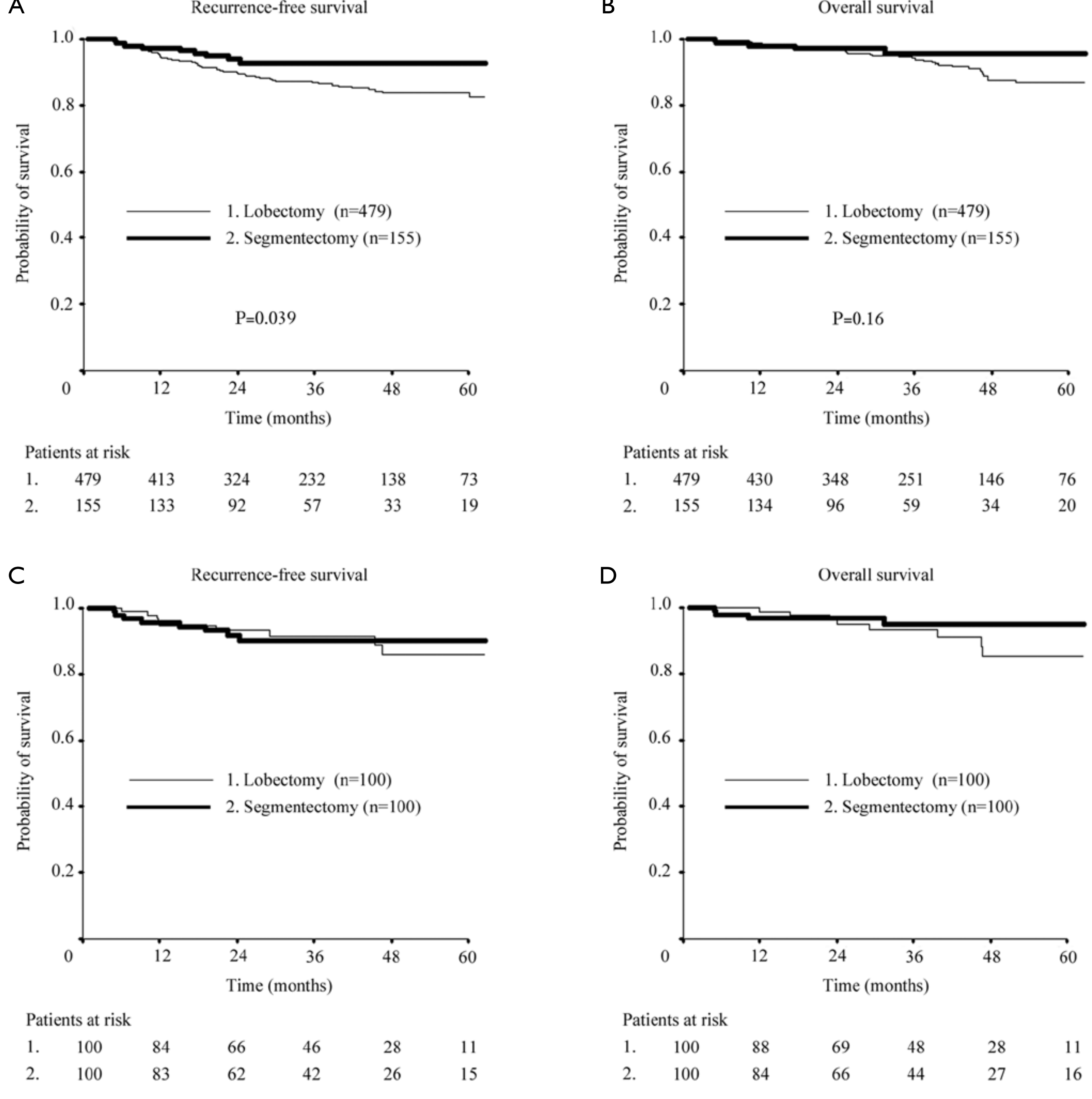

Figure 1 Recurrence-free (RFS) and overall survival (OS) curves of patients after lobectomy and segmentectomy. Three-year RFS (A) and OS (B) after lobectomy and segmentectomy were 86.9% *vs.* 92.7% (P=0.0394) and 94.1% *vs.* 95.7% (P=0.162), respectively, in all cohorts. Three-year RFS (C) and OS (D) in propensity score-matched patients after lobectomy and segmentectomy were 91.5% *vs.* 90.2% and 93.3% *vs.* 94.8%, respectively.

pathological factors of lymphatic, vascular or pleural invasion, or lymph node metastasis were similar in propensity score-matching analyses that matched for potentially confounding variables of age, sex, tumor size, SUVmax, tumor location to minimize selection bias. Only age and SUVmax significantly differed. The three-year RFS and OS rates after segmentectomy and lobectomy group were similar in the matched model, although the former

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Table 3 Multivariate analyses for RFS and OS

Variables	HR (95% CI)	P value
Multivariate analysis for RFS†		
Age	1.04 (1.01-1.07)	0.011
Gender		
Male *vs*. female	1.20 (0.74-1.93)	0.46
Tumor size (cm)	1.36 (0.86-2.14)	0.19
SUVmax‡	1.17 (1.09-1.25)	<0.001
Procedure		
Lobectomy *vs*. segmentectomy	0.72 (0.34-1.52)	0.39
Multivariate analysis for OS#		
Age	1.05 (1.01-1.09)	0.0082
Gender		
Male *vs*. female	1.10 (0.49-1.70)	0.78
Tumor size (cm)	1.23 (0.67-2.26)	0.50
SUVmax‡	1.13 (1.04-1.24)	0.0068
Procedure		
Lobectomy *vs*. segmentectomy	0.68 (0.25-1.82)	0.44

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. †, recurrence-free survival; ‡, maximum standardized uptake value; #, overall survival.

Table 4 Propensity score-matched comparison of clinical and pathologic factors between patients who underwent lobectomy and segmentectomy

Variables	Lobectomy (n=100)	Segmentectomy (n=100)	P value
Clinical factors			
Age	63 [33-82]	66 [32-89]	0.030
Gender			
Male	46 (46%)	50 (50%)	0.67
Tumor size (cm)	1.6 (0.7-3.0)	1.6 (0.6-3.0)	0.28
SUVmax†	1.2 (0-8.7)	1.2 (0-9.8)	0.047
Side			0.27
Right	62 (62%)	53 (53%)	
Lobe			0.10
Upper	62 (62%)	50 (50%)	
Lower	38 (38%)	50 (50%)	
Pathologic factors			
Lymphatic invasion	11 (11%)	7 (7%)	0.45
Vascular invasion	9 (9%)	9 (9%)	1.0
Pleural invasion	10 (10%)	7 (7%)	0.61
Lymph node metastasis	7 (7%)	3 (3%)	0.34

†, maximum standardized uptake value.

were significantly older and had a higher SUVmax. These data suggest that segmentectomy could be an alternative strategy for treating clinical stage IA lung adenocarcinoma when HRCT and FDG-PET/CT findings are taken into consideration.

This investigation has several limitations and the results should be interpreted with care. Information in the database analyzed herein included surgical procedures; however, further details such as indications for segmentectomy—that is, whether or not patients who were treated with segmentectomy could have tolerated lobectomy—are difficult to obtain. In addition, patients who underwent segmentectomy tended to have less invasive, smaller tumors, with small tumor size or low SUVmax, and thus a lower frequency of pathologically invasive factors such as lymphatic, vascular, pleural or nodal involvement. Therefore, we used propensity score-matched analysis to adjust the patients' backgrounds as much as possible. However, we could not compare the surgical outcomes of patients with a relatively low SUVmax, implying that patients with a high SUVmax require close scrutiny. The database also did not include information about lung function. The key advantage of segmentectomy is the preservation of lung function, and several studies have shown that segmentectomy has functional advantages over lobectomy (5,17,18).

The target tumors of most previous studies that compared the outcomes of segmentectomy and lobectomy were T1 N0 M0 NSCLC of ≤2 cm (4-6). However, the present study included patients with clinical T1b tumors of 2 to 3 cm. Patients with T1b lung adenocarcinomas with a sufficient surgical margin could be candidates for sublobar resection if selected based on HRCT and FDG-PET/CT findings (12).

The ongoing, multicenter phase III clinical trials of propriety of radical segmentectomy in the United States (CALGB-140503) and Japan (JCOG0802/WJOG4607L) should be carefully monitored. The primary end-point of the Japanese study is OS (disease-free survival in the US study), and wedge resection is not permitted as a sublobar resection, as it differs from radical segmentectomy. The Japanese study (19) aims to compare the surgical outcomes

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of lobectomy and segmentectomy for T1 N0 M0 NSCLC measuring ≤2 cm, excluding radiologically less-invasive tumors such as ground-glass opacity (GGO)-dominant tumors on HRCT (20), and thus can show the true colors of segmentectomy compared with lobectomy. Segmentectomy is more procedurally demanding than either lobectomy or wedge resection, and thus incorrect outcomes of these clinical trials due to technical errors, such as recurrence at resection lines or excessive loss of lung function, might be a concern. Surgeons must carefully avoid local failure at the margin and fully expand adjacent segments to maximize postoperative lung function.

Current understanding of radical segmentectomy can be summarized as follows. Firstly, the indication for segmentectomy should be limited to T1 tumors ≤3 cm in diameter, and HRCT and PET-CT findings must be taken into consideration, particularly for T1b tumors (21-23). Whenever nodal involvement or an insufficient margin is confirmed intraoperatively, segmentectomy should be converted to lobectomy with complete nodal dissection. Secondly, radical (intentional) and compromising indications for segmentectomy must be independently discussed. The former is for low-risk patients who can tolerate lobectomy. Thirdly, segmentectomy is more valuable than wedge resection from an oncological perspective because it allows nodal dissection at the hilum. Thus, the decision of the most suitable procedure, such as whether or not to intraoperatively convert to lobectomy, should consider precise staging and the lower rate of local recurrence resulting from sufficient surgical margins. Therefore, segmentectomy must be clearly separated from wedge resection amongst the categories of sublobar resection for lung cancer. Surgeons must become adept and master segmentectomy as a keynote procedure because small lung cancers are being detected with increasing frequency.

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Lobectomy *vs.* segmentectomy for NSCLC (T<2 cm)

Nestor Villamizar, Scott J. Swanson

Department of Thoracic Surgery, Brigham and Women's Hospital, Boston, Massachusetts, USA

Correspondence to: Nestor Villamizar, MD. Department of Thoracic Surgery, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA. Email: nvillamizarortiz@partners.org.

Abstract: The extent of surgical resection for peripheral clinical T1N0M0 non-small cell lung cancer (NSCLC) ≤2 cm continues to be a matter of debate. Eighteen years ago, a randomized controlled trial (RCT) established lobectomy as the standard of care for peripheral clinical T1N0M0 NSCLC. However, numerous publications since then have reported similar outcomes for patients treated with segmentectomy or lobectomy for peripheral clinical T1N0M0 NSCLC 2 cm or smaller in size. The majority of these publications are retrospective studies. Two ongoing RCTs aim to resolve this debate, one in Japan and the other in the United States. This manuscript is a comprehensive review of the literature that compares lobectomy to segmentectomy for peripheral clinical T1N0M0 NSCLC 2 cm or smaller in size. Until data from the ongoing RCTs become available, this literature review provides the best evidence to guide the thoracic surgeon in the management of these patients.

Keywords: Segmentectomy; lobectomy; lung cancer; 2 cm

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Lobectomy was established in 1995 as the standard of care for optimal oncologic resection of stage I non-small cell lung cancer (NSCLC), after the results of the Lung Cancer Study Group (LCSG) reported a significantly higher rate of recurrence and associated trend toward lower cancer-specific survival in patients undergoing sublobar resections (1). Since then, several investigators have challenged this dogma by demonstrating equivalent oncologic outcomes of segmentectomy and lobectomy for stage IA NSCLC. A large proportion of studies have integrated segmentectomy and wedge resection under the category of limited resection when making comparisons to lobectomy (2). However, recent publications have focused on comparisons between segmentectomy and lobectomy excluding cases of wedge resection (3-6).

Potential advantages of segmentectomy over lobectomy include preservation of lung function and reduced morbidity and disability. Preservation of lung function may be particularly important for elderly patients, those with borderline preoperative cardiopulmonary function, and patients with synchronous or metachronous cancers that would require repetitive resections over the course of their lifespan. The incidence of a second primary lung cancer may be as high as 3% per year (7); thus, patients who survive five or more years after their first resection would face a significant cumulative risk of second cancers. On the other hand, lobectomy may provide a lower recurrence rate that could translate into longer disease free survival, particularly in young patients who are good surgical candidates.

The main objective of this manuscript is to review the literature that compares lobectomy versus segmentectomy for NSCLC less than 2 cm in size. The data provided here is intended to help in the decision-making process about which of these two surgical approaches should be used based on tumor and patient characteristics.

Lung Cancer Study Group (LCSG) trial

This randomized controlled trial (RCT) enrolled patients from February 1982 through November 1988 and compared open lobectomy to sublobar resection for patients with lung cancer ≤3 cm with absence of lymph node involvement (1). There were 247 patients eligible for analysis: 122 received a limited resection and 125 underwent lobectomy. Of the 122

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patients who underwent a limited resection, 40 (32.8%) had a wedge resection and 82 (67.2%) had a segmentectomy. There were no significant differences for all stratification variables, selected prognostic factors, perioperative morbidity, mortality, or late pulmonary function. The rate of local recurrence in the limited resection group was 6.3%, which was significantly higher than the 2.1% observed in the lobectomy group (P=0.008), and the 5-year survival rate in the limited resection group was 83.1%, which was slightly poorer than the 89.1% observed in the lobectomy group. In addition, postoperative pulmonary function was not significantly different in the two groups, even at one year after surgery. The authors concluded that, compared with lobectomy, limited pulmonary resection does not confer improved perioperative morbidity, mortality, or late postoperative pulmonary function. Furthermore, due to higher death rates and locoregional recurrence rates associated with limited resection, lobectomy must be considered the surgical procedure of choice for patients with peripheral T1N0 NSCLC.

It must be acknowledged that a considerable number of wedge resections (32.8%) were included in the limited resection group; tumor sizes ranging from 2 to 3 cm were included in the analysis; and routine computed tomographic examination of the lung was not required either preoperatively or for postoperative surveillance. Several publications have demonstrated a lower rate of loco-regional recurrence after segmentectomy compared to wedge resection for stage IA NSCLC (8-10). An adequate body of literature has also demonstrated that T1b tumors (2-3 cm) have lower survival rates than T1a tumors (≤2 cm) (11,12). Moreover, advances in imaging and optimal pre-resection surgical mediastinal staging have improved staging accuracy since the LCSG trial was published (13). This trial was done in an earlier era when tumors were often more central, many were squamous cell cancers, and they were larger stage I tumors (14).

Extended segmentectomy for stage I lung cancer

Since the results of the LCSG were published, several Japanese investigators have studied the role of sublobar resection for stage I NSCLC. The Study Group of Extended Segmentectomy for Small Lung Tumors was created and their final report was published in 2002 (15). This prospective multicenter study enrolled 55 patients with peripheral clinical T1N0M0 (cT1N0M0) NSCLC (≤2 cm) from January 1992 to December 1994. All patients were in physical conditions to tolerate a lobectomy. Extended segmentectomy involves the development of the intersegmental plane, by keeping inflated the segment to be resected after ligation of the segmental bronchus, while the adjacent segments are collapsed. The resection is then performed on the side of the collapsed segments in order to optimize lateral margins, and a complete lymph node dissection including segmental, hilar and mediastinal lymph nodes is undertaken, as is performed during lobectomy (16). The patients were followed up at 1- or 3-month intervals for five years or more. The 5-year disease-free survival (DFS) rate was 91.8%. Postoperative loss of lung function was 11.3% in forced vital capacity (FVC) and 13.4% in forced expiratory volume in one second (FEV1). The authors concluded that extended segmentectomy is viable as a standard operation for patients with small peripheral lung tumors, and causes minimal loss of lung function.

More recently, Nomori *et al.* (17) also examined the outcomes of 179 patients who underwent intentional open radical segmentectomy with systematic lymph node dissection for peripheral cT1N0M0 NSCLC between 2005 and 2009 at a single institution. All analyzed patients had intraoperative frozen section to demonstrate surgical margins of at least 2 cm. Of these 179 patients, 134 (75%) had tumors ≤2 cm, and 45 (25%) had tumors 2.1 to 3 cm. The 5-year DFS was 95% for patients with tumors ≤2 cm and 79% for those who had tumors 2.1 to 3 cm. Postoperative pulmonary function (measured at least six months after surgery) was preserved at 90%±12% of preoperative levels.

The importance of lymph node dissection during segmentectomy has been demonstrated. The frequency of lymph node metastasis in patient with cT1N0M0 NSCLC is approximately 10% (18). A theoretical disadvantage of segmentectomy versus lobectomy is the potential presence of metastatic disease in level 13 lymph nodes in the preserved adjacent segments. Nomori *et al.* (19) investigated the distribution of subsegmental lymph nodes in resected and preserved segments during segmentectomy. Out of 94 patients with cT1N0M0 NSCLC treated with segmentectomy, segmental nodes at both the resected and nonresected segments could be dissected in 42 of the 94 patients. The authors concluded that segmental lymph nodes should be dissected at both the resected and nonresected segments during segmentectomy, especially for tumors in the anteriorly located segment.

Another factor that appears to play an important role in recurrence after segmentectomy is the surgical margin. Schuchert and colleagues (20) performed a retrospective review of 182 consecutive patients undergoing anatomic

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segmentectomy for stage I NSCLC from 2002 to 2006. The average surgical margin for segmentectomy was 18.2 mm. There were 32 recurrences after segmentectomy (17.6%) at a mean of 14.3 months (14 locoregional, 18 distant), and 89% of recurrences were seen when tumor margins were 2 cm or less. Margin/tumor diameter ratios exceeding 1 were associated with a significant reduction in recurrence rates, compared with ratios of less than 1 (25% versus 6.2%, P=0.0014).

Segmentectomy versus lobectomy for cT1N0M0 NSCLC ≤2 cm

In order to elucidate factors associated with survival, Okumura *et al*. (12) analyzed 144 patients who underwent segmentectomy and 1,241 who underwent lobectomy. The authors concluded that a favorable outcome would be obtained by a segmentectomy in patients with a maximum diameter of the tumor smaller than 2 cm, no nodal involvement, and non-large cell carcinoma. Five- and 10-year overall survival (OS) in patients who met those criteria were both 83%, which was significantly higher than that for those who did not (41%) (P<0.0001). In comparison, 5- and 10-year OS in patients who underwent lobectomy meeting the same criteria (non-large cell carcinoma at stage IA ≤2 cm) was 81% and 64% respectively (P=0.66). There were no 5-year survivors among the six patients with large cell carcinoma who underwent a segmentectomy. In contrast, there was no difference in survival among different histologic types when a lobectomy was performed. The authors concluded that lobectomy, but not a segmentectomy, is recommended for large cell carcinomas, even when the tumor diameter is 2 cm or smaller.

In another retrospective study, Yamato and colleagues (21) reviewed 523 cases of cT1N0M0 peripheral adenocarcinomas ≤2 cm between 1991 and 2004. The surgical procedure was a lobectomy in 277 patients, segmentectomy in 153 patients and wedge resection in 93 patients. The limited resection was intentional in 140 cases, and it was performed for compromised patients in 106 cases. The 5-year survival rate of the patients who underwent a wedge resection was 70.6%, which was significantly worse than the 87.5% after a segmentectomy and the 85.5% after a lobectomy.

A multicenter nonrandomized study comparing lobectomy to sublobar resection was conducted by Okada *et al*. (22) from 1992 to 2001 for patients with a first peripheral cT1N0M0 NSCLC ≤2 cm who were able to tolerate a lobectomy. During the operation, the tumor status was confirmed to be T1N0 on the basis of frozen-section analysis of sampled segmental, lobar, hilar, and mediastinal lymph nodes. For segmentectomy, a margin of at least 2 cm of healthy lung tissue was required. It was specified that when the surgical margin was less than 2 cm or a lymph node was positive, lobectomy had to be performed instead. Of the 567 patients enrolled, 214 patients underwent curative segmentectomy, 30 underwent wedge resection and 236 had lobectomy. DFS and OS were similar in all groups. Five-year DFS was 92.2% after segmentectomy and 91.5% after lobectomy (P=0.64). Five-year OS was 93.9% after segmentectomy and 95.3% after lobectomy (P=0.43).

More recently, Carr and coworkers (11) performed a retrospective review of 429 patients undergoing resection of pathologically confirmed stage IA NSCLC via lobectomy (251 patients) or anatomic segmentectomy (178 patients) from 2002 to 2009. Video-assisted thoracoscopic surgery (VATS) was the approach utilized in 59% of segmentectomies and 39.4% of lobectomies during the study period. The margin:tumor ratio was similar whether performing an anatomic segmentectomy or lobectomy for T1a or T1b tumors. There was no difference in mortality, recurrence rates (14% segmentectomy *vs*. 14.7% lobectomy, P=1.00), or 5-year cancer-specific survival (CSS) for T1a tumors (90% *vs*. 91%, P=0.984) when comparing segmentectomy and lobectomy. The authors concluded that anatomic segmentectomy may achieve equivalent recurrence and survival compared with lobectomy for patients with stage IA NSCLC.

A criticism of the literature comparing the efficacy of segmentectomy and lobectomy since 1995 is that the majority of publications have been limited to single-institution retrospective reviews. However, more recently some investigators have used the Surveillance Epidemiology and End Results (SEER) database to compare survival after lobectomy and limited resection in patients with stage IA NSCLC. Whitson *et al*. (23) analyzed the SEER database for stage I adenocarcinoma or squamous cell carcinoma in patients 40 years and older from 1998 through 2007. The analysis included 13,892 patients who underwent lobectomy and 581 who underwent segmentectomy. Even after stratifying by tumor size, the authors found that lobectomy was associated with more favorable 5-year OS (P=0.0002) and CSS (P=0.0047) rates for tumors ≤2 cm.

Yendamuri and coworkers (13) also used the SEER database to identify surgically treated patients with stage I NSCLC ≤2 cm in size from 1988 to 2008. The cohort included 2,161 patients undergoing sublobar resection and 6,636 patients undergoing lobectomy or greater resection. They grouped these patients into three temporal cohorts:

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the first included patients from 1988 to 1997 (early), the second was from 1998 to 2004 (intermediate) and the third was from 2005 to 2008 (late). In the early group, sublobar resection was associated with worse outcome. In the intermediate group, wedge resection but not segmentectomy was associated with a worse outcome compared with lobectomy. The association between extent of resection and OS completely disappeared in the late subgroup, in which neither wedge resection nor segmentectomy had an outcome worse than did lobectomy. The authors concluded that the survival advantage offered by lobectomy over sublobar resection in NSCLC patients with tumor size ≤2 cm has incrementally decreased over the past two decades.

A recent meta-analysis (24) included 24 studies (11,360 patients) published from 1990 to 2010 to compare OS and CSS of stage I NSCLC after sublobectomy or lobectomy. In stage IA patients with tumor ≤2 cm, there were no differences in OS between lobectomy and sublobectomy (HR 0.81; 95% CI, 0.39-1.71; P=0.58). For the comparison between lobectomy and segmentectomy, there was no significant difference on OS (HR 1.09; 95% CI, 0.85-1.40; P=0.45) and CSS (HR 0.99; 95% CI, 0.72-1.38; P=0.97) in stage I NSCLC.

Several studies have specifically limited their objective to compare outcomes between lobectomy and segmentectomy for NSCLC ≤2 cm, excluding larger tumors or wedge resections. Mattioli *et al*. (25) performed a retrospective investigation to compare anatomical segmentectomy and lobectomy for peripheral cT1N0M0 NSCLC ≤2 cm on preoperative CT scan, with regard to the number/station of lymph nodes resected, as well as survival. In this case-matched study, 46 intentional segmentectomy patients were matched with 46 lobectomy patients for age, anatomical segment, and size of the tumor. All patients were able to tolerate a lobectomy as evaluated by cardiopulmonary functional tests. Starting in January 2001, the authors offered anatomical segmentectomy as an alternative to lobectomy to patients affected by a peripheral cT1aN0M0 NSCLC. The cases in which lobectomy was performed within the same time period were retrospectively retrieved from the institutional electronic medical record system database. The approach for the resection was an axillary muscle-sparing thoracotomy. Radical dissection of lymph node stations 4, 5, 6 and 7 was identical in segmentectomies and lobectomies. Node stations 10, 11, 12 and the segmental 13 were also dissected carefully during segmentectomy and in the pathology laboratory after lobectomy. The median number of total dissected lymph nodes was 12 in anatomical segmentectomy compared with 13 in lobectomy (P=0.68), with the number of N1 nodes being 6 and 7, respectively (P=0.43), and N2 nodes 5.5 and 5 (P=0.88). No perioperative mortality was observed. Complications occurred in 13% of segmentectomies and in 15% of lobectomies (P=0.76). The median follow-up was 25 months for the segmentectomy group and 32 months for the lobectomy group. Freedom from recurrence at 36 months was 100% for anatomical segmentectomy and 93.5% for lobectomy (P=0.33)

Thoracoscopic segmentectomy *vs.* lobectomy

The vast majority of the evidence described above involves open procedures. However, a few recent studies have compared the outcomes of thoracoscopic segmentectomy and thoracoscopic lobectomy for small-sized stage IA lung cancer. Shapiro *et al*. (6) analyzed patients between January 2002 and February 2008. Indications for segmentectomy were tumor smaller than 3 cm, limited pulmonary reserve, comorbidities, and peripheral tumor location. Thirty-one patients underwent a segmentectomy and 113 had a lobectomy. Patients undergoing a segmentectomy had worse mean FEV1 than those having a lobectomy (83% *vs*. 92%, P=0.04). There were no differences in mean number of nodes (10) and nodal stations (5) resected. The mean follow-up was 21 months. There were 5 (17.2%) recurrences after segmentectomy and 23 (20.4%) after lobectomy (P=0.71), with locoregional recurrences rates of 3.5% and 3.6%, respectively. OS and DFS were similar between the groups. Zhong and colleagues (26) also compared outcomes between thoracoscopic segmentectomy and thoracoscopic lobectomy. Their inclusion criterion was limited to stage IA NSCLC ≤2 cm. The study period was between March 2006 and August 2011. A total of 39 segmentectomies and 81 lobectomies were analyzed. The two groups had a similar incidence of postoperative complications. The median follow-up was 26.5 months. Local recurrence rates were similar after segmentectomy (5.1%) and lobectomy (4.9%). No significant difference was observed in 5-year OS (79.9% *vs*. 81%) or DFS (59.4% *vs*. 64.2%).

Segmentectomy for clinical T1N0M0 ≤2 cm and ≥50% ground glass opacity component (GGO-dominant)

Tumor characteristics may also play an important role in deciding the extent of surgical resection. Tsutani *et al*. (27)

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evaluated 239 patients with GGO-dominant clinical stage IA lung adenocarcinoma from four institutions between August 2005 and June 2010. All patients underwent HRCT and FDG-PET/CT followed by curative R0 resection. The inclusion criteria were absence of >1 cm enlargement in mediastinal or hilar lymph nodes and an absence of >1.5 accumulation for maximum standardized uptake values (SUVmax) in these lymph nodes. Sublobar resection was allowed for a peripheral cT1N0M0 intraoperatively assessed as N0, using frozen section evaluation of enlarged lymph nodes or by ensuring that there was no obvious enlargement of lymph nodes in the thoracic cavity. Systematic lymph node dissection was performed during segmentectomy, but not during wedge resection. Follow-up included a chest CT every six months for the first two years postoperatively, and every year thereafter. Median follow-up period after surgery was 42.2 months. Lobectomy was performed in 90 patients, segmentectomy in 56, and wedge resection in 93. A total of 155 tumors were classified as T1a and 84 as T1b. There was no significant difference in 3-year DFS among patients with GGO-dominant tumors who underwent lobectomy (96.4%), segmentectomy (96.1%), and wedge resection (98.7%; P=0.44). A multivariate Cox proportional hazards model for DFS included variables of age, gender, clinical T descriptor, solid tumor size, SUVmax, and surgical procedure. However, none of these variables were independent prognostic factors.

Pulmonary function tests

With regards to the functional advantage of a limited resection, Harada *et al*. (28) analyzed PFT preoperatively and at two and six months after radical segmentectomy in 38 patients and lobectomy in 45 patients. Both groups were able to tolerate a lobectomy and had cT1N0M0 NSCLC ≤2 cm. The anatomic segmentectomy was made through video-assisted approach with minithoracotomy. They performed segmentectomy if the patient consented to the sublobar resection, and lobectomy if the patient did not. During the postoperative course, statistically significant differences were observed between the two groups in the ratio of postoperative to preoperative FVC (P=0.0006) and FEV1 (P=0.0007), whereas a marginal difference was seen in the ratio of postoperative to preoperative anaerobic threshold (P=0.616). Keenan and colleagues (29) retrospectively analyzed patients undergoing lobectomy (n=147) or segmentectomy (n=54) for stage I NSCLC between March 1996 and June 2001. From the pathologic analysis, there were 126 stage IA and 21 stage IB patients in the lobectomy group, and 47 stage IA and 7 stage IB patients in the segmentectomy group. PFT was obtained preoperatively and at one year. At one year, lobectomy patients experienced significant declines in FVC (85.5% to 81.1%), FEV1 (75.1% to 66.7%), and diffusing capacity (79.3% to 69.6%). In contrast, a decline in diffusing capacity was the only significant change seen after segmental resection. Actuarial survival in both groups was similar (P=0.406), with a 1-year survival of 95% for lobectomy and 92% for segmentectomy. Four-year survivals were 67% and 62%, respectively. Overall, the risk of any recurrence, whether local, regional, or systemic, was identical in the two groups (20.4% segmentectomy, 19% lobectomy). The authors concluded that for patients with stage I NSCLC, segmental resection offers preservation of pulmonary function compared with lobectomy and does not compromise survival.

Ongoing prospective RCTs

The controversy about the optimal extent of surgical resection for peripheral NSCLC ≤2 cm has led to several multicenter prospective RCTs. The JCOG0802/WJOG4607L trial (30) began in August 2009 in Japan to evaluate the non-inferiority in OS of segmentectomy compared with lobectomy in patients with peripheral NSCLC ≤2 cm. A total of 1,100 will be accrued from 71 institutions within three years. The inclusion criteria include age 20-79 years old, sufficient organ function, single tumor, ≤2 cm in maximum diameter, proportion of maximum diameter to consolidation >25%, center of tumor located in the outer third of the lung field, tumor not located at middle lobe, and no lymph node metastasis. The secondary endpoints include postoperative respiratory function, relapse-free survival, and proportion of local recurrence. The distance from the dissection margin to the tumor edge must be evaluated intra-operatively. If the distance is less than 2 cm, the absence of cancer cells in the resection margin must be histologically or cytologically confirmed before finishing surgery. When lymph node metastasis is present or resection margin is not cancer-free, the surgical procedure must be converted to a lobectomy. All randomized patients will be followed for at least five years. Tumor markers, CXR and chest CT is evaluated at least every six months during the first two years and at least every 12 months for the duration of follow-up.

Similarly, the CALGB 140503 study (31) aims to determine whether DFS after sublobar resection (segmentectomy or

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wedge) is non-inferior to that after lobectomy in patients with NSCLC ≤2 cm. A total of 692 patients will be accrued to the study and randomized intra-operatively to either lobectomy or limited resection. Prior to registration, patients must have a lung nodule measuring ≤2 cm on CT scan, presumed to be lung cancer and located in the outer third of the lung. Intraoperative histological confirmation of NSCLC must be obtained (if not done preoperatively), as well as confirmation of N0 status by frozen examination of levels 4, 7, and 10 on the right side and 5 or 6, 7 and 10 on the left side, either at the time of surgery or pre-operatively by mediastinoscopy within six weeks of the definitive procedure. Patients must also have a performance status of 0-2. Exclusion criteria include prior malignancy within five years, prior chemotherapy or radiation, and age <18 years.

Conclusions

The increasing use of CT scans and improvement in CT resolution has been associated with earlier detection of NSCLC with smaller tumor size. Also, the location and type of lung cancer has evolved over time such that smaller, peripheral adenocarcinomas are now among the most common presentation. An extensive body of literature mainly composed of retrospective studies supports the use of radical anatomical segmentectomy for peripheral cT1N0M0 NSCLC ≤2 cm, certainly for older patients with limited cardiopulmonary function. However, caution should be taken to promote a widespread indication for intentional segmentectomy in young good surgical candidates until the results of the ongoing RCTs become available. When expertise exists, the surgeon should use a minimally invasive approach to realize perioperative and functional patient benefits.

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State of the art in surgery for early stage NSCLC—does the number of resected lymph nodes matter?

Laura Romero Vielva[1], Manuel Wong Jaen[1], José A. Maestre Alcácer[2], Mecedes Canela Cardona[1]

[1]Thoracic Surgery Department, Vall d'Hebron University Hospital, Barcelona, Spain; [2]Instituto Oncológico, Dr. Rosell Quirón Dexeus University Hospital, Barcelona, Spain

Correspondence to: Dr. Laura Romero Vielva. Thoracic Surgery, Vall d'Hebron University Hospital, Passeig Vall d'Hebron 119-129, 08035, Barcelona, Spain. Email: lromero@vhebron.net.

Abstract: Surgery is the treatment of choice in patients with early stage NSCLC. However, the results remain poor in these patients. Lymph node involvement is the main prognostic factor in patients with NSCLC, but there is still no clear definition of the number of nodes required to consider a lymphadenectomy as complete. Although there is no defined minimum number of lymph nodes required for a complete lymphadenectomy, there are some recommendations to perform this procedure, published by different scientific societies. Current practice in thoracic surgery regarding lymphadenectomy, differs on some points from the guidelines recommendations, with data regarding patients with no mediastinal assessment between 30-45% according to some of the published data. Different studies have probed the fact that the probability of finding a positive node increases with the number of lymph nodes analyzed. Therefore, a complete lymphadenectomy provides proper staging, which helps to identify the patient's real prognosis. Several nonrandomized studies and retrospective series have shown that survival increases in the group of patients with a higher number of lymph nodes removed. There is no contraindication to performing a complete lymphadenectomy. The increase in survival in patients with a complete lymphadenectomy may be due to more accurate staging. Therefore, complete lymphadenectomy should be mandatory even in early stage patients.

Keywords: Early stage lung cancer; number of lymph nodes; lymphadenectomy; survival

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Introduction

Surgery is the treatment of choice in patients with early stage NSCLC. However, results remain poor in these patients, with 5-year survival rates ranging from 84% in stage I to 39% in stage IIIA, according to different studies (1).

The heterogeneity of patients in different stages and errors in staging may be related to these differences in survival.

Lymph node involvement is the main prognostic factor in patients with NSCLC, but there is still no clear definition of the number of nodes required to consider a lymphadenectomy as complete.

The objective of this article is to review the published literature presented at the 10th Congress on Lung Cancer of the Spanish Lung Cancer Group, in Barcelona, November 2013.

Lymph node involvement

Mediastinal lymph node involvement is the main prognostic factor in patients with NSCLC, and therefore one of the goals of surgical treatment is to diagnose such involvement and establish accurate staging to provide the patient with proper treatment. One of the aims of lymph node dissection, both hilar and mediastinal, is to achieve an intraoperative staging as accurate as possible, and to obtain local control of the disease (2).

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There is an ongoing debate about the extent to which a lymphadenectomy procedure can be performed in patients with stage I lung cancer. During the procedure, the surgeon can choose between different procedures: from not removing any nodes, to performing a non-systematic sampling or carrying out a bilateral complete mediastinal dissection, as is done by some Japanese teams (3,4). Only the pathologist can say what nodes are affected and detect the presence of micrometastases. The characteristics of the nodes, whether they are calcified for example, can determine the surgeon's attitude regarding what technique to choose, but, as Gaer and Goldstraw (5) proved, it is not possible to predict intraoperatively which lymph node will be affected based on its appearance. In 68% of patients in the series published by Takizawa, the surgeon was not able to determine whether the mediastinal nodes were involved or not because their macroscopic appearance was normal (6). In the series of Riquet, 20% of the positive nodes were perceived as possible negative by the surgeon, proving that ocular assessment is inexact (7). It is proved that non-systematic sampling is not reliable for proper staging (8,9).

Minimum number of lymph nodes

Unlike in lung cancer, in other tumors there does exist a defined number of nodes required to consider a lymphadenectomy as complete. In the case of colorectal cancer, survival has a direct relationship between the number of lymph nodes examined (10). Several authors have shown that twelve is the minimum number of lymph nodes needed to stage a patient as N0 (11,12). The American Society of Clinical Oncology (ASCO) recommends treatment with adjuvant chemotherapy for all patients staged as N0 with less than 12 lymph nodes resected, since they are at high risk of recurrence (13).

In lung cancer, the minimum number of lymph nodes necessary to consider a lymphadenectomy as complete is not clearly defined. Some authors have tried to state this in several studies. Doddoli considered a lymphadenectomy as complete when more than ten lymph nodes from at least two different mediastinal levels were removed (14). Wu *et al.* considered a lymphadenectomy as inadequate for proper staging and local control of the disease if less than 15 lymph nodes had been resected (15).

Guidelines recommendations

Although there is no defined minimum number of lymph nodes for a complete lymphadenectomy, there are some recommendations for performing this procedure published by different scientific societies. Thus, the European Society of Thoracic Surgeons (ESTS) recommends systematic nodal dissection in all cases (16). Ideally, this should be done as an en bloc resection of the upper mediastinal nodes on the right side stations (2R and 4R), any visible nodes stations 3a and 3p, and the lower mediastinum, (stations 7, 8, and 9). On the left side, removal of stations 5 and 6, and inferior paratracheal (4L) lymph nodes is minimally required. For a complete nodal dissection of the left upper mediastinum, it is recommended to perform a division of the ligamentum arteriosus allowing mobilization of the aortic arch. In addition, it is important that the highest mediastinal node removed should be identified, in order to assess whether the resection is complete (16).

For peripheral squamous T1 patients, a lobe-specific systematic nodal dissection is acceptable, if the hilar and interlobar lymph nodes are negative, because it has been shown that the probability of unforeseen N2 disease is very low (<5%) in such patients. In this case, it is necessary to remove three mediastinal stations, always including station 7, and at least six lymph nodes must be excised (16).

Common practice in thoracic surgery

Current practice in thoracic surgery regarding lymphadenectomy differs on some points to the guideline recommendations. A survey of surgeons in the UK revealed that 45% of them did not perform sampling if mediastinal nodes had a normal macroscopic appearance, and only 23% of them performed routine complete lymphadenectomy (17).

In another survey conducted in 2001 to examine patterns of treatment of 11,000 patients with NSCLC, the authors observed that 42.2% of the patients did not undergo any type of lymph node dissection (18).

Analysis of the Society of Thoracic Surgeons' database also showed similar data, with no mediastinal lymph node evaluation in 35% of patients (19).

More recently, two papers continue to show similar practices. The review of the SEER database confirmed that 48% of patients had no assessment of mediastinal lymph nodes (20), and a retrospective cohort study in the Netherlands in 216 patients, demonstrated that no lymphadenectomy was performed in 21 patients with suspicious mediastinum by CT scan or PET-CT (21). This series also showed that only eight patients met ESTS criteria (21).

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Table 1 Percentage of positive and negative lymph nodes according to the number of lymph nodes resected (27)

Resected lymph nodes	Negative lymph nodes	Positive lymph nodes
<10 (N=227)	166 (50.61%)	61 (38.85%)
≥10 (N=258)	162 (49.39%)	96 (61.14%)

Chi-square 5.89 (P<0.025).

Table 2 Hazard ratio of disease-free survival and overall survival of patients with more than ten lymph nodes resected (27)

Multivariate analysis	HR	CI 95%	P
Disease free survival	0.97	0.96-0.99	0.004
Overall survival	0.97	0.95-0.99	0.001

The more lymph nodes resected, the more positive lymph nodes identified

It seems obvious, from a logical point of view, to think that the probability of finding a positive node increases with the number of lymph nodes analyzed. This was proved in the Izbicki series, in which a complete lymphadenectomy increased the percentage of patients in whom positive mediastinal lymph nodes were detected (22).

Comparing complete lymphadenectomy to sampling, Bollen *et al.* showed that more positive lymph nodes can be diagnosed with complete lymphadenectomy (35%) than with sampling (13%), concluding that sampling is inadequate for accurate staging (23). Similar data were found by Keller *et al.* (24).

In the papers published by Naruke *et al.* (25) and Yoshino *et al.* (26), the results are similar: sampling detected between 9% and 17% of positive nodes, and complete lymphadenectomy obtained between 22% and 32% positive lymph nodes.

In a review of patients operated on in our department in Vall d'Hebron University Hospital, Barcelona, similar data were found, with a greater number of positive nodes in the group of patients with more than ten lymph nodes resected (27) (*Table 1*).

Therefore, a complete lymphadenectomy provides proper staging which helps to identify patients' real prognosis. Hence, professionals are able to offer appropriate treatment according to the patient's stage.

The more lymph nodes resected, the better the survival

Several nonrandomized studies and retrospective series have shown that survival increases in the group of patients with a higher number of lymph nodes removed.

Wu *et al.* published a series of 471 patients with stage I-IIIA NSCLC, comparing complete lymphadenectomy to systematic sampling. They observed that 5-year survival in the sampling group was 36.98%, compared to 48.37% in the lymphadenectomy group (statistically significant difference) (28).

In our data, the results were similar, identifying the number of nodes as a protective factor in the multivariate analysis (27) (*Table 2*).

In two articles published by Lardinois *et al.* (29) and Sakurai *et al.* (30), no significant difference in overall survival was observed between lymphadenectomy and sampling, but there was an increase in disease-free survival and in local recurrences if patients underwent complete lymphadenectomy.

A recent meta-analysis of three studies observed a benefit in survival in stage I, II and IIIA patients if lymphadenectomy was complete, and that this procedure reduced the risk of death in early stages (31).

Complete lymphadenectomy in stage I NSCLC patients

The improvement in survival in early stages is also seen in the series published by Wu *et al.*, with 5-year survival of 57.49% in the sample group compared to 82.16% if complete lymphadenectomy was performed (27).

In another study published in 2003 in which 321 stage I patients were analyzed, 5-year survival was significantly higher in the group with more than 15 lymph nodes resected (57.1% *vs.* 54.5%), concluding that the number of lymph resected could explain the differences in survival in stage I patients (15).

In a review of 442 stage I patients, those in whom less than six nodes were resected through lymphadenectomy showed higher recurrence and mortality rates than the group with complete lymphadenectomy (32).

Similar data were observed in a paper published by Ou *et al.*, analyzing stage IA patients. They found a 5-year

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survival rate of 75.9% in the group with more than 15 resected lymph nodes, compared to 57.9% in the group with less than 6 resected lymph nodes (33).

Xu *et al*. recently published a series of 203 stage IA patients with 5-year overall survival of 62%. They divided the series into three groups according to the number of lymph nodes resected (<10 nodes, 10 to 20, and >20). The analysis of disease-free survival showed statistically significant differences between the groups: 20.26%, 58.8% and 75% respectively. The differences remain significant in the analysis of the number of resected stations, and the data are very striking when analyzing the mediastinal stations resected (9.1% when less than three mediastinal stations were resected *vs*. 65.1% when more than 3) (34). The authors point out that if an adequate lymphadenectomy is not performed, the true N stage remains unknown, which can result in misclassification of stage IA (33).

In a randomized study comparing sampling *vs*. complete lymphadenectomy in patients with T1 and T2 N0 or N1 with no hilar involvement, the authors found no differences in long-term survival rate of local or regional recurrence (35). This study did not include patients with nodal involvement of station 10, so the possibility of mediastinal stations may be low, which could explain the results. The authors, however, conclude that a complete lymphadenectomy provides the most accurate staging. They also point out that current preoperative staging cannot accurately identify patients with mediastinal lymph node involvement. Another reason for this procedure is that patients with known hilar or N2 disease, or with T3/T4 tumors, may benefit from mediastinal lymph node dissection. Therefore, the authors still recommend that all patients with resectable NSCLC undergo mediastinal lymph node dissection because the procedure does not increase mortality or morbidity (34).

Conclusions

There is no contraindication for performing a complete lymphadenectomy. The increase in survival in patients with complete lymphadenectomy may be due to more accurate staging. Therefore, complete lymphadenectomy should be mandatory, even in early stage patients.

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Treatment of stage I lung cancer in high-risk and inoperable patients: SBRT *vs*. RFA *vs*. sublobar resection

Matthew J. Bott, Traves Crabtree

Division of Thoracic Surgery, Washington University School of Medicine, St. Louis, Missouri, USA

Correspondence to: Traves Crabtree. Division of Thoracic Surgery, Washington University School of Medicine, St. Louis, Missouri, USA. Email: crabtreet@wudosis.wustl.edu.

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Background

Although surgical resection for early stage lung cancer is the mainstay of treatment, many patients are inoperable at the time of presentation due to either disseminated disease or medical comorbidities (1). Novel strategies are currently being developed to treat early-stage non-small cell lung cancer (NSCLC) in this expanding population of high-risk and inoperable patients. Stereotactic body radiotherapy (SBRT) modifies traditional radiation techniques to provide a high-dose per fraction of radiation to the tumor which is administered over a few fractions. This allows for effective tumor ablation with preservation of the surrounding tissue due to steep dose gradients. Radiofrequency ablation (RFA) utilizes CT-guided placement of a radiofrequency-emitting probe. As frictional heat energy from the probe is transferred to the tumor, cancer cells undergo coagulation necrosis.

In an effort to expand the population of operable patients, many groups are currently exploring the use of sublobar resection to treat early stage tumors. Early evidence suggests that sublobar resection may provide satisfactory oncologic outcomes while avoiding the morbidity of standard lobectomy in patients with poor pulmonary reserve (2). Three major clinical trials have been developed to investigate the use of these different modalities to treat early stage lung cancer in inoperable or high-risk patients. A recently published trial, RTOG 0236, is a North American phase II trial of SBRT in patients with stage I NSCLC deemed inoperable by a surgeon or a pulmonologist. The study showed a local control rate of 90.6% at three years, and disease-free survival and overall survival at three years were 48.3% and 55.8%, respectively (3). ACOSOG Z4032 is a phase III randomized controlled trial that compared sublobar resection to sublobar resection with brachytherapy for the treatment of stage I NSCLC. Thirty- and 90-day outcomes from this study have recently been published (4). In addition, three-year results were presented at the 2013 American Society of Clinical Oncology (ASCO) meeting, showing a similar rate of local recurrence for those treated with sublobar resection (12.8%) versus sublobar resection with brachytherapy (12.5%) (5). Overall survival was comparable between the groups (sublobar resection =71%, sublobar resection with brachytherapy =72%). Lastly, ACOSOG Z4033 is a phase II prospective nonrandomized study examining high-risk patients with stage I NSCLC treated with RFA. This study has completed accrual, but survival and recurrence data have not yet matured. We conducted a comparison of selection criteria and short-term outcomes for these three studies.

Patients and setting

Patients

This study focuses on patients with stage I lung cancer that are high risk for surgical intervention due to medical co-morbidities.

Intervention(s)

We explore the selection criteria and short-term outcomes in high risk patients treated with three different treatment modalities: SBRT, sublobar resection, and RFA.

Objective(s)

We sought to compile data from three major North

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Table 1 Pre-treatment demographics and comorbidity profiles for RTOG 0236, ACOSOG Z4032, and ACOSOG Z4033

Pre-treatment characteristics	RTOG 0236 (SBRT)	ACOSOG Z4032 (sublobar resection)	ACOSOG Z4033 (RFA)	P value
N	55	211	51	
Age (mean)	72.5±8.8	70.2±8.5	75.6±7.5	0.0003[1]
Age >75	21 (38.9%)	79 (37.4%)	30 (58.8%)	0.02[2]
Female	34 (61.8%)	117 (55.5%)	28 (54.9%)	0.7
ECOG 1-2	43 (78.1%)	169 (80.1%)	42 (82.4%)	0.86
Race (white)	51 (92.7%)	199 (94.3%)	44 (86.3%)	0.02[3]
Clinical stage IA	44 (80%)	208 (98.6%)	51 (100%)	<0.0001[4]
Pulmonary hypertension	NR	5 (2.4%)	1 (2.0%)	0.86
Poor LV function	NR	12 (5.7%)	6 (11.8%)	0.12
MMRC dyspnea score	NR	46 (21.8%)	12 (23.5%)	0.79
pO_2 ≤55 mmHg or SpO_2 ≤88%	2 (3.7%)	10 (4.7%)	1 (2.0%)	0.66
pCO_2 >45 mmHg	8 (14.8%)	6 (2.8%)	0	0.0002[5]
DLCO%	61.6±30.2	46.4±15.6	43.7±18.0	0.001[6]
FEV1%	61.3±33.4	53.8±19.6	48.8±20.3	0.15
FVC%	79.8±23.2	74.8±17	NR	0.4

Values are mean ± SD or n (%) as appropriate. P values are from Chi-square or Kruskall-Wallis test. NR, not reported; [1], P<0.0001 Z4032 *vs*. Z4033; [2], P=0.04 RTOG 0236 *vs*. Z4033, P=0.005 Z4032 *vs*. Z4033; [3], P=0.04 RTOG 0236 *vs*. Z4032; [4], P<0.0001 RTOG *vs*. Z4032, P=0.0007 RTOG 0236 *vs*. Z4033; [5], P=0.0004 RTOG 0236 *vs*. Z4032, P=0.004 RTOG 0236 *vs*. Z4033; [6], P=0.0008 RTOG0236 *vs*. Z4032, P=0.001 RTOG 0236 *vs*. Z4033. ECOG, Eastern Cooperative Oncology Group; DLCO%, diffusing capacity of the lung; FEV1%, forced expiratory volume in one second.

American trials in order to compare the selection of patients for these three treatment options, and to provide some insight into the short-term morbidity and mortality associated with each.

Methodology

The study was a retrospective secondary analysis of prospectively collected data from three multicenter trials (RTOG trial 0236, ACOSOG trial Z4032, and ACOSOG Z4033). The data were formally requested from the RTOG and ACOSOG, and the analysis was approved by both organizations. We compared entry criteria and short-term outcomes using raw data from all three trials. Categorical data were compared using chi-square test and continuous data using the Kruskal-Wallis test. We then performed a propensity-matched analysis of patients treated with SBRT and sublobar resection (RTOG 0236 and ACOSOG Z4032). Variables including age, Eastern Cooperative Oncology Group (ECOG) performance status, percentage of predicted forced expiratory volume in one second (FEV1%), and percentage of predicted carbon monoxide diffusing capacity of the lung (DLCO%) were used to build a propensity score for patients with clinical stage IA NSCLC. These scores were developed to estimate the adjusted risks of short-term outcomes associated with the choice of treatment (SBRT or surgery).

Primary outcomes

Main results

There were 55 patients available for analysis from RTOG 0236 (SBRT), 211 from ACOSOG Z4032 (sublobar resection), and 51 from ACOSOG Z4033 (RFA). RFA patients were older than those undergoing sublobar resection or SBRT (mean age in years =75.6, 70.2, 72.5 respectively, P=0.02) (*Table 1*). Despite having been identified as medically inoperable according to study criteria, SBRT patients had superior DLCO% (61.6%) compared with sublobar resection (46.4%) and RFA (43.7%) (P=0.001). All patients had either T1 or T2 tumors. Twenty percent of patients treated with SBRT had T2

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disease (n=11), compared with 1.4% of those treated with sublobar resection (n=3). All patients treated with RFA had T1 tumors. SBRT patients received an average of 60 Gy of radiation. In patients undergoing surgical resection for clinical stage IA disease, 29.3% ultimately had a higher stage on final pathology (pIB in 25%, pIIA in 0.5%, pIIB in 1.6%, pIIIA in 1.1%, pIIIB in 0.5%, and IV in 1.1%).

Thirty- and 90-day outcomes are shown in *Table 1*. For RFA, only mortality data were available. There was no significant difference in 30-day, 90-day, or treatment-related mortality amongst the three modalities. There was, however, a higher incidence of grade 3+ events at 30 days in patients undergoing sublobar resection (28.0%) compared with SBRT (9.1%) (P=0.004). The incidence was equivalent at 90 days (33.2% for sublobar resection, and 21.8% for SBRT, P=0.24). A propensity-matched score was then used to compare SBRT (n=44) and sublobar resection (n=208) in patients with T1 lesions. In the propensity-matched analysis, there was no difference in 30- or 90-day grade 3+ adverse events between these two modalities. An additional analysis was performed examining pre- and post-treatment DLCO% and FEV1% in patients treated with SBRT and sublobar resection. After adjusting for pre-treatment values, there was no difference in DLCO%. However, post-treatment FEV1% was 6.4% greater in patients undergoing sublobar resection compared with those treated with SBRT.

Study limitations

Although each of the trials was designed to evaluate patients with early stage lung cancer, subtle underlying differences in the patient populations exist. Similarly, as long-term data has not yet matured, we cannot comment on the oncologic efficacy of the treatments. In addition, our propensity matched comparison may be underpowered to detect differences in morbidity and mortality. The current analysis was meant to provide preliminary insight and definite conclusions will best be made using specifically designed, randomized controlled data comparing the modalities directly.

Applicability to other populations

These trials were designed to evaluate treatment of early stage lung cancer in high-risk or non-operable patients. The data are not necessarily applicable to patients with more advanced disease or to those who are satisfactory operative candidates.

Conclusions

Variability in patient populations in these three studies underscores the need for more reliable, objective criteria to identify the inoperable patient, the high risk but potentially operable patient, and the very high risk patient that may have a relatively better risk/benefit ratio from non-operative therapy *vs*. operative therapy. Our propensity-matched analysis of high-risk or inoperable patients with clinical stage I lung cancer shows no difference in 30- or 90-day mortality and morbidity between SBRT and sublobar resection. These results emphasize the need for specifically designed randomized trials to compare these treatment modalities and further stratify patients considered high risk.

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Improving the pathologic evaluation of lung cancer resection specimens

Raymond U. Osarogiagbon[1], Holly L. Hilsenbeck[2], Elizabeth W. Sales[3], Allen Berry[4], Robert W. Jarrett Jr[5], Christopher S. Giampapa[6], Clara N. Finch-Cruz[7], David Spencer[8]

[1]Multidisciplinary Thoracic Oncology Program, Baptist Cancer Center, Memphis, TN, USA; [2]Duckworth Pathology Group, Memphis, TN, USA; [3]Doctors Anatomic Pathology, Jonesboro, AR, USA; [4]Department of Pathology, St. Francis Hospital, Memphis, TN, USA; [5]Pathology and Clinical Laboratories, North Mississippi Medical Center, Tupelo, MS, USA; [6]Medical Center Laboratory, Jackson-Madison County General Hospital, Jackson, TN, USA; [7]Pathology and Laboratory Medicine Service, Department of Veterans Affairs, VA Medical Center Memphis, TN, USA; [8]Trumbull Laboratories, LLC/Pathology Group of the Mid-South, Memphis, TN, USA

Correspondence to: Raymond U. Osarogiagbon, MBBS. Thoracic Oncology Research Group, Baptist Cancer Center, Memphis, TN 80 Humphreys Center Drive, Suite 220, Memphis, TN 38120, USA. Email: rosarogi@bmhcc.org.

Abstract: Accurate post-operative prognostication and management heavily depend on pathologic nodal stage. Patients with nodal metastasis benefit from post-operative adjuvant chemotherapy, those with mediastinal nodal involvement may also benefit from adjuvant radiation therapy. However, the quality of pathologic nodal staging varies significantly, with major survival implications in large populations of patients. We describe the quality gap in pathologic nodal staging, and provide evidence of its potential reversibility by targeted corrective interventions. One intervention, designed to improve the surgical lymphadenectomy, specimen labeling, and secure transfer between the operating theatre and the pathology laboratory, involves use of pre-labeled specimen collection kits. Another intervention involves application of an improved method of gross dissection of lung resection specimens, to reduce the inadvertent loss of intrapulmonary lymph nodes to histologic examination for metastasis. These corrective interventions are the subject of a regional dissemination and implementation project in diverse healthcare systems in a tri-state region of the United States with some of the highest lung cancer incidence and mortality rates. We discuss the potential of these interventions to significantly improve the accuracy of pathologic nodal staging, risk stratification, and the quality of specimens available for development of stage-independent prognostic markers in lung cancer.

Keywords: Quality of care; survival; nodal staging; lung cancer surgery

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Lung cancer is the most common cause of cancer-related mortality worldwide and in the US; non-small cell lung cancer (NSCLC) represents approximately 85% of all cases (1,2). Despite improvements in methods of diagnosis and treatment, the aggregate overall 5-year survival rate of all patients with lung cancer has only improved from 12% in the 1970s to 17% in contemporary times (2). This is largely because most patients present with advanced disease for which curative therapy is currently unavailable. However, patients with early stage disease who undergo definitive surgery or combined modality therapy may have long term survival. The most effective current prognostic tool is the Tumor, Node, and Metastasis (TNM) staging system which is currently in its 7th edition (3).

Staging, while of great prognostic value, is only as useful as the degree of thoroughness with which it is applied (4,5). Comparison of clinical and pathological (post-resection) staging survival curves on the same patients reveals greater separation between the pN0-3 subsets than the cN subsets, in part because pathologic staging defines a group of pN0 tumors with better survival and a group of pN3 tumors with worse survival than predicted by clinical staging

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alone (6). This reflects the fact that clinical staging tests have sensitivity and specificity limitations that impair their accuracy (7-9).

Pathologic nodal stage is the most important determinant of prognosis in patients who undergo resection for NSCLC, with survival ranging from 56% in patients with pN0 to 6% in pN3 (6). It is also the main driver of post-operative management. For example, patients with pN1-3 disease benefit from adjuvant chemotherapy (10-12), while those with mediastinal lymph node metastasis may benefit from radiation therapy in addition to chemotherapy (13). However, pN-stage is the TNM category most susceptible to variability in both surgical resection techniques and pathologic evaluation (14).

Examination of large databases, such as the California Cancer Registry, the Surveillance, Epidemiology, and End Results (SEER) database and the National Cancer Data Base (NCDB), reveals worrisome statistics about pathologic nodal staging of NSCLC: a median of five lymph nodes are examined in pN0 resection specimens (15); 12% of all resections (and 18% of all 'node-negative resections') have no lymph nodes examined (pathologic NX) (14-17); 12% of pN0 cases have no N1 lymph nodes examined (18); and 42% of resections (and 62% of 'mediastinal lymph node negative' cases) have no mediastinal lymph nodes examined (14,15,19,20).

Less than fastidious pathologic nodal staging has profound survival implications. For example, survival of patients with pN0 disease rises sequentially with the number of lymph nodes examined, until a maximal improvement is achieved at approximately 18-20 lymph nodes, suggesting the impact of sampling error when few lymph nodes are examined (21,22); patients who undergo pNX resections have a significantly worse survival than those with pN0, much more akin to the survival of those with pN1 disease (17); and failure to examine mediastinal lymph nodes is associated with a 14% survival deficit (20). Even in patients in whom lymph node metastasis is detected, examination of all available lymph nodes remains of prognostic value. As with many cancers, including colorectal, esophageal and gastric cancer, the prognosis of NSCLC worsens with increasing number of lymph node metastasis or a rising positive lymph node ratio (23-29). The number of N1 lymph node metastases is independently prognostic (30), but also correlates strongly with the likelihood of mediastinal lymph node metastasis (23).

Accurate pathologic lymph node staging involves three key processes: the intra-operative collection of the hilar (station 10) and mediastinal (stations 2-9) nodes; secure transfer, and accurate communication of the anatomic provenance, of all specimens between the operating room and pathology laboratory; and examination of all delivered specimens in the pathology laboratory, including the intrapulmonary lymph nodes (stations 11-14) retrieved by gross dissection of the lung resection specimen. The collection of hilar and mediastinal lymph nodes is the responsibility of the operating surgeon, without whose performance those specimens cannot be obtained; the extraction of intrapulmonary lymph nodes and the examination of all provided specimens is the responsibility of the pathologist; and the delivery of specimens in a secure, anatomically distinguishable fashion is the joint responsibility of the operating room and pathology teams.

Multiple efforts have been made to standardize the extent of the surgical lymph node harvest (31-35). Although the details differ slightly, it is generally agreed that a systematic collection of hilar and mediastinal lymph nodes should be attempted by the surgeon (*Table 1*). Some further advocate that the surgeon should collect stations 11 (interlobar) and 12 (lobar) lymph nodes (33). On the pathology side, standard recommendations call for examination of all lymph nodes in the resection specimen (36). While pathologists routinely examine all specifically identified specimens, retrieval of intrapulmonary nodes is dependent on the quality of the gross dissection of the resection specimen, warranting careful oversight of this aspect of the pathology examination.

Although techniques for gross dissection of intrapulmonary lymph nodes have been described, actual practice likely varies significantly, as evidenced by the fact that almost 50% of pNX cases are lobectomy or greater resections, suggesting that not only were hilar and mediastinal lymph nodes not provided from the operating room, but intrapulmonary lymph nodes were not retrieved during gross dissection of the resection specimen (17,37-40). More direct evidence comes from a study in which fastidious re-examination of discarded remnant lung resection specimens revealed a median of four N1 lymph nodes examined and a median of six discarded. Furthermore, 29% of patients in this study had discarded lymph nodes with metastasis, and 12% of pN0 specimens had discarded N1 lymph node metastasis (41).

The pathology examination ideally should indicate the anatomic source of each of the lymph nodes examined (lymph node mapping) in order to provide clinicians a clear idea of whether lymph nodes are from N1, N2 or N3

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Table 1 Minimum recommended surgical mediastinal lymph node staging quality parameters

Tumor location	Guideline group and recommended surgical lymph node collection stations				
	ACOSOG (33)	CoC (34)	ESTS (32)	IASLC (31)	NCCN (35)
Right lung					
Upper	2R, 4R, 7, 10R	≥10	2R, 4R, 7	3, 4R, 7	≥3 N2 stations
Middle	Same	Nodes*	Same	Same	
Lower	Same		4R, 7, 8, 9	3, 4R, 7, 8, 9	
Left lung					
Upper	5, 6, 7, 10L	≥10	5, 6, 7	3, 5, 6, 7	≥3 N2 stations
Lower	Same	Nodes*	7, 8, 9	7, 8, 9	

*, no nodal station specification. ACOSOG, American College of Surgeons Oncology Group; CoC, American College of Surgery Commission on Cancer; ESTS, European Society of Thoracic Surgeons; IASLC, International Association for the Study of Lung Cancer; NCCN, National Comprehensive Cancer Network; L, left; R, right.

stations. Because pathologists cannot identify the origin of lymph nodes provided by surgeons without accurate labeling, it is important for the communication between the operating room and the pathology laboratory to include unambiguous information on the source of all submitted specimens. The importance of this point is illustrated by an audit of mediastinal lymph node examination practices in a city-wide lung resection database, which revealed a 61% discordance between the procedure reported in operating surgeons' notes and the procedure determined from objective review of the pathology report using pre-specified criteria. Whereas operating surgeons claimed a systematic nodal dissection in 45% of cases, only 8% met pathology criteria for systematic nodal dissection (42).

However, a blinded independent surgical review of the narrative description of the operation indicated that 30% of resections had adequately described a systematic nodal dissection (42). This sharp discordance between the operation narrative and the pathology report, which has been described as a 'Tower of Babel', suggests a multifaceted etiology of poor lymph node staging, encompassing actions both in the operating room and the pathology laboratory (43). This especially highlights the need for secure specimen delivery and better communication between the operating room and the pathology laboratory (42,44). A follow-up study in the same community institutions revealed marked improvement in concordance rate to 80% when surgeons used a lymph node specimen collection kit and checklist (45).

These observations suggest certain opportunities for intervention. The surgical hilar and mediastinal lymphadenectomy can be significantly improved with the use of pre-labelled surgical specimen collection kits, which help remind surgeons of the recommended lymph node collection procedure, provide a vehicle for the secure transfer of lymph node specimens, and with station-specific pre-labeling, eliminate all ambiguity about the anatomic source of each specimen. Use of such a specimen collection kit significantly improved hilar and mediastinal lymph node staging in a pilot study, with the ultimate result of an increase in the detection of pN2 disease from 8% of controls to 18% of cases (46). Routine use of kits such as these can address the operating room and communication aspects of the lymph node staging problem.

The finding of a high number of un-retrieved intrapulmonary lymph nodes has led to efforts to develop a more thorough standardized gross dissection method. Such a dissection protocol must be easy to learn, reproducible, quick to execute and feasible for use on fresh resection specimens in order not to interrupt the work flow in busy anatomic pathology laboratories. Such a protocol, in which blunt dissection of lymph nodes in the peri-bronchus is performed starting from the hilar surface of the resection specimen and working towards the periphery, has been shown to be feasible (47). This technique is easily taught, can be carried out on fresh specimens, requires a median of 9 minutes, and yields significantly more N1 lymph nodes than the current routine dissection protocol.

The combined use of the surgical specimen collection kit and thorough intrapulmonary lymph node retrieval protocols increased the number of lymph nodes examined in lung resection specimens from a median of 5 to 18, eliminated the pNX phenomenon, and, most importantly,

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increased the proportion of patients with detected node positive disease (and therefore potentially benefited by life-saving post-operative adjuvant therapy) from 30% to 45% (48). The potential survival impact of these combined interventions is large. Additionally, the improvement in lymph node mapping allows easy identification and correction of any errors in stage attribution.

In addition to identifying more patients with lymph node metastases, more lymph nodes with metastasis are found per patient with 'node-positive disease', potentially facilitating definitive examination of the prognostic impact of a higher number of lymph node metastasis in NSCLC. Ultimately, it is hoped that thorough lymph node retrieval will facilitate the search for other prognostic factors such as the real prevalence and prognostic value of micro-metastatic lymph node disease (detected by immunohistochemical analysis) and prognostic/predictive gene/protein expression patterns in primary tumors (49-54).

The combination of these two interventions, the surgical specimen collection kit and the standardized lung specimen dissection protocol, will be the subject of the '*Strategies to Improve Lymph node Examination in Non-small cell lung cancer Trial*', an institutional randomization study with the acronym 'SILENT', which is currently in development. Objectives of this study are to test the impact of improved lymph node examination on stage distribution and survival, as well as the economic value associated with these corrective interventions.

Looking ahead, it is ultimately expected that molecular predictors of response to adjuvant therapy and independent molecular prognosticators can be identified from gene and protein expression profiles of primary tumors. However, optimal development and testing of such molecular markers will need accurately staged groups of patients (55,56). This will require marked improvement in the routine pathologic staging of resected lung cancer, to minimize the confounding of results caused by suboptimal use of the TNM staging system.

Major questions remain. How can we equitably compensate pathologists for any additional time, manpower, equipment and supply costs required to achieve more thorough examination? How can we successfully implement better pathology practices across the spectrum of practice environments? The first steps, possibly, are to universally acknowledge the existence of the gap in quality of pathologic staging, recognize the impact on survival, and commit to implementing corrective measures. Some measures, such as routine use of specimen collection kits, may be relatively easy to implement, while others might seem less so. Although improving the dissection and retrieval of intrapulmonary lymph nodes may require a bit more time and effort from pathologists, doing so will allow for more accurate identification of high-risk patients who will benefit from intensive post-operative intervention. This, in turn, is likely to provide a population-wide improvement in outcomes of resected early stage NSCLC.

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Footnote

Conflicts of Interest: Dr. Osarogiagbon has a patent application for a surgical specimen collection kit under review. All other authors have no conflicts of interest to declare.

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Novel radiotherapy approaches for lung cancer: combining radiation therapy with targeted and immunotherapies

Charles B. Simone II[1], Stuart H. Burri[2], John H. Heinzerling[2]

[1]Department of Radiation Oncology, Hospital of the University of Pennsylvania, Philadelphia, PA, USA; [2]Department of Radiation Oncology, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Contributions: (I) Conception and design: JH Heinzerling, CB Simone 2nd; (II) Administrative support: JH Heinzerling, CB Simone 2nd; (III) Provision of study materials or patients: JH Heinzerling, CB Simone 2nd; (IV) Collection and assembly of data: JH Heinzerling, CB Simone 2nd; (V) Data analysis and interpretation: JH Heinzerling, CB Simone 2nd; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: John H. Heinzerling, MD. Department of Radiation Oncology, Levine Cancer Institute, Carolinas Healthcare System, 1021 Morehead Medical Drive, Charlotte, NC 28204, USA. Email: John.Heinzerling@carolinashealthcare.org.

Abstract: Targeted therapies and immunotherapies have quickly become fixtures in the treatment armamentarium for metastatic non-small cell lung cancer (NSCLC). Targeted therapies directed against epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) translocations, and ROS-1 rearrangements have demonstrated improved progression free survival (PFS) and, in selected populations, improved overall survival (OS) compared with cytotoxic chemotherapy. Immunotherapies, including checkpoint inhibitor monoclonal antibodies against programmed death receptor 1 (PD-1) and programmed death ligand 1 (PD-L1), have now also demonstrated improved survival compared with chemotherapy. The use of these novel systemic agents in non-metastatic patient populations and in combination with radiation therapy is not well defined. As radiation therapy has become more effective and more conformal with fewer toxicities, it has increasingly been used in the oligometastatic or oligoprogression setting. This has allowed improvement in PFS and potentially OS, and in the oligoprogressive setting may overcome acquired drug resistance of a specific lesion(s) to allow patients to remain on their targeted therapies. Molecularly targeted therapies and immunotherapies for patients with metastatic NSCLC have demonstrated much success. Advances in radiation therapy and stereotactic body radiotherapy, radiation therapy have led to combination strategies with targeted therapies among patients with lung cancer. Radiation therapy has also been combined with immunotherapies predominantly in the metastatic setting. In the metastatic population, radiation therapy has the ability to provide durable local control and also augment the immune response of systemic agents, which may lead to an abscopal effect of immune-mediated tumor response in disease sites outside of the radiation field in select patients.

Keywords: Abscopal effect; immunotherapy; lung cancer; radiation therapy; targeted therapy

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Role of radiation in early stage and locally advanced non-small cell lung cancer (NSCLC)

Definitive radiation therapy has been part of the standard of care for patients with locally advanced NSCLC for almost 5 decades. Combined modality therapy with chemoradiation became the preferred treatment of these patients based on multiple clinical trials showing improved survival (1,2). Conventionally fractionated radiation therapy remains the standard, and attempts at dose escalation have failed to show a benefit in this patient population (3). Newer technologies such as intensity modulated radiation (4), image guided radiation therapy, and proton therapy (5-7) are increasingly being utilized or studied to lower rates of toxicity with combined modality therapy.

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Table 1 Classes of targeted therapies in clinical use in metastatic non-small cell lung cancer

Target	Currently available targeted therapies
EGFR	Erlotinib
	Afatinib
	Gefitinib
	Cetuximab
ALK	Crizotinib
	Ceritinib
ROS1	Crizotinib
MET	Crizotinib
VEGF	Bevacizumab
	Ramucirumab

EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS1, ROS proto-oncogene 1; VEGF, vascular endothelial growth factor.

Surgical resection has been the standard of care for patients with stage I NSCLC with 5 years survival rates of approximately 60-70% (8,9). While patients determined to be medically inoperable have been treated in the past with standard fractionated radiotherapy, newer technologies within radiation therapy have led to the standardization of high dose, ablative hypofractionated therapy termed stereotactic body radiation therapy (SBRT) or stereotactic ablative radiotherapy (SABR) (10). SBRT has allowed for improved dose conformity, improved local tumor control, and superior overall survival (OS) when compared to conventionally fractionated radiotherapy (11,12). Based on the improved outcomes with SBRT and the increased utilization of this technology, interest in its use for medically operable patients has emerged. A recently published pooled analysis of two randomized trials comparing surgery and SBRT for stage I NSCLC demonstrated that SBRT was highly effective and had a limited toxicity profile, and that there was equipoise between the two treatment options (13).

SBRT has also begun to be used more frequently in patients with oligometastatic disease, including lung, liver, and bone metastases. Recent data has shown excellent control rates with encouraging progression free survival (PFS) in patients with oligometastatic NSCLC (14,15). Conventionally fractionated radiotherapy, in combination with chemotherapy, can also be considered in patients with oligometastatic disease not amenable to treatment with SBRT and may improve survival in a select subset of patients with minimal extrathoracic disease (16).

Targeted therapy for advanced NSCLC

With the discovery of molecular pathways that correspond with tumor progression and growth, numerous potential targets have been identified and explored for potential therapeutics for advanced NSCLC (*Table 1*).

Epidermal growth factor receptor (EGFR) is an essential part of the oncogenic growth pathway and is expressed at higher levels in some lung cancers. EGFR as a molecular target has shown promising results in advanced lung cancer. Monoclonal antibodies, such as cetuximab and panitumumab, and tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, and afatinib, are available. Initial trials evaluating patients treated with cytotoxic chemotherapy either in combination or followed by EGFR pathway inhibitors without prior molecular mutation analyses demonstrated mixed results, although trials have generally demonstrated at least a benefit to PFS (17-23). Further subset analysis of many of these trials showed clear correlation between the presence of EGFR driver mutations and clinical benefit of these agents. This has led to the standardization of the use of EGFR TKIs in the first line setting for patients with EGFR mutations (24-30).

Vascular endothelial growth factor (VEGF) plays an essential part in tumor angiogenesis and is often expressed at higher rates in NSCLC, thus creating another molecular pathway target for therapy. The most well studied VEGF inhibitor in NSCLC, bevacizumab, has shown increased PFS and OS in patients with non-squamous NSCLC when added to standard cytotoxic chemotherapy (31-33). Ongoing trials are evaluating bevacizumab with other platinum combinations (NCT00150657, NCT00753909), as well as with other targeted agents such as erlotinib and ramucirumab (NCT01532089, NCT00257608, NCT00553800).

One of the most promising recent areas of new drug development in treatment of NSCLC has been anaplastic lymphoma kinase (ALK) inhibitors. These are targeted agents directed at the novel fusion oncogene echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK). The first available drug was crizotinib, an oral small-molecule inhibitor of ALK and c-Met tyrosine kinases. Crizotinib has shown favorable outcomes both in the second line setting, as well as in the primary treatment setting for patients that are positive for this rearrangement (34,35). Second generation TKI inhibitors of ALK include ceritinib and alectinib are undergoing investigation in national trials in ALK positive patients that have progressed, as well as the primary setting with pending

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Table 2 Checkpoint inhibitors in clinical use or under development for advanced or metastatic non-small cell lung cancer

Monoclonal antibody	Target	FDA approved
Ipilimumab	CTLA-4 on T cells	Melanoma
Nivolumab	PD-1 on T cells	Lung cancer, melanoma
Pembrolizumab	PD-1 on T cells	Melanoma
BMS-936559	PD-L1 on tumor cells	No
MEDI4736	PD-L1 on tumor cells	No
MPDL3280A	PD-L1 on tumor cells	No
Lirilumab	Killer-cell immunoglobulin-like receptor (KIR) on NK cells	No
BMS-986016	Lymphocyte-activation gene 3 (LAG3) on tumor infiltrating lymphocytes	No

FDA, Food and Drug Administration; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; NK, natural killer.

results (NCT02292550, NCT02393625, NCT02075840, NCT02271139). ALK inhibitors have also demonstrated efficacy in patients with chromosomal rearrangements of the gene encoding ROS1 proto-oncogene receptor tyrosine kinase, which occurs in 1-2% of patients with NSCLC (36).

Immunotherapy for advanced NSCLC

Utilizing the immune system as an effective oncologic tool to fight cancer has been the subject of preclinical and clinical research for several decades (37). Immunotherapy agents allow the immune system to recognize a patient's cancer cells as foreign, prompting an immune response resulting in tumor cell death and/or inhibition of tumor growth. Newer immunotherapy agents have been developed based on improved knowledge of the molecular process of the immune response, leading to a resurgence in investigative use of these agents for patients with NSCLC. Such checkpoint inhibitors include monoclonal antibodies to cytotoxic T-lymphocyte antigen 4 (CTLA-4) such as ipilimumab, as well as antibodies to programmed death receptor 1 (PD-1), such as nivolumab and pembrolizumab (*Table 2*).

CTLA-4 is responsible for regulation of early T cell activity. It becomes upregulated after antigen exposure and competes for binding with CD28, preventing the stimulatory signal needed for T cell activation. Thus, inhibition of this receptor allows T cell activation after tumor antigen presentation. PD-1 is also upregulated on T cells, but it is thought to play a role further down the immune response pathway within the tumor microenvironment. Binding of PD-1 to programmed death ligand 1 (PD-L1) leads to T cell inactivation, and antibodies to PD-1 allow activation to proceed at the site of direct anti-tumor immune response.

The majority of data for use of these newer immunotherapy agents in NSCLC have been studied in advanced, stage IV patients. Ipilimumab was developed as an IgG1 CTLA-4 monoclonal antibody and was originally investigated in metastatic melanoma. A phase II randomized trial combining ipilimumab with standard first line chemotherapy in patients with stage IIIB-IV NSCLC showed improvement of PFS with the addition of ipilimumab (38). Subset analysis showed that patients with squamous cell histology benefitted primarily from the addition of ipilimumab, prompting an ongoing phase III trial that is comparing standard first line chemotherapy with carboplatin and paclitaxel with or without the addition of ipilimumab in patients with advanced squamous cell NSCLC. Additional trials are evaluating its effectiveness in combination with other targeted or immunotherapy agents (39).

Anti PD-1 antibody agents have been more commonly studied in patients with progressive metastatic NSCLC and showed promising results with prolonged tumor responses (40). Based on the recently published data from the CheckMate 017 and 063 trials in 2014, nivolumab has now received Food and Drug Administration (FDA) approval for treatment of advanced squamous cell NSCLC. Checkmate 063 was a single arm phase II trial in patients that had progressed after at least two prior systemic treatments. Nivolumab achieved an encouraging 1 year survival rate of 41% in these heavily pretreated patients (41). The follow up phase III trial, CheckMate 017, randomized patients with metastatic squamous cell NSCLC who had progressed after doublet chemotherapy to nivolumab or and docetaxel. The trial was stopped early due to superior OS in the nivolumab arm with a median survival of 9.2 *vs.* 6 months

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in the docetaxel arm (P=0.00025). Nivolumab also showed a more favorable toxicity profile compared with docetaxel (42). Additional phase III trials are currently evaluating pembrolizumab monotherapy in both the first line and second line setting for advanced and metastatic NSCLC (NCT02220894, NCT02142738) (38).

Targeted therapy with radiation therapy for localized NSCLC

Many targeted therapies have been integrated into the treatment of localized NSCLC. While the data are much more limited than for the metastatic setting, targeted therapies have been used in combination with or concurrently with radiation therapy. The majority of this data are in conjunction with radiation therapy in the setting of locally advanced NSCLC classically treated with concurrent chemotherapy and radiation.

Preclinical data have shown biologic rationale for combining EGFR inhibitors and radiation therapy. Cetuximab has been combined with chemotherapy and radiation in treatment of locally advanced NSCLC in both phase II and phase III trials (3,43,44). In two sequential Radiation Therapy Oncology Group (RTOG) trials, cetuximab was combined with carboplatin/paclitaxel and radiation therapy for stage IIIA/IIIB lung cancer. While the median survival (22.7 months) and 24-month OS (49.3%) achieved in the phase II study (RTOG 0324) of cetuximab and concurrent chemoradiation were longer than any previously reported by the RTOG (43), the randomized phase III trial RTOG 0617 failed to show a benefit to the addition of cetuximab to chemoradiation in an unselected population (3). Among all patients, median OS in patients randomized to cetuximab was 25.0 *vs.* 24.0 months among those not receiving cetuximab (P=0.29). However, in a planned analysis of the association of EGFR expression and outcome, among patients with an EGFR H score of 200 or higher, cetuximab use was associated with improved OS (42.0 *vs.* 21.2 months, P=0.032) (3).

Gefitinib and erlotinib have also been integrated into both the concurrent chemoradiation setting, as well as a maintenance therapy after chemoradiation for locally advanced NSCLC (45-47). Again, phase III trials have failed to show a benefit to these agents in all subsets of patients, but they have shown improved outcomes in patients who had evidence of EGFR amplification or EGFR mutation, suggesting that in selected patients, these drugs may prolong PFS or OS in combination with chemotherapy and radiation therapy for non-metastatic patients. Newer studies are evaluating the use of these agents in patients with confirmed mutations (NCT01391260, NCT01822496, NCT02277457) (38).

Another area of clinical interest combining radiation and targeted therapy has been in the limited or oligometastatic setting. While the definition of oligometastatic has varied in the clinical literature, there has been increased use of local therapies for patients with limited sites of metastastic disease, especially as the ability to deliver effective local therapies with less morbidity has improved. Given the encouraging local control and limited toxicity profile of SBRT in both the lung and other organs commonly afflicted with metastasis from lung cancer, this remains an active area of research in treating patients with limited oligometastatic disease in combination with targeted agents. One recent published phase II trial showed encouraging results for PFS in advanced NSCLC patients with six or fewer sites of metastatic disease when they were treated with local SBRT to these sites in combination with second line erlotinib (7). Other active studies are similarly looking at this patient population in combination with other targeted as well as immunotherapeutic agents (NCT02450591, NCT0208672, NCT02444741).

As in the oligometastatic setting, the use of radiation therapy can be considered in the oligoprogression setting among patients being treated with TKIs for metastatic NSCLC. While patients with stage IV NSCLC and EGFR mutation or ALK rearrangement have achieved excellent PFS with targeted therapy, disease progression often occurs within a year of therapy initiation. While initial progression of EGFR- or ALK-directed therapy can be diffuse, many patients can have oligoprogression, or limited sites of progression, potentially due to acquired resistance from evolutionary selection on molecularly diverse tumors in which tumor clones in some sites of metastasis but not others develop resistance. Systemic options for such patients include increasing the dose of the targeted therapy they are progressing on, switching to another next-line targeted therapy, switching to cytotoxic chemotherapy, or adding chemotherapy to the targeted therapy (48). However, several groups have recently demonstrated that radiation therapy or other local therapies to sites of oligoprogression can also be considered and can achieve durable local control of the sites of progression and also allow for patients to be maintained on their existing TKI, thus saving alternative or next-line systemic therapy options for subsequent disease progression (49,50).

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Anti-angiogenesis agents typically targeting VEGF have become standard treatment components of therapy for advanced NSCLC. Bevacizumab has been studied in combination with radiation therapy, but this combination has shown a high incidence of tracheoesophageal fistula formation when given concurrently, especially among patients with squamous cell carcinoma and centrally located tumors being irradiated (51).

Given the favorable results in advanced lung cancer, integration of ALK inhibitors into the setting of locally advanced NSCLC has already entered ongoing randomized phase II trials, including NRG/RTOG 1306/NCT01822496, which is evaluating erlotinib and crizotinib as induction therapy followed by standard chemoradiation in patients with confirmed EGFR mutation or EML4-ALK fusion rearrangement, respectively (39).

Immunotherapy with radiation therapy for NSCLC

Although there is limited data to date combining radiation therapy and immunotherapy, this combination has the ability to achieve a synergistic therapeutic effect (52,53). As ionizing radiation can increase the production and presentation of tumor antigens, it can serve to augment the antitumor immune responses achieved by checkpoint inhibitors (54). Radiation therapy can augment immunomodulation by bolstering cytotoxic T-lymphocyte activity (53) and reduce myeloid-derived suppressor cells (55), allowing for synergism with checkpoint inhibitors.

SBRT may be the radiotherapy modality most optimally combined with immunotherapy since it can achieve a more robust immune response than conventionally fractionated radiotherapy. SBRT has been shown to induce cellular expression of major histocompatibility complex (MHC) I, inflammatory mediators, costimulatory molecules, heat shock proteins, immunomodulatory cytokines, adhesion molecules, and death receptors, all of which can enhance antitumor immune responses of systemic therapy (56).

There have been a number of reports in which a distant tumor mass regresses following the administration of radiation therapy before or after treatment with immunotherapy, known as the abscopal effect (57-59). In addition to the abscopal effect, radiation therapy may also allow for immune activation that leads to a more complete or accelerated clearance of the irradiated tumor, or sterilization of microscopic metastasis that were not clinically apparent at the time of irradiation. Aside from case reports, a number of prospective clinical trials have been completed that have combined anti-CTLA-4 therapy and radiotherapy for melanoma (60) and prostate cancer (61) with promising results. A phase I/II study in metastatic castration resistant prostate cancer combining ipilimumab in combination with radiation therapy showed 50% of patients having a decline in prostate-specific antigen (PSA) with one complete response (60). A phase I trial combining ipilimumab and radiation in melanoma showed a response rate of 18% and PFS of 3.8 months prompting further investigation into this combination in the clinical setting (62). To date, no prospective study combining radiation therapy with anti-CTLA-4, anti-PD-1, or anti-PD-L1 therapy has been completed for lung cancer.

Future directions

Targeted therapy and immunotherapy have become pillars of lung cancer treatment. As we gain a greater understanding of the molecular basis of lung cancer, additional targeted agents will become part of standard practice to expand the role beyond the currently limited proportion of lung cancer patients with a known targetable mutation or translocation. Additionally, with increasing knowledge of acquired mutations, second- and third-line targeted agents will become standard options over salvage cytotoxic chemotherapy offering the promise of greater effectiveness and less toxicity. Cooperative group studies combining targeted agents and radiotherapy for non-metastatic patients are ongoing (NCT01822496).

Similarly, immunotherapies will become more entrenched as standard therapy for second-line NSCLC and will be investigated in the first line setting. Combination therapies will increasingly be the subject of investigation, including the inhibition of both CTLA-4 and PD-1, or the use of an immunotherapy agent with a targeted therapy or with a cytotoxic chemotherapy. Toxicities to such combinations, however, may prove prohibitive.

While there is much excitement around the phenomenon of a radiotherapy-induced anticancer immune response and combining radiation therapy with immunotherapy, numerous questions remain before this combination can be exported to routine clinical practice. Additional research is needed to determine if conventionally fractionated irradiation, multi-fraction SBRT, or single fraction SBRT is most effectively combined with immunotherapy, and how radiotherapy and immunotherapy should be sequenced. Like with combination systemic therapies, combining

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radiotherapy with such novel immunotherapies and systemic therapies may result in overlapping toxicities of radiation therapy and immunotherapy. In addition to the immune modulators and checkpoint inhibitors discussed in this manuscript, additional ways to provide tumor-associated antigen to the immune system that can be combined with radiotherapy are currently being investigated, including recombinant vaccines, tumor lysates, and synthetic peptides. While early results are promising, studies combining radiation therapy with immunotherapy warrant careful consideration of toxicity and safety.

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Footnote

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Exploiting sensitization windows of opportunity in hyper and hypo-fractionated radiation therapy

Anish Prasanna[1], Mansoor M. Ahmed[1], Mohammed Mohiuddin[2], C. Norman Coleman[1]

[1]Radiation Research Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health, Rockville, MD, USA; [2]Oncology Centre, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia

Correspondence to: Mansoor M. Ahmed, PhD. Radiotherapy Development Branch (RDB), Radiation Research Program (RRP), Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute/National Institutes of Health, 9609 Medical Center Drive, Rm. 3W224, Rockville, MD 20850-7440, USA. Email: ahmedmm@mail.nih.gov.

Abstract: In contrast to the conventional radiotherapyemoradiotherapy paradigms used in the treatment of majority of cancer types, this review will describe two areas of radiobiology, hyperfractionated and hypofractionated radiation therapy, for cancer treatment focusing on application of novel concepts underlying these treatment modalities. The initial part of the review discusses the phenomenon of hyper-radiation sensitivity (HRS) at lower doses (0.1 to 0.6 Gy), describing the underlying mechanisms and how this could enhance the effects of chemotherapy, particularly, in hyperfractionated settings. The second part examines the radiobiological/physiological mechanisms underlying the effects of high-dose hypofractionated radiation therapy that can be exploited for tumor cure. These include abscopal/bystander effects, activation of immune system, endothelial cell death and effect of hypoxia with re-oxygenation. These biological properties along with targeted dose delivery and distribution to reduce normal tissue toxicity may make high-dose hypofractionation more effective than conventional radiation therapy for treatment of advanced cancers. The novel radiation physics based methods that take into consideration the tumor volume to be irradiated and normal tissue avoidance/tolerance can further improve treatment outcome and post-treatment quality of life. In conclusion, there is enough evidence to further explore novel avenues to exploit biological mechanisms from hyper-fractionation by enhancing the efficacy of chemotherapy and hypo-fractionated radiation therapy that could enhance tumor control and use imaging and technological advances to reduce toxicity.

Keywords: Low Doses Fractionated Radiation Therapy (LDFRT); hyper-radiation sensitivity (HRS); induced radiation resistance (IRR); hyperfractionation; chemopotentiation; stereotactic body radiation therapy (SBRT); stereotactic ablative radiosurgery (SARS); stereotactic ablative radiotherapy (SABR); stereotactic radiosurgery (SRS); spatially fractionated GRID radiotherapy (SFGRT); lattice

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Introduction

Approximately 60% of patients with solid tumors are treated with radiation therapy, which highlights its importance in cancer treatment. For 15% of patients radiation therapy is the only form of treatment and the remaining 45% are treated with radiation combined with chemotherapy. The latter includes breast, lung, prostate, head & neck, bladder, gynecological, pancreas, colorectal and anal cancers and brain tumors (1). The efficacy of radiation therapy, whether treated alone or in combination, can be further improved by adopting recent technological advances and biological approaches. These advances in technology include improved dose distribution with intensity modulated and image guided radiotherapy (IMRT and IGRT), dose escalation (higher dose) and dose intensification (higher and more focused dose). Biological approaches include (I) adopting

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time-honored, "classical" concepts such as DNA damage repair, tumor cell repopulation and cell cycle distribution; (II) exploiting tumor microenvironmental changes such as hypoxia, reoxygenation, vasculature, etc.; (III) use of different types of particles (e.g., protons and carbon ions), which may have a high-linear energy transfer for improved radiobiological effectiveness; (IV) use of altered dose and schedule such as hyper- and hypo-fractionation; and (V) use of radiation protectors and sensitizers including concurrent chemotherapy. In this paper, we define standard fractionation as conventional 1.8 to 2.2 Gy (one fraction per day, five days a week continuing for 3-7 weeks), hyperfractionation as 0.5 to 2.2 (two fractions per day, 2-5 days a week, for 2-4 weeks), and hypofractionation as doses of 3-20 Gy (one fraction a day given for 1-3 days for doses 8-20 Gy).

As with cancer treatment in general, progress in radiation therapy has been steady with much more organ preservation (e.g., head & neck cancer, anal and rectal cancer, esophageal cancer) because of (I) patient selection based on improved clinical parameters, mostly of tumor stage but some with biomarkers such as proliferation and metabolism (e.g., PET scanning); (II) modified surgical/radio-surgical approaches; and (III) use of chemo/hormonal therapy based on pathological and molecular subtype (e.g., breast cancer). Progress is likely to accelerate with the incorporation of emerging new knowledge in cancer biology including tumor classification by molecular characterization and precision medicine, i.e., providing right treatment to right patient. Key to progress relies on well done randomized clinical trials that need to be based on improved preclinical models and careful post-trial analysis because well-conceived hypotheses may not be confirmed for a variety of reasons (2).

It is always wise to exploit what can be exploited based on careful clinical observation—some of which may have been hypothesis driven but much of it may be hypothesis generating based on thorough observations and innovative analyses. Examples from clinical treatments based on so-called "classical" radiation biology includes modifying radiation dose and treatment volume based on the shape of the survival curve (alpha and beta components of the linear-quadratic curve) but it would be preferable to understand the benefits of a particular dose size at the molecular, cellular, and tissue levels. Understanding what happens in various tumor types and relevant normal tissues at the clinically relevant dose fractions of 2 Gy is important, as there are extensive historical clinical-outcome data over many decades. This may help identify targets such as radiation-induced pro-survival factors that can confer induced radiation resistance (IRR). Were those the situation, one could use a particular radiation dose window (below threshold IRR dose) and schedule it in such a way that it does not activate pro-survival events. Resistance to treatment could relate more to factors within the heterogeneous tumor microenvironment niche or to other factors that might benefit from the use of chemotherapy as part of the regimen. The first part of the review will focus on low-dose hyperfractionation (below IRR dose or HRS-inducing dose) and chemopotentiation providing evidence both at pre-clinical and clinical level. In the second part, we provide data that support the contention that high-dose radiation has the potency to induce a robust bystander effect, as well as abscopal (distant) effects (3). Since high-dose hypofractionation regimens are now commonly adopted in the clinic (such as stereotactic radiation surgery), is there a defined dose/fractionation window to exploit certain potential sensitization avenues initiated by abscopal factors that can be potentially combined with agents (including immune modulating agents) or subsequent radiotherapy?

Low-dose hyperfractionation and chemopotentiation

In the past 100 years, the biological effects of various size doses of low-LET radiation have been examined in the clinic as well as by *in vitro* clonogenic assay since first reported by Puck and Marcus in 1955. Radiation hormesis or an effect of radiation at very low doses which can stimulate the repair mechanisms on the cellular level and thereby potentially protect cells from future exposure, are known to be induced at 0.1 to 0.2 Gy (100 to 200 mGy) (4). There is controversy as to what is the lowest radiation dose that can produce radiation-inducible cancer however, at doses above 0.10 Gy there is a risk of radiation-induced carcinogenesis, which increases with dose (5). Generally, at doses above 1 Gy growth arrest occurs and cell killing predominates above 2 Gy. A daily dose size in the range of 2-3 Gy and multiple dose schedules had been empirically selected over the years based on both normal tissue sparing from fractionation and evidence of clinical efficacy. However, as the biological effects of dose have been examined, novel regimens are being explored.

Low dose hyper-radiosensitivity (HRS) and induced radiation resistance (IRR)

Although, there is an understanding of the mechanism of

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cell death by radiation at conventional doses (1.5-2.2 Gy per fraction), the mechanism of radiation effects at lower doses (<1 Gy) is still emerging (6). The initial slope of the radiation cell-survival curve (doses of 0.1-1 Gy) was presumed to be ineffective for human tumor therapy, however, with dynamic microscopic imaging to study the effects of low dose radiation on individual cells within a larger cell population, it was demonstrated that X-rays are effective at cell killing at very low doses, around 0.1 Gy, then become less effective as the dose increased with minimal effectiveness at about 0.6 Gy, and then becoming more effective again as the dose increased to 1.5 Gy and above. This phenomenon is referred to as hyper radiation sensitivity (HRS) (6,7). At doses <1 Gy, many cell lines show low dose HRS (8-10). Interestingly, the HRS is most pronounced in radio-resistant cells, defined in this case as those with mutant p53 expression (11,12). Enns *et al.* (13) examining the response of human A549 lung carcinoma, T98G glioma, and MCF7 breast carcinoma cell lines to gamma radiation in the dose range 0 to 2 Gy, showed marked HRS at doses below 0.5 Gy. It was further determined that low dose hypersensitivity is possibly related to p53-dependent apoptosis, as treatment of cells with Pifithrin, an inhibitor of p53 function, completely ablated HRS. Thus, the role of p53 function in HRS is still unclear and requires further investigation using p53 knockout cell lines and validation in GEMMs.

HRS is evident in murine models (14), but it appears to be an underexplored phenomenon in humans. Since development of resistance is a major cause of treatment failure, circumventing resistance by exploiting HRS would greatly benefit in the treatment of many cancer types. Further, as seen *in vitro* HRS does not involve activation of pro-survival pathways [found at higher doses (15)] (16), providing a mechanism to explain the efficacy of radiation at these low doses. However, as Short and Joiner have pointed out, in order to benefit from low dose-per-fraction radiation in the clinical setting, therapy needs to be extended over 7-12 weeks for sufficient total dose to be delivered. During this prolonged period of treatment, tumor proliferation can occur, which would abate the gain due to enhanced cell killing at HRS radiation doses (17). Prolonged treatment in clinic, lasting 7-12 weeks, will result in several logistic issues as well as increasing cost. Hence, it is logical to combine a radiation dose that results in HRS with chemotherapy to potentiate the effects of chemotherapy and also shorten the treatment time.

In summary, there is a functional evidence for the existence of HRS *in vitro* and its exploitation in the clinic can be challenging. One possibility is to benefit in the clinic from HRS is by using Low Doses Fractionated Radiation Therapy (LDFRT) as a potentiator of systemic chemotherapy that would not trigger the activation of pro-survival pathways in the tumors. Here below, we describe the preclinical evidence to this end.

HRS-inducing LDFRT as a potentiator of chemotherapy: preclinical evidence

Extensive data are available on the HRS/IRR phenomenon observed in more than 40 tumor cell lines in response to single low dose radiation (18,19). HRS occurs after fractionated low doses in *in vitro* (18,19). Pretreatment with paclitaxel followed by multi-fractionated low dose radiation (0.5- or 1-Gy fractions for a total dose of 2 Gy) significantly enhanced the radiosensitizing effect in both HCT-116 and HT-29 cells when compared to single fraction 2 Gy dose (12). LDFRT was found to potentiate the effects of taxanes in head and neck cancer cell lines *in vitro* (15,20) as well as cisplatin in lung cancer cells *in vitro* (21).

The molecular mechanisms underlying the process of chemopotentiation by LDFRT are shown in *Table 1*. In brief, there is involvement of NFκB, NF-Y, bcl-2, XIAP and MDR1 in IRR and at the same time p53, bax, and pro-apoptotic effectors such as cytochrome C seems to be involved (*Figure 1*). Further, in a recent meeting presentation, HDAC inhibitor SAHA (Vorinostat) was combined with LDFRT in GBM cells lines D54 and U118. Findings of this study demonstrated that LDFRT potentiated the effect of Vorinostat in p53 dependent manner with the requirement of PTEN (22). It is important to note that at doses of approximately 0.5 Gy, ATM autophosphorylation occurs in normal cells such as skin fibroblast (23) and peripheral blood lymphocytes (24) resulting in activation of DNA repair programs, but in cancer cells the dose to activate ATM pathways is >1 Gy (25). Thus, it appears that HRS is due to a lack of activation of ATM autophosphorylation pro-survival pathways (*Figure 1*) (modification of apoptosis, NFκB). Thus, these mechanistic data from cell culture studies indicate that chemopotentiation by LDFRT is primarily due to cell killing, thus leading to further studies *in vivo*.

HRS inducing doses in fractionation setting were tested alone or with combination of chemotherapy in several mouse models and the results have not always been reflective of data obtained using cell cultures. For

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Table 1 Potential underlying mechanisms in HRS and IRR doses and in chemopotentiation settings

Treatments	Mechanisms	
	Normal cells	Tumor cells
HRS LDFRT (<0.6 Gy)	ATM activation and DNA repair programs initiated.	Bax upregulation with bcl-2 down regulation; pro-apoptotic proteins upregulated
IRR dose (>1 Gy)	ATM activation and DNA repair programs initiated.	ATM activation, pro-survival transcription factors (NFκB and NF-Y) upregulated, MDR-1 upregulated
LDFRT + chemotherapy	No data	Bax upregulation with bcl-2 down regulation, cytochrome C release; several pro-apoptotic proteins are upregulated XIAP was downregulated, but upregulated in LDFRT-resistant cells
IRR dose + chemotherapy	No data	Bcl-2 and MDR1 protein increased; increase in NFκB and NF-Y activity XIAP is significantly upregulated

IRR, induced radiation resistance; HRS, hyper-radiation sensitivity; LDFRT, Low Doses Fractionated Radiation Therapy.

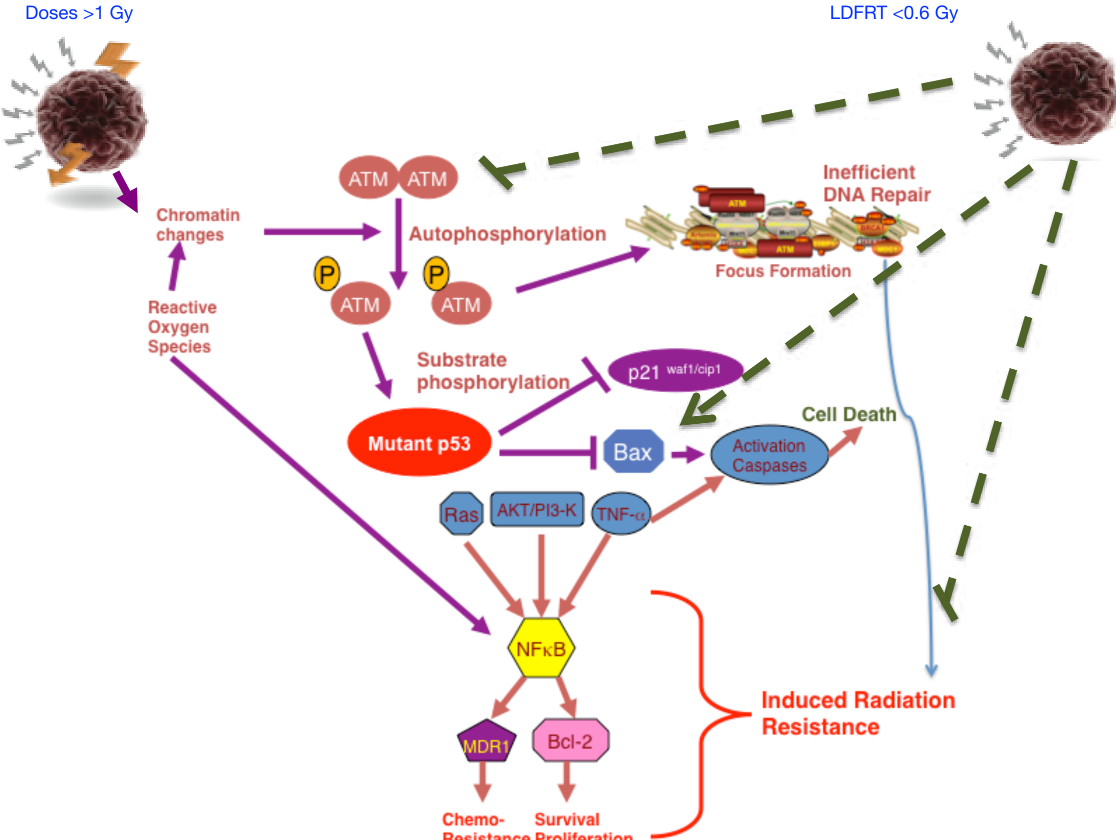

Figure 1 Reported molecular events in IRR and LDFRT. IRR is achieved similarly as DNA damage repair programs such as by activation of ATM, inefficient DNA repair, increase in NFκB, Bcl-2 and MDR1 (purple arrows); along with minimal extrinsic apoptotic induction via TNFα (orange arrows). In LDFRT settings in tumor cells (not in normal cells), ATM kinase is not activated and hence no DNA-repair, lack of increase in NFκB activity as well as in Bcl-2 and MDR1 proteins (green dashed lines). LDFRT activates directly bax to induce an intrinsic apoptotic killing (green dashed lines).

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example one study, compared the effect of low dose ultra-fractionation schedule (0.4 Gy/fraction—126 fractions in six weeks; an approach to exploit the HRS) with the conventional fractionation schedule (1.68 Gy/fraction, 30 fractions in six weeks) of a total dose of 50.4 Gy for inhibiting A7 tumor growth in nude mice (26). Although, ultrafractionation resulted in a significant decrease in tumor growth delay, it also showed a significant increase of the top-up TCD_{50} dose (the dose needed to cure 50% of animals) compared with conventional fractionation dose, but failed to prove the existence of HRS in *in vivo* (26). Thus, despite a pronounced HRS phenomenon observed *in vitro*, ultrafractionation appeared to be significantly less effective than conventional fractionation in the above nude mice xenograft model. The results from this study simply indicate that extrapolation of such data on single dose exposure or a few fractionated doses in *in vivo* is not always predictive of *in vitro* data and does not exclude the potential clinical value (27).

Low dose fractionation allows the delivery of a higher total radiation dose to the tumor for a better result as indicated in the studies below. In a mouse glioma tumor xenograft model, repeated irradiation with low dose (0.8 Gy 3 times/day × 4 days/wk × 2 wks, total dose of 19.2 Gy) was markedly more effective compared to a conventional fractionated dose schedule (2 Gy/day × 4 days/wk × 2 wks; total dose of 16 Gy) in inhibiting tumor growth (28). Similarly, Spring *et al.* (29) showed that LDFRT (0.5 Gy 2 times/day × 2 days/wk × 6 wks; total dose of 12 Gy) significantly prolonged tumor re-growth delay compared to a conventional fractionation dose schedule (2 Gy one fraction/day × 1 wk × 6 wks) in a SCCHN xenograft mouse model (29).

Recently, Tyagi *et al.* demonstrated the capability to deliver ten 0.2 Gy pulses in 8 mins [referred to as Pulsed Low-Dose Radiation (PLRT)] (30). This approach of dose-escalated PLRT was compared with standard radiation therapy (Std-RT), where 2 Gy fractions were delivered continuously in a single fraction in eight minutes, in an intracranial U87MG GBM nude mice tumor model (31). Both PLRT and Std-RT groups received treatments for 5 days/wk. One cohort of mice was treated with 20 Gy Std-RT or 20 Gy PLRT; a second cohort was treated with 30 Gy. Results showed that the mean survival was significantly better with 34.2 days for 30 Gy PLRT compared to 29 days with Std-RT, although there was no tumor cure in either of the groups.

Even though these results imply a minimally a better outcome when radiation is used alone as LDFRT in preclinical models, because of the existence of HRS at lower radiation doses as described above, there exists potential to benefit from the effects of chemotherapy when LDFRT is used in conjunction with chemotherapy. However, demonstration of efficacy of combination of chemotherapy with LDFRT in animal model(s) optimizing dose, time, and sequence is a critical prerequisite for a successful clinical translation.

Below we discuss three such studies in which combination of LDFRT or PLRT with chemotherapy has been used that substantiate potential opportunities for enhancing chemotherapy effects for better treatment outcome. (I) Complete tumor cure was demonstrated in the studies by Spring *et al.* (29), that evaluated the efficacy of LDFRT in potentiating tumoricidal properties of taxotere in SCCHN tumor xenograft animal model. Tumor regression was significant in all LDFRT groups. Mechanistic studies involving molecular analyses of resected tumor specimens showed an increase in Bax levels with an increase in cytochrome c release suggesting an apoptotic mode of cell death in LDFRT chemopotentiation of taxotere effects rather than clonogenic inhibition, albeit G2M cell cycle arrest by taxotere also appears to be an important sequencing component of chemopotentiation. (II) PLRT in combination with Temozolamide (TMZ) was more effective in reducing tumor volume and normal tissue damage and improving survival compared to standard fractionation RT with TMZ in an orthotopic GBM xenograft murine model (32). Increased and differential vascularization and significantly fewer degenerating neurons were seen in normal brain after PLRT with TMZ compared to standard RT with TMZ. (III) Similarly, in an on-going study in a mouse ovarian cancer model, combination of LDFRT with paclitaxel showed significantly improved survival over paclitaxel alone or LDRFT alone. A similar trend was noted when cisplatin was combined with LDFRT in the treatment of ovarian cancer (33) as well as when TMZ was used with LDFRT in the treatment of GBM in mouse models (unpublished observations).

The above preclinical *in vivo* studies assessing the benefit of combining LDFRT or PLRT with chemotherapy demonstrating improved efficacy and survival as well as reducing normal tissue toxicity together with supporting mechanistic evidences provided adequate rationale for conducting safety and efficacy trials in the clinic as these studies might unlock novel treatment avenues for radio-resistant and/or aggressive tumors with poor clinical

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Table 2 Reported clinical trials combining LDFRT with chemotherapy in solid tumors

Clinical trial parameters	Induction regimen	Phase I					Phase II
Site	Locally advanced SCCHN	Recurrent ovarian fallopian tube/ peritoneal cancers	Locally advanced pancreatic or small bowel adenocarcinoma	Stage III/IV endometrial carcinoma	Recurrent/ progressive GBM	Stage IIA/ B-IIIA breast cancer	Recurrent NSCLC
Design	Paclitaxel (225 mg/m^2), carboplatin (area under the curve of 6), and four 80-cGy fractions of radiotherapy (two each on days 1 and 2). This sequence was repeated on days 22 and 23	One of three dose levels of docetaxel (20, 25, or 30 mg/m^2) weekly with concurrent LDFRT given as 60 cGy bid 2 days weekly for 6 weeks	Gemcitabine 1,250 mg/m^2 at 10 mg/m^2/min on days 1 and 8 of a 3-week cycle. LDFRT at two dose levels: 60 cGy per fraction and 70 cGy per fraction on days 1, 2, 8, and 9 for 4 weeks	Six weekly cycles of FD-CDDP (40 mg/m^2, maximum 70 mg IV) + LDFRT at 0.5 Gy/fx (total 3 Gy) and 0.75 Gy/fx (total 4.5 Gy)	LDFRT 0.3 Gy twice daily with cisplatin and fotemustine if progressing on temozolomide, or 0.4 Gy twice daily with temozolomide if recurrent	LDFRT 0.4 Gy/per fraction, 2 fractions per day, for 2 days, every 21 days for 6-8 cycles) with non-pegylated liposomal doxorubicin and docetaxel	Pemetrexed (500 mg/m^2 IV) and concurrent LDFRT (40 cGy bid on days 1 and 2) was repeated fourfold every 21 days
Duration	5 years	2 years	37 months	27 months	20 months		2 years
Recruitment	40	13	10	12	26	10	19
References	Arnold *et al.* (34); Gleason Jr *et al.* (35)	Kunos *et al.* (36)	Regine *et al.* (37)	Wrenn *et al.* (38)	Balducci *et al.* (39)	Nardone *et al.* (40)	Mantini *et al.* (41)

LDFRT, Low Doses Fractionated Radiation Therapy.

outcome (e.g., GBM and ovarian cancers). LDFRT can be exploited to potentiate the effect of chemotherapy for achieving maximum tumor cell killing with significantly reduced toxicity and a favorable clinical translation of the HRS phenomenon observed at low radiation doses to help overcome IRR at radiation doses above 0.6 Gy seen in standard fractionated chemo-radiotherapy regimen. In summary, there is strong pre-clinical evidence and mechanistic reasoning for using HRS low-doses of radiation to potentiate the effects of chemotherapy particularly in hyperfractionated settings.

HRS-inducing LDFRT as a potentiator of chemotherapy: clinical evidence

Several clinical trials have been conducted to assess the benefit of combining LDFRT with standard chemotherapeutic agents for improved outcome (*Table 2*). Arnold *et al.* (34) studied LDFRT as a chemopotentiator of paclitaxel and carboplatin in 40 patients with locally advanced SCCHN. LDFRT was given in two doses of 0.80 Gy (based on the average dose that yielded maximal HRS in four SCCHN cell lines each on days 1 and 2, administered 4-6 hours apart, and the sequence was repeated on days 22 and 23. Definitive RT began three weeks after the last dose of chemotherapy and LDFRT. The combinations of LDFRT, carboplatin and paclitaxel were extremely well-tolerated, with toxicity comparable to that of carboplatin and paclitaxel alone in a similar patient cohort.

Recently, the Arnold group reported 5-year results of the above prospective Phase II SCCHN trial (35). After a median follow-up of 83 months, LRC was 80% and distant control was 77%. Out of 39 evaluable patients, 5-year OS, diseases specific survival (DSS), and PFS were 62%,

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66%, and 58%, respectively. These data strongly indicate a favorable outcome compared to historical controls and excellent compliance with definitive therapy.

In the above trial, the status of p16 was evaluated, which is a validated marker for HPV status and an important predictor of response to various treatment modalities for SCCHN (42). Immunohistochemistry analysis of available 42 pre-treatment specimens showed 15 HPV positive (ten were oropharynx sub group) and 27 (seven were oropharynx subgroup) were negative. Of 15 patients with p16 positive tumors CR, PR, SD and SD were 5 (33.3%), 8 (53.3%) 1 (6.7%), and 1 (6.7%) respectively, compared to 2 (7.4%), 18 (66.7%), 6 (22.2%) and no PD among 27 patients with p16 negative tumors (P=0.0616), respectively. Similar results were also found in HPV positive oropharynx subgroup. Two-year OS was 93.3% for p16 positive patients compared to 73.08% in p16 negative patients (P=0.0252); two-year PFS was 80% (p16 + ve) and 69.23% (p16 – ve). In oropharyngeal subgroup, the 2-year OS was 100% (p16 + ve) and 42.86% (p16 – ve) tumors respectively (P=0.001). These results stress the point that p16 status can be an important predictor of response to LDFRT mediated chemopotentiation induction treatment similar to that seen in standard of care, in head and neck cancer treatment an observation recently described (43,44).

Based on the pre-clinical data (33), the Gynecology Oncology Group (GOG) conducted a feasibility study (36), of whole abdomen LDFRT for patients with recurrent epithelial ovarian fallopian tube, or peritoneal cancers along with weekly treatment of docetaxel 25 mg/m^2. LDFRT was delivered in 60 cGy fractions, twice daily for two days, with a minimum of 4 hr inter-fraction interval, starting on day 1 of each chemotherapy cycle. Three out of four patients completed therapy and none of the toxicities were dose limiting. Another phase I study (38), delivering once a week for six consecutive weeks of morning cisplatin followed 6-8 hours later by afternoon low dose-whole abdomen radiation therapy (LD-WART), enrolled 12 patients with optimally debulked Stage III/IV endometrial cancer. The results suggested feasibility of using LD-WART as a novel chemopotentiator to cisplatin in combination therapy as an adjuvant regimen (38). This trial showed no dose-limiting toxicities with follow-up that ranged from 4-36 months (median: 14 months). These data as well as the data from the GOG trial does indicate that 0.60 Gy/fraction was well tolerated.

Regine *et al.* (37) studied upper abdominal LDFRT given as a chemopotentiator for gemcitabine in patients with locally advanced pancreatic or small bowel adenocarcinoma. Gemcitabine was given at 1,250 mg/m^2 at 10 mg/m^2/min on days 1 and 8 of a 3-week cycle. Low-dose fractionated radiotherapy was tested at two dose levels: 0.6 Gy and 0.7 Gy/fraction. Radiotherapy was given b.i.d. on days 1, 2, 8, and 9. Two of the four patients at dose level 0.7 Gy/fraction experienced dose-limiting toxicity, therefore 0.6 Gy/fraction was deemed the MTD.

Balducci *et al.* (39) reported a study of LDFRT and chemotherapy for recurrent or progressive GBM in 17 patients who had previously received radiotheraqpy and recurred: they received total LDFRT dose of 7.2 Gy in 0.3 Gy fractions with concomitant chemotherapy (TMZ and Fotemustine). LDFRT regimen was well tolerated. In reality, a robust randomized clinical is warranted to establish as a new treatment modality for GBM patients with poor prognosis.

In recurrent NSCLC, Mantini *et al.* (41) found that LDFRT was safe when added to 500 mg/m^2 Pemetrexed as a 10-minute intravenous infusion on day 1 of a 21-day cycle, concurrent with LDFRT on days 1 and 2 at 0.4 Gy twice daily with each faction given 5-6 hrs apart, and the median total dose was 6.40 Gy. LDFRT was also tested in combination with liposomal doxorubicin and docetaxel in stage IIA/B-IIIA breast cancer that led to higher histological response rates compared to the sequential application of the same two drugs (40).

There are three more clinical trials ongoing (http://www.clinicaltrials.gov), which are summarized in *Table 3*. Unfortunately, as with the trials discussed above, none of them is randomized for evaluating the efficacy of LDFRT using robust end-points such as survival or quality of life.

Summary of hyperfractionation

- Over the years clear evidence has emerged from the cell culture studies on the existence of HRS and IRR phenomena that have provided adequate mechanistic rationale for using radiation dose in the HRS range to potentiate the effects of chemotherapy.
- Preclinical *in vivo* animal studies using mouse xenograft tumor models, as discussed above, assessing the benefit of combining LDFRT or PLRT with chemotherapy demonstrate improved efficacy and survival as well as a reduction in normal tissue toxicity and have helped optimize dose, time, and sequence schedule in experimental setting and lead to clinical trials.
- Several Phase I/II clinical trials conducted in different

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Table 3 Open clinical trials combining LDFRT with chemotherapy in solid tumors

Clinical trial parameters	Phase II		
Site	Recurrent Anaplastic Astrocytoma and Glioblastoma Multiforme	Recurrent and Inoperable SCCHN	Recurrent Unresectable Locally Advanced SCCHN
Design	Temozolomide (150 to 200 mg per square meter for 5 days during each 28-day cycle). LDFRT 0.5 Gy of radiation therapy twice daily with the first six 28-day cycles of temozolomide	No description available	Erbitux 400 mg/m^2 as a loading dose one week prior to radiation and taxotere, and then at 250 mg/m^2 given weekly on Mondays. Taxotere 20 mg/m^2 IV once a week on Mondays on weeks 2 to 7. LDFRT 0.5 Gy per fraction BID at least 6 hours apart on Tuesday and Wednesday of weeks 2 to 7 for a total dose of 12 Gy
Duration	1 year	Not available	3.5 years
Recruitment	49	38	35
ClinicalTrials.gov identifier	NCT01466686	NCT01820312	NCT01794845

LDFRT, Low Doses Fractionated Radiation Therapy.

cancer organ sites, such as SCCHN, GBM, ovarian, pancreatic, breast and lung cancers, are in process for an optimized LDFRT dose and schedule in order to potentiate the effects of chemotherapeutic drugs such as cisplatin, taxanes, TMZ, and also demonstrated improved efficacy.

- More randomized clinical trials are warranted to study the role of LDFRT as an adjuvant for chemotherapy in definite settings rather than induction regimen.

In conclusion, LDFRT has some very intriguing preclinical data, however, despite the fact that about ten clinical trials have been or are being performed, at present, it can be concluded that this technique appears to be relatively safe. Based on the reported as well as on-going clinical trials, it still remains unclear whether the patients can be benefited from the addition of LDFRT to chemotherapy and hence better designed prospective trials (randomized against chemotherapy-only controls, and with more robust endpoints such as survival and quality of life) must be conducted to ascertain the value of LDFRT in the management of solid tumors.

Hypofractionation: novel windows of opportunity

To take advantage of the technological ability to deliver precision radiation therapy and to utilize the biological effects of a large dose per fraction as well as the smaller dose per fraction just described, hypofractionated radiation therapy can provide a different pathway of biological effects either used alone or combined with chemoradiotherapy. A potential advantage of hypofractionated radiation therapy, which makes it an attractive approach for the management of advanced cancers, is the reduction in treatment time and cost and reduced burden of frequent and numerous radiotherapy sessions.

Hypofractionated radiation therapy can be approached in two different ways: (I) is to consider α/β ratio and Biologically Effective Dose (BED), where the "classical" concepts of repair, re-assortment, re-oxygenation and re-population (4-Rs) are applicable. This is a categorical approach for hypofractionated radiotherapy that uses 3 to 6 Gy dose fractions; (II) Hypofractionation schedule that uses above 8 Gy doses/fraction in radiotherapy, in which the biological changes different than the "classical" 4-Rs are felt to be applicable, generally known as high-dose hypofractionation radiation therapy (HDHRT). This section of the review will focus HDHRT with more detailed understanding of new radiobiology.

There are data to suggest that the use of HDHRT radiation is effective as an alternative means of dose escalation with conventional fractionation treatment schedule. The results with HDHRT in the early-stage lung cancer population have thus far been very encouraging with local control rates up to 90% (45,46), being superior to the control rates obtained with conventionally fractionated radiation. Biologically, new mechanistic insights suggest that HDHRT may cause four unique effects that can be further exploited for sensitization. HDHRT can (I) cause

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non-targeted pharmacodynamics effects (such as intratumoral bystander as well as abscopal effects) mediated by TNF-α, TRAIL, PAR-4 and ceramide (47-49); (II) robustly induce tumor endothelial death at doses above 8-11 Gy (50); (III) increase host immune recognition of radiation-induced enhanced antigen presentation, such that a single fraction may incite an immune response that enhances the effects of radiation (51); and (IV) result in a better response of that tumors that are heterogeneous with different cell populations, whose clonal radiosensitivity considerably differ (52).

The interaction between HDHRT and hypoxia needs to more fully understood. The effects would depend in part on the initial hypoxic fraction, the dose size used and fractionation, as reoxygenation could occur. Brown *et al.* (53), Song *et al.* (54), suggest the need for drugs to treat the hypoxic fraction whereas Meyer *et al.* (55) suggest that reoxygenation and the selection of a dose at the "hypoxia transition zone" could overcome hypoxia. With other potential mechanism of action of HDHRT, as noted above, studies that determine changes in hypoxia including imaging and biomarkers of hypoxia, as well as studies to modify hypoxia and or use cytotoxic agents would be needed to dissect out the complexity of the effect of hypoxia. Another interesting consideration could be the use of conventional radiation therapy following single high dose or high dose in combination with chemotherapeutic drugs to improve the response of tumors to treatment. There are strong biological data to suggest that a large induction dose of radiation preceding conventional fractionated radiation therapy results in significantly greater tumor regression (56,57). However, high doses of radiation prescribed uniformly to large tumor volumes are generally associated with significant side effects and potentially serious late toxicity, which can take many years to be manifest. At this point in time, there is limited use of high-dose-per-fraction radiation to smaller targets, as in the case of SABR for T1-2N0 lung cancer. In patients with stage III lung cancer, high-dose-per-fraction radiation to the entire target volume is precluded due to normal tissue tolerance. Therefore, future approaches could combine the capability of new imaging and treatment technology for target selection, including novel approaches described next, including HDHRT and its biological properties.

Technical aspects of hypofractionated radiation therapy

The challenges of hypofractionated radiotherapy for better treatment outcome primarily include development of optimal radiation dose delivery techniques. We provide a very brief account of technical development of SRS, SBRT and 3D lattice radiotherapy (LRT), with the understanding that high-dose rate brachytherapy with radionuclides or miniature X-ray source can also be an effective way of delivering highly localized radiation.

Traditionally SRS refers to single fraction stereotactic delivery of an intended ablative dose (58). The first full-scale successful radiosurgery system, Leksell Gamma Knife, was developed in the late 1960s. Since then its successful clinical utilization has established the foundations for intracranial radiosurgery and radiosurgery, in general. Following its success, a number of LINAC-based systems were developed since 1980s (59) and protons beams are also being used (60).

The concept of intracranial radiosurgery was first applied to other body sites in the early 1990s using modified conventional LINACs. The introduction of dedicated radiosurgery systems has widened the application, most noticeably from the early 2000s and clinical efficacy has been well demonstrated (61). In current terminology, SBRT refers to stereotactic body radiation treatments delivered in more than one fraction. While the term SBRT has been widely adopted, it is noteworthy that the difference between radiotherapy and radiosurgery is in the fractional-dose size that ostensibly leads to their differences in therapeutic effects—as a result of different radiobiological effects. The term stereotactic only indicates the method of target localization.

The goal of SABR is to administer a markedly higher dose to the treatment target volume without damaging the surrounding normal tissue thereby achieving enhanced local control and less normal tissue toxicity compared to conventional radiotherapy. The unique physical characteristics of traditional SRS are: high precision (sub-millimeter), highly-focused dose distribution (about a 10% dose fall-off per millimeter outside the treatment margin) and high dose (10 Gy and higher) (58).

In traditional SRS or SBRT, the coverage of prescribed dose to the treatment target volume is to be maximized. In contrast, the spatially fractionated high dose radiation therapy delivered in forms of spatially fractionated GRID radiotherapy (SFGRT) technique covers only partial tumor volume with the prescribed dose (48,49,62).

In the last decade, improvements in GRID design, ability to deliver higher tumor dose by improved target penetration along with reduced normal tissue damage

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as well as superior dosimetry have resulted in dramatic improvements in clinical responses (62-67). Unnecessary high dose exposure of the surrounding normal tissue can be significantly reduced by reconfiguring the GRID treatment into a 3D GRID dose in form of LATTICE. We now define 3D GRID as LATTICE which is a new approach to spatially fractionated radiation that takes advantage of modern-era technology of SABR systems in a safer and efficient way (68). The difference in the dose delivery is shown at the URL (http://assets.cureus.com/uploads/figure/file/538/13fig1.png) published by Wu *et al.* (68). Using this technique, high doses of radiation are concentrated at vertices within the tumor volume, with drastically lower dose between vertices (peak-to-valley effect) and leaving anything outside of tumor volume minimally exposed. Because more pronounced radiation dose peaks and valleys are generated using LATTICE technique compared to 2D-GRID, it may be more radio-biologically effective, with lower radiation dose to adjacent normal tissues resulting in a reduction in normal tissue toxicity.

Hypofractionation and normal tissue toxicity

The α/β ratios derived from linear quadratic model of the radiation survival curve describes the effectiveness of the dose and is used to model cell survival at different conventional doses used in radiation therapy (69). A similar approach has also been adapted to model cell survival with the large doses for hypofractionation studies (70,71). However, this approach may overestimate tumor control. Because of the improvements in radiotherapy planning and delivery, targeting accuracy of radiation to the tumor is also improved with a reduction in surrounding normal tissue damage. It is feasible to use higher doses of radiation per fraction without inducing significant acute and late radiation induced toxicity with SABR. However, concerns still remain on the late toxicity with high dose hypofractionation and it must be emphasized that these may take many years or a decade or more to be seen. An intriguing concept for both technological limitations and capabilities and also for biological advantages is to consider irradiating only limited portions of the tumor and still achieve similar or better outcomes with SABR as discussed next.

When large doses of radiation are delivered to only a fraction of the target volume, scaling back on the irradiated tumor volume invariably results in a reduction of dose to the adjacent normal tissues. Such scaling back of target treatment volume may not compromise the benefits of high dose per fraction for better control because underlying radiobiological mechanisms of damage by large dose per fractions remain the same. SFGRT (2D-Grid) and now LATTICE (3D-Grid), results in a better dose distribution in tumor spatially rather than temporally, which results in significantly improved sparing of normal tissue achieving a better tumor control.

Next we discuss the role of three underlying radiobiological mechanisms of bystander/abscopal effects, activation of immune system, and damage to endothelial cells, that might contributing to a better tumor control with SFGRT and LATTICE in salvage settings, however, needs randomized trials for definitive treatment practices.

High dose radiation-induces factors leading to bystander/abscopal effects

Brooks *et al.* reported the first observation of radiation-induced non-targeted effects in a hamster model (72). Although evidences for these effects have accumulated over time, the exact mechanisms by which they cause tumor regression distant to site of irradiation remains somewhat speculative. A few major mechanistic categories have been proposed to account for abscopal effects based on studies involving different malignancies: immune system, cytokines and pseudo-abscopal effect (73).

Cell-cell communication appears to play an important role in mediating the bystander effect, and there may also be contributions from the transfers of soluble mediators generated in irradiated medium. It is most likely that multiple mechanisms are involved in bystander effects. The presence of gap junctions is not essential. Transfer of radiation-conditioned medium (RCM) from confluent cell culture is more effective, a phenomenon that is termed as "indirect radiation effects" (74-77). Irradiated cells may release clastogenic factors into serum that will induce chromosomal damage when transferred to cultured cells from unirradiated donors (78-80). In a study in rats, for example, clastogenic activity persisted in circulating plasma of irradiated animals for the 10-week duration of the study, and was not abrogated by diluting with non-irradiated serum. Serum irradiated *in vitro* was not clastogenic suggesting that these factors were released from the irradiated cells (81).

Although evidence for the presence of these factors has been accumulating over past decades, their exact nature as well as the mechanisms by which they cause the distant bystander effects (more of an abscopal effect) has proven elusive. One such mechanism might be through radiation-

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induced early genes and induction of cytokines. Indeed, TNF-α and TRAIL are directly involved in apoptosis and are induced by ionizing radiation (82-86). There is a demonstrated correlation of therapeutic efficacy following SFGRT with TNF-α induction in the serum obtained from these patients as well as ceramide production (48,49).

For SFGRT, the "bystander effect" is within the GRID irradiated tumor volume that falls directly under shielded regions (low-dose regions) of the GRID. Bystander factors, such as TNF-α shown by Sathishkumar *et al.* (49) and Shareef *et al.* (47); TRAIL shown by Shareef *et al.* (47) and ceramide shown by Sathishkumar *et al.* (48) are induced in cells that are under the open field of the high-dose GRID areas and are hypothesized to be responsible for initiating the cell death cascade both in the epithelial and endothelial compartments of the tumor micro-environment. Recent reports have demonstrated the presence of radiation-induce signal transduction leading to significant DNA damage and cellular stress (87,88). In addition to the bystander effect within the GRID-irradiated tumor, Peters *et al.* (3) reported that there is robust "abscopal effect" in distant tumors or metastatic lesions that are not irradiated or treated and has been reported clinically with the use of large doses (89).

In this respect, recently using SFGRT we found both bystander and abscopal effects in mice bearing A549 lung adenocarcinoma xenograft contra-lateral tumors (90). Maximal abscopal effect was observed in unirradiated right tumor when mice was exposed to 15 Gy SFGRT followed by 5 fractions of 2 Gy to the left tumor suggesting that the abscopal effect can be amplified by sequential combination of SFGRT with conventional fractionation. More recently, using LATTICE therapy we obtained similar results in mice bearing syngenic Lewis Lung Carcinoma (LLC) contra-lateral tumors (91). These findings strongly suggest that SFGRT is more potent in eliciting evident abscopal effect in the un-irradiated tumor than conventional dosimetric approaches.

High dose radiation activates immune system

There are quite a few reports that support the important role of immune factors in mediating the abscopal effects (92,93). In contrast to the generally believed notion that radiation therapy is immunosuppressive, recent reports indicate ablative high dose radiation therapy could activate immune system and reduce the primary tumor burden as well as distant metastasis (51,94). These effects were mediated by radiation therapy induced disruption of physical and immunologic barriers, stimulation of danger signaling pathways, increase in dendritic cells cross-presentation of tumor antigen, and possibly reversal of T-cell unresponsiveness in tumor-bearing hosts, leading to a rejection of local and distant tumors (51). Subsequently these authors demonstrated that IFN-α/β produced by tumor-infiltrating myeloid cells in an autocrine fashion is required to endow tumor-infiltrating dendritic cells with T-cell cross-priming capacity following local RT; however, T cells do not need to bear the type I IFN receptor to mediate tumor rejection (94). Together, these results score the importance of cytotoxic T-cell mediated antitumor immunity that mediates tumor regression. Our unpublished results show that RCM obtained from lymphoblasts is able to induce killing of lung cancer (A549) cells, suggesting that the immune factors in addition to cytokines and ceramide pathway may be involved. However, in our contra-lateral tumor xenograft athymic nude mice, we observed significant bystander and abscopal effects indicating that not only the T-cell mediated immune factors but also humoral immunity may play an important role in the radiation-induced abscopal effects. These observations suggest potential therapeutic role for immune factors.

Lee *et al.* (51) reported that reduction of tumor burden after ablative radiation depends largely on T-cell responses as it dramatically increases T-cell priming in draining lymphoid tissues, leading to reduction/eradication of the primary tumor or distant metastasis in a CD8(+) T cell-dependent fashion. Interestingly, this study observed that ablative radiation-initiated immune responses and tumor reduction are abrogated by conventional fractionated RT or adjuvant chemotherapy (if given after a week of single ablative dose) but greatly amplified by local immunotherapy. However, in SFGRT settings we observed significant enhanced response when the high dose radiation was followed by fractionated 2 Gy fractions (given after 24 hrs), implying that spatial fractionation of radiation delivery might activate immune factors that can synergize with the conventional fractionated radiation. These results strongly argue for more detailed investigations to elucidate the role of immune factors in radiation therapy.

High dose radiation induces damage to endothelium

Engagement of the vascular component in tumor response to radiation therapy has been a topic of interest in recent literature. However, in addition to release of cytokines, impaired blood vessel formation and induction of endothelial cell death in tumors not exposed to radiation have been demonstrated to play a role in abscopal effect (95).

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Endothelial cells generate 20-fold more of a unique form of acid sphingomyelinase (ASMase), termed Secretory ASMase, than any other cell type in the body. Secretory ASMase activation is required for ionizing radiation to kill endothelium (96), as endothelium in lung, gut, and brain are totally resistant to radiation-induced apoptotic death in the absence of ASMase. Garcia-Barros *et al.* (50) have postulated that high dose radiation-induced damage (15 Gy) to the endothelial cells could convert Potentially Lethal Damage (PLD) in tumor cells and cancer stem cells to lethal damage resulting in tumor cell death. Animal studies have shown that radiation at doses higher than 10 Gy induces endothelial apoptosis by activation of acid sphingomyelinase (ASMase) and ceramide generation (50,96-99); these effects that are not observed with conventional radiation doses. Findings by Garcia-Barros *et al.* (50) suggest that high-dose radiation-induced tumor regression can be entirely dependent on tumor endothelium apoptosis since these effects were abolished in ASMase knockout animals implanted with functional ASMase MCA/129 fibrosarcomas and B16F1 melanomas and restored upon bone marrow transplantation of ASMase functional stem cells. Further, elevated sphingomyelinase activity and ceramide concentration in the serum of patients undergoing high dose spatially fractionated radiation treatment were observed (48). Our unpublished findings in A549 xenografts showed increased elevation of ceramide in the serum of nude mice treated with SFGRT (90).

Although direct killing effect of tumor cells with SFGRT occurs, it cannot completely account for tumor regression observed after treatment. Recently, we demonstrated that treatment of 11 patients with various types of cancer with 15 Gy SFGRT therapy followed by multiple consecutive doses of 2 Gy each led to an increase in the activity of ASMase in serum and a corresponding elevation in the concentration of LDL-enriched ceramide. These changes correlated with the clinical outcome of the treatment, as they were found only in the 76% of patients with CR or PR and not in non-responders (48). It is evident that there is a biologic/therapeutic consequence of this response, whereby high single dose radiotherapy requires ceramide-driven endothelial apoptosis for tumor cure (50,100). This observation has broad implications for cancer treatment and is a subject of active debate in the field, as it is generally believed that radiation therapy works by partly targeting tumor stem cells and it is unclear which components of tumor microenvironment play important role in radiation cure.

There exists data on ceramide production, its relation to endothelial apoptosis and induction of abscopal regression of distant tumor with radiation exposure, however, there is little or any information available on the impact of negative regulators of ceramide pathway in radioresistance/radiosensitivity, their association with release of cytokines, and finally any possible cross-talks during cellular events associated with abscopal phenomena.

Hypofractionation and hypoxia

Tumor hypoxia has been observed in many human cancers and has been a major impediment for the success of radiotherapy. Generally, the phenomenon of re-oxygenation of hypoxic cells between several fractions of conventionally fractionated radiation therapy is considered to increase the sensitivity of the cells that were previously hypoxic. With the encouraging results using SABR or other hypofractionation strategies, this is a point of considerable debate whether the issue of hypoxia under such therapy settings. Taking into account several factors such as the potential over-estimation of cell killing and tumor control by the linear quadratic model at large doses, high dose hypofractionation has actually resulted in greater than expected tumor control. It is possible that single dose hypofractionation induced specific mechanisms abate hypoxia, or that the extreme ablative doses currently used in many SABR protocols are already high enough to overcome hypoxic radioresistance or both. The latter hypothesis implies that concurrent strategies (such as hypoxic cytotoxin) targeted directly at hypoxic cells might improve the therapeutic ratio of SABR and allow clinicians to treat with a larger fraction in the patient population.

Fractional doses in hypofractionation schemes vary significantly in clinical practice, from 3 Gy/fraction to 20 Gy/fraction. There are a number of processes that will be effected by dose size and fractionation that could be exploited, including changes in the "4-R's" (repair, repopulation, redistribution and reoxygenation), consequence of endothelial damage (which could worsen hypoxia) or tumor shrinkage (which could lessen hypoxia) and impact of the high dose on factors secreted by the tumor.

An example of the latter comes from our unpublished results (101). In two lung cancer cell lines, we observed that conditioned media collected from 10 Gy-irradiated hypoxic A549 cells (H-RCM) showed highly reduced cell proliferation effect on normoxic A549 cells when compared

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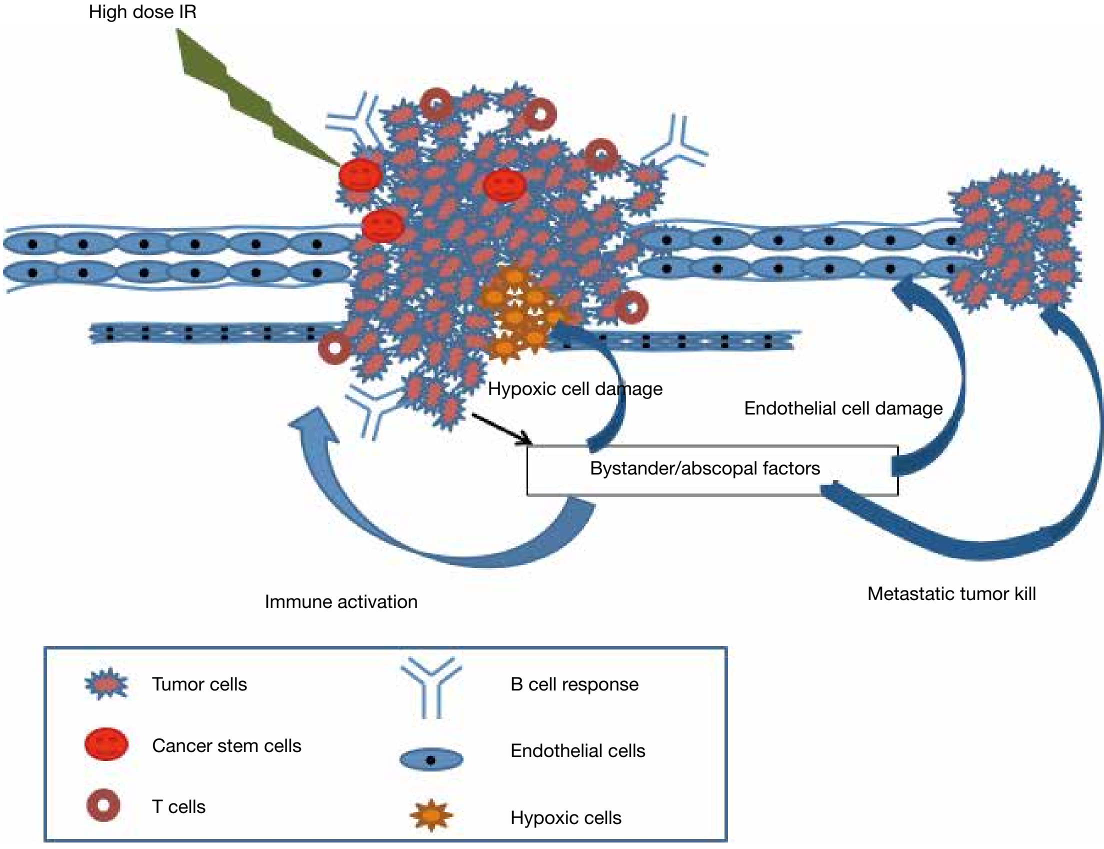

Figure 2 Impact of high-dose ablative RT on tumor micro-environment components. High-dose ablative RT given in lattice (2 vertices) to the tumor induces bystander/abscopal factors, endothelial cell death coupled with immune activation. The underlying radiobiological mechanisms for improved outcome obtained by high dose hypofractionated radiation therapy could be multifactorial. The differential effects on tumor endothelium and cancer stem cells could be responsible for this enhanced response. Further, complex immunological pathways could be linked to high dose radiation-induced mechanisms. All of these pathways could be affected by the bystander/abscopal factors released from the tumor following spatially fractionated radiation therapy. An animation of these events can be found at URL: http://youtu.be/KvQ8z91J6A8.

to media collected from irradiated normoxic A549 cells (N-RCM). Interestingly, with H-RCM obtained from 10 Gy irradiated hypoxic H-460 cells showed a significantly decreased cell proliferation in H460 cells but such reduced cell proliferation was absent with H-RCM obtained from 2 Gy irradiated hypoxic H-460 cells (101). This suggests that oxygen may potentially negate bystander effect. Nonetheless more data are needed, including modeling that would help define the potential complexities, for example, one recently published that aims to account for intercellular signaling (102).

How to best take advantage of the high dose effect but also not damage normal tissue remains to be established. This could include partial treatment of the tumor to high dose using a variety of technique such as the high-dose LATTICE approach. That might have positive effects on damaging the endothelial compartment and/or immune activation. Another important aspect that is not discussed in detail could be differential effect of hypofractionation on cancer stem cells.

Summary of new biology of hypofractionation

- Hypofractionated radiotherapy (>12 Gy) is an attractive approach in the management of cancer although long-term toxicity in patients with curative tumors remains to be evaluated as series mature.
- Success of hypofractionated radiotherapy is dependent

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on its ability to deliver a markedly higher dose to the target volume without damage to surrounding normal tissue. Over the last decade, technological improvement in terms of dose delivery and intra-tumoral spatial distribution of dose seems to have been achieved, with long-term data needed to see if the spatial distribution of dose can reduce normal tissue injury and maintain or even improve tumor control.

- The underlying radiobiological mechanisms for improved outcome obtained by high dose hypofractionated radiation therapy could be multifactorial, which include differential endothelial and cancer stem cell killing, overcoming hypoxic radioresistance, activation of complex immunological pathways, and bystander/abscopal tumoricidal effects, resulting in improved treatment outcome (*Figure 2*).
- There appears to be opportunities to achieve better response of tumors to high dose fractionated radiotherapy by the use of chemotherapeutic drugs or hypoxic cell radiosensitizers.
- While speculative, the use of spatial fractionation in the form of 2D SFGRT and 3D LATTICE in combination with conventional fractionated radiation therapy or chemotherapeutic drugs or hypoxic cytotoxins might be able to counteract the effects of hypoxia with simultaneous normal tissue sparing. In conclusion, ablative hypofractionation schemes are effective in certain solid tumors that may take advantage of new aspects of radiation biology by involving certain components of tumor microenvironment such as effects on vasculature as well as immunologic modulation.SFGRT provided some mechanistic insights pre-clinically as well as from patients (who received SFGRT as salvage therapy), however, to bring SFGRT in the mainstream needs more well designed trials Lattice (3D-Grid) has some promise in the main realm of definitive treatment, however, this approach warrants robust randomized trials. Overall, it is the ablative dose (delivery approaches may differ with or without homogenous dose distribution) that needs further exploration based on clinical observation of its efficacy and preclinical studies.

Overall conclusions

While hyper- and hypo-fractionation are presented as distinctly different, a key point to emphasize is that radiation fraction size and schedule have properties that can be exploited using radiation alone and in combination with immunotherapy, molecular target treatment and cytotoxic chemotherapy. Improvements in imaging and technology of treatment delivery can allow improvement in anatomical targeting and also in treating based on the physiological and biological processes as they present and evolve. New techniques such as LATTICE may be able to take advantage of heterogeneous dose delivery.

While there is a good deal of new and exciting data there is much research to do and, of course, the ultimate proof will be from well-designed clinical trials. Radiation therapy and radiation biology are far from static and with the ability for precision targeting and dose delivery, radiation “as a drug” can have a major impact in multi-modality cancer treatment.

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Improving radiotherapy planning, delivery accuracy, and normal tissue sparing using cutting edge technologies

Carri K. Glide-Hurst, Indrin J. Chetty

Henry Ford Health Systems, Detroit, MI, USA

Correspondence to: Indrin J. Chetty. Henry Ford Health Systems, Detroit, MI, USA. Email: ichetty1@hfhs.org.

Abstract: In the United States, more than half of all new invasive cancers diagnosed are non-small cell lung cancer, with a significant number of these cases presenting at locally advanced stages, resulting in about one-third of all cancer deaths. While the advent of stereotactic ablative radiation therapy (SABR, also known as stereotactic body radiotherapy, or SBRT) for early-staged patients has improved local tumor control to >90%, survival results for locally advanced stage lung cancer remain grim. Significant challenges exist in lung cancer radiation therapy including tumor motion, accurate dose calculation in low density media, limiting dose to nearby organs at risk, and changing anatomy over the treatment course. However, many recent technological advancements have been introduced that can meet these challenges, including four-dimensional computed tomography (4DCT) and volumetric cone-beam computed tomography (CBCT) to enable more accurate target definition and precise tumor localization during radiation, respectively. In addition, advances in dose calculation algorithms have allowed for more accurate dosimetry in heterogeneous media, and intensity modulated and arc delivery techniques can help spare organs at risk. New delivery approaches, such as tumor tracking and gating, offer additional potential for further reducing target margins. Image-guided adaptive radiation therapy (IGART) introduces the potential for individualized plan adaptation based on imaging feedback, including bulky residual disease, tumor progression, and physiological changes that occur during the treatment course. This review provides an overview of the current state of the art technology for lung cancer volume definition, treatment planning, localization, and treatment plan adaptation.

Keywords: Lung cancer; motion management; dose calculation; treatment planning

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Introduction

In the United States, lung cancer constitutes 56% of all new invasive cancers diagnosed, accounting for ~30% of deaths resulting from all cancers (1). Non-small cell lung cancers (NSCLC) account for 80-85% of all lung cancers (2), with locally advanced, stage III disease representing about 40% of the total cases. The prognosis of these patients, even with aggressive chemoradiation techniques, is quite poor, with 5-year overall survival rates of only 10-15% (3). Given the recent seminal finding that low-dose computed tomography (CT) for lung cancer screening reduces lung cancer mortality ~20% when compared to radiography (4), with widespread acceptance, it may be postulated that lung cancers will be found more frequently, and at earlier stages. For early-stage, medically inoperable NSCLC, stereotactic ablative radiation therapy (SABR, also known as stereotactic body radiotherapy, SBRT) has shown remarkable promise, yielding ~90% local tumor control and, in one study, ~55% overall survival at a time point of three years (5).

Recent retrospective research has shown a dose-effect correlation for lung tumors (6-8), however safe radiation dose escalation is complicated by the close proximity of critical organs, and is further complicated by respiration-induced tumor displacement. However, interim analysis of Radiation Therapy Oncology Group (RTOG) 0617, comparing high dose (74 Gy) versus standard dose (60 Gy) radiation therapy (RT) with and without Cetuximab for Stage III NSCLC patients (9), revealed that the high dose

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arm did not improve overall survival, with no significant differences in toxicity between treatment arms (10). While mature results are still lacking, the results of this clinical trial prompted a considerable amount of uncertainty in the Radiation Oncology community (11). It has been suggested that requiring the use of technical advances such as image-guided radiation therapy (IGRT), patient-specific dose levels based on nearby organs at risk (i.e., healthy lung tissue and heart), and motion management may be advantageous in future trials (11,12). Motion management is currently recommended on a patient-specific basis for tumor excursions greater than 5 mm in any direction (13). To further facilitate dose escalation and increase local control, considerable effort has been made to characterize patient-specific tumor motion using the tumor (14-16), the organ in which it is embedded (17), implanted fiducial markers (18,19), or another part of the anatomy presumed to be related to tumor motion (i.e., diaphragm or abdomen surface) (20-22).

Advances in imaging, including four-dimensional computed tomography (4DCT) and volumetric cone-beam computed tomography (CBCT) have enabled more accurate target definition and precise tumor localization for both advanced stage lung cancer treatment and SBRT to further support dose escalation efforts while sparing nearby organs at risk. In addition, advances in dose calculation algorithms have allowed for more accurate dosimetry in heterogeneous media, thereby providing a clearer picture of dose distributions. Finally, new delivery approaches, such as tumor tracking or gating, offer additional mechanisms to reduce target margins. This work will provide an overview of the current state of the art for lung cancer volume definition, treatment planning, localization, and treatment plan adaptation.

Internal target volume (ITV)

In 1999, ICRU Report 62 introduced the concept of the "internal margin", which is meant to incorporate uncertainties arising from physiological variations, such as respiratory motion (23). When the internal margin is combined with the clinical target volume, or CTV, the ITV is formed, which represents the "envelope" encompassing tumor movement determined during the simulation 4D-CT acquisition. The internal margin is expanded to form the planning target volume (PTV), which accounts for geometric variation in the CTV due to day-to-day (interfraction) uncertainties in the patient setup. A margin (planning risk volume, PRV) should also be added to an organ-at-risk to account for interfraction variation in the OAR position (23). Margins for the PTV must be designed with an understanding of the random and systematic errors associated with patient setup (24). For locally advanced stage NSCLC, typical margins for the PTV are on the order of 5-10 mm if an ITV is used for motion compensation and daily IGRT is often employed during treatment. In the absence of motion compensation or IGRT, margins should be much larger (10-20 mm) to minimize the chance of missing the target as a result of motion.

The American Association of Physicists in Medicine (AAPM) Task Group Report No. 76 (13) recommends a variety of approaches to account for respiratory motion. One such example is respiratory-correlated or 4DCT (14,25-27), where organ and tumor motion are both inherently provided during different phases of the respiratory cycle, often sampling data over 10-20 breathing cycles. *Figure 1A* and *1B* illustrate the end-inhale and end-exhale phases of respiratory motion, respectively, for a highly mobile lung tumor. Tumors can be delineated on all 4DCT phases, and a union can be derived to generate the ITV as shown in *Figure 1C*. By contrast, conventional free-breathing CTs (FBCTs) are acquired at arbitrary states of the breathing cycle, during which tumors, nearby critical structures, and corresponding tissue densities are not static, as shown in *Figure 2*. Furthermore, due to the fast acquisition time of FBCT, it is possible to acquire imaging data at an extreme phase of the breathing cycle (i.e., end-inhale or end-exhale). Typically, conventional CT-simulator software employs retrospective temporal (i.e., phase-based) 4DCT sorting into 2-10 different phases, although artifact reduction has been realized through the use of amplitude-based 4DCT binning, particularly for irregular breathing patterns (28). Ten-phase 4DCTs often contain >1,000 CT slices, and may result in reconstruction and sorting artifacts introduced by varied respiratory patterns during a single 4DCT acquisition. This is of particular consequence in lung cancer radiotherapy due to patients presenting with compromised pulmonary function. 4DCT artifacts can lead to discrepancies in target and critical structure delineation, as well as impact the accuracy of dose calculation.

Furthermore, the vast amount of data generated via 4DCT may substantially increase the time needed for image review and target/critical structure delineation. Therefore, a problem arises in how to fully exploit 4DCT data for treatment planning with an emphasis on clinical efficiency without compromising accuracy. To reduce the workload

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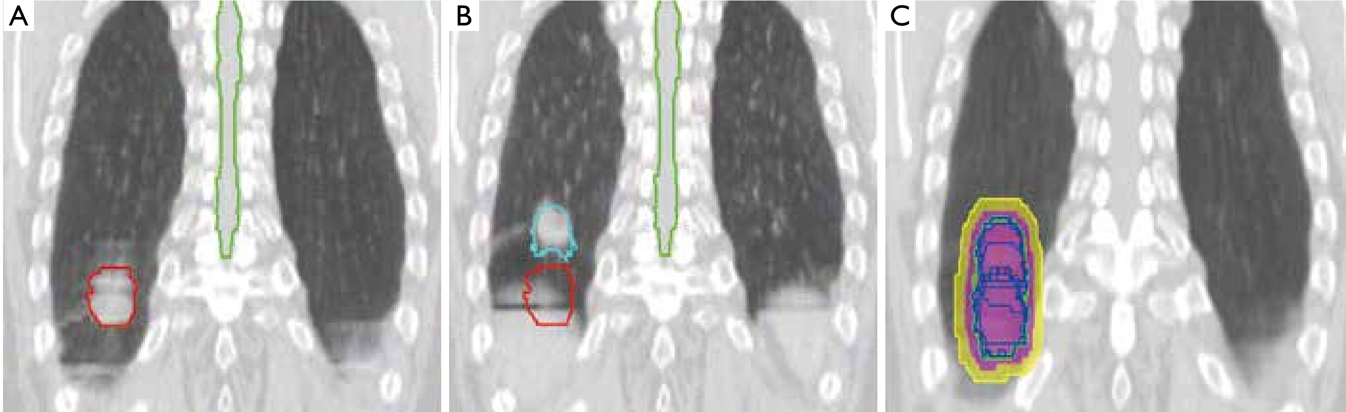

Figure 1 4DCT images of an early-stage lung cancer patient at end-inhalation (A); end exhalation (B); and contours from all 10 phases of the 4DCT combined (C). Abbreviation: 4DCT, four-dimensional computed tomography.

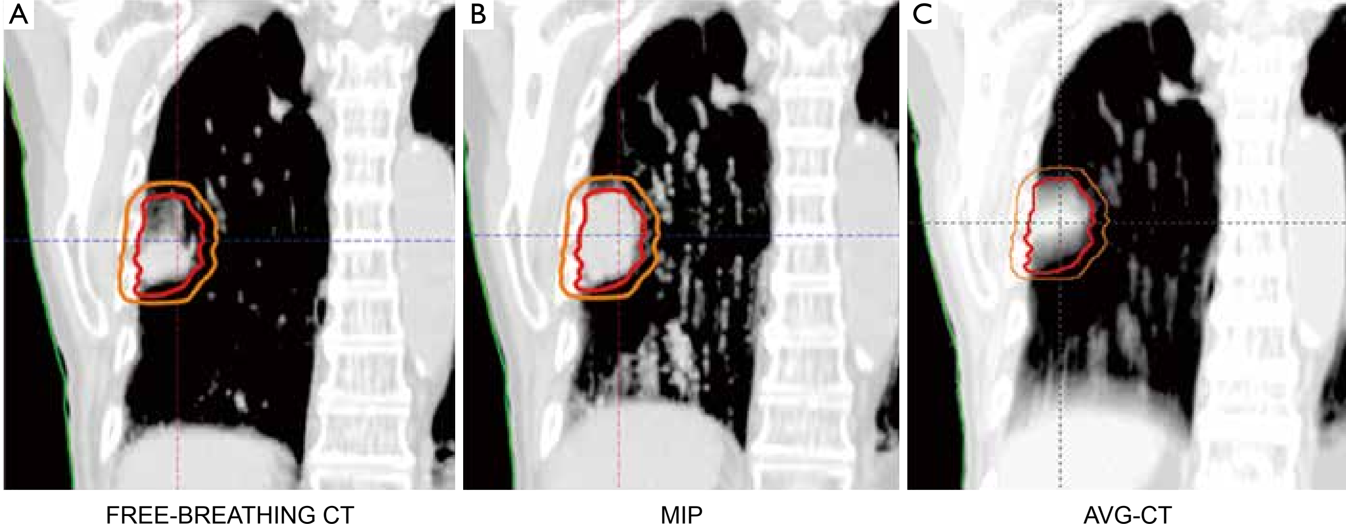

Figure 2 (A) Positional differences between the tumor position on the free-breathing CT; (B) maximum intensity projection (MIP); and (C) AVG-CT, indicating that the FBCT was acquired at an extreme phase of the breathing cycle. Contours show the ITV and PTV. Abbreviations: AVG-CT, average computed tomography; ITV, internal target volume; PTV, planning target volume.

of contouring multiple target volumes in 4DCT, post-processing can be conducted to generate derivative datasets such as the average CT (AVG-CT) and maximum intensity projection (MIP). The AVG-CT data set provides a 3DCT scan with voxels equal to the arithmetic mean of the 4DCT, while the MIP image corresponds to the greatest voxel intensity values throughout the 4DCT. Another commonly used dataset is the mid-ventilation CT scan, corresponding to the specific 4DCT phase with the tumor center of mass closely representing the time-averaged position over the respiratory cycle (29). To further address large 4DCT datasets, several groups have worked toward developing automated contour delineation (30,31), deformable image registration (DIR) techniques (32-34), treatment planning on fewer breathing phases (35), the mid-ventilation phase (29,36), or AVG-CT over the entire breathing cycle (37,38). If 4DCT is not available, end-inspiration and end-exhalation images can be acquired to assess tumor excursion, or the tumor can be observed under fluoroscopy, such as with a conventional simulator.

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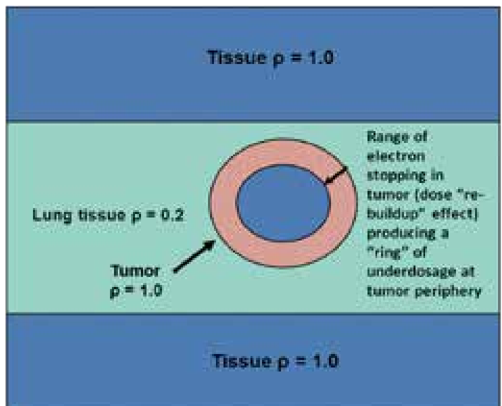

Figure 3 Geometry of an "island-like" lung tumor where electrons scatter laterally into lower density lung tissue, carrying dose away from the tumor. Electrons "stopping" within the tumor deposit dose over a finite range, resulting in an underdosage at the periphery of the tumor. Dose algorithms incorporating 3D scatter corrections, including the effects of electron scattering, must be used to properly characterize dose deposition within the tumor and surrounding healthy lung tissue. Abbreviation: 3D, three-dimensional.

Dose calculation

Dose calculation accuracy is of paramount importance in the clinical treatment process. The AAPM Report No. 85 (39) on Tissue Inhomogeneity Corrections for Megavoltage (MV) Beams notes that a 5% change in dose may result in a 10% to 20% change in tumor control probability (TCP) at 50%, and 20% to 30% impact on normal tissue complication probabilities (NTCP). The report further cites two examples where a 7% difference in dose delivered to different groups of patients was discovered by a radiation oncologist through clinical observations (39).

Dosimetric considerations

The presence of low-density lung tissue surrounding thoracic tumors complicates radiation dose computation in lung cancer treatment planning. Conditions of loss of charged-particle equilibrium (CPE) are produced when the field size is reduced such that the lateral ranges of the secondary electrons become comparable to (or greater than) the field size; such conditions occur for larger field sizes in lung than in water-equivalent tissues due to the increased electron range in lung. Under such circumstances, the dose to the target is determined primarily by secondary electron interactions and dose deposition. Because conventional dose algorithms do not explicitly account for transport of secondary electrons, they can be severely limited in accuracy under non-equilibrium conditions. In low density, lung-equivalent tissues, the reduction of dose due to electron scattering in the lung and the "re-buildup" of dose in the tumor at the lung-tumor interface, as electrons begin to stop in the tumor over a finite range, can produce significant underdosage at the tumor periphery (*Figure 3*). The reduction of dose at the tumor periphery is also exacerbated at higher beam energies, due to the increased electron range. Based on these dosimetric considerations, the RTOG No. 0236 (40) excluded the use of radiation field sizes less than 3.5 cm and restricted the use of beam energies above 10 MV. The article by Reynaert *et al.* (41) and the AAPM Task Group No. 105 (42) provide examples of numerous studies reported on the inaccuracies associated with conventional algorithms for dose calculations in the lung. For lung cancer treatment planning, and especially when dealing with smaller tumors with field sizes <5×5 cm^2, algorithms including three-dimensional (3D) scatter integration such as convolution/superposition, or the Monte Carlo (MC) method are necessary-the latter accounts explicitly for electron transport (43,44).

The AAPM TG Report No. 101 (43) and other articles (45) recommend that pencil-beam algorithms not be utilized for SBRT-based lung dose calculations. The report also states that for the most complex situations, involving small, peripheral lung tumors, surrounded entirely by lung ("island-like" lesions), the MC method is ideal (43). *Figure 4* provides a comparison of the 100% isodose line in a treatment plan for a patient with locally advanced stage NSCLC. Dose calculations were performed using a pencil-beam-type algorithm (dashed line) and the MC method (solid line). Whereas the pencil-beam-based calculation shows good dose coverage of the PTV, significant underdosage is noted with the MC algorithm. This example illustrates that PB-based algorithms are relatively insensitive to the presence of low-density lung tissue and do not account for electron scattering within the surrounding lung tissues. Consequently dose to the tumor is overestimated using PB algorithms, and the "actual" dose delivered, as properly predicted with the MC method, is much lower.

Figure 5 shows dose volume histograms (DVHs) for the PTV for a peripherally located lung tumor with PTV dimensions of ~4.5 cm planned with six MV photons. The prescription dose was 48 Gy (delivered in four 12 Gy fractions) to the 95% line. The initial 3D conformal

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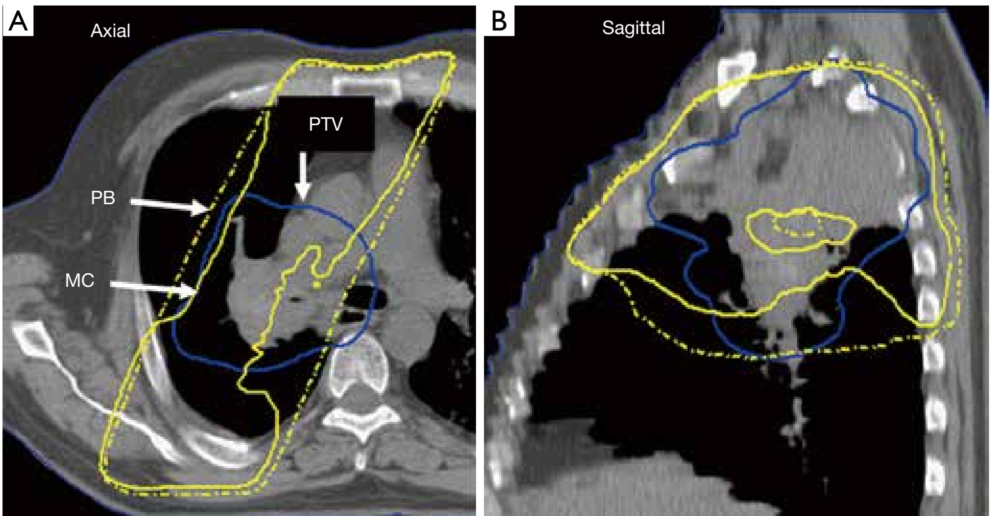

Figure 4 Comparison of 100 % isodose line in a treatment plan for a patient with locally advanced stage non-small cell lung cancer, shown in the axial (A) and sagittal (B) views. Dose calculations performed using a pencil-beam-type algorithm (dashed line) and the Monte Carlo (MC) method (solid line). Significant underdosage of the PTV (solid line) is noted with the MC algorithm using UMPlan (University of Michigan) treatment planning system.

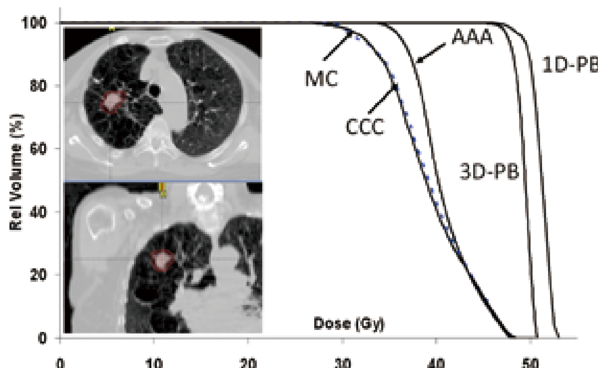

Figure 5 Dose volume histograms (DVHs) for the planning target volume (PTV) for a peripherally located lung tumor with PTV dimensions of ~4.5 cm planned with 6 MV photons. Algorithms include pencil beam-type (1D-PB and 3D-PB), convolution/ superposition type (AAA and CCC) and Monte Carlo (MC). All calculations were done using treatment planning systems at the Henry Ford Hospital. Figure adapted from reference 46.

(3D-CRT) treatment plan was computed with the 1-D PB algorithm. When re-computed with the convolution/ superposition and MC-type algorithms, the "actual" dose to the PTV was much lower than that predicted with the PB algorithm. Both the MC and CCC algorithms show underdosage of the minimum PTV dose of 75% relative to PB (27 *vs.* 48 Gy). Differences in the minimum PTV dose of 25% were noted between MC or CCC and the AAA algorithm; the former which were lower. The substantial differences observed between pencil beam and convolution/ superposition or MC-based algorithms for this particular case can be attributed to several factors, including "island-like" geometry (where the tumor is surrounded entirely by lung), relatively small tumor size, and beam arrangements/ trajectories. Such conditions amplify the effects of electron scattering and the importance of electron transport; differences are therefore not unexpected.

Table 1 provides the results of a retrospective dose calculation study consisting of 135 patients with early stage NSCLC treated with SBRT (46). As in the example provided in *Figure 5*, doses were planned initially using a 1D-PB algorithm to a total dose of 48 Gy (in 12 Gy fractions); treatment plans were recomputed using convolution/superposition type and MC-based algorithms. A recently available algorithm, AcurosXB, uses a discrete-ordinates approach to solve the radiation transport equation. It is similar to the MC method but is deterministic in nature. Results in *Table 1* show that the convolution/superposition, MC and discrete ordinates algorithms predict differences of ~–10% and ~–20% in the PTV mean and dose to 95% of the volume (D95) values relative to the 1D-PB algorithm. 1D and 3D PB algorithms

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Table 1 Absolute dose values (in Gy) of the PTV mean (Dmean), D95, and MLD early stage NSCLC treatment plans treated with SBRT

Algorithm	Dmean (Gy)		D95 (Gy)		MLD (Gy)	
	Avg.	Range	Avg.	Range	Avg.	Range
EPL-1D	49.2	46.8-53.6	48.0	38.5-51.8	3.0	0.6-10.3
EPL-3D	47.9	44.3-53.4	45.9	38.7-51.4	3.0	0.4-10.6
AAA	44.7	37.9-52.5	40.8	31.5-48.7	2.8	0.5-9.7
CCC	45.1	37.4-52.8	40.9	30.0-48.6	2.9	0.5-10.1
AcurosXB	44.3	34.2-52.1	39.8	29.8-47.6	3.0	0.5-10.4
MC	45.0	36.2-52.4	40.9	30.5-49.0	2.9	0.5-10.6

Abbreviations: PTV, planning target volume; D95, dose corresponding to 95% of the volume; MLD, mean lung dose. Both average dose and the range are presented for the EPL-1D (pencil beam 1D), EPL-3D (pencil beam 3D), AAA (convolution/superposition type), CCC (convolution/superposition type), AcurosXB (discrete ordinates-type), and Monte Carlo (MC) algorithms. The dose prescription was 48 Gy (in 12 Gy per fraction) to the 95% line, computed initially using the 1D-PB algorithm. The same monitor units and plan parameters as in the 1D-PB plan were used for computation with all other algorithms. All calculations were done using treatment planning systems at the Henry Ford Hospital, adapted from Reference (46).

are generally within 5% agreement. Differences in mean lung dose (MLD) are not significant, in part because the MLD values are low (~3 Gy). These results confirm that pencil-beam type algorithms should be avoided for thoracic cancer treatment planning, particularly for SBRT.

Treatment planning considerations

Beam arrangements for treatment planning of lung cancers can range from simple two-field, parallel opposed fields (e.g., anterior-posterior, opposed, AP/PA) for late stage NSCLC to complex multiple gantry angle, intensity modulated beams for local or locally advanced disease. Beams are shaped with a multileaf collimator (MLC) which enables conformation of radiation to the target. Treatment plans should be designed to minimize dose to surrounding normal organs and thereby limit the risk of treatment toxicity, implying sharp gradients in the dose fall-off outside the target (43). AP/PA fields may be considered with more extensive, centrally located disease to help reduce dose to the unaffected lung volume. The goal in such cases is to produce a homogeneous dose distribution across the treated volume to encompass the extent of the disease. However, AP/PA beams can only be used for cumulative PTV doses in the range of 45-50 Gy (in 1.8-2 Gy per fraction) due to spinal cord tolerance. "Off-cord" fields are required beyond 45-50 Gy. When treating large volumes of lung, it is especially important to design treatment plans that adhere to normal lung tolerance doses. Dose indices, such as V20, V5 and MLD must be closely observed to avoid radiation pneumonitis and other catastrophic consequences (47,48). For treatment planning of local or locally advanced NSCLC, more conformal dose distributions employing multiple beam angles are warranted. Treatment plans can be developed using 3D-CRT or intensity modulated radiation therapy (IMRT) techniques and must include beams from multiple gantry angles (five or more beams), particularly in the context of SBRT (43), to limit normal tissue sequelae, such as skin erythema, which has been observed clinically.

For IMRT-based planning, one must bear in mind the interplay effect, which describes the interplay between a given MLC position and instance of radiation delivery with the position of the tumor in the respiratory-induced motion cycle at the same instance (49). For conventional 3D treatment, small dose gradients can be expected and moving anatomy within the treatment field will blur the dose distribution, effectively increasing the beam penumbra (13). Conversely, for IMRT, this effect is more marked due to the interplay between the MLC leaf motions and the target motion perpendicular to the treatment beam. To account for this, the dose deposited for each respiratory phase can be computed by the subset of MLC sequences delivered to that specific phase, rather than by the entire MLC sequence delivered in aggregate. The interplay effect has been evaluated for intra-fraction cumulative dose and while the interplay effect was significant for individual phases, it "washed out" in dose accumulation over ten phases. The interplay effect caused less than 1% discrepancy in the PTV and ITV minimum doses using an energy mapping algorithm (50). imilarly, the interplay effect averages out

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over 30 or more treatment fractions (49,51). However, in the SBRT setting, where 3-5 dose fractions are delivered, it is not clear how the interplay will impact dose distributions.

Treatment planning for SBRT must be done with an understanding of the dose gradients so as to develop dose distributions with sharp gradients. This is typically achieved using multiple non-overlapping, and non-coplanar beams as necessary, and a MLC with 5 mm or smaller leaf width (43). The dose prescription line can be low (e.g., 80%) with much smaller margins for beam penumbra ("block edge") than conventional radiotherapy; the motivation is to produce a faster dose falloff and thereby improve sparing of surrounding healthy tissues (43). AAPM Task Group No. 101 discourages the use of calculation grid sizes greater than 3 mm for SBRT planning (43).

Recently, volumetric modulated arc therapies (VMAT) have become available for SBRT-based treatments. The delivery of radiation in significantly less time with VMAT is likely to substantially mitigate patient movement on the treatment table as a result of discomfort during a long treatment procedure, and thereby improve delivery quality (52). Another advantage of VMAT is the ability to deliver multiple beams in different directions and preferentially spare neighboring critical structures. However, one must be cognizant of "low-dose" spread with VMAT, which may be higher than IMRT due to the rotational delivery. As such, parameters such as V5 to the healthy lung tissue must be carefully assessed when using VMAT. Nevertheless, comparisons of VMAT and 3DCRT have revealed no early clinical or radiographic changes in the lung post-treatment (53). Also, as with conventional IMRT, VMAT-based plans are subject to the interplay effect, which must be considered depending on the mobility of the tumor and the degree of modulation of the MLC fields.

4D dose accumulation

With widespread 4DCT implementation, a natural progression has been made to estimating the delivered dose during respiration through the use of 4D treatment planning and dose accumulation (32,54,55). Because the tumor and nearby organs at risk change in density and shape during the different phases of respiration, it is advantageous to calculate dose on each, or a subset, of breathing phases, and accumulate the dose to a reference phase. To accomplish this, DIR is necessary to generate the displacement vector field (DVF) between the source and reference images. DVFs describe the voxel-by-voxel correlation across multiple CT sets, and can be used to map the doses deposited during other phases back to the reference phase. The most straightforward, although not efficient, implementation of 4D dose accumulation is to perform a full 4D dose calculation and calculate the weighted average over the breathing course (35). In an effort to simplify 4D dose calculation and computational expense, reduction in datasets have been proposed such as coupling the DVFs with the AVG-CT to estimate cumulative dose (56), using fewer breathing phases (35), or using the midventilation phase (54,57). All of these approaches have revealed close approximations to a full 4D dose accumulation, thereby supporting integration of cumulative dose into clinical treatment planning. For example, in a patient case that was considered to be the worst-case scenario (tumor abutted the diaphragm with ~2 cm of superior-inferior motion), the largest deviation observed between DIR coupled with full 4D dose accumulation or the AVG-CT was 2% for the maximum dose and dose to 1% of the gross target volume (56) as shown in *Figure 6*.

Another method that has been proposed is to determine the actual energy and mass transferred to that voxel, and then divide the energy by mass to get the dose (termed energy/mass transfer mapping) (58-61). A comparison of direct dose mapping and energy/mass transfer mapping in ten patients with demonstrable tumor excursion revealed similar cumulative doses to the ITV and PTV, although minimum dose differences of up to 11% in the PTV and 4% in the ITV minimum doses were observed between the two dose mapping algorithms with treatment plans computed with AAA (62).

While DIR facilitates cumulative dose estimation, propagated DIR errors will lead to irregularities in automatic contouring, dose warping, and overall dose accumulation. However, verification of DIR is challenging due to the absence of "ground truth". Commonly, visual assessment of the DIR results is conducted, sometimes evaluating propagated contours or the deformed image set (63,64). Others have evaluated DIR performance against physician delineations or noted landmarks (65,66). However, large registration errors are often observed in regions of uniform intensity, and errors estimated by feature-guided evaluation methods may not represent voxel registration accuracy away from those landmarks. Approaches such as evaluating the curl vector (67) or warping images with known DVFs and evaluate the recovered deformations have been implemented (64). Stanley *et al.* benchmarked and evaluated DIR algorithms using patient-specific finite element

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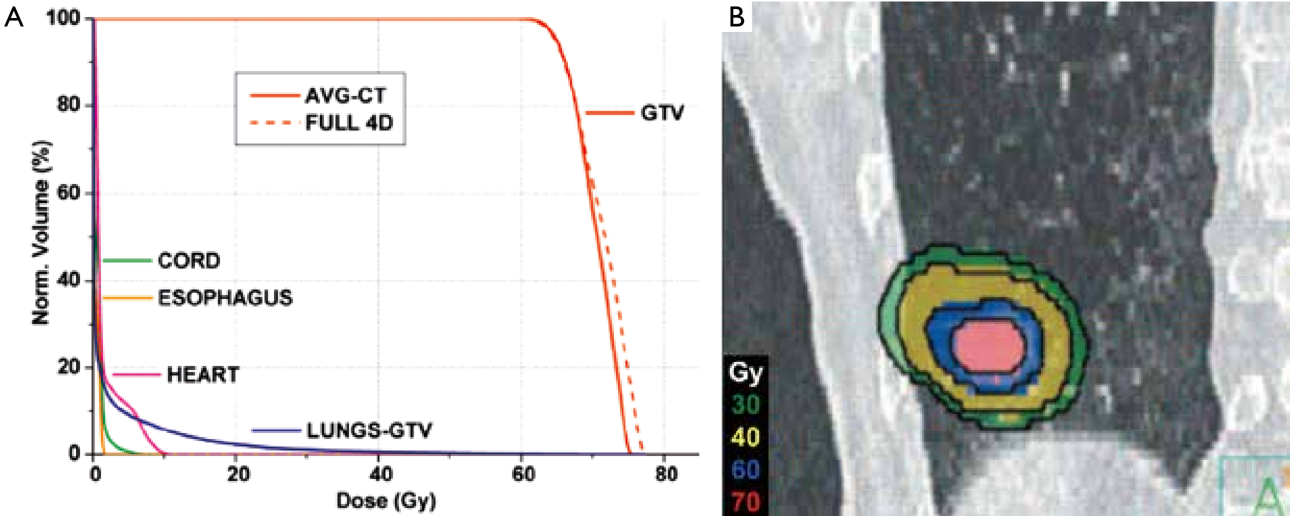

Figure 6 Dose volume histogram (A) and coronal 4DCT data set (B) demonstrating the close association between deformable image registration coupled with full 4D dose summation or using the AVG-CT as an approximation for a patient with 2 cm superior-inferior tumor excursion. Isodose washes represent the AVG-CT approximation while the black isodose lines represent the corresponding full 4D dose summation. Figure adapted from Ref (56). Abbreviations: 4DCT, four-dimensional computed tomography; AVG-CT, average computed tomography; 4D, four-dimensional.

models (FEM) and a physical deformable phantom (68). *Figure 7A* shows a programmable deformable phantom that contains a heterogeneous sponge with average density equivalent to lung (*Figure 7B*) that can be deformed. The modular phantom can be disassembled to insert film or thermoluminescent dosimeters for 4D dose verification.

On-line IGRT

On-line IGRT verifies the target volume and organ at risk locations before daily treatment (inter-fraction) and can also be used to monitor the target during treatment (intra-fraction). Daily IGRT-based setup has been shown to significantly reduce residual errors, and consequently planning margins (69,70). For SBRT-based treatments, where motion management and IGRT are the recommended standard-of-care (43), PTV margins can range from 3-6 mm (69,71-73). On-board imaging can include a kilovoltage (kV) source and flat-panel detector mounted orthogonal to the MV therapy beam axis on the linear accelerator gantry. Image acquisition includes planar radiographic (i.e., kV images), fluoroscopic (cine loops of triggered planar kV images), and volumetric (series of angular projection images reconstructed to generate CBCT datasets (74-78). A chief advantage of kV imaging, particularly CBCT, is the soft tissue visibility, which has been a key component of implementing lung SBRT (70,79,80). Furthermore, because CBCTs are acquired over ~1 minute, the 3D volume represents a time-averaged scan, often indicating the average position of the tumor. Most linear accelerators are also equipped with MV electronic portal imaging devices (EPIDs) mounted at the exit of the treatment beam, which can be used to verify bony landmarks. MV CBCT is also available using an EPID mounted on the treatment beam axis, allowing for volumetric MV imaging.

At Henry Ford Hospital, volumetric CBCT-based imaging is employed to visualize the tumor with respect to organs at risk, for lung SBRT cases. The localization procedure includes setting the patient to tattoos, acquiring a CBCT image, and using automatic image registration tools to align the CBCT to the reference CT. Bony alignment is first verified by the physicist, and manually adjusted if deemed necessary. The physician and physicist then review the registration using soft-tissue window/level and verify that the ITV contour encompasses the lesion. If the lesion falls outside the ITV contour, the physician will manually adjust the registration until the targets are aligned. The image registration is then approved by the physician, and resulting couch corrections are applied. Verification imaging is performed via an orthogonal pair of MV/kV images that are automatically registered to the digitally reconstructed

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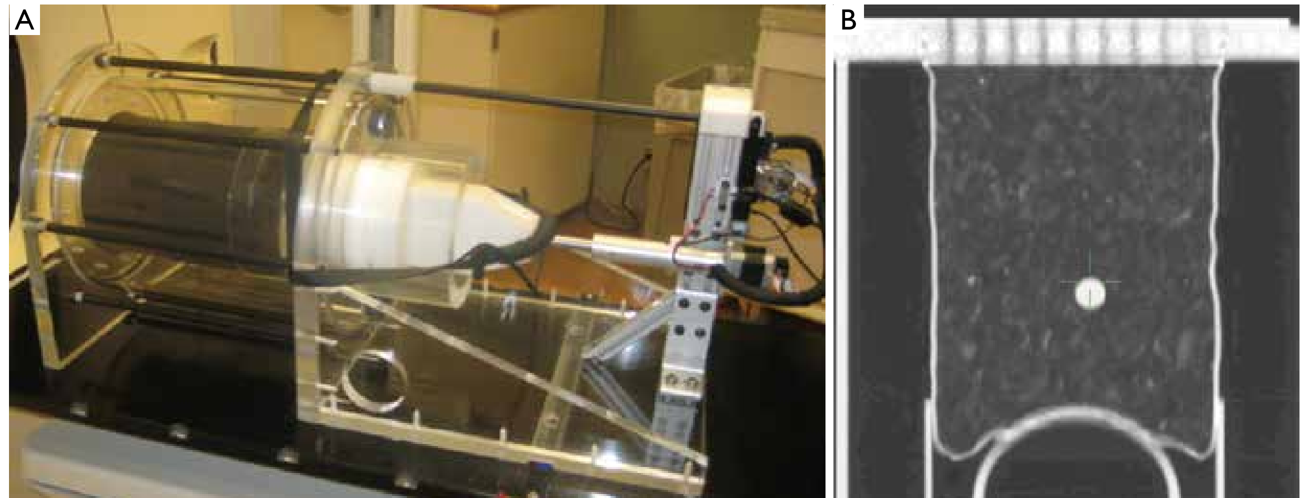

Figure 7 In-house developed deformable lung phantom (A) and coronal cross section (B) showing implanted tumor embedded in the lung material (Courtesy of Hualiang Zhong, Henry Ford Health System).

radiograph (DRR). MV/kV matching ensures the proper couch shift has been applied and the patient has not moved between the original CBCT acquisition and treatment. If the registration result is <2 mm/1 degree (not including shifts made for soft tissue matching in the previous step), treatment commences at the CBCT position. Otherwise, another CBCT is performed and the process is repeated.

Ideally, respiratory-correlated CBCT (or 4D-CBCT) would be implemented to mitigate breathing artifacts while providing the tumor mean position, trajectory, and shape over respiration (81). While the feasibility of 4D-CBCT has been demonstrated on different linear accelerators (82,83), scan times can be on the order of four minutes, yielding ~700 projections of data for sorting, and delivering 2-4 cGy/scan depending on area of interest evaluated (81). Another solution that has been integrated into some clinical workflows include a multiple breath-hold CBCT, often called the "stop and go" CBCT (84,85). Here, CBCT acquisition is paused over multiple breath-holds and the resulting datasets are combined into one final reconstruction.

Tracking

Tumor tracking

Lung tumor motion can be measured and monitored using techniques such as fluoroscopy (15,86), real-time tumor tracking radiotherapy (RT-RT) (18,19), or using implanted fiducials. An example of an in-house analysis program designed to track the tumor and diaphragm in fluoroscopy frames is shown in *Figure 8A* and *B*, respectively. Details and validation can be found elsewhere (20,36), but briefly, a region of interest (ROI) is contoured on a single frame, and a template-matching technique using rigid-body registration and nearest-neighbor interpolation propagated the ROI to all other frames. For patients, ROIs can include the tumor or nearby ROI, apex of the diaphragm, or any other anatomy of interest. Centroids of the propagated contours can then be exported to generate the tumor or surrogate trajectories over fluoroscopic frames.

The fluoroscopic real-time tumor-tracking system (RTRT system) (Mitsubishi Electronics Co. Ltd., Tokyo, Japan) uses four sets of diagnostic X-ray systems oriented with the central axis at isocenter to track gold markers implanted at or near moving tumors (15,87-90). 3D marker positioning is determined via a template-matching algorithm applied to the digital images, and if the measured and expected marker positions do not match inside pre-determined tolerances, a machine interlock is asserted. Clinical outcome data suggests similar local control and overall survival rates for RTRT as compared to SBRT without gating (91). One caveat is that significant skin surface doses (29-1,182 mGy/h) have been reported (92).

Another external-internal tumor tracking modality is the Synchrony™ Respiratory Tracking System (Accuray, Inc., Sunnyvale, CA, USA) integrated with the CyberKnife robotic linear accelerator. Briefly, the Synchrony camera

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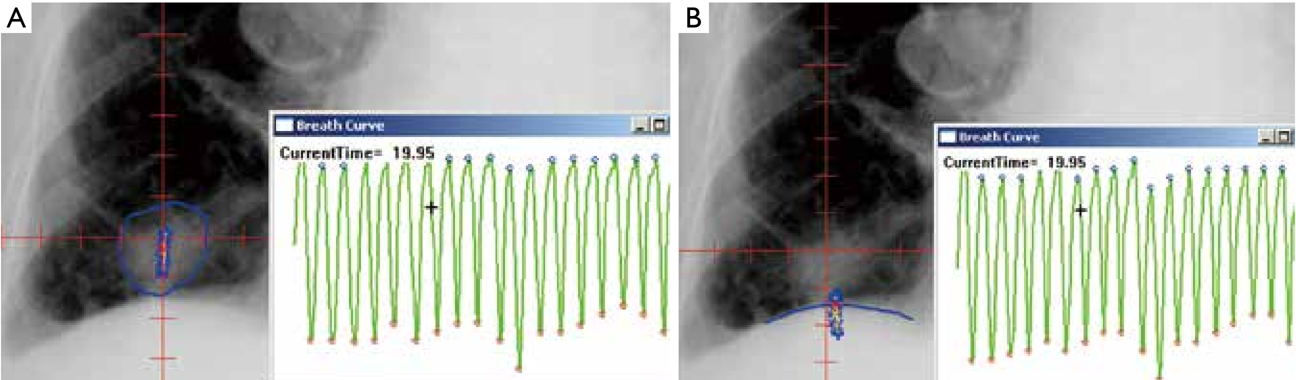

Figure 8 AP fluoroscopy images of an advanced stage lung cancer patient with the tumor (A) and diaphragm (B) tracked using automated in-house software [Courtesy of Jian Liang, William Beaumont Hospital, adapted from Reference (86)].

array tracks three external LED markers affixed to the patient's chest while orthogonal stereoscopic X-ray images are obtained to localize two to four fiducial markers implanted at or near the tumor (93). Real-time feedback from patient monitoring is used to develop a correspondence model, inferring internal tumor positioning from the external surrogates. The correspondence model predicts tumor position, sends feedback to the robotic linear accelerator, and the robot realigns the beam with the tumor. A soft-tissue tracking algorithm has also been reported that can be used for peripheral tumors (diameter >15 mm) in the lung (94). A few disadvantages include the use of ionizing radiation and the additional margin required to account for deformation (94).

The implantation of electromagnetic transponders [e.g., Calypso wireless transponders (Beacons™) currently part of Varian Medical Systems, Palo Alto, CA] at or near the tumor has been widely implemented in prostate cancer RT (95). Briefly, the system uses an array of AC magnetic coils to generate a resonant response in implanted transponders (8 mm length, 2 mm diameter) subsequently detected using a separate array of receiver coils. Beacons' coordinates are identified on a treatment planning CT, and the offset between the beacons' centroid and intended isocenter is reported. During treatment, the Calypso system continuously monitors and reports the 3D offset between the actual and desired isocenter locations at a frequency of 10 Hz. Transponders have been implanted into canine lungs, although migration and transponder expulsion were challenges for the original beacon design (96,97). As a result, a new anchored beacon was devised under an Investigational Device Exemption (IDE) granted by the FDA, and clinical trials are currently underway (98). While tracking implanted markers within the tumor is optimal, the invasiveness of implantation, increased risk of pneumothorax (99), and potential "dropping" or migration of markers from the implantation location (87) can also be deterrents.

External surrogate tracking

External surrogates can infer tumor motion, although they can be limited by the need to verify the relationship with the tumor motion, the potential for external marker placement to affect this correlation (100), and time-dependent characteristics (101). External surrogates of the abdomen can be derived from pressure-sensitive belts, infrared blocks, or surface images. One such example is the Real-Time Position Management Respiratory Gating System (RPM) (Varian Medical Systems, Palo Alto, CA, USA). Briefly, the RPM system uses a plastic block containing two to six markers that reflect infrared light (*Figure 9A*). These markers are subsequently tracked with an infrared-sensitive charge-coupled device camera, and this video signal is transferred back to the RPM computer. RPM can be used for 4DCT sorting, or coupled with respiratory gating with linear accelerators. Another device that derives an external surrogate includes a pneumatic belt (bellows) (Philips Medical Systems, Cleveland, OH, USA) consisting of a rubber belt that expands and contracts as patients' breathing volumes change (*Figure 9A*). Changes in the pressure are converted via a transducer to a voltage signal that is then digitized and sent to the CT scanner system for 4DCT sorting. In a simultaneous comparison of bellows and

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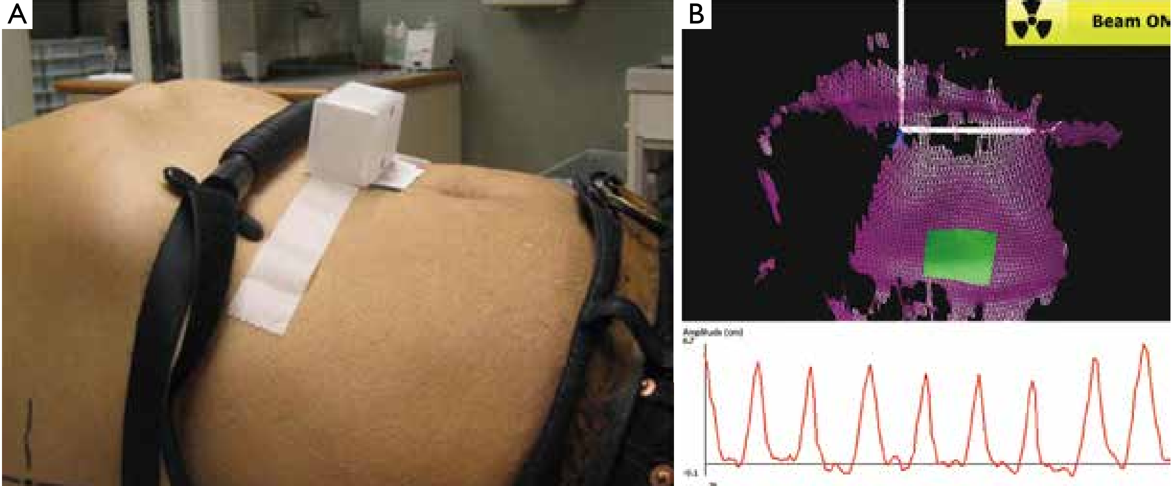

Figure 9 Examples of external surrogates used for patient monitoring. (A) Pneumatic belt placed superiorly of the RPM block; (B) surface images obtained from AlignRT [adapted from Reference (86)]. Abbreviation: RPM, Respiratory Gating System.

RPM, slight differences in waveform and latency analyses were observed, particularly for low amplitude motions. However, these did not adversely impact image quality or delineations (102). Another example of a pressure sensor is Anzai Medical's small pneumatic sensor.

Video camera-based, 3D imaging systems are available that are used to derive 3D surface images during RT, for example AlignRT (VisionRT Ltd., London, UK) and C-Rad Sentinel^{TM} (C-RAD AB, Uppsala, Sweden). AlignRT uses two or three cameras combined with a projected speckled-light pattern to derive 3D surface images (shown in *Figure 9B*), whereas C-Rad uses a line scanning mode with a single camera and laser system. Reference datasets can be derived from RT structure sets (i.e., a CT external structure) or from a previously acquired 3D surface acquisition. Rigid body transformations are used by the systems to perform a least square fit to minimize the difference between the planned 3D model of the patient relative to isocenter and the observed surface model of the patient (103). In a study of simultaneous surface imaging and kV fluoroscopy acquisition of three lung cancer patients in the treatment position, most patient fractions studied showed associations between the abdomen and tumor were equivalent or better than those observed between the diaphragm and tumor. Improved internal-to-external associations have been observed when multiple markers or deformed surface images were used as external surrogates (104-106), although these approaches can be computationally expensive and are not currently incorporated into standard clinical practice. One study explored implementing multiple internal surrogates, such as the air content, lung area, lung density, and body area for 4D CT sorting, and found strong agreement with external surrogates recorded by RPM (107).

Image-guided adaptive radiation therapy (IGART)

While IGRT, such as CBCT, has improved target localization accuracy by providing daily positional information used for online repositioning, daily target and critical structure deformation cannot be fully accounted for using IGRT alone. To combat this, IGART can be implemented. IGART uses patient-specific dynamic/temporal information for potential treatment plan modification during the treatment course (108-110). IGART can address tumor volume and positional changes, as well as other pathologic changes and deformations occurring during the RT treatment course. For lung cancer, inter-fraction baseline variability in lung tumor position, its respiratory trajectory, and normal structures relative to the bony anatomy have been observed (20,36,111-115). Without adjustment, marginal misses can occur. Two cases in point are where a bronchial obstruction is relieved and collapsed lung is re-expanded, resulting in possible tumor shift (116) or in a patient with fluid accumulation in the lungs over the treatment course due to pneumonia (115). Significant reduction in tumor size, particularly for large tumors, has been observed throughout treatment for conventional fractionated radiotherapy of NSCLC

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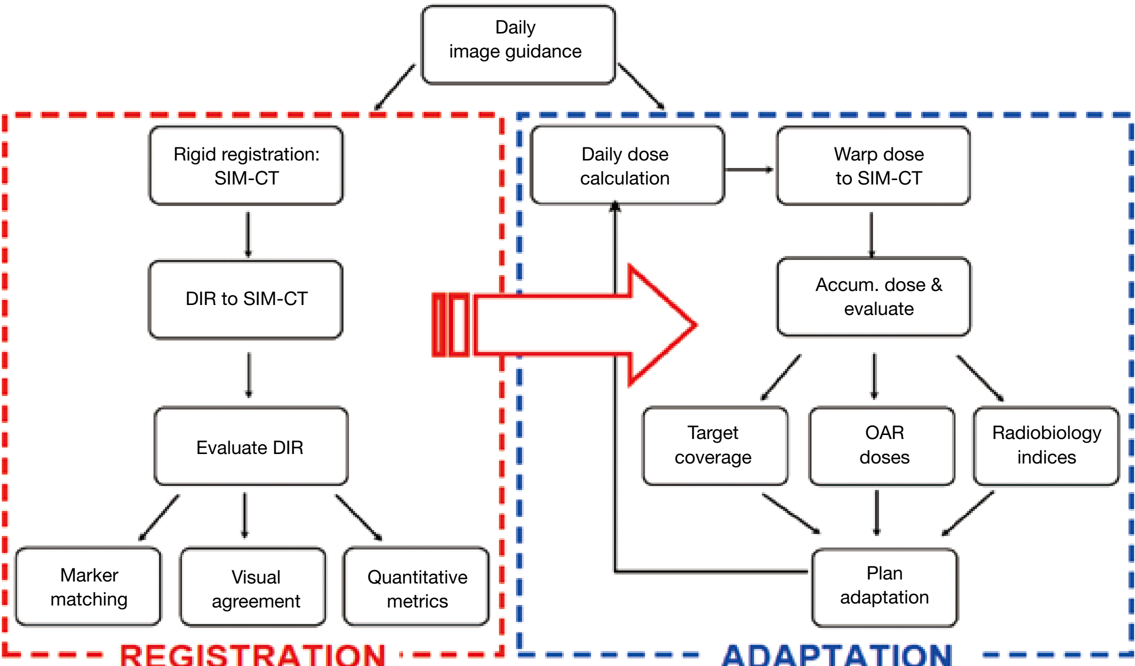

Figure 10 Image-guided adaptive radiation therapy framework developed at Henry Ford Health System. Figure adapted from Ref (120).

(117,118), suggesting that this lung cancer population may benefit most from ART techniques. Conversely, for SBRT, ART has been shown to offer limited value due to the small amount of target volume changes over the shortened time course (119).

To accomplish IGART, a workflow is needed that includes high-quality, temporal volumetric information that is used as a feedback loop in the DIR, dose reconstruction, dose accumulation, and plan adaptation processes (120) as shown in *Figure 10*. An offline IGART framework has been implemented consisting of a closed-loop system incorporating feedback from updated patient geometry (i.e., CBCTs) and anatomical information to recompute dose and determine the actual dose delivered to the target and surrounding healthy tissues (120). Similar concepts have been proposed previously (108,121), although a unique feature of the presented framework is that it includes a systematic validation of the DIR algorithm and dose accumulation techniques.

On-line plan re-optimization using an "anatomy of the day" approach has also been implemented. Li *et al.* have developed new IMRT plans using daily IGRT images using a two-step process: segment aperture morphing (SAM), to correct for target deformation/translation using the MLC, and segment weight optimization (SWO), to determine the optimal MU for each segment (122). Full plan re-optimization can be accomplished in ~10 minutes. While this would be challenging to implement in the clinic, on-line IGART is becoming more realistic due to recent advances in computing such as implementing the graphics processing unit (GPU) (123-125), which has reduced online optimization time from minutes down to seconds.

A prospective, randomized, multi-institutional clinical trial is currently underway to incorporate a during-RT PET/CT-adapted boost for patients with large lung tumors that may potentially benefit from dose escalation (12). In this manner, individualized ART will be performed for patients with inoperable or unresectable stage III NSCLC, a population in which overall prognosis currently remains quite poor despite advances in RT techniques including IMRT and IGRT. Controlled clinical trials such as this will help streamline IGART approaches into clinical practice.

Conclusions and future directions

Lung cancer RT is complicated by tumor motion, challenges of accurate dose calculation in low density media, and changing anatomy over the treatment course, in addition to radiobiologic and individual patient-response-specific issues. As tumor localization improves, whether via high quality

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daily IGRT images or tumor tracking, margin reduction and further dose escalation is possible. Furthermore, dose calculation accuracy has substantially improved in recent years, including the ability to incorporate 3D scatter and implement MC for modeling electron transport, and these algorithms are now available in the clinic. 4DCT and DIR have made dose accumulation and IGART possible, and advances in computational speed will continue to make on-line IGART more clinically plausible over the treatment course.

Some promising new techniques currently being evaluated include incorporating biological feedback into treatment planning, such as dynamic contrast-enhanced MRI (DCE-MRI) as an early indicator of treatment response and perfusion changes (126,127), exploring the role of nanoparticles in lung cancer (128), and exploiting radiosensitizers during RT (129). Finding new ways to assess dose response, normal tissue sparing, and identify opportunities for dose escalation, particularly for advanced stage lung cancer patients, is advantageous.

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Imaging techniques for tumour delineation and heterogeneity quantification of lung cancer: overview of current possibilities

Wouter van Elmpt[1], Catharina M. L. Zegers[1], Marco Das[2], Dirk De Ruysscher[3]

[1]Department of Radiation Oncology (MAASTRO), [2]Department of Radiology, GROW, School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands; [3]Radiation Oncology, University Hospitals Leuven/KU Leuven, Leuven, Belgium

Correspondence to: Wouter van Elmpt, Ph.D. Department of Radiation Oncology (MAASTRO), GROW, School for Oncology and Developmental Biology, Maastricht University Medical Centre, Dr. Tanslaan 12, NL-6229 ET Maastricht, The Netherlands. Email: wouter.vanelmpt@maastro.nl.

Abstract: Imaging techniques for the characterization and delineation of primary lung tumours and lymph nodes are a prerequisite for adequate radiotherapy. Numerous imaging modalities have been proposed for this purpose, but only computed tomography (CT) and FDG-PET have been implemented in clinical routine. Hypoxia PET, dynamic contrast-enhanced CT (DCE-CT),
dual energy CT (DECT) and (functional) magnetic resonance imaging (MRI) hold promise for the future. Besides information on the primary tumour, these techniques can be used for quantification of tissue heterogeneity and response. In the future, treatment strategies may be designed which are based on imaging techniques to optimize individual treatment.

Keywords: Lung cancer; imaging; tumour delineation; heterogeneity

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Introduction

Lung cancer is the single most important cause of cancer deaths in all developed countries (1). In the upcoming countries such as China, it is expected that lung cancer will have epidemic proportions within a few decades (2). Radiotherapy plays an increasing role in all stages of lung cancer: stage I non-small cell lung cancer (NSCLC) is treated with stereotactic body radiotherapy (SBRT) (3), also called stereotactic ablative radiotherapy or SABR with results that equal those of surgery. Stage III NSCLC and small cell lung cancer (SCLC) is most often treated with combined chemotherapy and radiotherapy and patients with oligometastases may experience long-term disease-free survival with treatment that includes radiotherapy (4,5).

However, a thorough definition of the tumour to be irradiated is a prerequisite for successful radiotherapy. Visualisation of the tumour boundaries using morphological imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) are of importance, but also the biological characteristics of the cancer and of the organs at risk (OAR) can nowadays be visualized using molecular imaging e.g., positron emission tomography (PET) techniques. Assessment of this biological heterogeneity of tumours using imaging may lead to more individualized therapy. Using the knowledge of characteristics of the tumour and of the OARs should enable an optimised therapeutic ratio. Although seemingly obvious, reality shows that achieving this goal has been proven to be difficult. Definition of the tumour boundaries with high accuracy and low inter- and intra-observed variability is hampered by the lack of validated automated systems that work well for complicated volumes that are surrounded by OARs with similar densities. Biological characteristics can be imaged, but their implementation in standard practice requires prospective clinical studies showing improved outcomes.

The present manuscript will focus on the delineation and characterization of primary tumour and lymph node involvement in lung cancer patients using the latest available imaging techniques. Some of these techniques are already applied in clinical practice and some of them are

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still on a research level. Furthermore, an outlook is given how to use these methods in the future to individualize lung cancer treatment and to optimize the balance between local tumour control and organ toxicity.

Imaging modalities for target volume delineation and quantification

FDG-PET/CT

The accuracy of FDG-PET is higher than CT for the staging of mediastinal lymph nodes in advanced stage lung cancer. Hence, the incorporation of PET in the treatment planning process of radiotherapy is logical. In many planning studies in NSCLC, the use of FDG-PET has resulted in a decrease of the irradiated volumes of the OARs, which may lead to less side effects or to the possibility of radiation dose-escalation with the aim to improve local tumour control (6,7). Prospective studies both in NSCLC and in SCLC indeed showed that selective mediastinal node irradiation based on FDG-PET scans did not lead to higher isolated nodal recurrences (8-10).

The use of FDG-PET in radiotherapy planning was shown to reduce variability of tumour delineation amongst radiation oncologists and allows automatic tumour delineation that can be followed with manual editing if required (11-13). To use PET/CT equipment directly for radiotherapy treatment planning purposes, some additional criteria have to be considered. A detailed overview on the basic technical aspects and recommendations for radiotherapy treatment planning is described in Thorwarth *et al.* (14). On a standard 3D PET/CT acquisition, small lesions might be difficult to detect due to the intrinsic blurring of breathing motion and might also lead to inaccurate quantification of the standardized uptake value (SUV) compared to respiratory correlated 4D acquisitions (15). PET/CT scanners have options for acquiring the images in a respiration correlated (4D) mode to compensate for breathing motion in thorax. Furthermore, several publications have shown that 4D PET indeed improves lesion detectability (16,17). The 4D scan is usually reconstructed as a set of 5, 8 or 10 3D PET/CT scans representing the different phases of the respiratory cycle (18). Acquiring such a 4D PET scan together with a 4D CT scan is however not yet widely implemented in practice. A drawback of the 4D image acquisition is the somewhat prolonged acquisition times that might limit throughput on the PET/CT scanners and not all software systems are able to visualize this large amount of imaging data. However by using more advanced reconstruction algorithms that use only the part of the acquisition without breathing motion (e.g., the exhale phase) (19,20) or (non-rigidly) register the various breathing phases of the PET image to a single image (21) the workflow might be improved.

Tumour delineation for radiotherapy treatment planning purposes is a time-consuming manual procedure that is associated with a lot of intra- and inter-observer variability (22). Although the use of strict delineation protocols decrease variability (23), the time investment for delineation still remains and is limiting for adaptation protocols as well. As in radiotherapy the CT scan is used as the primary dataset because of the accurate quantification of (electron) density necessary for the dose calculation of the radiotherapy treatment plan, automatic segmentation based on CT scans are logical. Moreover, 4D-CT scans have been implemented in routine practice and this movement information can readily be accounted for in automatic delineation protocols. On the other hand, FDG-PET scans do correlate better with anatomical boundaries than CT if the tumour is surrounded by lung (24). Combining CT and FDG-PET is therefore logical and automatic segmentation methods could reduce delineation time. However, only few studies have validated their automated segmentation method with pathology (22,25-28) and there is a lack of technical validation and accuracy as well (29,30). Fully automated tumour segmentation has therefore not been implemented in routine clinical practice.

Hypoxia PET

Tumour cell hypoxia is a known characteristic of solid tumour lesions, which negatively influences treatment efficacy (31). Accurate identification of tumour hypoxia is of importance to select patients which will benefit from specific anti-hypoxic treatments. The use of the Eppendorf electrode is the gold standard to assess tumour hypoxia, however this method has the disadvantage to be invasive, limiting its use to well accessible superficial tumours (32). Hypoxia PET imaging allows a non-invasive detection and quantification of tumour hypoxia and it provides the opportunity to display the spatial distribution of hypoxia, which is essential for its integration in radiation dose distribution. The most common mechanism to detect tumour hypoxia is the use of 2-nitroimidazoles PET tracers which show a selective binding and retention in the hypoxic tumour cells.

Several 2-nitroimidazoles, labelled with fluor-18 [^{18}F], have already been applied in patients to identify hypoxia.

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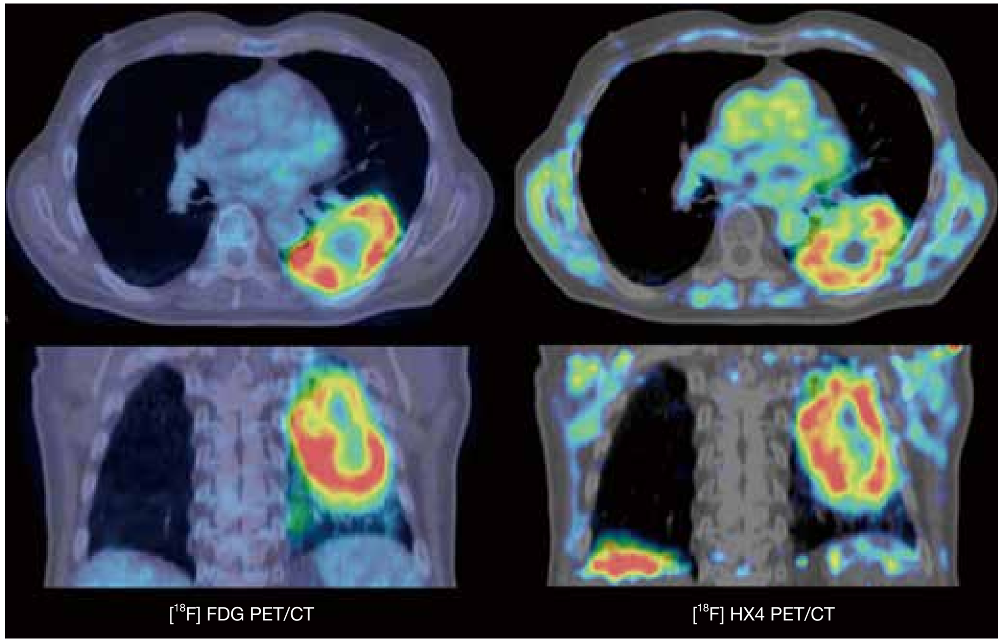

Figure 1 Example of a NSCLC patient having both an FDG-PET/CT scan (left) and a hypoxia HX4-PET/CT scan. Clearly visible is the tumour heterogeneity both on the metabolic (FDG) and hypoxic (HX4) PET image.

The first and most familiar hypoxia PET tracer is [^{18}F]MISO, however, a slow accumulation in the hypoxic lesions and limited normal tissue clearance limits its clinical use (33). Therefore, alternative tracers are developed to improve the pharmacokinetic properties of the hypoxia tracer by enhancing the hydrophilicity and clearance of the tracer, examples are [^{18}F]AZA, [^{18}F]ETNIM, [^{18}F]EF3, [^{18}F]HX4 and the nucleoside conjugate Cu-ATSM.

Quantification of tumour hypoxia based on PET imaging can be performed on static images, acquired at a certain time-point post-injection, or based on dynamic acquisitions, which takes also perfusion of the lesion into account (34). *Figure 1* shows an example of a lung cancer patient having both an FDG-PET/CT scan and an hypoxia [^{18}F]HX4-PET/CT scan. In NSCLC patients, hypoxia PET has shown to be correlated with prognosis and to give different information than FDG uptake (35,36). Studies with hypoxia PET imaging show the presence of tumour cell hypoxia in the majority of NSCLC lesions (37-40). The extent of tumour hypoxia correlates with tumour response and risk of relapse after radiotherapy (41,42). Recent theoretical studies show that boosting or dose painting by numbers based on hypoxia imaging is feasible and that an increased radiation dose to the radio-resistant/hypoxic areas may result in an increased local control (43-45).

MRI

MRI provides high-resolution anatomical information with excellent soft-tissue contrast. Its use for delineation of the tumour and lymph nodes has been investigated. A major issue is obviously the movement of tumours that may cause significant artefacts. To deal with motion, two particular acquisition sequences have been useful: fast low-angle shot (FLASH) and true fast imaging with steady-state precession (TrueFISP) (46,47). Both techniques showed regular and synchronous diaphragm and chest-wall motion of diagnostic quality. Dynamic MRI can be used to define an Internal Target Volume (ITV) as it allows imaging of the entire lung volume over the breathing cycle. However, dynamic MRI scans of the lung are still prone to artefacts, which affect registration accuracy.

To the best of our knowledge, there have been no contouring studies comparing MRI to CT or FDG-PET-CT in lung cancer, neither have there been validation studies with pathology. Nevertheless, to differentiate benign from malignant nodules, Diffusion Weighted MRI (DW-MRI) may have similar accuracy as FDG-PET scans (48).

Dynamic contrast-enhanced CT (DCE-CT)

DCE-CT (or perfusion CT) imaging is a relatively new

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method for tumour characterization. It offers a fast way to assess functional parameters in lung cancer patients. To date DCE-CT is still a research tool, but initial results are showing promising results for the future. DCE-CT scans give information on the blood flow (BF), blood volume (BV) and permeability of the vessels (49-52). Whereas in the literature some DCE-CT studies were hampered by the limited field-of-view (e.g., 3-5 cm) of the scanner in the cranial-caudal direction, the technical infrastructure nowadays has the ability to capture DCE-CT scans of large volumes up to 12 cm. The reproducibility of the extracted parameters of the DCE-CT scan is also within an acceptable range (49,50,53) and allows larger patient studies to look at prognostic factors for treatment outcome. These parameters are related to accessibility for chemotherapy or anti-angiogenesis drugs (54) and shown to be different between treatment responders and non-responders (53). In some series, DCE-CT extracted values correlated with prognosis and with the histological subtype of NSCLC (55). DCE-CT values give other information than FDG uptake and therefore may be complementary to characterise tumours. The clinical and prognostic implications are not yet fully understood and the number of patients who have been studied with DCE-CT is still low. Thus further clinical studies are need to assess the value of DCE-CT for the future individualized treatment and prognosis. In a recent study by Mandeville *et al.* DCE-CT parameters were evaluated in relation to markers of hypoxia (56). It was shown that BV and BF was inversely correlated to immuno-histochemical markers for hypoxia. Recently it has been shown by Lee at al. that reproducibility is high in DCE-CT (57). If DCE-CT is used to measure enhancement curves over time Hwang *et al.* could show that enhancement patterns correspond to tumor staging (58). Interestingly, looking into other body regions DCE-CT parameters might be able to predict survival, as, e.g., was shown by Koh *et al.* in patients with colorectal cancer (59). Spira *et al.* evaluated DCE-CT parameters in correlated these to histopathological findings, showing good correlation especially for microvascular density (MVD) (60). Fraioli *et al.* could demonstrate the correlation between altered perfusion parameters after treatment–indicating treatment response (61).

Dual energy CT (DECT)

Newest CT scanner technology is capable of applying two different kV setting simultaneously or rapidly after each other. The two different resulting scans can be used for tissue characterization and iodine mapping. Some studies tried to use iodine mapping for lung tumour characterization, showing initially promising results (62-64). Initial differentiation between benign and malignant pulmonary nodules seems possible, but the number of studied patients is still too low and the real clinical problem of small pulmonary nodules <8 mm currently cannot be solved sufficiently (65-67).

Imaging modalities for normal tissue characterization

Radiotherapy is always pushing the optimization of maximum tumour control with an accepted (low) level of side-effects. Radiation induced lung toxicity (RILT) is one of the major dose limiting factor in escalating the dose to lung tumours; Therefor assessment of the lung function could potentially play an important role in the design of the treatment plan. Various imaging techniques can be utilized to quantify the lung function also on a local scale, besides the general pulmonary lung function tests that only give a global assessment of the lung function.

SPECT/CT

The use of SPECT/CT for quantification of perfusion and ventilation defects in the lung is a frequently used modality for assessing lung function using imaging although the spatial resolution of the SPECT scan is limited. Radiotherapy has been shown to cause lung perfusion alterations in NSCLC patients with perfusion (68-70). Knowledge about the regional sensitivity and functioning of the lung may also guide the treatment plan design to avoid highly functioning regions inside the lung (71-74). However the hypothesis of reduced lung toxicity still has to be validated in clinical trials.

CT

CT density changes have been described after radiotherapy and show remarkable variability between patients (75,76). In depth analysis of CT characteristics of the lungs may lead to the definition of risk groups for radiation-induced lung damage.

PET/CT

The uptake of FDG in the lungs probably reflects the

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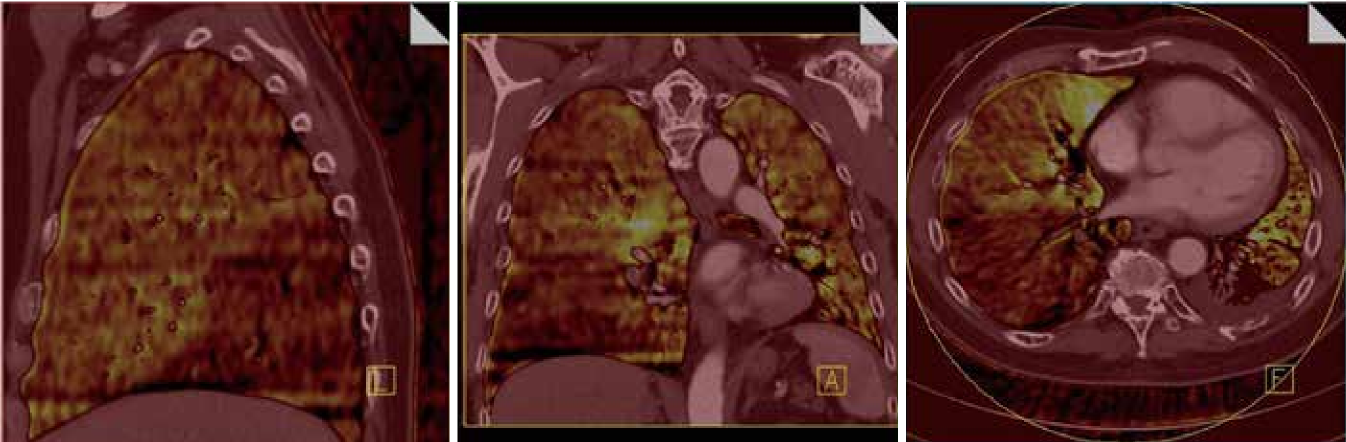

Figure 2 An example of a patient with emboli in the segmental arteries causing a large perfusion defect of the right lower lobe.

inflammatory status. It was found that a high FDG uptake in the lungs before radiotherapy is an independent risk factor to develop subsequent radiation pneumonitis (77). FDG-avid areas in the lungs were at the highest susceptibility for pneumonitis. Further studies are needed to elaborate on these findings before this can be used to change radiation dose distributions in the lungs on the basis of FDG uptake patterns.

MRI

MRI scans using inert hyperpolarised helium-3 gas that is inhaled by the patient show ventilated areas in the lungs (78). Non-ventilated regions do not show an MRI signal. In theoretical studies, the incorporation of this information decreased the V20 of the lungs significantly (78). However, this strategy was never investigated in prospective trials and thus remains investigational.

DECT

DECT for visualizing lung perfusion is often used in the context of the detection of pulmonary embolism (PE) (79-83). An iodine contrast material (CM) is administered and using 2 energy settings of the CT scanner (usually 80/140 kV) it is possible to visualize the distribution of iodine in the lungs. CT is the method of choice to rule out acute PE, nicely showing the emboli up to the sub-segmental level. With the use of DECT it has become possible not only to show the embolus, but also to show corresponding perfusion defects. This is of clinical importance, as was shown in earlier studies—single sub-segmental emboli (not causing significant perfusion defects) can be left untreated (84). Based on the assumption that radiation therapy of the lung may also alter CM perfusion in the lung, this technique offers potential for further assessment of patients treated for lung cancer with radiotherapy. *Figure 2* shows an example of a PE in the right lower lobe causing a large perfusion defect.

While DECT is primarily used for iodine perfusion maps of the lung, Xenon ventilation consequently adds the missing part of ventilation maps for the patients. In the last years some study groups could show that the use of Xenon ventilation is feasible and safe and could also show that ventilation maps may add additional value in different pathologies such as asthma, in intensive care patients or even in children (85-93).

Treatment individualization using imaging

The next major step forward that is currently tested in clinical trials is the dose-painting hypothesis (94,95). The rationale for this is the heterogeneous nature of tumours. Differences in biological characteristics throughout tumours make them respond non-uniformly to treatment (96). Hence treatment resistant parts of the tumours are with the current homogeneous irradiation treatment techniques not optimally treated. Individualizing the treatment by using imaging information to guide or define the actual dose-response relationship is the next phase of treatment individualization (97). A currently on-going multi-centric trial in advanced NSCLC is testing the hypothesis whether a uniform dose or a boost dose to the high metabolic active

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volumes gives rise to better local control rates (98).

Another way of using imaging information to individualize treatment is in the context of response assessment. Using repeated imaging during treatment may provide predictive information to treatment success. Hypoxic (e.g., HX4, FAZA, FMISO), metabolic (e.g., FDG) or proliferation [e.g., FLT, (99)] PET tracers allow early in the course of treatment already an assessment of treatment (100). MRI scans can be used to evaluate changes in tumours during radiotherapy as well (101). DW-MRI derived ADC (apparent diffusion coefficient) values changes correlate well with survival. However, ADC and FDG changes also correlate significantly. It remains unclear what the clinical value is of these predictive parameters.

With the current fractionated radiotherapy schedules in lung cancer of 4-6 weeks, there is still room for adaptation of the treatment. As previously stated, these adaptations of the treatment plan can be based either on reducing side-effects or increasing the chance of local tumour control.

Conclusions

Imaging is an integral part of target volume delineation used in current clinical practice. Tumour characterization is the next step that needs to be exploited. To fully optimize the therapeutic ratio also normal tissue toxicity is of importance. Assessment of imaging features to characterize tissue functioning should be explored as well in the context of individualized treatment optimization.

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Hyperfractionated and accelerated radiotherapy in non-small cell lung cancer

Kate Haslett[1], Christoph Pöttgen[2], Martin Stuschke[2], Corinne Faivre-Finn[1,3]

[1]Radiotherapy Related Research, The Christie NHS Foundation Trust, Manchester, UK; [2]University of Duisburg-Essen, Department of Radiotherapy, Essen, Germany; [3]The University of Manchester, Oxford Road, Greater Manchester, UK

Correspondence to: Dr. Corinne Faivre-Finn. Radiotherapy Related Research, The Christie NHS Foundation Trust, Manchester. M20 4BX, UK. Email: corinne.finn@christie.nhs.uk.

Abstract: Radical radiotherapy plays a major role in the treatment of non-small cell lung cancer (NSCLC) due to the fact that many patients are medically or surgically inoperable. Advances in technology and radiotherapy delivery allow targeted treatment of the disease, whilst minimizing the dose to organs at risk. This in turn creates an opportunity for dose escalation and the prospect of tailoring radiotherapy treatment to each patient. This is especially important in patients deemed unsuitable for chemotherapy or surgery, where there is a need to increase the therapeutic gain from radical radiotherapy alone. Recent research into fractionation schedules, with hyperfractionated and accelerated radiotherapy regimes has been promising. How to combine these new fractionated schedules with dose escalation and chemotherapy remains open to debate and there is local, national and international variation in management with a lack of overall consensus. An overview of the current literature on hyperfractionated and accelerated radiotherapy in NSCLC is provided.

Keywords: Accelerated radiotherapy; hyperfractionated radiotherapy; non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer is a major public health concern worldwide. Progress in improving 5-year survival is lagging behind comparable survival rates in other common cancers. Population-based lung cancer registry data analysis shows only a minimal increase in survival from 7-16% between 1995-1999 to 8-18% between 2005-2007 (1).

The majority of patients with locally advanced non-small cell lung cancer (NSCLC) are not suitable for surgical resection, often due to pre-existing co-morbidities and poor performance status. The international standard of care is concurrent chemo-radiotherapy which is associated with a 5-year survival of 20-30% and a median survival of 17-28 months (2-6). Due to the potential toxicity of concurrent chemo-radiotherapy patient selection is important. Patients with a good performance status, without major co-morbidities and assuming an acceptable radiation dose to normal tissues are eligible for this intensive treatment (7,8). Alternative treatment options are sequential chemo-radiotherapy or radiotherapy alone. Radiotherapy alone is associated with a 5-year survival of less than 5% due to local, regional and distant relapse. Local control with standard 3D conformal radiotherapy remains poor, with reported two years loco-regional control rates of 20-44% (9-11).

However, recent studies have shown that better local control of lung cancer can lead to an improvement in overall survival (10), prompting interest in altering radiotherapy delivery regimes. High dose stereotactic ablative body radiotherapy typically delivering >100 Gy biologically effective dose (BED) in 3-8 fractions is associated with very high in-field local control rates, but such doses cannot be delivered safely to locally advanced tumours due to the proximity of organs at risk such as the proximal bronchial tree, heart and spinal cord. A gap between radiation

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fractions allows recovery of damage in normal tissues and may also increase the sensitivity of the tumour cells to radiation by processes such as reoxygenation (12). If the individual fraction size is reduced and the fractions delivered closer together (e.g., twice daily), it may be possible to increase the dose without detriment to normal tissues.

One of the strategies to improve local control is dose escalation. Evidence gathered from the standard radiation schedules utilised in NSCLC over the past 40 years have confirmed the importance of total dose as a factor in tumour response (13). These schedules often use a single treatment of 1.8-2 Gy fractions per day over 5 days per week for a period of 5-7 weeks.

The RTOG 0617 study has evaluated dose escalation in the context of standard fractionation (2 Gy/day) and concurrent chemo-radiotherapy (5). Unfortunately the study was closed early due to futility indicating the absence of a survival benefit to high dose radiotherapy (74 Gy in 37 fractions delivered over 7.5 weeks) compared to standard dose (60 Gy in 30 fractions delivered over 6 weeks) (5).

An alternative approach to increasing the biological tumour dose in NSCLC is to develop new fractionation regimes, most commonly by hyperfractionation or acceleration. Hyperfractionation is a radiation treatment in which the total dose of radiation delivered is divided into smaller doses and treatments are given more than once a day (typically 2-3 a day). Acceleration means radiation treatment in which the total dose of radiation is given over a shorter period of time (fewer days) compared to standard radiation therapy. A recent meta-analysis by Mauguen and co-workers, evaluated ten trials including 2,000 patients and concluded that modifying the radiotherapy schedule by hyperfractionation, acceleration or both resulted in an increase in overall survival (14). The use of modified radiotherapy led to a 12% reduction in the risk of death (P=0.009). The absolute increase in overall survival in the NSCLC patients was by 3.8% at three years and 2.5% at five years, improving the survival rate from 15.9% to 19.7% at three years and from 8.3% to 10.8% at five years (14). Modified radiotherapy increased the risk of acute severe oesophagitis from 9% to 19% (P<0.001), and as expected the most accelerated regimes were associated with the most severe toxicity. However, at least 90% of patients completed the planned radiotherapy, with compliance in the experimental arms similar to the control arms. A summary of both hyperfractionation and acceleration is presented below.

Hyperfractionation

Early clinical trials evaluating hyperfractionation in the late 1980's and early 1990's investigated the benefit of adding chemotherapy to radiotherapy. The RTOG 8808-ECOG 4588 randomised 458 patients to two months of induction chemotherapy with cisplatin and vinblastine, followed by conventional radiotherapy (60 Gy in 2 Gy per fraction), or radiotherapy alone, with either the same radiotherapy regime or a hyperfractionated regime of 1.2 Gy per fraction delivered twice daily to a total dose of 69.6 Gy (15,16). This study showed that patients receiving induction chemotherapy did best, with a median survival of 13.2 months and a 5-year overall survival of 8% (P=0.04). Although the twice-daily radiation arm performed slightly better compared with the conventional radiation arm, the difference was not statistically significant (median survival 12 *vs.* 11.4 months, 5-year overall survival 6% *vs.* 5%).

The trials evaluating hyperfractionated radiotherapy are summarised in *Table 1*. One of these pivotal trials in demonstrating the advantage of concurrent over sequential chemo-radiotherapy was the RTOG 9410 study (17). It also addressed the important question of overall treatment time in the management of stage III NSCLC. This 3-arm study randomised patients to sequential chemo-radiotherapy with cisplatin/vinblastine followed by radiotherapy (60 Gy in 30 fractions of 2 Gy over six weeks) beginning on day 50 (arm 1); concurrent chemo-radiotherapy with combination cisplatin/vinblastine and the same radiotherapy beginning on day 1 (arm 2); *vs.* concurrent chemo-radiotherapy using combination cisplatin/etoposide with hyperfractionated radiotherapy beginning on day 1 (69.6 Gy in 58 fractions of 1.2 Gy twice daily, over six weeks) (arm 3). Phase II data suggested that the hyperfractionated regimen in arm 3 would be superior (17). However survival in the RTOG 9410 study was actually higher for patients treated with the concurrent regimen with once-daily radiotherapy (arm 2) compared with the concurrent regimen using twice-daily radiotherapy (arm 3) (P=0.046) (17). Median survival times were 14.6%, 17% and 15.6 %, with five years survival of 10%, 16% and 13% for arms 1-3, respectively (P=0.046). This trial highlighted that dose escalation by a hyperfractionation regime delivered over a standard overall treatment time does not improve survival. In addition the results supported the use of concurrent chemo-radiotherapy with conventional fractionation, which has since become the gold standard treatment in good performance status stage III patients (3).

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Table 1 Description of included trials using hyperfractionation radiotherapy schedule in non-small cell lung cancer

Trial	No. patients randomised	Inclusion period	RT dose/no. of fractions	Dose per fraction	Duration (weeks)	Chemotherapy
RTOG 8808-ECOG 4588 (15,16)	326	1989-1992	Control arm: 60 Gy/30	2 Gy OD	6	None
			Experimental arm: 69.6 Gy/58	1.2 Gy BID	6	None
RTOG 9410 (17)	610	1994-1998	Study 1: 63 Gy/34	1.8 Gy ×25, 2.0 Gy ×9 OD	7	Sequential
			Study 2: 63 Gy/34	1.8 Gy ×25, 2.0 Gy ×9 OD	7	Concurrent
			Study 3: 69.6 Gy/58	1.2 Gy BID	6	Concurrent

Abbreviations: RT, radiotherapy; BID, RT given twice a day; ECOG, Eastern Cooperative Oncology Group; No, number; OD, RT given once a day; RTOG, Radiation Therapy Oncology Group.

Accelerated hyperfractionation

Three fractions per day regime

Treatment using continuous hyperfractionated accelerated radiotherapy (CHART) was shown to be of significant benefit by improving local control and overall survival (18,19). The randomised trial recruited 563 patients, PS 0-1, medically inoperable, and compared CHART (54 Gy in 36 fractions of 1.5 Gy 3 times per day over 12 consecutive days) to conventionally fractionated radiotherapy (60 Gy in 30 once daily fractions of 2 Gy over six weeks). As anticipated the main toxicity during treatment was dysphagia, which was more severe in the CHART patients, with 19% experiencing severe dysphagia, compared with 3% in the conventional group. Overall there was a 24% reduction in the relative risk of death in the CHART arm and the overall survival rates were significantly higher: 30% *vs.* 21% at two years and 12% *vs.* 7% at five years respectively for the CHART and conventional radiotherapy arm (P=0.004) (18,19). On subgroup analysis, CHART demonstrated an even greater improvement for squamous cell carcinomas, with an overall survival at three years of 21% compared with 11% for the conventional regime (P=0.0007). This evidence suggests that reducing overall treatment time in an effort to reduce tumour repopulation plays a key role in tumour control and treatment of NSCLC. Meanwhile, it should be noted that (I) the control arm of CHART would not be considered current standard of care as chemotherapy is not delivered with radiotherapy (either sequentially or concurrently) and (II) a large percentage of patients had stage I-II disease (36%) who would nowadays be considered for a surgical approach or in some cases stereotactic ablative body radiotherapy. Despite the overall benefit seen with hyperfractionated accelerated radiotherapy in the CHART trial, this has not become standard practice. Recently published data gathered from a survey of UK clinical oncologists (20), revealed 55 Gy in 20 daily fractions as the commonest fractionation schedule for NSCLC in the UK, followed by 66 Gy in 33 daily fractions. Only 14/50 centres offered CHART despite the National Institute for Health and Clinical Excellence (NICE) recommending CHART as highly cost-effective (21). It is widely recognised that the schedule is demanding for patients and requires flexible and ad hoc radiotherapy department staffing willing to work extended day. If patients are unable to travel this treatment often necessitates a 12-day inpatient stay.

Between 1991 and 1994, Fu *et al.* conducted a phase I/II trial evaluating hyperfractionated accelerated radiation therapy (HART) which was published as a comparative cohort study. HART was delivered by 1.1 Gy per fraction, three fractions per day at intervals of four hours with five treatment days per week (22). The clinical disease was irradiated to 74.3 Gy delivered in 66-69 fractions over 33 days (not corrected for lung density), and the subclinical disease to 50.0 Gy delivered in 44-46 fractions over 33 days. There were 60 patients in the HART group and their survival and local control results were compared to those of 50 patients treated by conventional fractionated irradiation during the same period. Survival and local control were improved in the HART group. Three-year survival was 28% *vs.* 6% (P<0.001). Three-year local control was 29% *vs.* 5% (P=0.008). Median survival for HART was 22.6 months compared with 14.0 months for standard radiotherapy patients (P<0.05).

The evolving evidence in favour of concurrent chemo-radiotherapy led to the premature closure of a number of clinical trials evaluating accelerated and hyperfractionated

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Table 2 Description of included trials using acceleration or hyperfractionation radiotherapy schedules in non-small cell lung cancer

Trial	No. patients randomised	Inclusion period	RT dose/no. of fractions	Dose per fraction	Duration (weeks)	Chemotherapy
Ball 1999 (23)	204	1989-1995	Control arm: 60 Gy/30	2 Gy OD	6	+/- concurrent
			Experimental arm: 60 Gy/30	2 Gy BID	3	+/- concurrent
CHART (18,19)	563	1990-1995	Control arm: 60 Gy/30	2 Gy OD	6	None
			Experimental arm: 54 Gy/36	1.5 Gy TID	1.5	None
Fu 1997 (22)	69	1991-1994	Control arm: 60-64 Gy/32-34	1.8-2.0 Gy OD	7	Adjuvant or none
			Experimental arm: 74.3 Gy/66-69	1.1 Gy TID	6.5	Adjuvant or none
CHARTWEL-trial (ARO 97-1) (24)	406	1997-2005	Control arm: 66 Gy/33	2 Gy OD	6.5	Induction or none
			Experimental arm: 60 Gy/40	1.5 Gy TID	2.5	Induction or none
ECOG 2597 (25)	119	1998-2001	Control arm: 64 Gy/32	2 Gy OD	6.5	Induction
			Experimental arm: 57.6 Gy/36	1.6 Gy TID	2.5	Induction
Nyman 2009 (26)	152	2002-2005	Control arm: 60 Gy/30	2 Gy OD	6	Induction & concurrent
			Control arm: 60 Gy/30	2 Gy OD	6	Induction & concurrent
			Experimental arm: 64.6 Gy/38	1.7 Gy BID	4.5	Induction & concurrent
Van Baardwijk 2012 (27)	137	2006-2009	Total dose 51-69 Gy		Total 6-7	Concurrent
			Study dose: phase 1 45 Gy/30	1.5 Gy BID	3	
			Study dose: phase 2 isotoxic	2 Gy OD for remainder	3-4	

Abbreviations: CHART, Continuous Hyperfractionated Accelerated Radiation Therapy; CHARTWEL, CHART Week-End Less; ECOG, Eastern Cooperative Oncology Group; No, Number; RT, Radiotherapy; OD, RT given once a day; RTOG, Radiation Therapy Oncology Group; BID, RT given twice a day; TID, RT given three times a day.

regimen. The trials which evaluated both these fractionation schedules as the primary treatment modality are summarised in *Table 2*. The ECOG 2,597 trial was closed in June 2001 when 141 patients had been recruited, reaching 42% of the overall target (25). This trial randomly assigned stage III NSCLC patients to induction chemotherapy followed by standard thoracic radiotherapy (64 Gy, 2 Gy once daily over 6.5 weeks), *vs.* induction chemotherapy followed by HART (57.6 Gy, 1.5 Gy in three daily fractions over 2.5 weeks, with weekend breaks). Although not statistically significant there was an improvement in survival with HART (20.3 *vs.* 14.9 months; P=0.28).

The CHART schedule was logistically difficult for radiotherapy departments to implement due to the additional weekend and evening treatments. This led to the CHARTWEL-trial evaluating hyper-fractionated accelerated radiotherapy which omitted weekend treatments (24). The CHARTWEL-trial compared 60 Gy in 1.5 Gy fractions, delivered 3 times per day, on the 5 weekdays, over an average of 17 days *vs.* conventional treatment of 66 Gy in 33 fractions delivered once daily over 45 days. The study found no significant difference between the two arms, with two years survival rates of 32% in the conventional arm and 31% in the CHARTWEL arm (P=0.43). However, this study confirmed the importance of a time factor in this disease as the lower total dose in the CHARTWEL arm was compensated by the shorter overall treatment time.

Another strategy is to dose escalate CHART. Continuous hyperfractionated accelerated radiotherapy escalated dose (CHART-ED) was a multi-centre phase I feasibility study which completed recruitment in September 2012. It compared dose-escalated CHART, adding twice daily fractions after completion of 54 Gy in 36 fractions over 12 days (28). Patients were treated on day 15 in group 1 (total dose 57.6 Gy in 38 fractions), days 15-16 in group 2 (total dose 61.2 Gy in 40 fractions) and days 15-17 in group 3 (total

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Table 3 Neoadjuvant chemo-radiotherapy trials prior to surgery using accelerated hyperfractionation radiotherapy schedules in non-small cell lung cancer

Trial	No. patients randomised	Inclusion period	RT dose/no. of fractions	Dose per fraction	Duration (weeks)	Chemotherapy
Thomas (29)	558	1993-2003	Control arm: post-op RT 54-68.4 Gy/30-38	1.8 Gy OD	6-7.5	Induction
			Experimental arm: pre-op 45 Gy/30	1.5 Gy BID	3	Induction & concurrent
			Experimental arm post-op: none or 24 Gy/16	1.5 Gy BID	1.5	No adjuvant
Pöttgen 2013 (30)	239	2000-2012	Control arm: 46 Gy/23	2 Gy OD	4.5	Induction & concurrent
			Experimental arm: 45/30	1.5 Gy BID	3	Induction & concurrent
Pöttgen 2010 (31)	135	2004-2008	Experimental arm 45 Gy/30	1.5 Gy BID	3	Induction & concurrent

Abbreviations: No, number; RT, Radiotherapy; OD, RT given once a day; BID, RT given twice a day.

dose 64.8 Gy in 42 fractions). The incidence and grade of potentially dose-limiting toxicities will be assessed to determine whether dose escalation of around 6-10 Gy using this approach is safe, and the data is currently awaited.

Two fractions per day regime

An Australian study by Ball *et al.* used a 2×2 factorial design to evaluate shortening of the overall treatment time and the addition of carboplatin in patients with inoperable NSCLC (23). The trial randomised 204 patients between conventional radiotherapy (60 Gy in 30 fractions, once daily over six weeks) or accelerated radiotherapy (60 Gy in 30 fractions, twice daily, over three weeks) with or without concurrent carboplatin chemotherapy. Oesophageal toxicity was significantly higher in the three week radiotherapy arms and no significant survival difference between the groups was found.

Between June 2002 and May 2005 152 patients with stage III NSCLC, PS 0-1 were randomised in a Swedish 3-arm (A, B and C) phase II study by Nyman *et al.* (26). All arms started with two cycles of induction chemotherapy (carboplatin/paclitaxel), a third cycle was given concomitant with the start of accelerated radiotherapy in arm A (64.6 Gy in 1.7 Gy twice-daily fractions over 4.5 weeks), while in the remaining arms (B and C) conventional radiotherapy (60 Gy in 2 Gy daily fractions over 6 weeks) was combined with daily or weekly chemotherapy. Toxicity for all arms was similar and manageable with 12% grades 3-4 esophagitis, 1% grades 3-4 pneumonitis (all arms combined). Median survival was 17.8 (14.4-23.7) months (17.7, 17.7 and 20.6 months for A, B and C respectively). The 1-, 3- and 5-year overall survival was 63%, 31% and 24%. This study demonstrated that similar survival results could be achieved by intensifying treatment with either accelerated fractionated radiotherapy or concomitant chemo-radiotherapy.

Between 1995 and 2003 the German Lung Cancer Co-operative Group (GLCCG) evaluated the role of accelerated hyperfractionated chemo-radiotherapy regimes in the pre-operative setting (29). The trials which included this fractionation schedule in the neoadjuvant setting are summarised in *Table 3*. 558 patients with stage IIIA-IIIB NSCLC were randomised between pre-operative chemo-radiotherapy and chemotherapy alone. In the control arm three cycles of cisplatin and etoposide chemotherapy were delivered followed by surgical resection, then adjuvant radiotherapy at 1.8 Gy daily fractions, the total dose dependent on surgical resection margins (54 Gy for negative margins, 68.4 Gy for positive margins). In the experimental arm the same induction chemotherapy was delivered, but followed by concurrent chemo-radiotherapy 45 Gy at 1.5 Gy twice daily fractions with carboplatin and vindesine, prior to surgical resection. If the margins were negative no further radiotherapy was given. But in the presence of positive margins, additional radiotherapy of 24 Gy at 1.5 Gy twice daily fractions was delivered. Pneumonectomies were performed in 35% of the patients in each group, with an increase in treatment-associated mortality seen in the experimental arm. Overall a similar number of patients

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underwent surgery, with a slightly higher complete resection rate in the experimental arm of 37% compared with 32% in the control arm. However there was no difference in progression free survival, the primary endpoint of this trial (29).

Pöttgen *et al.* also evaluated neo-adjuvant accelerated hyperfractionated chemo-radiotherapy. In an observational study, 239 patients with stage III NSCLC were treated with neoadjuvant radiochemotherapy using either accelerated hyperfractionation (45 Gy in 1.5 Gy twice-daily fractions over three weeks) or conventional fractionation (46 Gy in 2 Gy once daily fractions over 4.5 weeks) prior to thoracotomy (30). The crude pathological complete response (pCR) rates of 37% and 24% were seen in the accelerated hyperfractionated group and conventional fractionated group respectively, with a significant relationship between pCR rates and the BED suggesting an improvement in local effectiveness of accelerated hyperfractionation in lung cancer.

This accelerated regimen was further evaluated in a prospective trial by the same group in stage III NSCLC patients not deemed resectable, mainly stage IIIB (31). After three cycles of induction chemotherapy (cisplatin/paclitaxel) concurrent chemo-radiotherapy was delivered (accelerated hyperfractionated, 45 Gy in 1.5 Gy twice daily fractions over three weeks, with cisplatin/vinorelbine). Once 45 Gy was reached, a multidisciplinary panel decision was made regarding operability. Inoperable patients received definitive radiotherapy (total dose 65 or 71 Gy, depending on the mean lung dose) with additional concurrent chemotherapy (cisplatin/vinorelbine). The majority (21 of 28 patients) received 71 Gy. Oesophagitis Grade 3+ was observed in 18% and pneumonitis Grade 3+ in 4% of the patients. At three years, the loco-regional control rate was 52% (95% CI, 29-75%). In an exploratory analysis, those patients receiving 71 Gy had a loco-regional control at two and three years of 74% (95% CI: 51.2-96.3%) and 63% (95% CI: 36.1-90.4%), while in those patients receiving the lower total dose (65 Gy), loco-regional control at two and three years was 18% (95% CI: 0-49.2%; P=0.001, Wilcoxon test), respectively. Overall survival at three years was 31% (95% CI: 12-50%) for all patients. This study led to the ESPATÜ trial, a phase III multicentre study that compared induction chemotherapy followed by definitive concurrent chemo-radiotherapy to trimodality treatment (induction chemotherapy followed by concurrent chemo-radiotherapy followed by surgery). The study recently closed and results are awaited.

Given the evidence in favour of hyperfractionation and acceleration, this has been taken a step further with specifically tailored regimes. The MAASTRO group have pioneered the concept of "isotoxic" radiotherapy allowing for individualised dose escalation in stage I-III patients based on dose delivered to organs at risk (such as lung and spinal cord), using hyperfractionated accelerated radiotherapy (32). In the first MAASTRO study 166 NSCLC patients (59% stage III) not suitable for concurrent chemo-radiotherapy received an individualised dose of radiotherapy alone or after induction chemotherapy (55% of patients). Using 3D conformal therapy, the total dose delivered was between 50.4-79.2 Gy (delivered within an accelerated schedule of 1.5 Gy twice daily). With a median follow-up of 31.6 months, the median overall survival was 21.0 months—95% CI, 15.8 to 26.2 months, (stage IIIA 16.2 months—95% CI, 7.6 to 24.8 months; stage IIIB, 17.2 months—95% CI, 8.4 to 26.0 months) with a 2-year overall survival of 45.0%. Only eight patients (4.8%) developed acute grade 3 dysphagia. Less than 10% of patients with stage III received the maximum dose as per protocol of 79.2 Gy.

A further MAASTRO study, evaluated the same strategy in the concurrent setting (27), only in stage III NSCLC patients. One hundred and thirty seven patients were included in this phase II study and treated with 3D conformal radiotherapy. The individually prescribed dose was based on mean lung dose of 19 Gy, spinal cord dose of 54 Gy, brachial plexus dose of 66 Gy and central mediastinal structure dose of 74 Gy. A total dose between 51 and 69 Gy was delivered in 1.5 Gy twice daily up to 45 Gy, followed by 2 Gy once daily and radiotherapy was started at the 2nd or 3rd course of chemotherapy. The median dose was 65.0±6.0 Gy delivered in 35±5.7 days. With a median follow-up of 30.9 months, the median overall survival was 25.0 months (95% CI: 19.8-30.3 months) and 2-year overall survival 52.4%. Thirty five patients (25.5%) developed G3+ dysphagia.

It should be noted that patients in the two MAASTRO group studies were treated with 3DCRT, probably limiting individualised dose escalation. The use of Intensity Modulated Radiotherapy (IMRT) could potentially allow for further dose escalation. IMRT modulates the intensity profile of radiation delivered to the patient, permitting improved targeting of the radiation dose, and in the thorax leads to a reduction in dose to organs at risk. This could therefore lead to increased tumour control probability yet with the same normal tissue complication probability (33). A planning study by The Christie using IMRT and twice

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daily fractionation for stage II/III NSCLC showed that this had potential to allow a further individual dose escalation in this group of patients (34). The starting point for dose escalation in this study was 55.8 Gy in 1.8 Gy per fraction delivered twice daily. The number of fractions was then increased until one or more organ at risk (OAR) tolerance dose was exceeded or a maximum dose of 79.2 Gy (i.e., 44 fraction of 1.8 Gy BD) was reached. IMRT allowed a significant dose increase in comparison to other methods ($P<0.0001$) while no difference was found between 3D conformal planning and inverse planning ($P=0.06$).

This regime will be assessed in a UK feasibility multicentre study of isotoxic hyperfractionated accelerated radiotherapy in stage III NSCLC patients not suitable for concurrent chemo-radiotherapy (ClinicalTrials.gov Identifier: NCT01836692). If isotoxic IMRT is proven to be feasible this regimen will be compared to standard sequential chemo-radiotherapy in a national phase II "pick-the-winner" trial alongside three other dose-escalated regimens currently being evaluated in the UK.

The use of concurrent chemo-radiotherapy with accelerated hyperfractionated schedules is compromised by high rates of acute mucosal toxicity which can be challenging for both patient and clinicians, however these side effects are usually transient and resolve within a few weeks of completion of radiotherapy. The Bortfeld group have raised the interesting issue that the optimal fractionation schedule (hypofractionated *vs.* hyperfractionated) may depend on the OAR doses (35). For larger tumours, their model which minimizes maximum BED within a serial organ suggests hyperfractionation. Thus, accelerated hyperfractionation may eventually turn out as an ideal alternative to pure dose-escalation in locally advanced NSCLC and should deserve further evaluation within properly designed randomised trials.

Conclusions

There is significant evidence that prolonging the overall treatment time, can allow cancer stem cells to repopulate, and thus be detrimental to disease outcome (36). CHART has shown improved survival over standard radiotherapy, in patients with unresectable stage I-III NSCLC. Selected patients (with ECOG performance status 1 who do not fit the criteria for sequential or concurrent chemotherapy or patients who prefer radiotherapy only) may be considered for CHART (7,8).

Within the field of thoracic oncology evidence is emerging to suggest that an accelerated hyperfractionated radiotherapy schedule may be superior to conventional treatment. We believe that such treatment should be closely combined with other strategies in order to improve local control and survival. Dose escalation and individualised radiation doses facilitated by the use of IMRT should be combined in order to increase local control and survival. This is an exciting time for thoracic radiotherapy with these developments leading towards the goal of personalised treatment.

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Radiation dose effect in locally advanced non-small cell lung cancer

Feng-Ming (Spring) Kong[1], Jing Zhao[1,2], Jingbo Wang[3], Corrine Faivre-Finn[4]

[1]Department of Radiation Oncology, GRU Cancer Center, Georgia Regents University, Augusta, Georgia, USA; [2]Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; [3]Cancer Hospital/Institutes of Peking Union Medical College, Beijing 100730, China; [4]Faculty Institute of Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, UK

Correspondence to: Feng-Ming (Spring) Kong, MD, PhD. Department of Radiation Oncology, GRU Cancer Center, Georgia Regents University, Augusta, Georgia, USA. Email: fkong@gru.edu.

Abstract: Radiation is the foundation of treatment for locally advanced non-small cell lung cancer (NSCLC), and as such, optimal radiation dose is essential for successful treatment. This article will briefly review biological considerations of radiation dose and their effect in the context of three-dimensional conformal radiation therapy (3D-CRT) including intensity modulated radiation therapy (IMRT) and stereotactic body radiation therapy (SBRT) for NSCLC. It will focus on literature review and discussions regarding radiation dose effect in locally advanced NSCLC including potential severe and lethal toxicities of high dose radiation given with concurrent chemotherapy. Potential new approaches for delivering safe and effective doses by individualizing treatment based on functional imaging are being applied in studies such as the PET boost trial and RTOG1106. The RTOG concept of delivering high dose radiation to the more resistant tumors with the use of isotoxic dose prescription and adaptive planning will also be discussed in detail.

Keywords: Non-small cell lung cancer (NSCLC); radiation dose; concurrent chemotherapy

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Introduction

Radiotherapy (RT) is needed in over 60% of patients with lung cancer at least once during the course of disease, adequate dose is an essential element for successful treatment of patients with non-small cell lung cancer (NSCLC). This article will briefly review biological considerations of radiation dose and their effect in the context of three-dimensional conformal radiation therapy (3D-CRT) including intensity modulated radiation therapy (IMRT) and stereotactic body radiation therapy (SBRT) for NSCLC. It will focus on literature review and discussions regarding radiation dose effect in locally advanced NSCLC including potential severe and lethal toxicities of high dose radiation given with concurrent chemotherapy. Potential approaches for delivering safe and effective doses by individualizing treatment are being applied in studies such as RTOG1106. The concept of delivering high dose radiation to the most resistant tumors with the use of isotoxic dose prescription and adaptive approaches will also be discussed in this paper.

Radiation dose effect: biology consideration

In the laboratory, from a biological effectiveness perspective, efficacy of radiation cell killing is directly correlated with the dose delivered. According to the basic principle of the linear-quadratic model, lethal radiation damage is created in one of two ways: as a consequence of a single ionizing event of double-strand breaks in the DNA or as a consequence of two, separate, sub-lethal ionizing events which interact pairwise to create lethal damage. As a result, the biological effect (E) of RT depends on the dose in a

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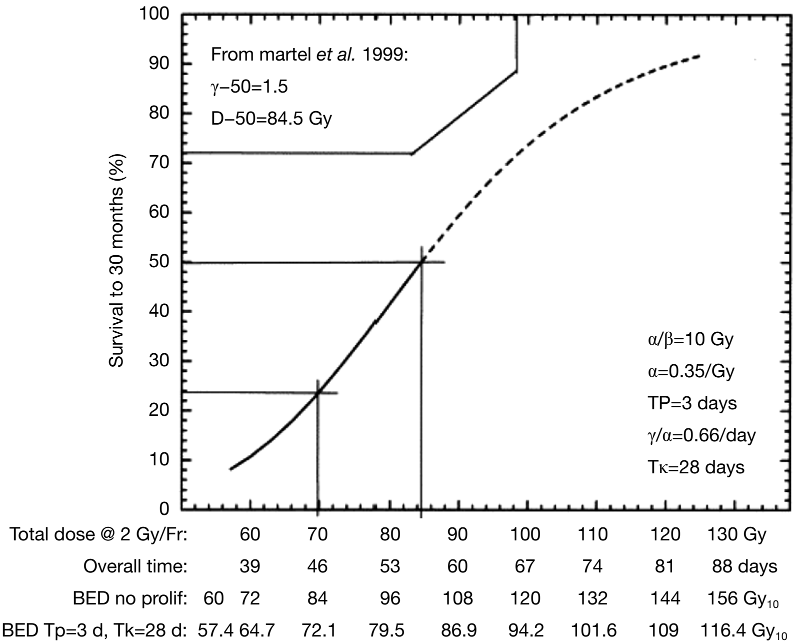

Figure 1 Tumor control probability and biological effective dose. The dose response relationship is sigmoidal in one of the early dose escalation studies of non-small cell lung cancer (NSCLC) performed in University of Michigan.

linear and quadratic fashion: $E = n(\alpha d+\beta d^2)$ with n being the number of fractions, d being the dose per fraction, and α and β being parameters that determine the initial slope and curvature of the underlying cell-survival curve. From this equation, the biological effect dose (BED) can be calculated as: $BED = nd\ [1+d/(\alpha/\beta)]$ (1). BED varies according to dose per fraction, number of fractions and characteristics of the tissue contributing to the α/β ratio. BED is used to estimate the effect or risk of radiation in current practice of radiation oncology. When effects of equivalent total doses with different fractionation schemes are compared, they produce unequal biological effects (1). In lung cancer, early evidence suggests that the tumor control rate increases with escalation of BED (*Figure 1*) (2).

RT dose effect in NSCLC treated with conventionally fractionated 3D-CRT

While traditional radiation was previously more limited by technology for normal tissue sparing, modern 3D-CRT is able to deliver high-dose radiation to the tumor target areas while minimizing dose to surrounding tissues, allowing greater RT dose for early stage inoperable NSCLC patients (3-7). Dose has been escalated to up to 102.9 Gy while limiting lung dosimetry with most patients tolerating treatment, and post treatment radiation injuries considered to be acceptable (8). Increasing the dose of radiation improves local control and overall survival in most studies reported. In RTOG protocol 73-01 (9) it was found that the in-field failure rate decreased from 58% to 35% as the dose was increased from 40 to 60 Gy. In a phase I dose-escalation study reported by Rosenzweig *et al.* (10) the 2-year overall survival (OS) rate for patients with stage I-II disease who received <80 Gy was 60%, compared with 66% for patients who received >80 Gy (P<0.05), with a median survival time of 25.0 months versus 53.6 months, respectively. A prospective study reported by Kong *et al.* (3) found that the 5-year local-regional progression-free survival (PFS) rates were 12%, 35%, and 49% for groups treated with 67, 80, and 97 Gy, respectively. Median survival (5-year OS) in this study was 12 months (4%), 27 months (22%), and 22 months (28%) for dose levels of 63-69 Gy (mean =67 Gy), 74-84 Gy (mean =80 Gy) and 92-102 Gy (mean =97 Gy), respectively (P<0.0002) (*Figure 2*) (8). The dose response curve for local tumor control was steeper for five years than that of three or four years. Kong *et al.* from University of Michigan (8) demonstrated that high-dose radiation is more vital for patients with larger tumors and may be effective

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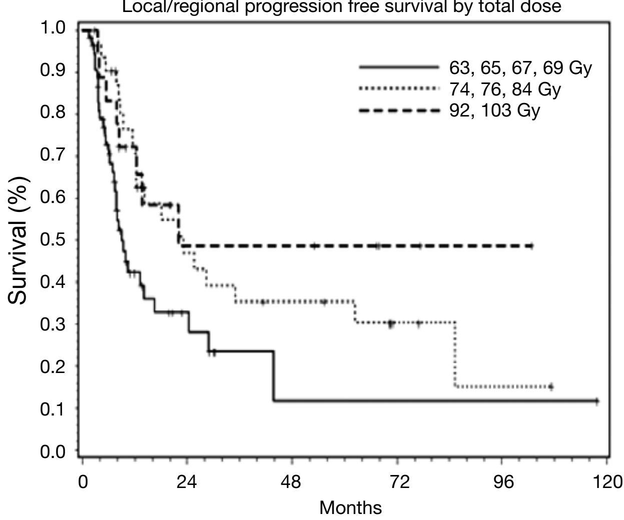

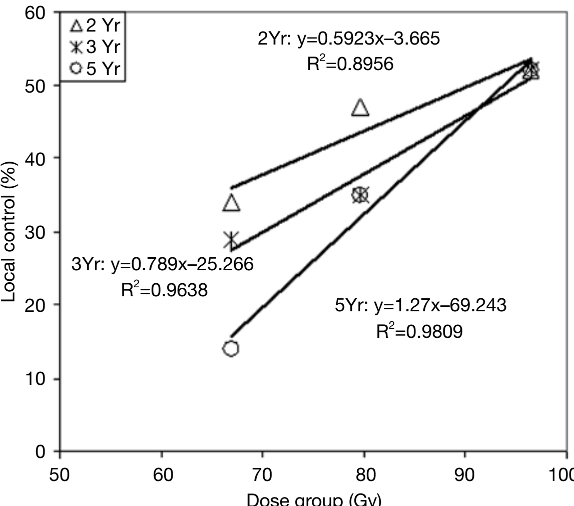

Figure 2 Local tumor control increases with higher dose radiation. Radiation dose is associated with long-term tumor control. Dose response relationship is steeper for longer follow-up.

in reducing the adverse outcome associated with a large GTV in early stage NSCLC treated with conventionally fractionated radiation.

RT dose effect in early stage NSCLC treated with hypo-fractionated SBRT

A promising new technique, SBRT normally delivers much higher BED than conventionally fractionated 3D-CRT (typically BED of 70-85 Gy), and has generated outstanding tumor control in early stage NSCLC. High BED often contributes to long survival and good local tumor control. Studies from Japan, Germany and China all reported that SBRT with BED ≥100 Gy was associated with significantly better local control and long-term survival. In patients who received a BED ≥100 Gy, local tumor control was over 90%. A multicenter study (11) reviewed 257 patients treated at 14 institutions in Japan using a number of different treatment doses and delivery approaches. At median follow up of 38 months, local recurrence rate was 8.4% in patients who were treated to a BED ≥100 Gy. A recent German study also reported that BED ≥100 Gy is critical for achieving good local control (12). A Chinese study applied daily fractionated SBRT with a total BED of up to 115 Gy and reported 3- and 5-year OS rates for T1-3 patients of 57.3% and 35.1%, respectively, and 60.2 and 36.5% 3- and 5-year OS rates for stage T1-2 patients respectively (13). Studies from the U.S. suggest that patients who receive 16 Gy ×3 (BED =124 Gy) have significantly better local control than those who receive lower doses (14). Dose response analysis showed that the outcome plateaued around 120 Gy BED. In Guckenberger's study (12), a PTV-encompassing dose of ≥100 Gy BED was estimated to be required for local tumor control rates >90%. RTOG 0236 (15), using 18 Gy ×3, equating to a BED of 180 Gy to tumor, represented the First National Cancer Institute cooperative group trial using SBRT for early NSCLC. The study reported 98% tumor control rate at three years. Updated Japanese (16) and German (17) studies of BED above 100 Gy confirmed over 90% local tumor control for T1 tumors. However, there is no randomized trial to compare different dose regimens for SBRT. In a meta-analysis containing 34 published SBRT datasets (18), observed 5-year OS and cancer specific survival (CSS) was best in those treated to medium BED (around 100 Gy).

Modern technology also allows SBRT delivery of very high radiation dose to the target volume, in as few as one single fraction. However, the effects of radiation after SBRT in a single fraction are not well known. In lung metastases patients receiving a dose of 30 Gy in a single fraction therapy It was reported that LC rates at one and two years were 89.1% and 82.1%, OS rates were 76.4% and 31.2%, CCS rates were 78.5% and 35.4%, and PFS rates were 53.9% and 22%, respectively (19). Interestingly, Guckenberger *et al.* (20) reported that the dose-response relationship was limited in fractionated SBRT: LC was

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independent from the irradiation dose in the subgroup of patients treated with single-fraction SBRT. Nevertheless, adequate radiation dose is important for good tumor control and survival in early stage NSCLC and the success of hypofractionated high dose SBRT is a strong testimony for radiation dose effect in patients treated with hypofractionated techniques (3 to 8 fractions).

RT dose effect in locally advanced NSCLC treated with chemoradiation

In locally advanced NSCLC, there are two important aspects to consider: (I) does local regional tumor control impact survival in patients with locally advanced disease, with high risk of distant disease spread? (II) with extensive tumor involvement in the chest which hosts critical structures, would high dose radiation cause significant toxicity adversely impacting patients? Ultimately, it is important to address whether high dose radiation improves overall survival and quality of life.

Local-regional tumor control and overall survival in locally advanced NSCLC

Local tumor progression is common, and remains a major problem after radiation-based non-surgical treatment in locally advanced NSCLC, despite of advances in radiation technology. Using modern techniques, current radiation therapy applying a uniform dose prescription of 60 Gy or slightly higher generates local control rates of less than 50% and a 5-year overall survival rate of about 10-15% (8,21,22). After RT with or without neoadjuvant chemotherapy, Kong *et al.* in a University of Michigan trial reported ultimate local failure in 70% of patients (8). After neoadjuvant chemoradiotherapy in CALGB 9431 (23), 90% of patients ultimately failed locally, with 45% having local failure alone. After neoadjuvant and concurrent chemotherapy with radiation doses of 60-74 Gy, Socinski *et al.* (24) reported that 46% of patients initially had local failure. Evaluation by bronchoscopy and biopsy one year after treatment completion revealed pathologic local control rates of only 15-17% after 65 Gy of radiation with neoadjuvant therapy (25). After chemoradiation with RT doses of 60 Gy in 2 Gy daily fractions or 69.6 Gy in 1.2 Gy twice daily fractions, a secondary analysis of 11 RTOG trials (9/11 had concurrent chemoradiation) with 1,356 patients reported 2- and 5-year survival rates of 38% and 15%, with 2- and 5-year local-regional failure (LRF) rates of 46% and 52%, respectively (26).

Local-regional disease not only leads to death due to local effects within the chest, but also can serve as a source for metastatic dissemination. In patients with locally advanced disease, Arriagada (27) concluded that the main cause of failure is the absence of local control, and local progression or relapse correlated with poorer survival. In RTOG 73-01 (9), the death rate in patients with intra-thoracic failure was similar to that of patients with distant metastases, and increased survival was observed in patients with complete tumor response (28). In the CHART trial, local control rates of 20% and 29% were associated with median survivals of 9.9 and 27.9 months, respectively (29). In an EORTC trial, Schaake-Koning *et al.* (30) demonstrated a similar correlation between LRC and survival. Reviewing mature results of ten randomized phase III trials with inclusion of concurrent chemoradiation, Auperin *et al.* (31) reported local or local regional control along with overall survival; there seemed significant correlation between LRC and survival rates (*Figure 3*) (32-37).

RT dose, fraction and survival in locally advanced NSCLC

In locally advanced NSCLC, 5-year OS rate is only about 15% after conventionally fractionated 60 Gy radiation. Dose escalation trials using involved field radiation therapy have demonstrated improved outcomes for patients treated to higher radiation doses, however only a few studies have investigated efficacy and tolerance. The Memorial Sloan Kettering Cancer Center (MSKCC) conducted a phase I dose escalation study of stage IIIA/B patients who received radiation dose of 70.2 to 84 Gy in 1.8 Gy fractions; the OS was significantly superior in patients who received ≥80 Gy (38). In a randomized trial from China, 5-year LC and 2-year OS improved significantly in stage III patients treated with total dose of 68-74 Gy compared with those treated to 60-64 Gy (51% *vs.* 36%, P=0.032; 39.4% *vs.* 25.6%, P=0.048) (39). Hypo-fractionated RT regimens can also increase the dose to the tumor volume based on the concept that a higher dose per fraction can increase BED, though there are no randomized trials comparing benefits and tolerance among Hypo-fractionated RT and standard schedules. A study by Zhu *et al.* (40) performed dose escalation up to 65-68 Gy in 22 to 23 fractions in 34 NSCLC patients with stage III at diagnosis. 2-year OS, PFS, and LPFS rates were 38%, 30%, and 61%, respectively. In a recent study (41) reported by Osti *et al.*, 24 stage IIIA/B patients had a median OS of 13 months (16 months for

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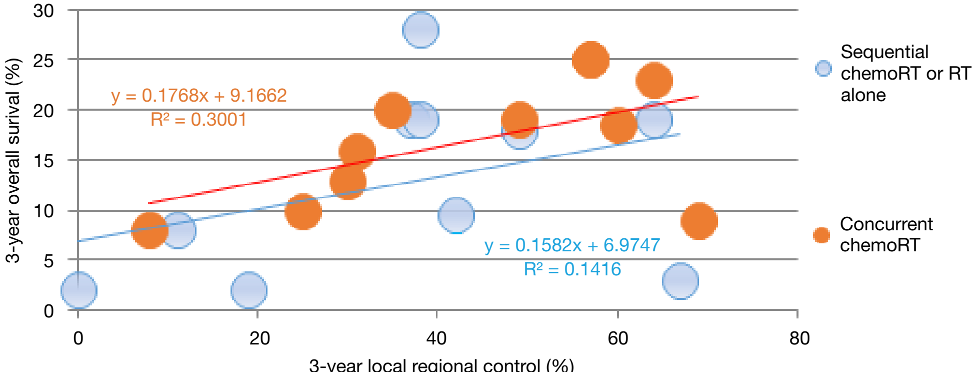

Figure 3 Correlation between local regional tumor control and overall survival in locally advanced non-small cell lung cancer (NSCLC). Data presented are reported individual results from 10 phase III trials comparing sequential chemoradiation with concurrent chemoradiation.

IIIA; 13 months for IIIB), with a range of 4 to 56 months. BED >55 Gy was significantly associated with survival benefit (P<0.001). Another hypo-fractionated RT study (42) included 37 stage III patients without administration of concurrent chemotherapy. All patients were treated with 25 fractions, with dose per fraction ranging from 2.28 to 3.22 Gy. The outcome data showed that 17% of patients achieved complete response, the actuarial 2-year OS calculated to be 46.8%±9.7%, with median survival of 18 months. Hyper-fractionated accelerated RT is another method to elevate BED to the tumor. In order to increase total dose to tumor while shortening treatment duration and decreasing late effects, hyper-fractionated-accelerated RT has been attempted in IIIA/B NSCLC patients. In 127 patients receiving hyper-fractionated-accelerated RT, Jeremić *et al.* (43) reported 5-year OS, local PFS and distant metastasis-free survival of 7%, 16%, and 36%, respectively. After two cycles of chemotherapy, stage III NSCLC patients in the DART-bid trial (44) had median OS of 24.3 months, and 2-/5-year OS rates to 51% and 18%, respectively. In a randomized phase III trial reported by Baumann *et al.* (45), survival after conventional RT and Hyper-fractionated-accelerated RT was not different, while local control after Hyper-fractionated-accelerated RT was significantly better than control after conventional RT in patients who had received chemotherapy before RT (P=0.019).

RT dose effect in locally advanced NSCLC treated with concurrent chemoradiation

In the standard care for locally advanced NSCLC: platinum based chemotherapy concurrent with RT, local tumor control and overall survival remain poor. After neo-adjuvant and concurrent chemotherapy with radiation doses of 60-74 Gy, Socinski *et al.* (46) reported that 46% of patients initially had local failure. A secondary analysis of 11 RTOG trials (9/11 had concurrent chemoradiation) with 1,356 patients treated with chemoradiation with RT doses of 60 Gy in 2 Gy daily fractions or 69.6 Gy in 1.2 Gy twice daily fractions reported 2- and 5-year OS rates of 38% and 15%, with 2- and 5-year LRF rates of 46% and 52%, respectively (25). With concurrent chemotherapy, RTOG 92-04 reported that 2- and 4-year in-field progression (TTPs) were 26% and 30% in the patients receiving radiation dose of 69.6 Gy, compared to 45% and 49% in the 63 Gy arms (47).

RT dose may be an important factor for local tumor control and perhaps survival in this patient population. A good example is a report of 237 patients with stage III NSCLC treated with radiation +/– chemotherapy between 1992 and 2002 at the University of Michigan which showed that BED was the most significant prognostic factor associated with the risk of death (HR =0.96 for each Gy, 95% CI: 0.95-0.97, P<0.001). For patients who received concurrent chemotherapy, the hazard ratio of BED for the risk of death was 0.97 per Gy (95% CI: 0.95-0.99, P=0.013). One Gy of dose escalation was associated with a 3% reduction in the risk of death. BED remained a significant independent prognostic factor in patients treated with chemoradiation in the dose range of 60-66 Gy (HR =0.91, 95% CI: 0.84-0.99, P=0.041) (48). The RTOG secondary analysis of 1,356 patients treated with chemoradiation between1988 to 2002 serves as a good example of this as well. This study analyzed for BED effect (1,348 for

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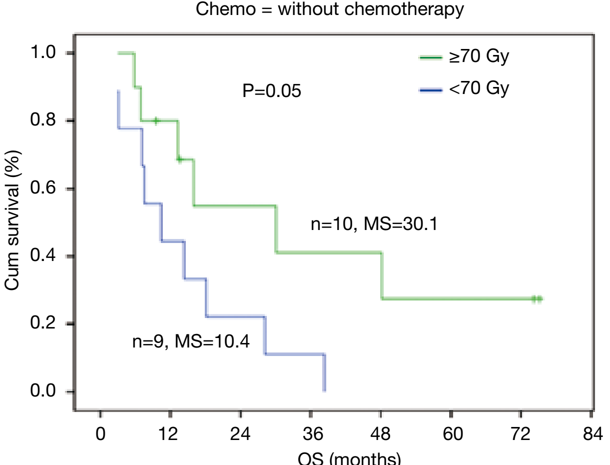

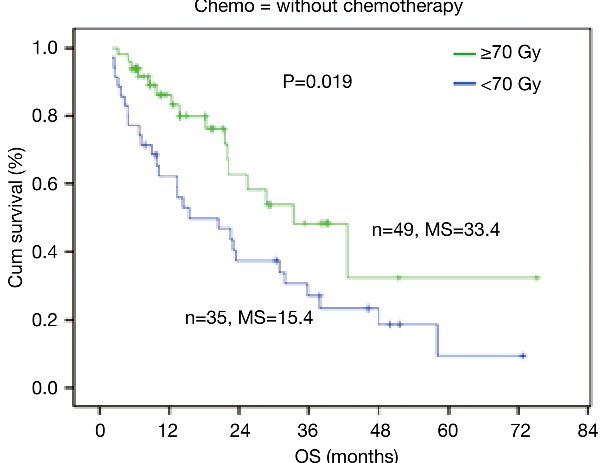

Figure 4 Radiation dose and survival in non-small cell lung cancer (NSCLC) in patients treated with or without concurrent chemotherapy. High dose group has better overall survival in both Chemo+ and Chemo- groups.

treatment time adjusted BED~tBED) in the range of 60 Gy in 2 Gy fractions and 69.6 Gy in 1.2 Gy fractions. The 2- and 5-year OS rates were 38% and 15%, respectively. The 2- and 5-year LRF rates were 46% and 52%, respectively. BED (and tBED) was significantly associated with both OS and LRF, with or without adjustment for other covariates on multivariate analysis (P<0.0001). A 1-Gy BED increase in RT dose intensity was significantly associated with approximately 4% relative improvement in survival (HR for death =0.96) and 3% relative improvement (HR =0.97) in local-regional control (26).

Overall, radiation dose escalation may improve local regional control and overall survival in patients with stage III NSCLC, based on the results of non-randomized trials (8,48-50) and an RTOG secondary analysis (26) of over 1,300 cases treated with chemoradiation. Regarding the dose effect of >70 Gy with concurrent chemoradiation, investigators from University of Michigan reported results on patients treated in the dose range of 60-100 Gy with concurrent and adjuvant carboplatin and paclitaxel (51). The median local-regional PFS was 10.7 (range: 8.4-13.0) months and has not yet been reached (14.1 to date) (P=0.001) for physical doses <70 and >70 Gy, respectively. The median survival was 15.5 (range: 6.5-24.4) months and 41.9 (range: 18.3-65.5) months (P=0.003), for physical doses less than and greater than 70 Gy, respectively. The RT dose effect was statistically significant for patients treated with or without concurrent chemotherapy (*Figure 4*).

Challenges in delivering high dose radiation in locally advanced NSCLC

Treatment effect and toxicity after dose escalated RT

It is a remarkable challenge to deliver high dose radiation in patients with advanced NSCLC. A dose escalation study of 79 patients with locally advanced NSCLC treated without chemothereapy reported a maximum tolerance dose og 63.25 Gy in 25 daily fractions over five weeks using intensity-modulated RT to limit severe toxicity to 20%. Grade 4 to 5 late toxicities were attributable to damage to central and perihilar structures and correlated with dose to the proximal bronchial tree (52-54). A trial from University of Michigan with concurrent carboplatin and paclitaxel (UMCC 2003-073) was stopped prematurely due to lack of dose escalation in 60% of patients limited by clinical lung toxicity at 15%. RTOG 0117, a phase I/II dose escalation study with concurrent and adjuvant carboplatin and paclitaxel, reported two acute, treatment-related dose limiting toxicities (DLTs) in the 1st cohort of 17 patients and 6/8 (75%) grade ≥3 events during long-term follow up. The protocol was revised to de-escalate the radiation therapy dose (74 Gy in 37 fractions). In the new cohort of seven patients, treated with 74 Gy, there was 1 DLT in the first five patients and no DLTs in the next two patients. The maximum tolerable dose was thus determined to be 74 Gy in 37 fractions (2 Gy per fraction) using 3D-CRT with concurrent paclitaxel and carboplatin therapy (55). The CALBG 30105 trial (11) studied induction chemotherapy

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followed by concurrent chemoradiotherapy in stage III NSCLC patients randomised between two different chemotherapy regimens delivered concurrently with dose-escalated thoracic conformal RT (74 Gy, once daily, 2 Gy per fraction) in both arms. The carboplatin/gemcitabine arm closed prematurely due to a high rate of grade 4 to 5 pulmonary toxicity. However the carboplatin/paclitaxel arm demonstrated a median survival of 24 months with a 12% rate of grade 3 or higher pulmonary toxicity.

These trial results compared favorably to the historical standard concurrent chemoradiotherapy doses of 60-66 Gy in 2 Gy fractions.and formed the basis for the experimental arm in the recently closed phase III RTOG 0617 trial. In this 2×2 factorial design trial patients with stage III NSCLC were treated with weekly carboplatin-paclitaxel chemotherapy and concurrent RT in 2 Gy fractions. Patients were randomised to receive 60 or 74 Gy RT, with or without cetuximab. After RT, all patients received a further two cycles of consolidation chemotherapy, with or without cetuximab. A planned interim analysis after 85 documented events demonstrated a non-superior median survival in the high dose arms which were closed due to a low likelihood of survival benefit from high dose RT with additional accrual and follow up. An updated analysis of the data after 207 events demonstrated a significant increased risk of death in the high dose arms [median survival 28.7 (60 Gy arm) *vs.* 19.5 months (74 Gy), P=0.0007; HR =1.56, 95% CI: 1.19-2.06], with a 37% increased risk of local failure in the high dose arms (HR =1.37, 95% CI: 0.99 to 1.89, P=0.0319). There were more treatment related deaths in the high dose arms (10 *vs.* 2) but this did not reach statistical significance. The worse local control and survival of the high dose arms of RTOG 0617 trial has challenged the assumption that RT dose escalation using conventional dose/fractionation regimens with concurrent chemotherapy will improve outcome in stage III NSCLC. At the time of writing this article, the reasons for the underperformance of the 74 Gy arm are still unclear and the analysis of the individual RT plans by RTOG is ongoing. Hypotheses for the worse local control in the 74 Gy arms include issues with the assessment of local progression versus fibrosis, chemotherapy and RT dose delivery and compliance, issues with RT planning and quality assurance (particularly since IMRT was only used in 46% of centers) and accelerated repopulation due to the prolongation of the overall treatment time. This is supported by an early analysis estimating that tumor control probability of NSCLC decreases 1.6% per day after a six-week duration of RT, and according to a secondary analysis of three RTOG trials for stage III NSCLC patients treated with concurrent chemoradiotherapy, showing that prolonged treatment time translated into a 2% increase in the risk of death for each day of prolongation in therapy (56). A combination of factors probably account for the survival results of RTOG 0617, including inferior local control in the 74 Gy arms; but unreported treatment-related deaths (cardiac and pulmonary) are likely to be one of the major causes for the inferior survival in the 74 Gy arms. Indeed the multivariate survival analysis reported that V5 and V50 heart were both associated with worse survival. This study highlights the need for stricter constraints to adjacent critical organs at risk such as heart, lung, proximal bronchial tree and RT quality assurance programs in future studies and institutional protocols. The current view in the radiation oncology community is that radiation dose escalation with conventional fractionation and concurrent CT is not the way forward, but treatment intensification should be pursued, including studies of altered fractionation and individualization of dose (57-59).

Currently, there are investigative efforts to increase daily fraction size to escalate total radiation dose without extending the treatment duration. One approach involves dose escalation using 2.25 Gy daily fractions (once or twice daily) while limiting treatment duration to six weeks (60). This approach was used to escalate to 87.8 Gy in patients with limited lung volumes without concurrent chemotherapy. Another approach is to use a higher dose fraction every day while limiting the treatment duration to five weeks without concurrent chemotherapy (61). UMCC 200373 and UMCC2007123 limited treating duration to six weeks while delivering RT dose escalation with concurrent chemotherapy, and achieved promising results (51).

Treatment related death after RT based treatment

Treatment related severe toxicities can be fatal. For example, a recent meta-analysis reported 1.9% grade 5 pneumonitis after concurrent chemoradiotherapy (62). Radiation pneumonitis attributed death occurred in up to 10% (35,63,64) of patients treated with concurrent chemoradiation, and up to 4.3% of patients treated with radiation alone (35,65,66). Critical organs at risk include the heart, lung and esophagus. Grade 5 adverse events were reported in 1.7% (range, 1-3%) (67,68), and 2.5% (range, 1.2-8.2%) (69,70), for patients treated with concurrent chemotherapy with conventional doses (60-63 Gy) and concurrent chemotherapy with escalated doses (>63 Gy). It

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Table 1 Grade 5 events in reported clinical trials

Trials	RT total dose (Gy)	Number of Fx	Number of patients	Grade 5 events (%)	Chemoregimens
Dose escalation radiation with concurrent chemotherapy					
RTOG 0617, Bradley *et al.*, 2013 (56)	74	37	208	8.2	TC
	60	30	216	3.2	TC
RTOG 9410, Curran *et al.*, 2011 (71)	63	34	195	3.6	Vinblastine, cisplatin
	69.6	58	382	1.8	EP
Salama *et al.*, 2011 (11)	74	37	26	7.7	Gemcitabine, carboplatin
Uitterhoeve, 2007 (72)	66	24	56	1.8	cisplatin
Berghmans *et al.*, 2009 (73)	66	33	48	6.3	Gemcitabien, cisplatin, vinorelbine
Movsas *et al.*, 2005 (74)	69.6	58	242	1.2	TC
LAMP trial, Belani *et al.*, 2005 (75)	63	34	166	1.8	TC
NPC 95-01, Fournel *et al.*, 2005 (35)	66	33	100	10	EP
Conventional dose radiation concurrent with chemotherapy					
RTOG 0617, Bradley *et al.*, 2013 (56)	60	30	216	3.2	TC
Albain *et al.*, 2009 (76)	61	NR	194	1.5	EP
SWOG S0023, Kelly *et al.*, 2008 (34)	61	33	543	1.1	EP
NCCTG 90-24-51, NCCTG 94-24-52, Schild *et al.*, 2007 (65)	60	20 or 40	129	1.6	EP
Radiation alone					
NCCTG 90-24-51, NCCTG 94-24-52, Schild *et al.*, 2007 (65)	60	20 or 40	37	2.7	—
JCOG9812, Atagi *et al.*, 2005 (36)	60	30	23	4.3	—
ECOG, Clamon *et al.*, 1999 (66)	60	30	120	1.7	—

RT, radiotherapy; EP, etoposide and cisplatin; TC, paclitaxel and carboplatin.

is possible that these increased events were due to treatment toxicity, though some of them were not identified as such. Another ongoing issue with the reporting of treatment related deaths is that many patients die at home or at local community hospitals, leading to probable underreporting of grade 5 events. These treatment toxicities often arise as a consequence of the challenges of delivering high dose radiation to locally advanced disease without incidentally delivering high dose to the OARs (*Table 1*).

Potential strategies to improve therapeutic gain in NSCLC

It is imperative to pursue new strategies to increase the dose ratio of tumor target over critical structures. Radiation physics and technology advancements such as IMRT, IGRT, and volume based planning are important for delivery of radiation precisely to the target, though this will not be discussed in this review. Knowledge of tumor target gained from tools such as Positron Emission Tomography helps define the target more accurately. Individualized radiation with isotoxicity prescription is a promising strategy. For traditional adaptive radiation plan, prescription dose is required to cover the whole GTV and CTV determined according to images simulated before therapy. To obtain the best LRC and OS from radiation, higher total dose while limiting total treatment duration less than six weeks and dosimetric factors such as V20 and MLD should be seriously considered especially for larger tumors (diameter >5 cm). An ongoing European phase II PET-boost trial (ClinicalTrials.gov Identifier: NCT01024829) randomises patients with stage IB-III NSCLC to dose-escalation

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starting from 66 Gy given in 24 fractions of 2.75 Gy with an integrated boost to either the entire primary tumour or to >50% of the maximum Standardised Uptake Volume (SUV_{max}) area of the primary tumor, while limiting MLD to 20 Gy. Preliminary results from the first 20 randomised patients showed that this was feasible and did not exceed pre-defined normal tissue constraints. Recent studies from Kong *et al.* at University of Michigan (3) demonstrated that there is a significant decrease in tumor size and FDG activity after radiation dose of 45 Gy. According to this result, we could adapt targeting to the decreased tumor defined on FDG-PET/CT after 45 Gy with a fixed composite MLD limit of 20 Gy while allowing remarkable escalation of total dose to the tumor. Kong *et al.* have demonstrated that tumor volume reduces significantly more on FDG PET than on CT at 40-50 Gy (4-5 weeks during the course of fractionated RT) (77). Using the reduced volume identified on during-RT PET, dose to active and resistent tumor was significantly escalated while dose to the normal tissues were either reduced (due to adaptive shrinking fields) or unchanged (78). The ongoing RTOG1106 trial (http://www.rtog.org/ClinicalTrials/ProtocolTable/StudyDetails.aspx?study=1106) adopted this concept, and will use this approach to obtain FDG-PET/CT during the course of chemoradiation to adapt their plan to a tumor target smaller than that from before therapy to escalate dose to as high as 80.4 Gy delivered in six weeks without increasing doses to the OARs. The total dose for each patient in the experimental arm will be determined by the dose corresponding to a MLD of 20 Gy (equivalent to a 15-17% probability of grade >2 lung toxicity based on the current NTCP model). The study hypothesized that the during-treatment PET/CT-based adaptive therapy will allow us to dose escalate (i.e., raise the daily dose to the reduced target volume for the remainder of the treatment) in the majority of patients and meet the dose limits of normal structures, thus improving LRC without increasing normal tissue toxicity. This will also allow us to use the lung dose limits to individualize adaptive dose escalation to residual active tumor regions and limit the incidence of pneumonitis and other toxicities simultaneously.

Conclusions

In summary, there is a clear radiation dose effect in NSCLC patients. Although the benefit of high dose radiation has been demonstrated in early stage patients, the clinical benefit of high dose radiation in patients has been challenged by preliminary results from RTOG0617. Treatment related toxicity can be a major reason for failure of high dose radiation. Future study of radiation therapy may benefit from individualized radiation dose prescription based on the sensitivity of tumor and critical organs of each individual patient. Studies from Europe will individualize doses based on FDG intensity at baseline while limiting treatment duration to five weeks. RTOG1106, an ongoing randomized phase II study, will examine the effect of individualized adaptive radiation therapy (over an uniform 60 Gy) by targeting high dose radiation to most resistant tumor while keeping doses to critical structures strictly controlled in locally advanced NSCLC patients.

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Accelerated dose escalation with proton beam therapy for non-small cell lung cancer

Daniel R. Gomez, Joe Y. Chang

Department of Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Correspondence to: Daniel Gomez, MD. The University of Texas MD Anderson Cancer Center, 1840 Old Spanish Trail, Houston, TX, USA. Email: dgomez@mdanderson.org

Abstract: Local tumor control remains challenging in many cases of non-small cell lung cancer (NSCLC), particularly those that involve large or centrally located tumors. Concurrent chemotherapy and radiation can maximize tumor control and survival for patients with locally advanced disease, but a substantial proportion of such patients cannot tolerate this therapy, and sequential chemoradiation regimens or radiation given alone at conventionally fractionated doses produces suboptimal results. An alternative approach is the use of hypofractionated proton beam therapy (PBT). The energy distribution of protons can be exploited to reduce involuntary irradiation of normal tissues, particularly the low-dose irradiation problematic in intensity-modulated (photon) radiation therapy (IMRT). Here we summarize current evidence on the use of hypofractionated PBT for both early-stage and locally advanced NSCLC, and the possibility of using hypofractionated regimens for patients who are not candidates for concurrent chemotherapy.

Keywords: Hypofractionation; early-stage disease; locally advanced disease; proton beam therapy (PBT); stereotactic ablative body radiation (SABR); intensity-modulated proton therapy (IMPT); passive scattering; dosimetric comparisons; clinical studies

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Introduction

Local tumor control remains a substantial challenge in many cases of non-small cell lung cancer (NSCLC). For patients with early-stage disease, the advent of stereotactic ablative body radiation (SABR) for definitive therapy has drastically reduced the rate of locoregional recurrence (1), but some tumors, particularly those that are large or centrally located, remain challenging to treat because of the risk of severe toxicity (2). For patients with locally advanced disease, concurrent chemotherapy and radiation have been shown to maximize control and survival outcomes, but many patients are not candidates for this approach because of age, the presence of comorbid conditions, or poor performance status (3,4), and for such patients sequential chemoradiation regimens or radiation given alone at conventionally fractionated doses produces suboptimal results.

Thus, more effective and safe radiation therapy regimens are needed for subsets of patients with early-stage or locally advanced NSCLC. An approach that has been increasingly explored over the past decade has been the use of hypofractionated proton beam therapy (PBT). The energy distribution of protons [as opposed to photon (X-ray- or gamma-ray-) based irradiation] has theoretical advantages over that of photons because of the Bragg peak characteristic of proton particles, which can be exploited to reduce exposure of normal tissues to radiation, particularly at low doses. Under this premise, emerging dosimetric and clinical studies are being undertaken to assess the role of PBT, including hypofractionated regimens as appropriate, for carefully chosen patients.

This review summarizes current evidence regarding the use of hypofractionated PBT for early-stage NSCLC, including use of PBT as an alternative to SABR for patients with T1-T2 node-negative tumors, followed by a discussion of PBT for locally advanced disease, including tumors that involve the mediastinum, and the possibility

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of using hypofractionated regimens for patients who are not candidates for concurrent chemotherapy. We have endeavored to convey a level-of-evidence-based approach to applying these concepts for specific cases and to outline future paths for research to better determine which patients would derive the greatest benefit from hypofractionated PBT.

Hypofractionated proton beam therapy for early-stage NSCLC

Dosimetric analyses

Several treatment-planning studies have been done to compare the radiation dose that would be delivered to tumors and surrounding normal structures with PBT *vs.* with photon techniques for early-stage tumors. In one of the earliest analyses, investigators from the University of Florida and the Mayo Clinic assessed eight patients with medically inoperable, peripherally located lesions that had initially been treated with SABR to 48 Gy in 12 fractions. An additional set of treatment plans at the equivalent dose was then generated to identify possible differences in dose distribution to normal structures if the treatment had been passive-scattering PBT instead of SABR. The median relative difference in lung dose between the two modalities was 2-10% depending on the parameter of interest, with low-dose regions being affected more than higher doses [median difference in the volume receiving at least 5 Gy (V_5) =10.4%; in V_{20} =2.1%; and in V_{40} =1.5%]; the median difference in mean lung dose was 2.2 Gy. Depending on the location of the lesion, PBT was also beneficial in other dose-volume parameters of the heart, esophagus, and bronchus. The investigators concluded from these findings that normal structure dosing was superior with PBT compared with SABR for early-stage, peripheral tumors (5).

A similar analysis done by authors from the University of Nagoya in Japan involved 21 patients with peripheral stage I NSCLC for whom plans were generated for both SABR and stereotactic body proton therapy (SBPT) to 66 Gy (RBE) in ten fractions. Again, the investigators found differences in several lung, heart, spinal cord, and esophagus doses, with the advantage from PBT again being more pronounced in the lower-dose than in the higher-dose regions in the lung. They further found that incremental increases in the tumor/target volume led to sharper rates of increase in V_5 for SABR versus SBPT, but these differences were attenuated for V_{15}-V_{20}. Overall, because the differences in low-dose regions were more substantial when planning target volumes were larger, this group concluded that SBPT seemed to be more advantageous for larger tumors (6).

Finally, researchers at The University of Texas MD Anderson Cancer Center examined the role of SBPT for particularly challenging cases of early-stage disease, specifically tumors that were centrally or superiorly located. They compared plans for SABR, given as either passive scattering SBPT or intensity-modulated proton therapy (IMPT), for 15 patients with tumors located within 2 cm of a critical structure. They found that SABR plans could be created that would meet dose constraints for normal structures in 6 of the 15 patients, passive scattering SBPT for 12 patients, and IMPT for 14 of the 15 patients. Moreover, the proton techniques were associated with considerable improvements in target coverage when tumors were within 2 cm of the following structures: aorta, brachial plexus, heart, pulmonary vessels, and spinal cord (7) (*Figure 1*). Collectively, these studies demonstrated that hypofractionated PBT was dosimetrically superior to SABR for most patients with early-stage NSCLC, and that this superiority was substantially enhanced (as was the potential clinical benefit) for patients with larger, superiorly or centrally located tumors within 2 cm of a critical structure.

Clinical analyses

Although the sum total of clinical experience with hypofractionated PBT is still relatively limited at this time, several institutions have reported their experiences with this technique, and all showed similarly promising outcomes. These studies are summarized in *Table 1*. The experience with the longest follow-up comes from Loma Linda University, which has published several studies on toxicity and survival among patients with node-negative disease who underwent definitive treatment with PBT (8,13,14). In the most recent analysis, these investigators published their 12-year findings on the use of PBT to treat patients with T1-T2N0M0 peripheral NSCLC tumors (60%) or centrally located NSCLC tumors who could not undergo surgery for medical reasons or who declined resection. All patients received PBT in a dose-escalating fashion starting at 50 Gy (RBE) and increasing to 70 Gy (RBE) in ten fractions. At a median follow-up time of 48 months for the 111 patients so treated (mean tumor size, 3.6 cm), overall survival was significantly improved in patients who received 70 Gy (RBE) compared with those treated to 51 or 60 Gy (RBE) in ten fractions. Moreover, although local control rates were excellent at about 85-90% for patients with T1

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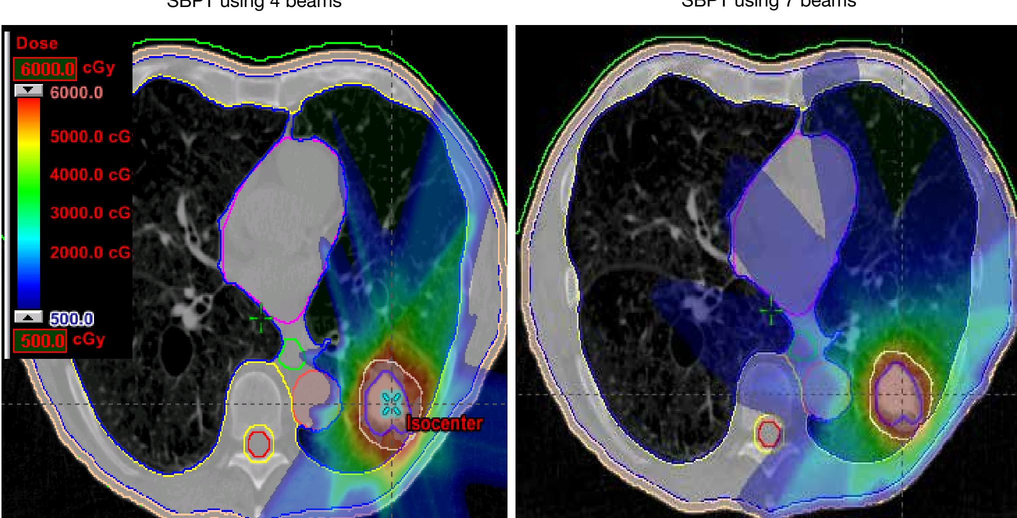

Figure 1 Comparison of stereotactic body proton therapy (SBPT) and stereotactic ablative body radiation (SABR) plans for early-stage lung cancer.

Table 1 Selected studies of accelerated proton beam therapy for early-stage non-small cell lung cancer

Study and reference	Year	No. of patients	Regimen	Toxicity	Control and survival rates
Bush *et al.* (8)	2013	111	Dose escalation (50-70 Gy in 10 fractions)	No patients with grade ≥2 RP; 4 patients with rib fractures	4-year outcomes for 70 Gy: OS 51%; DSS 74%; LC 86-91% for T1 tumors, 45-74% for T2 tumors
Hata *et al.* (9)	2007	21	50-60 Gy in 10 fractions	1 patient with grade 2 RP; 1 patient with painful subcutaneous induration; 1 patient with chest wall myositis	2-year outcomes: OS 74%; DSS 86%; LC 95%
Iwata *et al.* (10)	2010	57 (23 with carbon therapy)	60 Gy in 10 fractions	13% grade ≥2 RP; 16% grade 2 dermatitis; 4% grade 3 dermatitis; 23% grade 2 rib fracture; 6% grade 2 fibrosis of soft tissue	3-year outcomes: OS 75%; DSS 86%; LC 82%
Chang *et al.* (11)	2011	13	87.5 Gy in 35 fractions	11% grade 2 RP; 1 patient with grade 2 esophagitis; 67% grade 2 dermatitis; 17% grade 3 dermatitis	2-year outcomes: OS 55%; DFS 46%
Westover *et al.* (12)	2012	15 (20 tumors)	42-50 Gy in 3-5 fractions	1 patient with grade 2 fatigue; 1 patient with grade 2 dermatitis; 3 patients with rib fracture; 1 patient with grade 3 RP	2-year outcomes: OS 64%; LC 64%

Abbreviations: RP, radiation pneumonitis; OS, overall survival; DSS, disease-specific survival; LC, local control; DFS, disease-free survival.

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tumors, the difference in control was much more significant for those with T2 lesions (4-year local control rates of 45% for those receiving 60 Gy *vs.* 74% for 70 Gy). Analysis of outcomes among patients who were also thought to be candidates for SABR revealed excellent rates of local control rate (96%) and overall survival (80%) at four years. Finally, treatment-related toxicity with PBT was minimal, with no patients experiencing radiation pneumonitis requiring intervention, and pulmonary function, as measured by forced expiratory volume in one second (FEV_1), was largely maintained. These investigators concluded that PBT was feasible, safe, and effective for either peripheral or centrally located lesions, and that use of higher radiation doses was beneficial in terms of local control, particularly for larger tumors (8).

Other institutions have also reported outcomes with use of PBT, although the follow-up time in most studies has been shorter. Investigators from the University of Tsukuba in Japan published an initial analysis (9) and then follow-up data (15) on patients with medically inoperable stage I NSCLC treated to either 66 Gy (RBE) in ten fractions for peripherally located lesions or 72 Gy (RBE) in 22 fractions for central lesions. In the most recent report, at a median follow-up time of 17 months, the progression-free survival rates were 88.7% at two years and 78.9% at three years, with no differences found between T1 *vs.* T2 tumors or between central *vs.* peripheral lesions. Of the seven recurrences in this group of 55 patients, one was local, three were in the mediastinum or lymph nodes, and three were at other locations within the lung. Two patients experienced grade 3 pneumonitis, two grade 2, and one grade 1. One patient was noted to have a rib fracture. These investigators concluded, as did those in the Loma Linda study, that PBT was safe and feasible for patients with medically inoperable stage I disease (15).

Investigators from several institutions in Japan have reported their results PBT or carbon therapy to treat stage I NSCLC. Patients treated with PBT initially received 80 Gy (RBE) in 20 fractions, and this regimen was subsequently changed to a more aggressive alternative of 60 Gy (RBE) in ten fractions. As initially reported, at a median follow-up of approximately three years for living patients, the 3-year local control rate was 82%, with an overall survival rate at three years of 75%. Of the 80 treated patients, only one experienced grade 3 pulmonary toxicity (10). A subsequent report of outcomes among 70 patients with T2 tumors (43 treated with PBT), with the hypothesis being that control rates and toxicity would be better for this subset of patients with PBT than with SABR revealed that, at a median follow-up time of 51 months, the 4-year rates of overall survival, local control, and progression-free survival for the 70 patients were 58%, 75%, and 46%. Notably, 11 of 70 patients had mediastinal or hilar recurrences; another 12 patients with T2a or T2b tumors had similar control rates, and 2 of 70 patients experienced grade ≥3 radiation pneumonitis. Five patients had grade 3 or 4 dermatitis, and one rib fracture was reported. These investigators concluded that PBT or carbon ion therapy was well tolerated by patients with T2 disease but given the relatively high rate of distant and regional metastases, the addition of systemic therapy should be considered as well (16).

An analysis of patients treated with SBPT at Massachusetts General Hospital from 2008 through 2010 revealed a 2-year overall survival rates of 64% but a local control rate of 100% (12). Finally, in a phase I/II trial at MD Anderson Cancer Center, patients with early-stage disease who were not candidates for SABR (i.e., those with central or superior lesions or tumors >3 cm) were treated with a hypofractionated regimen of 87.5 Gy (RBE) in 35 fractions. In the first report from this trial, 18 patients had been treated at a median follow-up time of 16.3 months; no patient had experienced grade 4 or 5 toxicity, and the most common grade ≥3 adverse event was dermatitis (17%). No patient experienced grade 3 or higher pneumonitis or esophagitis. The local control rate was 89%, with 11% of patients experiencing local-regional recurrence and 28% distant metastasis. Conclusions from this study were that this regimen was well tolerated and was promising in terms of local control. Notably, the dermatitis was probably related, at least in part, to the use of two or three beams in the treatment plan (*vs.* using more than three beams to distribute the dose to the skin and chest wall over a larger area) (11), and thus the current practice at MD Anderson for hypofractionated regimens is to use four to six beams to minimize hot spots in that region.

Hypofractionated PBT for locally advanced NSCLC

Dosimetric analyses

Few studies to date have explored dosimetric differences between tumor targets and normal structures when hypofractionated dosing regimens are used for locally advanced disease. Therefore, such comparisons must be extrapolated from the literature on use of PBT at

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conventionally fractionated doses. For instance, investigators from MD Anderson Cancer Center compared dose-volume histograms in patients with stage III NSCLC treated with either PBT or (photon) IMRT and found that lung tissue parameters such as mean lung dose, V_5, V_{10}, and V_{20} were all improved with PBT as compared with IMRT. Doses to the lung, spinal cord, heart, and esophagus were also improved with PBT relative to IMRT (17). Similarly, a study from the University of Florida examined whether PBT could reduce the radiation dose to the lung and bone marrow [compared with 3-dimensional conformal radiation therapy (3D-CRT) or IMRT] in patients with stage III NSCLC. In plan comparisons for eight patients, PBT was associated with a median reduction of 29% in lung V_{20} and a 30% reduction in bone marrow V_{10} compared with 3D-CRT. These advantages were maintained when PBT was compared with IMRT, with PBT showing an improvement of 26% in lung V_{20} and 27% in bone marrow V_{10}. In a correlative study, the same authors found that PBT could cover "high-risk" lymph nodes (mediastinal, hilar, or supraclavicular nodal regions anatomically adjacent to involved regions according to positron emission tomography) with a lung dose approximating that of photon plans that covered only involved lymph nodes, leading the authors to include that PBT could be used to expand coverage to at-risk regions without substantially increasing lung dose (18). Presumably the dosimetric advantages demonstrated in studies of locally advanced disease such as these can be extrapolated to hypofractionated therapy as well, because the proportional differences should hold with the change in fraction size.

Clinical analyses

Use of hypofractionated 3D-CRT or IMRT regimens for locally advanced disease has been evaluated by several groups; these regimens tend to involve moderate hypofractionation, with smaller fractions used than for early-stage disease because of the risks of irradiating mediastinal structures and the greater degree of lung involvement in many patients. For example, investigators from the University of Wisconsin conducted a dose-escalation study in radiation was given in 25 fractions ranging from 2.28 to 3.22 Gy. Toxicity was acceptable, with no incidences of grade ≥3 pneumonitis and 15% of patients developing grade 2 radiation pneumonitis (19). Similarly, investigators at Fudan University in Shanghai treated 34 patients with stage III NSCLC with 3D-CRT in accelerated hypofractionation, with an initial dose of 50 Gy in 20 fractions ultimately escalated to a total dose of 68 Gy after two cycles of induction chemotherapy. At three years, the median progression-free survival rate was 32% and the overall survival rate 30%, but the local-regional control rate at that time was a remarkable 61%, demonstrating that induction chemotherapy followed by hypofractionated RT is promising for such cases (20).

Another group at MD Anderson published their findings from the use of 45 Gy, delivered in 3-Gy fractions, for 26 patients with stage I-IIIB disease with involved nodes and borderline performance status, defined as a Karnofsky Performance Status (KPS) score of 60-70 or weight loss of >5%. These authors found that this regimen produced comparable survival outcomes (local control, freedom from progression) and toxicity for these patients relative to patients with higher performance status (KPS >70 and with weight loss of ≤5%) who were treated to 60-66 Gy in a standard fractionation regimen over 6 to 6.5 weeks, leading them to conclude that the accelerated treatment regimen was a reasonable alternative to conventionally fractionated doses for patients who could not tolerate concurrent chemotherapy (21). This analysis was updated after its initial publication to include 119 patients in the accelerated-treatment group and again showed no differences with regard to local or distant control compared with patients given standard fractionation regimens (22).

With these prior results, investigators at MD Anderson undertook the first dedicated study of hypofractionated PBT that included patients with locally advanced disease. In this phase I trial, 25 patients were treated in a dose-escalating manner with fifteen 3-, 3.5-, and 4-Gy fractions, yielding total doses of 45-60 Gy, with the dose being escalated in a 3+3 design. Thus 3 patients were treated to 45 Gy, 4 patients to 52.5 Gy, and 18 patients to 60 Gy. At a median follow-up time of 13 months for patients who were alive at the time of analysis, the authors found that only two patients had experienced dose-limiting toxicity, one with grade 3 infectious pneumonia after receiving a dose of 60 Gy in 4 Gy fractions and the other with a grade 5 tracheoesophageal fistula developing nine months after PBT to 52.5 Gy in 3.5-Gy fractions (23). However, the latter patient had also received bevacizumab, which has been shown to cause fistulas (24,25), at one month before developing the fistula. These investigators concluded that hypofractionated PBT to the thorax was well tolerated even when significant doses were delivered to the lung and central structures such as the bronchus and esophagus. This analysis also involved the development of unique

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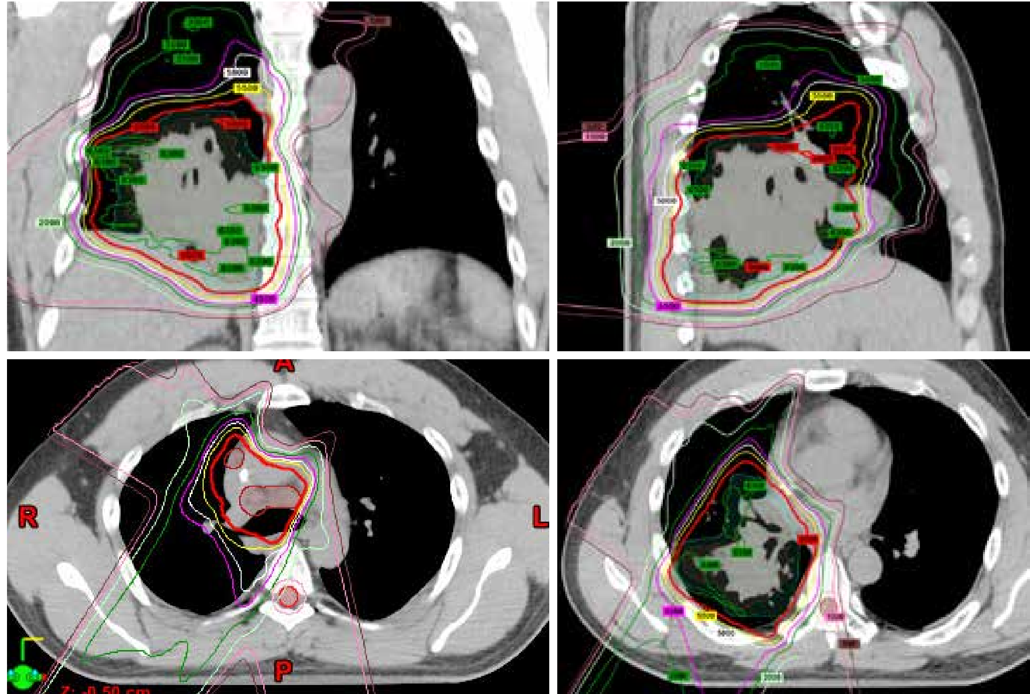

Figure 2 Dose distributions for a patient who received proton-beam therapy for a T3N2 adenocarcinoma of the right lower lobe in a prospective phase I trial. The contralateral lung is almost completely spared.

					50 Gy (RBE) in 4 consecutive fractions
R	S			R	
E	T	Cohort 1		A	Cohort 1 A
G	R	Central		N	SBPT
I	A			D	Cohort 1 B
S	T			O	SBRT
T	I			M	
E	F	Cohort 2		I	Cohort 2 A
R	Y	Recurrent		Z	SBPT
				E	Cohort 2 B
					SBRT

Figure 3 Schema for ongoing trial of stereotactic ablative body radiotherapy (SABR) *vs.* stereotactic body proton therapy (SBPT) for centrally located or recurrent NSCLC. The primary outcome is 2-year toxicity, with a target accrual of 120 patients.

dose constraints, based on extrapolations of those used in standard fractionated regimens and adjusted for biologically equivalent dose, which can be used as a foundation for future trials examining analogous regimens for mediastinal disease. Representative dose distributions for a patient treated to 60 Gy in 4 Gy fractions in that study are shown in *Figure 2*.

Conclusions and future directions

The feasibility of hypofractionated dose-escalated PBT for NSCLC has been demonstrated by several groups at a variety of institutions. The evidence is stronger for early-stage disease, as more studies have focused solely on PBT. The clinical benefit of PBT remains to be seen; SABR, particularly for small, peripherally located lesions, appears to produce excellent results, with local control rates often exceeding 95% and modest toxicity (1). The benefit of hypofractionated SABR in this context may be limited to patients with larger or centrally or superiorly located lesions or patients with recurrent disease. To address this possibility, investigators from MD Anderson and Massachusetts General Hospital have begun a randomized phase II study comparing SABR with SPBT for patients with centrally located stage I, selected stage II, or recurrent NSCLC (*Figure 3*). Candidates for this study must have primary tumors located within 2 cm of the bronchial tree,

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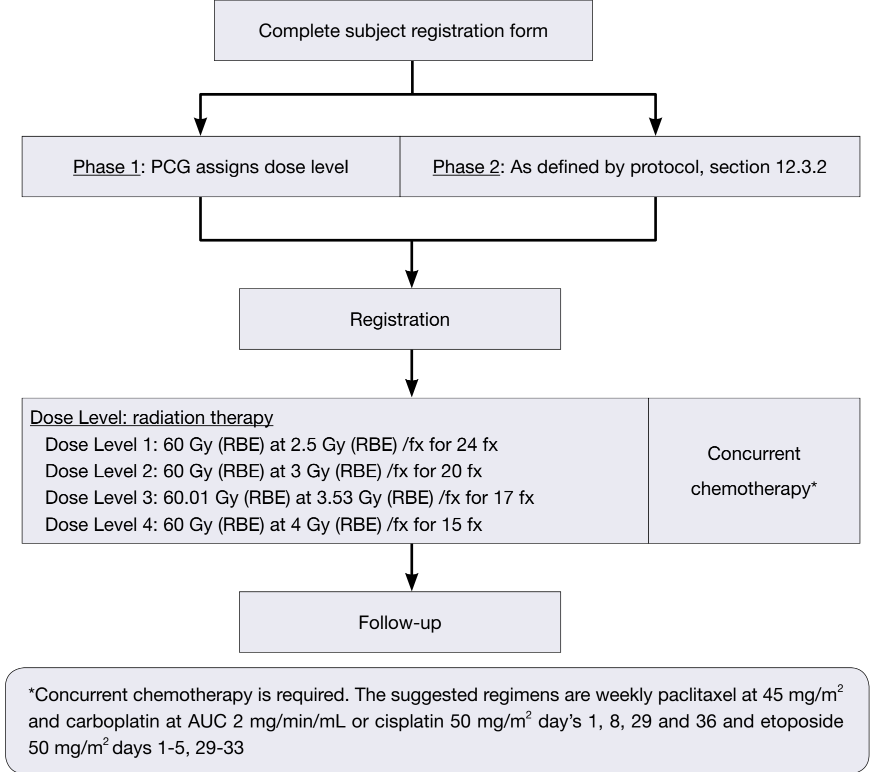

Figure 4 Schema for prospective phase I/II study of hypofractionated PBT, with concurrent chemotherapy, for stage II-III non-small cell lung cancer. This dose-escalation study will enroll 28 patients in the phase I component and 61 in the phase II component. Abbreviations: PCG, Proton Cooperative Group; fx, fractions.

major vessels, or mediastinal structures; or T2/T3 lesions with involvement of the mediastinal pleura or pericardium; or recurrent disease. Patients are randomly assigned to receive SBRT or SBPT to a total dose of 50 Gy in four fractions, and the primary outcome is a reduction in the 2-year toxicity rate. This study will provide valuable information to address the question of whether patients with more challenging tumors would benefit more from SBRT or PBT.

Regarding hypofractionated PBT for locally advanced disease, dosimetric analyses have shown a benefit for PBT over 3D-CRT or IMRT in select cases, and this advantage can reasonably be extrapolated to the hypofractionated context. Several phase I and phase II trials have also demonstrated the feasibility of hypofractionated regimens for patients with stage II-III disease who are not candidates for concurrent chemoradiation, with promising local control rates and acceptable toxicity. However, dose-escalation regimens in such cases have been somewhat limited by normal tissue constraints and the degree to which mediastinal structures can be spared. Ideally, the dosimetric advantages of PBT would translate into the ability to prescribe increasing fraction sizes, which would maintain reasonable rates of adverse events while improving local control. To date, only one published study has focused solely on hypofractionated PBT for NSCLC, and this analysis showed limited toxicity. However, much more information is needed regarding the safety of hypofractionated PBT before it can be widely adopted, and long-term follow-up is urgently needed to assess chronic toxicities (those appearing more than 12 months after treatment) and rates of disease control and survival compared with conventionally fractionated regimens and prior studies using photon techniques. In a phase I/II study recently opened through the Proton Cooperative Group (*Figure 4*), patients are to receive concurrent chemotherapy at escalating doses of hypofractionation; this regimen is intended for patients with higher performance status who are also candidates for systemic therapy. The concept is that the increased sparing of normal tissues afforded by PBT will

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allow more aggressive approaches to be used. Over the next several years, given the growing number of PBT facilities, collaborative efforts in prospective, ideally randomized studies will be crucial for developing appropriately individualized treatments that can take advantage of PBT, a valuable yet limited, resource-intensive, and costly modality, in the hypofractionated setting.

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Combining targeted agents and hypo- and hyper-fractionated radiotherapy in NSCLC

Fiona McDonald, Sanjay Popat

The Royal Marsden NHS Foundation Trust, UK

Correspondence to: Dr. Fiona McDonald. The Royal Marsden NHS Foundation Trust, Downs Road, Sutton, Surrey, SM2 5PT, UK. Email: Fiona.mcdonald@rmh.nhs.uk.

Abstract: Radical radiotherapy remains the cornerstone of treatment for patients with unresectable locally advanced non-small cell lung cancer (NSCLC) either as single modality treatment for poor performance status patients or with sequential or concomitant chemotherapy for good performance status patients. Advances in understanding of tumour molecular biology, targeted drug development and experiences of novel agents in the advanced disease setting have brought targeted agents into the NSCLC clinic. In parallel experience using modified accelerated fractionation schedules in locally advanced disease have demonstrated improved outcomes compared to conventional fractionation in the single modality and sequential chemo-radiotherapy settings. Early studies of targeted agents combined with (chemo-) radiotherapy in locally advanced disease in different clinical settings are discussed below and important areas for future studies are high-lighted.

Keywords: Non-small cell lung cancer (NSCLC); radical radiotherapy; modified fractionation; targeted agents

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Introduction

Arguably one of the most important objectives for cancer researchers remains the reduction in the millions of years of healthy life lost to lung cancer worldwide each year [estimated at 24.5 million in 2008 (1)] with little impact made on the poor relative survival in recent years (2) and improvements in survival trailing behind other cancers (3). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Approximately one third of these patients have early stage disease (stages I and II) at the time of presentation and are usually treated surgically, with radiotherapy being reserved for those who are medically inoperable. Another one third of patients present with advanced disease and radiotherapy is reserved for palliation of symptoms. The remainder of patients present with locally advanced disease (stage III) with the majority being unresectable and the mainstay of treatment is radical intent radiotherapy.

In good performance status patients, the addition of sequentially or concomitant platinum-based chemotherapy is considered as the standard of care in patients with locally advanced disease due to the associated improved outcome (4,5). Importantly, a meta-analysis of over 1,200 patients from six trials comparing concomitant to sequential chemo-radiotherapy reveals the concomitant approach is associated with lower loco-regional disease progression (absolute decrease of 6.1% at five years, from 35.0% to 28.9%) but similar distant disease progression (40.6% and 39.5%, respectively) compared to sequential (6). This suggests an important temporal relationship between the two treatment modalities. The consequent 4.5% increase in 5-year overall survival from 10.6% with sequential to 15.1% with concomitant chemotherapy highlights the opportunity for radio-sensitisation with systemic agents and the relevance of improved local disease control on long term outcome.

However, an estimated 60% of patients with locally advanced disease are not fit enough for concomitant chemo-radiotherapy due to poor performance status and co-morbidities (7). In addition to the less toxic alternative of sequential chemo-radiotherapy, radiotherapy dose

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escalation has been explored, given conventional doses achieve sub-optimal rates of local disease control with estimates of pathologically persistent tumour following treatment in 60% of patients (8). Tumour control probability modelling suggests that using conventional fractionation (1.8 to 2 Gy daily), a dose of 84 Gy is required to achieve 50% probability of tumour control at three years (9), some 18-24 Gy higher than the standard dose radiotherapy. Unfortunately, preliminary clinical data from the RTOG 0617 randomised phase III trial of conventionally fractionated radiotherapy (with concurrent and consolidation platinum-based chemotherapy +/– cetuximab) comparing standard dose (60 Gy) to high dose (74 Gy) has revealed the conventionally fractionated high dose arm is associated with a higher rate of local disease progression (34% compared to 25%) and shorter median survival (19.5 months compared to 28.7 months) compared to standard dose (10). It is as yet unclear the reason for the detrimental effect of the higher dose arm, but the extended duration of treatment by dose escalating using conventionally fractionated may be an important factor.

The alternative strategy is to intensify radiotherapy dose using modified fractionation schedules and reduced overall length of the treatment course with the aim of reducing the effect of accelerated tumour cell repopulation during treatment (11,12). The number of fractions given each day can be increased from one to two or three with at least a 6-hour gap in-between (hyper-fractionation) or the number of daily fractions given can be decreased by increasing the dose per fraction (hypo-fractionation). Such schedules increase the biologically effective dose (BED) (13) delivered to the tumour. Experience with extreme hypo-fractionation in stereotactic ablative radiotherapy for early stage disease demonstrates that a BED of over 100 Gy (using a ratio of 10 for tumour linear to quadratic radio-sensitivity) is required to achieve local disease control rates in excess of 90% (14,15). A recent meta-analysis of over 2,000 patients, of which >80% had stage III disease, from eight trials comparing modified to conventional fractionation radiotherapy schedules reveals modified fractionation is associated with improved overall survival at five years (absolute increase of 2.5%, from 8.3% to 10.8%) compared to standard fractionation schedules and importantly, good compliance with the modified regimens (16). Additionally accelerated radiotherapy is associated with higher pathological complete resection rates than conventional fractionation in patients with stage III NSCLC treated with tri-modality therapy (17). The optimal modified fractionation schedule is yet to be clarified, however accelerated schedules to a total dose of 60-66 Gy are considered optimal for patients considered unsuitable for concomitant chemo-radiotherapy (18).

With the recent increase in understanding of the molecular biology of NSCLC and experience of the use of targeted agents in the advanced disease setting, a number of published studies report on combining targeted agents into radical treatment schedules for locally advanced disease, from addition to concomitant chemo-radiotherapy in good performance status patients to combination with radiotherapy alone in elderly or poor performance status patients. Published studies in the various clinical settings are discussed below.

Molecular biology of NSCLC and epidermal growth factor receptor (EGFR) inhibitors

EGFR is one of a family of four structurally similar tyrosine kinase-associated receptors which comprise the human epidermal growth factor receptor (HER) family. EGFR (HER1 or ERBB1) was the first to be described in humans, and identified to be a protein comprising an extracellular ligand-binding domains, trans-membrane domain and an intracellular tyrosine kinase domain (19). Each receptor must homo- or hetero-dimerise to activate the intrinsic kinase activity and phosphorylate tyrosine residues on the C-terminal tail, activating intracellular signalling pathways. Epidermal growth factor expression has long been regarded as a poor prognostic factor in NSCLC, suggesting its potential as a therapeutic target (20,21).

Since then, a number of small molecule reversible and more recently irreversible EGFR tyrosine kinase motif inhibitors (TKIs) have been developed, with gefitinib and erlotinib both demonstrating modest activity in EGFR wild-type advanced NSCLC (22,23), leading to licensing for erlotinib. The discovery of constitutionally activating somatic EGFR mutations mapping to the kinase domain in 2004 (24,25) changed drug development strategies, with gefitinib, erlotinib and afatinib now licensed for EGFR TKI naïve advanced NSCLC, with an overwhelming consistent evidence from eight randomized trials demonstrating their superior efficacy over chemotherapy in advanced NSCLC. In this setting, toxicities of EGFR TKIs are more manageable than chemotherapy, and toxic fatalities rare usually at up to 3%. Moreover, there seems to be no obvious difference in proportion of grade 3-5 toxicities between the three agents. The most significant serious adverse

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event reported in EGFR-TKI development was initially pneumonitis. However, with greater experience of use of these agents in the advanced disease setting, rates of grade 3-5 pneumonitis are routinely observed at up to 3% of most trial series, with no clear differences between the agents, but a possible geographical distribution, with increased events reported from East Asian series (26). Whether this reflects pharmacogenomic differences or differing clinical diagnostic interpretation remains unresolved.

Unlike the success of the EGFR-TKIs, targeting through antibody inhibition has proven more problematic in advanced NSCLC. Whilst preclinical models demonstrated the activity of anti-EGFR monoclonal antibodies (MAbs) against several carcinoma cell lines, with synergistic activity in combination with cisplatin (27), despite encouraging phase II studies (28) two large randomized phase III trials in advanced NSCLC (29,30) demonstrated little or no survival advantage for the addition of cetuximab to standard platinum-doublet chemotherapy, although subsequent post-hoc analyses suggested potential activity contingent on extent of EGFR expression (31). EGFR MAbs are therefore not standard in advanced NSCLC.

For stage III NSCLC, the combination of EGFR inhibitors and radiotherapy has considerable scientific rationale, despite some of the efficacy concerns identified through advanced disease trials. A positive correlation has been demonstrated between EGFR expression and tumour radio-resistance (32) and the magnitude of over-expression has been correlated with the degree of resistance (33). Radiation damage results in increased EGFR expression and subsequent augmentation of down-stream pathways (34,35). Pre-clinical evidence suggests EGFR blockade potentiates tumour radio-sensitivity. Cetuximab has demonstrated the ability to modulate tumour proliferation, apoptosis and inhibit deoxyribonucleic acid (DNA) repair following irradiation (36-39). Gefitinib has been shown to inhibit the radiation-induced activation of DNA-dependent protein kinase and potentiate radiation response (40,41). Erlotinib similarly causes radio-sensitization potentially through a number of effects including increased apoptosis, cell cycle arrest, and DNA damage repair changes (42). Other mechanisms postulated include micro-environmental changes mediated through decreased vascular endothelial growth factor messenger ribonucleic acid (VEGF mRNA) and protein expression, and blunted hypoxia-inducible factor 1-alpha (HIF-1α) induction (43), with studies of gefitinib (44) and cetuximab (45) demonstrating improved oxygenation.

EGFR inhibitors with conventional fractionation radical radiotherapy alone

In the clinical setting, subsequent to the encouraging improved outcomes with minimal additional toxicity in locally advanced head and neck cancer patients treated with radical radiotherapy combined with cetuximab compared to radiotherapy alone (46), similar studies have been carried out in patients with locally advanced NSCLC. Given the patient population offered radiotherapy alone tend to be elderly and/or with poor performance status, the N0422 phase II single arm study of radical radiotherapy (60 Gy) combined with concomitant cetuximab is interesting (47) (*Table 1*). The cohort of 57 patients with stage III NSCLC who were considered unfit for combined chemo-radiotherapy included either patients aged 65 years or older with an ECOG performance status of 0-1 or patients of any age with a performance status of 2. Fifty patients (86%) completed the entire treatment and there were no treatment related deaths. Grade 3/4 toxicities were experienced by 31 (54%) patients, with the most common side effects being fatigue (9%) and dyspnoea (9%). The median survival of the cohort was 15.1 (95% CI: 31.1-19.3) months. Of note, patients in this study were not staged with positron emission tomography (PET) scans and outdated radiotherapy techniques were used. A similar smaller single arm phase II study, the Near trial, treated 30 patients with stage III NSCLC, who were considered unfit for or who had refused combined chemo-radiotherapy, with radical radiotherapy (66 Gy) combined with concomitant cetuximab followed by maintenance cetuximab (48) (*Table 1*). The median age of this cohort was younger at 71 years and all patients had a Karnofsky performance status of ≥70%, however, the median survival was encouraging at 19.6 (95% CI: 11.5-24.7) months. Treatment completion rate and grade 3/4 toxicity rates were similar at 90% (27 patients) and 40% (12 patients), respectively, with the most common side effect being pneumonia (10%). There were however three deaths (myocardial infarction, bacterial endocarditis related sepsis, pulmonary embolus following deep vein thrombosis) reported as unlikely related to the treatment. Both studies included elective nodal irradiation up to 40-50 Gy, however in contrast to the first study, patients in the Near trial were staged with PET scans and modern radiotherapy techniques were used, including intensity modulated radiotherapy (IMRT) and cone beam CT image guided delivery. It is also noted that while the median percentage of normal lung planned to receive 20 Gy (V_{20}) in this cohort of patients was

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Table 1 Published studies of EGFR inhibitors with conventional fractionation radical radiotherapy alone

Ref	Patients	Disease	Induction	Target dose/ fractionation	RT planning/delivery	Concomitant	Consolidation/ maintenance	Compliance/toxicity	Median survival (months)
(47) Ph II	Number: 57; Age: 77 [60-87]; M/F: 60/40; PS: 22/57/21	Path: 38/43/19; Stage: 0/59/41/0; PET: N	–	60 Gy 30#; Once daily; ENI to 44 Gy	Planning: 2D; Verification: IGRT N	Cetuximab	–	Compliance 86% overall; G3/4 54%; Overall G5 0%; Oesoph G3/4 7%; Pulmon G3/4 9%	15.1
(48) Ph II	Number: 30; Age: 71 [57-82]; M/F: 77/23; PS: (Karnofsky ≥70%)	Path: 33/57/10; Stage: 6/57/37/0; PET: Y	–	66 Gy 33#; Once daily; ENI to 40-50 Gy	Planning: 4D IMRT; PTV: 254 [46-529]; Lung V_{20}: 26% [15-60]; Verification: IGRT Y	Cetuximab	Cetuximab	Compliance 90% overall; G3/4 40%; Overall G5 10%; Oesoph G3/4 3%; Pulmon G3/4 23%	19.6
(49) Ph I	Number: 9; Age: 63 [56-71]; M/F: 89/11; PS: (All 0-1)	Path: 72/14/14; Stage: 0/55/44/0; PET: N	–	60 Gy 30#; Once daily; ENI to 40 Gy	Planning: 3D; Lung V_{20}: All ≤35%; Verification: IGRT N	Gefitinib	–	Compliance 44% overall; G3/4 44%; Overall G5 0%; Oesoph G3/4 0%; Pulmon G3/4 11%	–
(50) Ph I	Number: 26; Age: 56 [30-84]; M/F: 42/58; PS: 4/85/11	Path: 73/15/12;Stage: 0/8/11/81; PET: Optional	54% prior systemic chemotherapy	'Individualised' GTV 70 Gy 30#; PTV 50 Gy 30# Once daily; ± SABR to 1-3 metastatic sites	Planning: 3DCRT/ IMRT; GTV: 56 [5-420]; Lung V_{20}: 14% [3-28]; Verification: IGRT Y	Gefitinib or Erlotinib	69% maintenance median 7.3 months	Compliance 96% overall; G3/4 NR; Overall G5 0%; Oesoph G3/4 4%; Pulmon G3/4 4%	21.8

Abbreviations: Ref, Reference; Ph, phase; Number, number of patients; Age, median age of patients in years [range]; M/F, percentage of males to females; PS, percentage of patients with performance status of 0/1/2; Path, percentage of patients with histological adenocarcinoma/squamous cell carcinoma/other subtypes of NSCLC; Stage, percentage of patients with stage II/IIIA/IIIB/IV disease; PET Y/N, Yes or No to mandatory use of PET for staging; ENI, elective nodal irradiation; 2D, 2 dimensional; 3D, 3 dimensional; 4D, 4 dimensional; CRT, conformal radiotherapy; IMRT, intensity modulated radiotherapy; IGRT Y/N, Yes or No to use of image-guided radiotherapy delivery; GTV/PTV, gross or planning target volume median in cm^3 (range); Lung V_{20}, median percentage of total lung volume receiving at least 20 Gy (range); Toxicity G3/4/5, rates of grades of toxicity; NR, not reported; Oesoph, oesophageal; Pulmon, pulmonary; DLT, dose limiting toxicity; Medial survival, overall median survival in months.

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26%, the range extended up to 60% and therefore included patients at high risk for pulmonary complications due to the radiotherapy (51). Given the skin toxicity rates associated with cetuximab, there is interest in newer EGFR MAbs that demonstrate a lower incidence of skin complications, with phase I studies of nimotuzumab in the palliative radiotherapy setting for NSCLC patients demonstrating feasibility and tolerance (52,53).

Studies of erlotinib and gefitinib in combination with radical radiotherapy alone in locally advanced NSCLC have raised concerns about pulmonary toxicity. In particular, a phase II study from Japan (49) (*Table 1*) on good performance status patients with a median age of 54 years was closed early due to toxicity concerns. Of the nine patients with stage III NSCLC recruited to the study, seven received gefitinib concurrently with thoracic radiotherapy (60 Gy). Three dimensional (3D) conformal planning was used and all plans had a lung $V_{20} \leq 35\%$. Despite this, two of these patients experienced acute pulmonary toxicity (grade 1 and 3) after approximately 30 Gy had been delivered. In contrast, another phase II study from China (50) (*Table 1*) studied 26 patients with stage III or IV disease, treated with 'individualised' radical radiotherapy in combination with either erlotinib or gefitinib. The patients were a heterogeneous group with only 5 (19%) patients having stage III disease. The 21 (81%) patients with stage IV disease had up to three organs treated with stereotactic ablative radiotherapy in addition to radical thoracic radiotherapy given concurrently with the EGFR tyrosine kinase inhibitor. However, treatment was completed as planned in 96% of patients and grade 3/4 pulmonary toxicity rates were acceptable at 4%. The whole cohort had a promising median survival of 21.8 (95% CI: 8.5-35.1) months. Additional toxicity concerns with erlotinib, published in abstract only, come from a small phase I/II Canadian study of erlotinib given concurrently with radical radiotherapy (60 Gy) in poor risk patients with PS 2 or weight loss >5% (54). This study was terminated early due to grade 3-5 pulmonary toxicity in two of five patients.

EGFR inhibitors with conventional fractionation sequential chemo-radiotherapy

An early phase I study demonstrated the safety of combining cetuximab with radical radiotherapy (64 Gy) following induction platinum-based chemotherapy in 12 patients with stage III NSCLC (55) (*Table 2*). One patient died of bronchopneumonia during treatment and two others experienced grade 3 toxicity (a fatigue and a pneumonitis). All patients radiotherapy plans had a lung V_{20} <30% (median 22%).

Subsequently a single arm phase II study, the Satellite trial, treated 71 patients with stage III NSCLC using a combination of cetuximab and radical radiotherapy (68 Gy) following induction chemotherapy (56) (*Table 2*). The patients were of good performance status [0-1] with a relatively low median age of 62 years, however 37% had significant weight loss prior to treatment, a documented poor prognostic factor (60,61). Interestingly, this study omitted elective nodal irradiation, yet despite this PTV volumes up to 1,543 cm^3 (median 586 cm^3) were treated and lung V_{20} parameters up to 54% (median 33%) were documented. Importantly, the study reports high compliance rates, low severe toxicity and a median overall survival of 17 (95% CI: 14.0-23.0) months in the whole cohort and a median survival of 24 months in the patients with <5% weight loss prior to treatment. Impact on health related quality of life with the combination also appears reasonable (62). Of note, the one patient with grade 5 toxicity developed pneumonitis soon after treatment and had a lung V_{20} of 41%, higher than the recommended QUANTEC constraint of 35% (51). Recently a further phase II study of 40 patients with stage II NSCLC reported on experience of cetuximab with concurrent radiotherapy (73.5 Gy) followed by cetuximab and consolidation chemotherapy with paclitaxel and carboplatin (57) (*Table 2*). The radiotherapy volumes and normal tissue constraints are not reported however one patient died from pneumonitis after 56 Gy of radiotherapy. Overall median survival was 19.4 (95% CI: 15.4-26) months and interestingly no oesophageal toxicity > grade 2 was observed.

Again concerns over pulmonary toxicity have been raised in studies of EGFR TKIs in combination with radical radiotherapy given sequentially with systemic chemotherapy. A Japanese phase II study, JCOG 0402 trial, in 38 good performance status patients with stage III NSCLC and median age of 60 years received gefitinib concurrently with radical radiotherapy (60 Gy) following two cycles of platinum-based induction chemotherapy (58) (*Table 2*). Compliance with completing the planned concomitant phase of treatment was low at 63% and a patient (3%) developed grade 3 pneumonitis. However, a promising median survival rate of 28.5 (95% CI: 22.5-38.2) months was reported. The CALEB 30106 phase II study evaluated the addition of gefitinib concurrently with radical sequential or concomitant chemo-radiotherapy to patients with stage

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Table 2 Published studies of EGFR inhibitors with conventional fractionation radical sequential chemo-radiotherapy

Ref	Patients	Disease	Induction	Target dose/ fractionation	RT planning/delivery	Concomitant	Consolidation/ maintenance	Compliance/ toxicity	Median survival (months)
(55) Ph I	Number: 12; Age: 68 [58-76]; M/F: 74/25; PS: 42/58/0	Path: 33/50/17; Stage: 40/60; PET: N	≤4 cycles platinum doublet	64 Gy 32#; Once daily; ENI to 50 Gy	Planning: 3D; Verification: IGRT N; Lung V_{20}: 22% [14-29]	Cetuximab;	–	Compliance 75%; Overall G3/4 17%; Overall G5 8%; Oesoph G3/4 0%; Pulmon G3/4 8%	–
(56) Ph II	Number: 71; Age: 62 [42-81]; M/F: 50/50; PS: 62/38/0; >5% wt lo: 37%	Path: 49/39/12; Stage: 37/63; PET: N	2 cycles cisplatin docetaxel	68 Gy 34#; Once daily; No ENI	Planning: 3D; Verification: IGRT N; GTV: 91 [9-499]; PTV: 586 [135-1,543]; Lung V_{20}: 33% [12-54]	Cetuximab	–	Compliance 82%; Overall G3/4 NR; Overall G5 1%; Oesoph G3/4 1%; Pulmon G3/4 4%	17.0
(57) Ph II	Number: 40; Age: 67 [40-82]; M/F: 65/35; PS: All 0-1	Path: 37/27/35; Stage: 32/64; PET: N	–	73.5 Gy 35#; Once daily; No ENI	Planning: 2D/3D; Verification: IGRT N;	Cetuximab	Paclitaxel carboplatin cetuximab	Compliance 84%; Overall G3/4 NR; Overall G5 3%; Oesoph G3/4 0%; Pulmon G3/4 11%	19.4
(58) Ph I/II	Number: 38; Age: 60 [30-69]; M/F: 37/63; PS: 76/24/0; >5% wt lo: 5%	Path: 97/0/3; Stage: 58/42; PET: N	2 cycles cisplatin vinorelbine	60 Gy 30#; Once daily; ENI to 40 Gy	Planning: 2D/3D; Verification: IGRT N	Gefitinib	Gefitinib	Compliance 63%/24%; Overall G3/4 NR; Overall G5 0%; Oesoph G3/4 0%; Pulmon G3/4 3%	28.5
(59) Ph II	'Poor risk' arm; Number: 21; Age: 68 [41-82]; M/F: 76/24; PS: 0/62/38; >5% wt lo:≥62%	Path: 32/48/20; Stage: 43/57; PET: N	2 cycles carboplatin Paclitaxel	66 Gy 33#; Once daily; ENI to 44 Gy	Planning: 2D/3D; Verification: IGRT N	Gefitinib	Gefitinib	Compliance NR; Overall G3/4 71%; Overall G5 5%; Oesoph G3/4 19%; Pulmon G3/4 10%	19.0

Abbreviations: Ref, reference; Ph, phase; Number, number of patients; Age, median age of patients in years (range); M/F, percentage of males to females; PS, percentage of patients with performance status of 0/1/2; >5% wt lo, percentage of patients with >5% weight loss; Path, percentage of patients with histological adenocarcinoma/squamous cell carcinoma/other subtypes of NSCLC; Stage, percentage of patients with stage IIIA/IIIB disease; PET Y/N, Yes or No to mandatory use of PET for staging; ENI, elective nodal irradiation; 2D, 2 dimensional; 3D, 3 dimensional; 4D, 4 dimensional; CRT, conformal radiotherapy; IMRT, intensity modulated radiotherapy; IGRT Y/N, Yes or No to use of image-guided radiotherapy delivery; GTV/PTV, gross or planning target volume median in cm^3 (range); Lung V_{20}, median percentage of total lung volume receiving at least 20 Gy (range); Toxicity G3/4/5, rates of grades of toxicity; NR, not reported; Oesoph, oesophageal; Pulmon, pulmonary; DLT, dose limiting toxicity; Medial survival, overall median survival in months.

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III NSCLC, based on initial assessment of prognositic factors (59). Patients considered as 'poor risk' in the study were those with a PS of 2 and/or weight loss of ≥5%. These patients were treated similarly to in the Japanese study, with two cycles of platinum-based chemotherapy followed by gefitinib given concurrently with radical radiotherapy (66 Gy). The grade 3/4 pulmonary toxicity rate was 10% with grade 5 pulmonary toxicity rate of 5%. The median survival was 19 (95% CI: 9.9-28.4) months. In both studies PET staging was not mandated and 2D radiotherapy planning was permitted with comparable elective nodal irradiation included to 40-44 Gy. An additional confounding factor for the studies is that in both protocols patients were additionally offered maintenance gefitinib. These studies were designed prior to the reporting of the randomised phase III SWOG S0023 trial of concurrent chemo-radiotherapy and consolidation docetaxel with or without maintenance gefitinib in stage III NSCLC, demonstrating inferior survival for the maintenance gefitinib arm (63).

EGFR inhibitors with conventional fractionation concomitant chemo-radiotherapy

The addition of cetuximab to concomitant chemo-radiotherapy has also been studied in patients with locally advanced NSCLC. The phase II RTOG 0324 study treated 87 good performance status patients radical radiotherapy (63 Gy) and concomitant and consolidation carboplatin, paclitaxel and cetuximab (64) (*Table 3*). The majority of patients were staged with PET and all had 3D conformal radiotherapy. Compliance with treatment was 68% and grade 3/4 toxicity rates were acceptable, however there were six deaths (7%) considered as related to the treatment and at leastthree of these were pulmonary in nature. The median survival was encouraging at 22.7 (95% CI: 15.3-30.4) months. Another phase II study in 101 good performance status patients with locally advanced NSCLC compared high-dose radical radiotherapy (70 Gy) given with concomitant carboplatin and pemetrexed chemotherapy with or without cetuximab, followed by maintenance pemetrexed. PET staging was mandated and 3D or 4D radiotherapy was used without elective nodal irradiation. Compliance was similarly just over 50% in both arms with acceptable grade 3/4 toxicity rates. There were two (4%) patients with grade 5 toxicities in the arm without cetuximab and three (6%) patients in the cetuximab arm, all pulmonary related. The median survival rates were 21.2 and 25.2 months in the non-cetuximab versus cetuximab arms, respectively. The patients were highly selected which may account in part for the higher than anticipated median survival in the non-cetuximab arm. It is important to note this study was designed before lack of efficacy of pemetrexed in squamous histology was known (70). Also there is concern about the effect of the high-dose of radiotherapy used in this study, given in standard 2 Gy daily fractions, due to the recent preliminary results from the subsequent phase III RTOG 0617 study. The RTOG 0617 trial treated 544 patients with locally advanced NSCLC using radical radiotherapy with concomitant carboplatin and paclitaxel chemotherapy followed by consolidation chemotherapy and randomised patients in a 2×2 factorial design between an escalated dose of 74 Gy compared to 60 Gy in 2 Gy daily fractions and between concomitant cetuximab or not. The initial results of the radiotherapy dose analyses demonstrated a worse prognosis in the high-dose compared to standard-dose radiotherapy arm (10), with an 18-month overall survival of 53.9% versus 66.9 %, respectively. Recently, the initial results of the cetuximab analyses were also presented (10) and unfortunately no significant difference was observed in median survival or 18 month overall survival between the cetuximab and non-cetuximab arms (23.1 versus 23.5 months and 60.8% versus 60.2%, respectively). The addition of cetuximab was however associated with increase toxicity compared to the non-cetuximab arm (≥ grade 3 non-haematological 70.5% versus 50.7% and ≥ grade 4 35.8% versus 28.2%, respectively).

Phase I studies of erlotinib and gefitinib given with concomitant chemo-radiotherapy for locally advanced disease have demonstrated feasibility of the combination with both standard (68,69) and high-dose (66,67) conventionally fractionated radiotherapy, although the associated medial survivals reported in these studies have been disappointing (~12-16 months) (*Table 3*). Again confounding factors are noted including for example, lack of PET staging and use of maintenance gefitinib (63) in some studies. In addition, the CALEB 30106 phase II study discussed above in relation to combination of gefitinib given with sequential chemo-radiotherapy, treated the 'good-risk' patients, defined as PS 0-1 with <5% weight loss, with two cycles of induction carboplatin and paclitaxel chemotherapy followed by concomitant gefitinib and chemo-radiotherapy to 66 Gy in standard fractionation, followed by maintenance gefitinib. The median overall survival was poor at 13 (95% CI: 8.5-17.2) months and worse than the median survival of 19 (95% CI: 9.9-28.4) months observed in the 'poor-risk'

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Table 3 Published studies of EGFR inhibitors with conventional fractionation radical concomittant chemo-radiotherapy

Ref	Patients	Disease	Induction	Target dose/ fractionation	RT planning/delivery	Concomitant	Consolidation/ maintenance	Compliance/toxicity	Median survival (months)
(64) Ph II	Number: 87; Age: 64 [42-85]; M/F: 57/43; PS: 47/53/0; >5% wt lo: 0%	Path: NR; Stage: 46/54; PET: 64%	–	63 Gy 35#; Once daily; ENI to 45 Gy	Planning: 3D; Verification: IGRT N	Carboplatin paclitaxel weekly + Cetuximab	2 cycles Carboplatin paclitaxel + Cetuximab	Compliance 68%; NH G3/4 68%; Overall G5 7%; Oesoph G3/4 7%; Pulmon G3/4 9%	22.7
(65) Ph II	Arm A Number: 48; Age: 65 [41-79]; M/F: 56/44; PS: 58/42/0 Arm B Number: 53; Age: 66 [32-81]; M/F: 64/36; PS: 34/66/0	Arm A Path: 46/35/19; Stage: 60/38 Arm B Path: 42/34/24; Stage: 51/45; PET: Y	–	70 Gy 35 #; Once daily; No ENI	Planning: 3D/4D; Verification: IGRT N	4 cycles Carboplatin Pemetrexed Arm A; Carboplatin Pemetrexed + Cetuximab Arm B	≤4 cycles Pemetrexed	Arm A Compliance: 54%; NH G3/4 52%; Overall G5 4%; Oesoph G3/4 16%; Pulmon G3/4 12% Arm B Compliance: 53%; NH G3/4 62%; Overall G5 6%; Oesoph G3/4 13%; Pulmon G3/4 11%	Arm A 21.2 Arm B 25.2
(59) Ph II	'Good risk' arm Number: 39; Age: 64 [44-82]; M/F: 72/28; PS: 46/54/0; >5% wt lo: 0%	Path: 33/41/26; Stage: 54/46; PET: N	2 cycles carboplatin paclitaxel	66 Gy 33#; Once daily; ENI to 44 Gy	Planning: 2D/3D; Verification: IGRT N	Carboplatin paclitaxel gefitinib	Gefitinib	Compliance NR; Overall G3/4 86%; Overall G5 5%; Oesoph G3/4 31%; Pulmon G3/4 11%	13.0
(66) Ph I	Number: 23; Age: 62 [44-82]; M/F: 48/52; PS: 60/40/0; >5% wt lo: 17%	Path: 52/44/4; Stage: 60/40; PET: 91%	2 cycles carboplatin paclitaxel irinotecan	74 Gy 37#; Once daily; ENI to 44 Gy	Planning: 2D/3D; Verification: IGRT N	Carboplatin paclitaxel gefitinib	—	Compliance 86%; Overall G5 0%; Oesoph G3/4 5%; Pulmon G3/4 10%	16.0

Table 3 *(continued)*

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Table 3 *(continued)*

Ref	Patients	Disease	Induction	Target dose/ fractionation	RT planning/delivery	Concomitant	Consolidation/ maintenance	Compliance/toxicity	Median survival (months)
(67) Ph I	Number: 16; Age: 64 [43-79]; M/F: 56/44; PS: 6/94/0	Path: NR; Stage: NR; PET: N	—	70 Gy 35#; Once daily; ENI to 40 Gy	Planning: 3D; Verification: IGRT N	Gefitinib + Dose-escalating docetaxel	2 cycles Docetaxel + Gefitinib	Compliance 88%; Overall G5 19%; Oesoph G3/4 19%; Pulmon G3/4 6%	21.0
(68) Ph I	Step 1 Number: 5; Step 2 Number: 9 Steps 1+2 Age: 60 [38-74]; M/F: 79/21; PS: 93/7/0	Path: NR; Stage: NR; PET: 'optimal'	Variable	63 Gy 34#; Once daily; ENI to 45 Gy	Planning: 3D; Verification: IGRT N	Step 1 gefitinib Step 2 Csplatin + Gefitinib	Gefitinib	Step 1 Overall G5 0%; Oesoph G3/4 0%; Pulmon G3/4 0% Step 2 Overall G5 0%; Oesoph G3/4 22%; Pulmon G3/4 11%	Steps 1+2 12.5
(69) Ph I	Arm A Number: 17 Arm B Number: 17 Arms A+B Age: 63 [39-78]; M/F: 59/41; PS: 71/29	Arms A+B Path: 21/29/50 Stage: 29/71 PET: N	Arm B Carboplatin paclitaxel	66 Gy 33#; Once daily; ENI to 44 Gy	Planning: 2D/3D; Verification: IGRT N	Arm A Cisplatin etoposide erlotinib; Arm B carboplatin paclitaxel erlotinib	Arm A docetaxel	Arm A Overall G5 0%; Oesoph G3/4 18%; Pulmon G3/4 6% Arm B Overall G5 0%; Oesoph G3/4 35%; Pulmon G3/4 0%	Arm A 10.2 Arm B 13.7

Abbreviations: Ref, reference; Ph, phase; Number, number of patients; Age, median age of patients in years (range); M/F, percentage of males to females; PS, percentage of patients with performance status of 0/1/2; >5% wt lo, percentage of patients with >5% weight loss; Path, percentage of patients with histological adenocarcinoma/ squamous cell carcinoma/other subtypes of NSCLC; Stage, percentage of patients with stage IIIA/IIIB disease; PET Y/N, Yes or No to mandatory use of PET for staging; ENI, elective nodal irradiation; 2D, 2 dimensional; 3D, 3 dimensional; 4D, 4 dimensional; CRT, conformal radiotherapy; IMRT, intensity modulated radiotherapy; IGRT Y/N, Yes or No to use of image-guided radiotherapy delivery; GTV/PTV, gross or planning target volume median in cm^3 (range); Lung V_{20}, median percentage of total lung volume receiving at least 20 Gy (range); Toxicity G3/4/5, rates of grades of toxicity; NR, not reported; NH, non-haematological toxicity; Oesoph, oesophageal; Pulmon, pulmonary; DLT, dose limiting toxicity; Medial survival, overall median survival in months.

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patients treated sequentially.

Other targeted agents and radiotherapy for NSCLC

Considerable pre-clinical rationale exists to combine other targeted therapeutics with radiotherapy. The phosphoinositol 3-kinase (PI3K)/Akt/mTOR pathway is transforming for some NSCLC and a number of inhibitors of components of this pathway are in development for advanced NSCLC. Some of these have been shown to be radio-sensitizers in non-NSCLC models (71). Perhaps the best investigated includes abrogation of the tumour microvasculature by vascular disrupting agents (e.g., ZD6126) or anti-angiogenic agents (e.g., bevacizumab). VEGF is known to be upregulated by irradiation and VEGF inhibition is associated with increased tumour control after irradiation in pre-clinical models (72). However, early phase studies have raised toxicity concerns about combinations of agents targeting tumour vasculature or angiogenesis with radiotherapy in NSCLC patients (73) whereas early phase studies of radiotherapy combined with agents targeting tumour cell proliferation and survival pathways demonstrate feasibility (74,75). A recent review highlights the number of pre-clinical and ongoing early phase clinical studies assessing targeting agents in NSCLC patients (76). With the rapidly expanding availability of novel targeted agents and growing experience of these agents in the advanced disease setting, careful consideration of the optimal agents to combine with radiation and study design remains paramount to maximise therapeutic gain and avoid undue toxicity. Guidelines have been published to provide a framework for assessment of novel radio-sensitizers in the pre-clinical and early phase clinical setting (77).

Of the different exploitable mechanisms (78) by which a drug may interact with radiotherapy to improve the therapeutic ratio, it may be that NSCLC patients identified as harbouring an oncogenic driver mutation that confers sensitivity to a specific targeted agent [e.g., echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase gene translocation (EML4-ALK) and ALK TKI crizotinib] will benefit from treatment schedule aimed at maximising spatial co-operation of treatment modalities whereas those without an identifiable mutation may derive benefit from a schedule aimed at maximising the concomitant radio-sensitising approach of combining novel agent with radiotherapy. The central role of DNA damage response to radiotherapy and whether this effect can be modulated by targeted agents remains an important area of research (79). Modulation of the effect of radiation rather than targeting specific driver mutations is also of research interest given the emerging issues of tumour heterogeneity (80).

Targeted agents with altered fractionation radiotherapy in NSCLC

Whilst the majority of studies of targeted agents with radiotherapy in NSCLC have also included concomitant chemotherapy, it is important to maintain a focus on studies of radiotherapy and targeted agent without additional chemotherapy or with sequential chemotherapy for the important group of patients with locally advanced NSCLC who are elderly, have poor performance status or multiple co-morbidities (7). With evidence that modified fractionation schedules are associated with improved outcome compared to conventional fractionation in NSCLC (16) and the experience to date of combining cetuximab with conventionally fractionated radiotherapy alone or sequential chemo-radiotherapy suggesting feasibility with acceptable toxicity, studies of cetuximab with modified fractionation radiotherapy in these settings are warranted. Patient selection remains important with accurate staging and reporting of important prognositic factors in addition to patient demographics to assist the reproducibility of treatment results in the wider population.

Given the initial results from the phase III RTOG 0617 study, there does not appear to be a role for the additional of cetuximab in combination with standard dose concurrent chemo-radiotherapy using conventional fractionation. Interestingly, no significant interaction between the radiotherapy dose and the addition of cetuximab were observed. The question remains as to whether cetuximab can be safely added to modified fractionation schedule chemo-radiotherapy and whether this provides any benefit.

Additional considerations

When considering the total dose of radiation prescribed for a given schedule, it is important to consider that locally advanced NSCLC encompasses a heterogenous population of individuals with differing volume, location and extent of disease. Recently the concept of isotoxic dose escalation was introduced, moving away from a fixed radiotherapy dose prescription for all patients to a tailored prescription based on the surrounding normal tissue dose constraints,

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predicting a certain acceptable probability of toxicity (81). Use of this approach in modified fractionation radiotherapy with sequential or concomitant chemotherapy demonstrates promising results the in phase II setting (82-84). The study of the addition of targeted agents to isotoxic dose escalated accelerated radiotherapy schedules is an interesting area of ongoing research.

For trial design, patient selection remains important and patients need to be optimally staged and stratified based on prognostic variables to ensure the results are repeatable in the wider patient population. State-of-the-art radiotherapy techniques for planning and delivery, including IMRT and image-guided radiotherapy (IGRT), stand to optimise the therapeutic window. Detailed reporting of radiotherapy planning and delivery parameters will reduce the heterogeneity in studies discussed above and permit optimal comparison between studies and reproducibility of outcomes.

Further work is required to improve understanding of the mechanisms of response and toxicity using targeted agents with radiation and to assess for early predictors of response and toxicity, particularly with respect to fraction-size sensitivity with the increasing use of altered fractionation radiotherapy schedules.

Conclusions

Advances in the molecular understanding of NSCLC have accelerated in recent years and the era of personalised medicine in systemic treatment, particularly in advanced disease, has become a reality. At the same time, advances in technology and imaging have led to improvements in patient selection and in accuracy of radical radiotherapy planning and delivery for locally advanced NSCLC. The combination of individualised biological optimisation using novel targeted agents with physical optimisation using state-of-the-art radical (chemo-) radiotherapy, including accelerated-fractionation schedules and individualised radiotherapy dose-prescriptions, stands to improve outcomes in the heterogeneous population of patients with unresectable locally advanced NSCLC.

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Treatment of Lung Cancer

New techniques for assessing response after hypofractionated radiotherapy for lung cancer

Sarah A. Mattonen[1], Kitty Huang[2,3], Aaron D. Ward[1,2], Suresh Senan[4], David A. Palma[2,3]

[1]Department of Medical Biophysics, [2]Department of Oncology, The University of Western Ontario, London, ON, Canada; [3]Division of Radiation Oncology, London Regional Cancer Program, London, ON, Canada; [4]Department of Radiation Oncology, VU University Medical Center, Amsterdam, Netherlands

Correspondence to: Dr. David A. Palma. London Regional Cancer Program, 790 Commissioners Rd E, London, Ontario, Canada. Email: david.palma@lhsc.on.ca.

Abstract: Hypofractionated radiotherapy (HFRT) is an effective and increasingly-used treatment for early stage non-small cell lung cancer (NSCLC). Stereotactic ablative radiotherapy (SABR) is a form of HFRT and delivers biologically effective doses (BEDs) in excess of 100 Gy10 in 3-8 fractions. Excellent long-term outcomes have been reported; however, response assessment following SABR is complicated as radiation induced lung injury can appear similar to a recurring tumor on CT. Current approaches to scoring treatment responses include Response Evaluation Criteria in Solid Tumors (RECIST) and positron emission tomography (PET), both of which appear to have a limited role in detecting recurrences following SABR. Novel approaches to assess response are required, but new techniques should be easily standardized across centers, cost effective, with sensitivity and specificity that improves on current CT and PET approaches. This review examines potential novel approaches, focusing on the emerging field of quantitative image feature analysis, to distinguish recurrence from fibrosis after SABR.

Keywords: Lung cancer; stereotactic radiotherapy; hypofractionated radiotherapy (HFRT); image feature analysis; positron emission tomography (PET)

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Hypofractionated radiotherapy (HFRT) for early-stage lung cancer

HFRT is an effective and well-tolerated treatment for early stage non-small cell lung cancer (NSCLC) (1,2). Although several HFRT schemes have been used historically in the treatment of T1N0 or T2N0 NSCLC, ranging from mildly hypofractionated regimens [e.g., 55 Gy in 20 fractions (3)], to more potent stereotactic regimens (e.g., 54 Gy in 3 fractions), evidence suggests that a biologically effective dose (BED) in excess of 100 Gy_{10} is required for optimal local control (4). Such stereotactic regimens, referred to as stereotactic ablative radiotherapy (SABR) or stereotactic body radiotherapy (SBRT), have been rapidly adopted into clinical use in the last decade (5). SABR is a guideline-recommended treatment for T1/T2 N0 NSCLC when surgery, the gold standard treatment, is not an option due to patient comorbidities or refusal (6-8). SABR is arguably one of the largest medical breakthroughs in the curative treatment of early stage NSCLC in the last two decades, with improved population-based survival rates demonstrated after the implementation of SABR (9-11).

Excellent long-term outcomes support this increasing popularity of SABR as a treatment option for lung cancer. SABR outcomes appear not only superior to more fractionated HFRT regimens (12), but are comparable to standard surgical resection, as supported by retrospective, single- or multi-institution, and modeling studies, with the largest single-institution retrospective study reporting a 5-year local control rate of 89.5% (13-15). Although three randomized studies comparing surgery to SABR have failed to accrue, propensity score matched analyses are available, and have shown comparable, if not superior outcomes post-

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SABR (16,17). In high-risk patients with severe pulmonary comorbidities, SABR offers comparable rates of local control without the attendant short-term mortality risks of surgery (18). In the operable patient population, promising outcomes are reported by two prospective clinical trials: RTOG 0618, reporting a primary tumor failure rate of 7.7% (19), and JCOG 0403, reporting a preliminary 3-year tumor control rate of 86% (20). For institutions without the capability to deliver SABR, other HFRT regimens can also achieve reasonable local control at early time-points: a recent Canadian multicenter study of HFRT delivering 60 Gy in 15 fractions (BED of 75 Gy_{10}) achieved a two-year local control rate of 88% (21).

Response assessment: lung injury after SABR

Response assessment following SABR is complicated by the frequent presence of benign lung injury on follow-up CT. Ablative doses of radiation delivered to the tumor and surrounding lung parenchyma nearly always result in radiologic lung injury (pneumonitis and fibrosis), appearing as an increased density and opacity on CT in the area of the high-dose region, and occasionally a corresponding increase in metabolic activity on functional imaging in the months following SABR (22,23). Such CT changes correlate closely with local delivered dose (24). Such findings are not unique to lung SABR; they have also been described in other organs treated with stereotactic radiotherapy including brain and liver (25,26). From histopathological studies obtained after resection for false-positive imaging studies, these areas of lung injury are made up of a benign mixture of inflammatory cells, fibrocytes and other benign features (27). The appearance of fibrosis is very common, occurring in 62% of patients within six months of treatment (acute) and 91% thereafter (late), as classified by a common classification scheme (22,23). This scheme classifies acute radiation pneumonitis into consolidative or ground-glass opacity changes, which can further be subdivided into diffuse (>5 cm) or patchy (≤5 cm). Late radiation fibrosis can be categorized into modified conventional, mass-like, or scar-like patterns. Although this classification scheme is used to categorize radiological changes following SABR, it is not used to distinguish recurrence from fibrosis. Morphologic patterns of fibrosis can also vary with treatment type; patients that underwent arc-based SABR had a predicted probability of a modified conventional pattern of 96.3% versus 68.9% for those who underwent fixed-beam treatment (28). Although such radiologic lung injury occurs in nearly all patients by two years (22), only a small minority of patients develop clinical symptoms.

Against this background of asymptomatic radiation-induced lung injury, accurate assessment of local recurrence is of paramount importance. Misclassification of a recurrence as “benign fibrosis” can result in a missed window of opportunity for curative-intent salvage treatment. Conversely, misclassification of fibrosis as a recurrence may lead to unnecessary interventions, such as biopsy, imaging, chemotherapy, and even surgery, exposing patients to unnecessary risks and morbidity (27,29-32). The ability to accurately assess response is particularly important in light of the changing practice patterns for early stage NSCLC. As a growing number of patients are being treated by SABR (5), this clinical scenario will become more common. The treatment of a fitter patient population may result in a larger proportion of patients who are candidates for salvage treatment in the case of recurrence. Finally, since recent data on potentially operable SABR patients suggest that failure may be higher than in the inoperable SABR cohort [with two-year lobar failure rates in one recent multicenter study (defined as recurrence anywhere in the irradiated lobe) as high as 19.2% (19)], accurate distinction between recurrence and fibrosis to permit early salvage is a pressing clinical problem.

Distinguishing a recurrent tumor from fibrotic lung changes on CT can be challenging for several reasons (*Figure 1*). Both radiation-induced lung injury and recurrent disease follow a similar temporal course, with lung fibrosis continuing to evolve two years after treatment, during which time, the majority of local recurrences occur (22,33). In contrast to lung injury following traditional 3D-CRT, which was often characterized by straight edges that conform to treatment portals (34) (*Figure 2*), the pattern of lung injury on CT following SABR can be mass-like, due to the conformal nature of SABR (22,31,35). Fibrosis may even appear on CT as an enlarging density and therefore can the mimic the growth of a local recurrence (31).

Current clinical approach for assessing response

Current recommendations for imaging follow-up after SABR are generally based on retrospective evidence and expert opinion, rather than randomized data. Such follow-up serves three major goals: detection of local recurrence, detection of regional recurrence that may be amenable to salvage, and detection of new primary lung tumors, which

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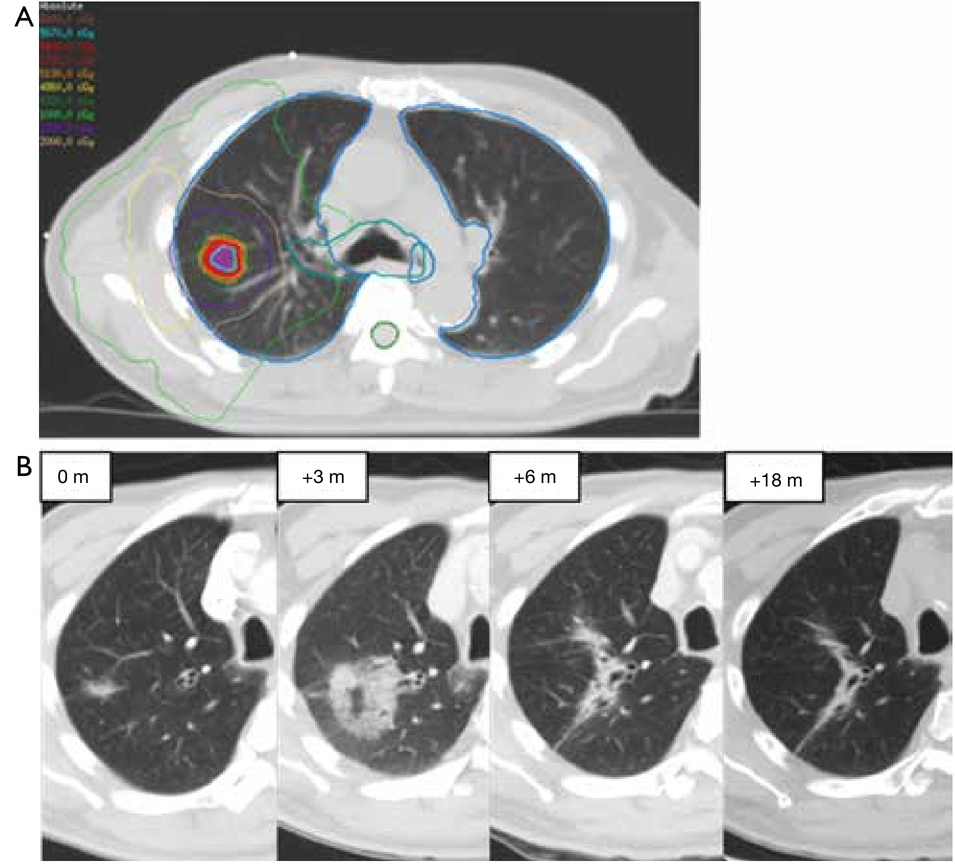

Figure 1 Radiological changes following SABR for an 85-year-old gentleman with biopsy proven adenocarcinoma. This patient received 54 Gy in 3 fractions with the treatment plan shown in (A). Radiological changes are seen (B) where 0 m indicates the pre-treatment lesion measuring 2.0 cm. At 3 months post-SABR, further enlargement of a ground-glass semi-solid opacity measuring 4.3 cm and at 6 months there is interval reduction in size and a decrease in ground-glass opacity, with ongoing reduction in size by 18 months.

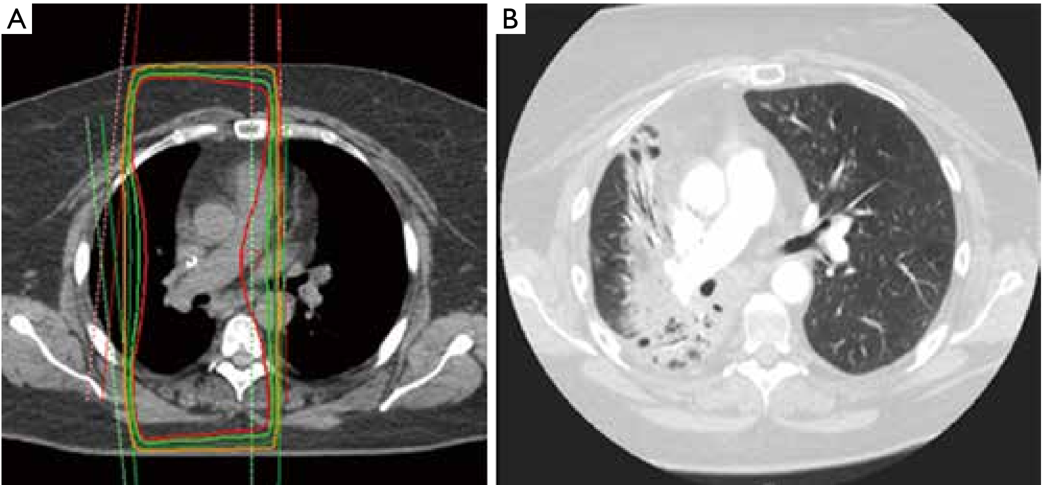

Figure 2 Radiation induced lung injury following a traditional anterior/posterior parallel opposed pair (treatment plan shown in Box A); (B) The resulting benign injury conforms to the treatment portals and is easily distinguished by a straight line.

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Table 1 Selected studies using FDG-PET for detecting recurrence following SABR

Study	Number of patients	Number of recurrences [proportion pathology proven %]	SUV_{max} cutoff	Sensitivity (%)	Specificity (%)	Definition of local recurrence if not biopsied
Essler, *et al.* (50)	29	6 [NR]	5.48	NR	NR	Increase in tumor volume of more than 25% on CT, accompanied by metabolic activity in FDG-PET
Bollineni, *et al.* (49)	132	6 [50]	5.0	NR	NR	Based on growth by more than 20% of the tumor diameter compared with the pretreatment
Zhang, *et al.* (47)	128	9 [78]	5.0	100	91	PET/CT
Takeda *et al.* (48)	154	17 [18]	3.2 (early) 4.2 (late)	100	96-98	Increase in the cross-sectional tumor size of >25% on successive CT scans at least three times over a 6-month period

NR, not reported.

Table 2 High-risk features for recurrence on CT. Data from reference (51)

High-risk feature	Sensitivity (%)	Specificity (%)
Enlarging opacity	92	67
Sequential enlargement	67	100
Enlargement after 12 months	100	83
Bulging margin	83	83
Linear margin disappearance	42	100
Loss air bronchogram	67	96
Cranio-caudal growth of ≥5 mm and ≥20%	92	83

occur at a rate of 2-10% per person-year (33,36). Based on the results of the National Lung Screening Trial (37), the American Association for Thoracic Surgery guidelines recommends four years of CT follow-up for patients who have undergone treatment for lung cancer and are eligible for additional treatment (38).

Tumor response assessment following definitive treatment is typically categorized according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria (39) as complete (disappearance of the target), partial (≥30% decrease), stable disease, or progression (≥20% increase) according to the diameter of the target tumor. However, RECIST 1.1 has limited use in the post-SABR lung setting, since the target lesion may actually represent lung fibrosis, and response may be mis-categorised (11,40). Re-evaluation of RECIST 1.1 has been proposed (41).

Although FDG-PET scans are recommended in lung cancer diagnosis and re-staging (42), functional imaging currently has a limited role in the evaluation of tumor response and detection of local recurrence. Lung injury following ablative radiation doses can commonly result in a metabolically active FDG-avid lesion, which may rise transiently immediately post-SABR and persist after 12 months (43-45). False-positive PET SUV_{max} readings as high as 7.0 have been reported (27,46). Most evidence supports a SUV_{max} of approximately 5.0 as a clinically useful threshold for the distinction between recurrence and fibrosis (47-50). *Table 1* summarizes selected studies using FDG-PET to assess treatment response post-SABR.

Following SABR, recommended surveillance for patients eligible for salvage treatment is routine CT imaging, often at 3-6-month intervals in the first year, then annually thereafter (8,38). A systematic review of the literature on the role of imaging in discriminating recurrence from fibrosis provides structured recommendations based on the available evidence, citing high-risk features (HRFs, *Table 2*) on CT (31,35,52) and specific SUV_{max} thresholds to estimate the probability of recurrence and appropriate investigations into "no-risk" "low-risk" and "high-risk" categories (23). The clinical performance of the HRFs was validated by a blinded assessment of matched CT datasets from pathology-proven recurrences and non-recurrences (51). The concurrent presence of ≥3 HRFs provides a useful cutoff (sensitivity and specificity both >90%) for detection

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of recurrence.

There are several advantages to the use of CT, rather than routine functional imaging, in assessment of response post-SABR. In contrast to FDG-PET imaging, CT is more accessible and inexpensive, does not rely on isotopes with short half-lives, and is already part of standard-of-care follow-up for patients who have received curative treatment for early-stage lung cancer, and who are eligible for salvage. Importantly, standardization of CT across centres is much less complex than standardization of PET/CT. Lack of PET/CT standardization can be an important confounder: measured SUVs can be affected by multiple factors, including technical, physical, and biologic (53). In order to generalize PET/CT findings, minimum performance or harmonizing standards are needed for many factors including uptake period, patient motion, inflammation, blood glucose level correction, as well as scan acquisition and reconstruction parameters. Standard machine settings and reconstruction algorithms are widely available for CT imaging of the chest, increasing the generalizability of any follow-up recommendations. As such, new algorithms for early detection of recurrence based on standard-of-care CT imaging could be easily integrated into current clinical practice. However, novel imaging techniques must move beyond qualitative image analysis and simple RECIST measurements.

Quantitative image feature analysis

In contrast to qualitative image assessment described above, quantitative image feature analysis extracts measurable information from within an image, such as intensities or densities, shape or morphology, or texture. Intensity refers the brightness of an individual voxel; in CT imaging this can also be described as density and is quantified in Hounsfield Units (HUs). HUs measure the attenuation of a material relative to water (HU =0). The shape or morphology of a region describes the geometry of the external boundary. “CT image texture” is a set of more complex measurements which describe local brightness variation or the spatial arrangement of intensities in an image (54,55).

Image feature analysis has emerging roles in general medicine and oncology. Numerous imaging modalities can be used for quantitative image analysis at different body sites, including CT, magnetic resonance imaging (MRI), ultrasound, and mammography (56,57). Applications in oncology include the computer-aided detection or diagnosis of diseases such as breast and bladder cancer (56,57). Texture analysis of the liver has suggested that texture parameters may distinguish high-risk from low-risk colorectal cancer patients (58). Texture analysis on MRI, CT, and PET has been able to diagnose and characterize tumor heterogeneity for several tumor types and is showing promise in response assessment and as a predictive biomarker (59,60). In the thorax, the use of quantitative image feature analysis on CT has been widely investigated in many benign diseases, including characterizing pulmonary infections as well as varying benign lung disease patterns (61-63). Texture analysis, specifically the product of tumor uniformity and gray-level, has also been correlated with tumor response following chemotherapy in advanced stage NSCLC (64).

Quantitative image analysis workflow

Figure 3 demonstrates the typical workflow for quantitative image feature analysis. In general, image acquisition should be standardized to minimize any variability between scanners, imaging parameters, or reconstruction techniques. Standardization includes the use of the same scan protocol for imaging acquisition, with consistencies in settings such as kV, mAs, slice collimation, and slice thickness. Breathing instructions and the use of intravenous contrast should also be consistent across all patients, although patients with contra-indications to contrast injection must be noted and studies analyzing the effect of contrast on image feature analysis should be performed. Reconstruction kernels or filters are used to determine image quality of a CT scan and are chosen based on the intended clinical application of the scan. Such decisions are a compromise between spatial resolution and noise, and depending on the organ being scanned, may require a smoother image with less noise or a sharper image with higher noise. Reconstruction kernels should also be consistent across all images and a higher sharpness thorax kernel should be used when available. However, optimal scan parameters and reconstruction kernels must be investigated for the effect of variations among these settings on quantitative image feature analysis.

Image feature analysis can be performed on any region of interest (ROI), such as tumor, normal lung, or fibrotic regions; such ROIs can be selected by means of manual, semi-automated, or fully-automated methods. A manual method involves delineation of an ROI by an investigator on each individual slice using imaging software. Manual methods do not require specialized algorithms, but can be tedious and time consuming, and are subject to intra- and inter-observer variability (65). A semi-automated

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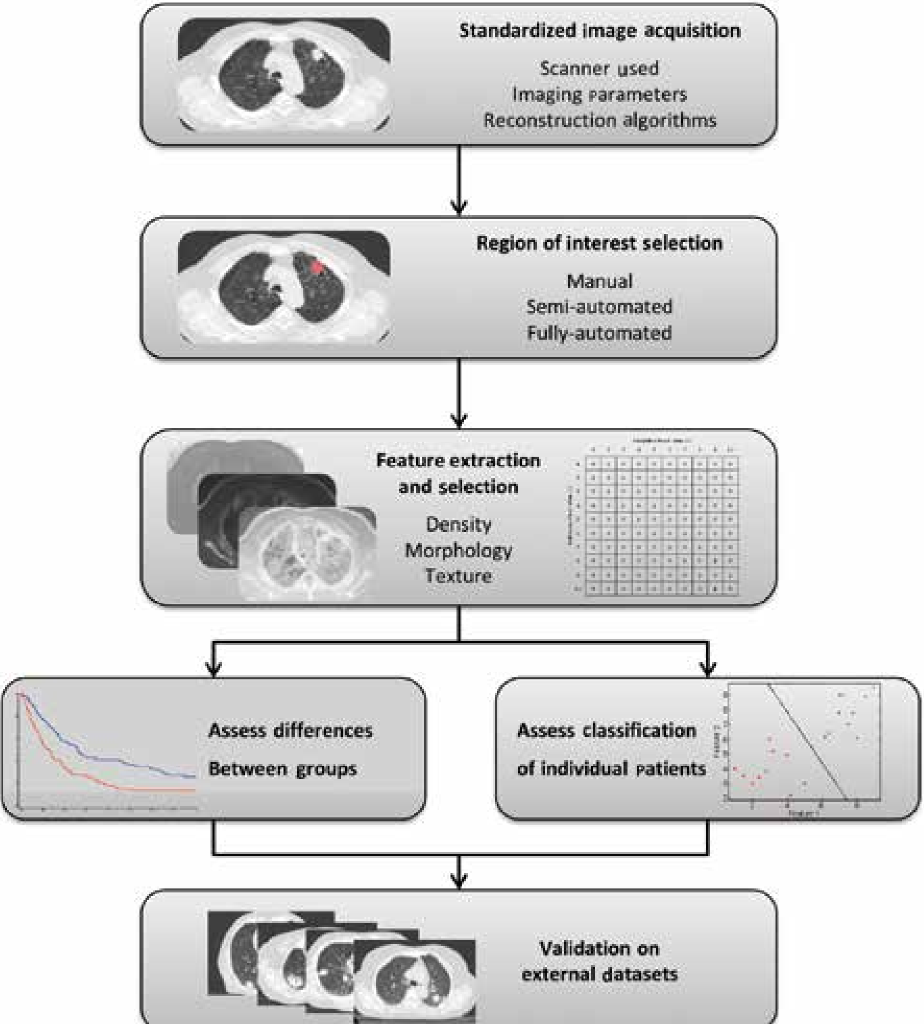

Figure 3 Typical workflow for image feature analysis.

method requires a smaller amount of user input, and may require a user to initialize the segmentation by selecting a point or ROI. A fully automated approach requires no user interaction or input and the image is automatically segmented based on a series of predetermined parameters. This makes a fully automated approach quick and reproducible; however the lack of user input or knowledge can be an issue in terms of reliability. Therefore, semi-automated approaches to segmentation have become increasingly popular as they are reproducible, fast, and require minimal user input or knowledge (66).

After ROIs are delineated, quantitative measures can then be extracted including measures such as density, morphology, or texture, and these measures can be evaluated as predictive or prognostic biomarkers. Extracted measures can be calculated with a variety of input parameters and settings specific to each case. Such measures range from simple first-order assessments such as the mean HU density within a region, to complex measures of the spatial relationship of voxel intensities, for example analyzing neighboring voxels of varying distances apart.

Optimal features or sets of features for predictive or prognostic biomarkers must be determined and validated through training and testing on multiple data sets. This can include analyzing individual features alone or a combination of these features together. Due to the large number of metrics available as well as the large number of possible combinations of these metrics, the high-risk of type I error

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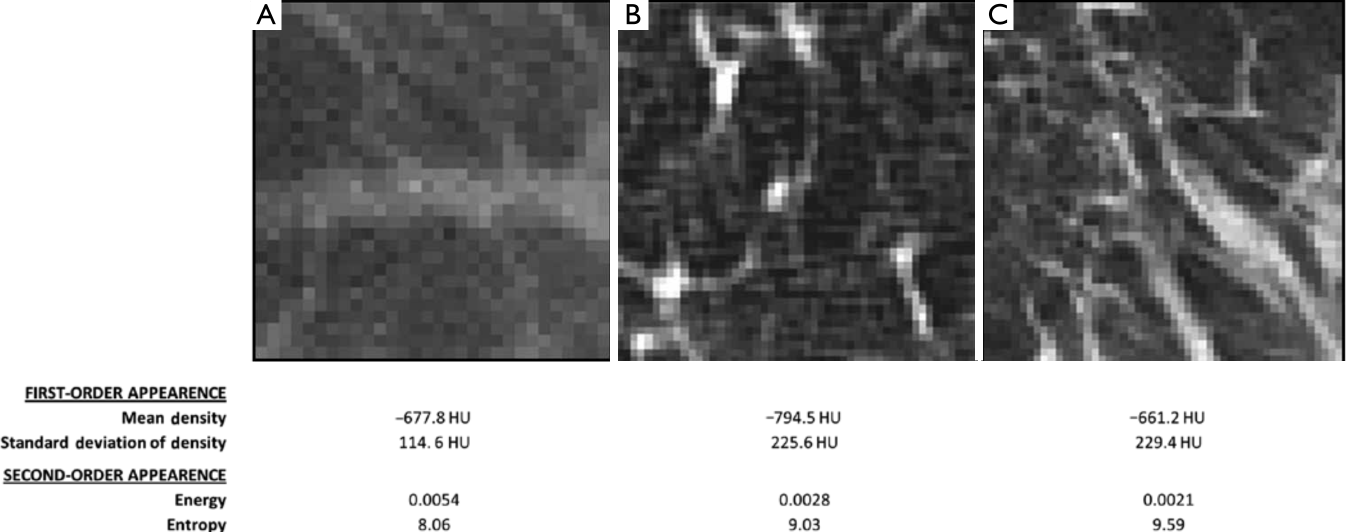

Figure 4 Sample lung images showing the variations in two first-order appearance measures [mean density and standard deviation of density (first-order texture analysis)] and two second-order appearance measures, energy and entropy. (A) and (C) have similar mean densities, but are better differentiated by the first- and second-order texture measures. (B) and (C) have similar first-order texture values, but are better differentiated by the second-order measures.

must be recognized when comparisons and cross-validations are performed. As a result, initial exploratory studies must be considered hypothesis-generating, and validation on external datasets is crucial.

Common metrics used for image feature analysis

Image feature analysis metrics can be defined as first-order, second-order, and third-order. First-order image appearance features measure the global appearance of a ROI and do not take into consideration relationships between adjacent voxels. A common example includes the mean density based on CT HU. The standard deviation of density can be used as a first-order texture feature, which shows the global variability of densities within a region (*Figure 4*). Second-order appearance measures characterize the intensity relationships between voxels pairs in an image, whereas third-order measures (which are less commonly used) consider the spatial relationship of three or more voxels in an image. Extraction of second and third-order texture features can be performed in many ways, including statistical methods, structural methods, model-based methods, and transform-based methods (67).

Statistical texture analysis is the most frequently cited method of texture analysis. This approach describes texture through high-order statistics of an image intensity histogram (67). This analysis typically assesses neighboring voxel pairs; however it can be done with multiple spatial directions and distances. Second-order statistical texture features are typically computed with the use of a grey-level co-occurrence matrix (GLCM). As shown in *Figure 5*, A GLCM is a square two-dimensional matrix g, in which the row and columns correspond to image intensity values. Each element in the matrix $g(i,j)$ contains a non-negative integer corresponding to the number of voxel pairs whose intensity values are i and j. A variety of texture measures can be calculated from the GLCM, such as energy, entropy, inverse difference moment (IDM), inertia, cluster shade, and cluster prominence (68-70). In general, energy and entropy measure the orderliness of the GLCM, or the homogeneity of the image. IDM and inertia measure the contrast of the image, and cluster shade and cluster prominence measure the symmetry of an image.

An example of images with their corresponding first-order and second-order appearance measures is seen in *Figure 4*. The variation in the number and distribution of vessels in the image results in differences in feature measurements. For example, *Figure 4A* and *C* have similar mean densities but are better differentiated by the texture measures, both first-order and second-order. *Figure 4B* and C have similar first-order texture feature measurements but are differentiated by the second-order measures of energy

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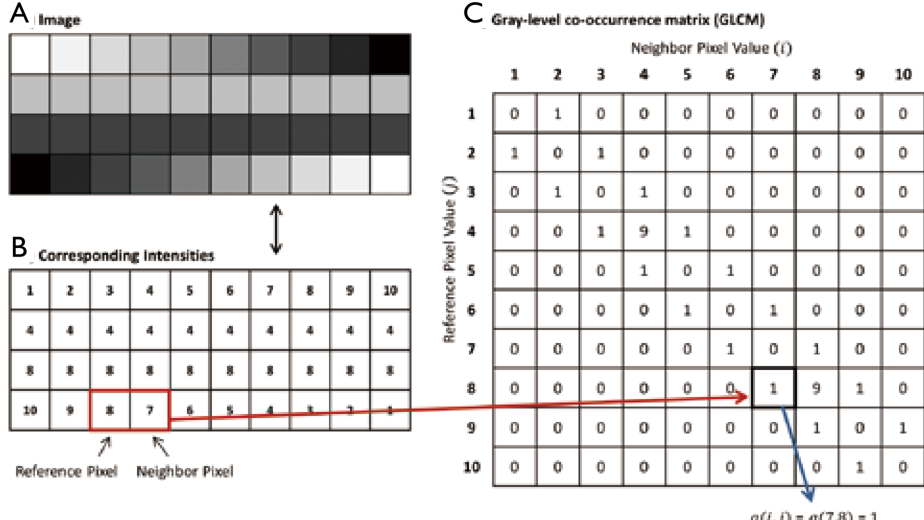

Figure 5 A sample image (A) with its corresponding numerical intensity values (B). The gray-level co-occurrence matrix (GLCM) for this image can be seen in (C), with the pixel relationship for analysis being one voxel to the right, as indicated by the reference and neighbor pixel.

and entropy. Each measure can extract specific information from the image, and overall first-order measures are less sensitive to spatial variations in intensities whereas second-order appearance measures are taking neighboring voxels into account and are therefore sensitive to the relationship of voxels.

Image feature analysis post-SABR

Several studies have examined simple dose-response relationships of HU changes following SABR. Increasing densities on CT post-SABR are seen with larger planning target volumes and longer time post-SABR, and these are most evident in regions receiving doses greater than 20 Gy (24). Density changes post-SABR have also been shown to linearly increase to doses of 35-40 Gy and then plateau thereafter (24,71). The spatial location of fibrosis following SABR is on average 2.6 cm from the gross tumor volume (GTV) position, although displacement of the fibrotic changes of >5 cm can also be observed (72).

Quantitative image analysis has been investigated for distinguishing RILI and recurrence following SABR (*Figure 6*). A preliminary study of 13 RILI lesions and 11 recurrent lesions (8 biopsy proven) suggested that first-order appearance measures could significantly distinguish RILI and recurrence patient groups at 9 months following treatment, with recurrence patients having significantly brighter consolidative changes (73). The standard deviation of densities within regions of GGO (first-order texture analysis) could also distinguish the groups at nine months, with recurrence patients having a larger standard deviation (variability) of densities. This indicates that these patients have a more variegated texture within the GGO, as seen in *Figure 4*. In contrast, size measures (RECIST or 3D volume) could not differentiate the groups until 15 months post-treatment. A preliminary study of predictive abilities of these measures has shown that the first-order texture analysis within the GGO was the best predictor of recurrence at nine months post-SABR with accuracies of 74% (74).

Further investigation has evaluated texture changes in the immediate post-SABR period. At 2-5 months post-SABR, preliminary analysis suggests that the basic measure of ground-glass texture alone can predict recurrence with 81% accuracy (75). Several second-order texture features have also shown promise, including energy and entropy, with leave-one-out cross validation accuracies of 81% and AUCs of 0.79-0.81 (75). Patients with recurrence had significantly higher entropy and lower energy values. In contrast, traditional measures of response such as RECIST performed inferiorly, with accuracy of 61% and an AUC of 0.72. These results suggest that early quantitative appearance changes may precede any changes in size, and as such may serve as early biomarkers of recurrence in

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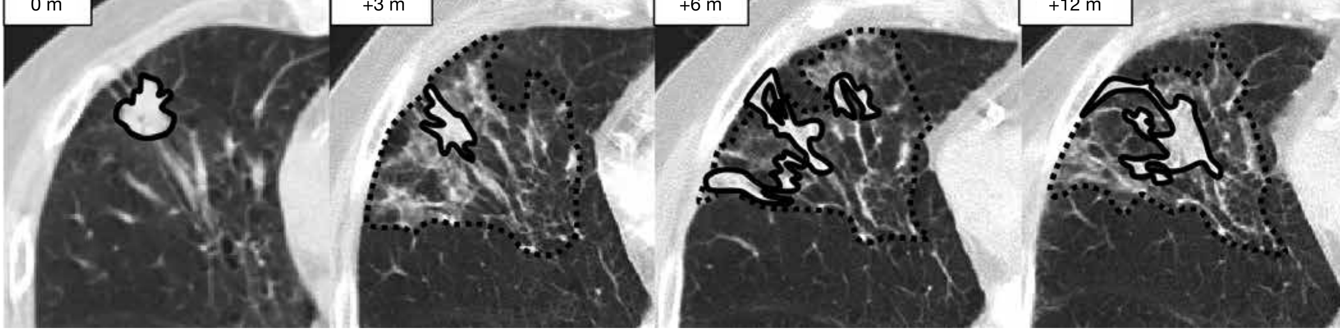

Figure 6 Post-SABR consolidative and ground-glass opacity findings throughout follow-up for a patient with radiation-induced lung injury (A) and recurrence (B). The zero-month (0 m) time point indicates the pre-treatment lesion. The solid lines enclose consolidative regions and the dashed lines enclose ground-glass opacity regions.

individual patients. Quantitative image analysis allows for maximal information to be obtained from images already being performed in clinical practice, and can easily be translated into a useful clinical tool to aid in treatment response assessment. Further quantitative metrics, including additional second-order textural features and shape analysis, should be investigated and validated for early prediction of recurrence following SABR.

Future directions and potential pitfalls

Novel imaging modalities may allow for better assessment of treatment responses following SABR or HFRT. In addition to standard FDG-PET reporting SUV_{max} values, functional imaging with additional metrics such as metabolic tumor burden markers may show improvement for assessing response. Preliminary studies have investigated using pre-treatment measures such as metabolic tumor volume and total lesion glycolysis for assessing clinical outcomes after SABR, however further studies with larger samples and follow-up periods are needed (76). Additional PET tracers such as 18-fluoroazomycin-arabinoside (FAZA) and 18F-fluoromisonidazole (F-MISO) are used for imaging hypoxia in head and neck cancers (77,78) and could also be investigated for assessing response following HFRT.

Perfusion imaging, such as dynamic-contrast-enhanced-CT (DCE-CT) or MRI (DCE-MRI) characterizes vascular properties of a tissue and can quantitatively map their spatial distributions. Measures such as blood volume, blood flow, permeability, and mean transit time can be calculated after administration of a contrast agent. Both DCE-CT and DCE-MRI have shown promise as prognostic or predictive biomarkers in oncology, and their value in assessing response after SABR warrants investigation (79,80).

Several potential pitfalls must be considered when evaluating novel imaging modalities for response assessment. First, the gold-standard definition of “recurrence” varies across studies, and many studies use

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imaging-based definitions of recurrence, rather than pathologic confirmation. Such imaging-based definitions of the endpoint may introduce substantial bias and create a self-fulfilling prophesy: if imaging features are used to define "recurrence" (e.g., sequential growth of lesion) and then the same features are assessed to predict these "recurrences", their performance may be artificially inflated. The majority of studies include only a small number of biopsy-proven recurrences, with remainder of patients defined as recurrence based on an increase in tumor size on successive CT scans (48,49,81). Many also use a modified progression criterion of two consecutive enlargements on CT to define recurrence, which hampers response assessment at an early time point, and suggesting that and that the usefulness of PET is limited. Since recurrences are uncommon after SABR, large databases are required to have sufficient events for analysis, and any new promising markers require robust external validation, since the chances of type I error are high when multiple features are being assessed. Variations in standardization of imaging protocols in both CT and PET studies must be assessed for their impact on predictive ability. Finally, post-SABR surgical studies, including registration of digitized histology to CT, would be valuable for correlating imaging findings at the voxel level with true pathologic outcome.

Conclusions

Distinguishing recurrence from fibrosis following SABR for early-stage lung cancer is expected to become an increasingly common clinical problem. Although recommendations exist for CT- and PET/CT-based follow-up after SABR, better metrics are required for early detection of recurrence, to allow for salvage, and to avoid unnecessary investigations in patients with benign radiation-induced lung injury. Promising new techniques may involve more robust analysis of currently-obtained imaging, such as CT texture analysis, or introduction of novel imaging modalities into routine clinical practice. Large imaging datasets are required for assessment and subsequent independent validation of novel new imaging biomarkers.

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Molecular markers to predict clinical outcome and radiation induced toxicity in lung cancer

Joshua D. Palmer[1], Nicholas G. Zaorsky[1,2], Matthew Witek[1], Bo Lu[1]

[1]Department of Radiation Oncology, Kimmel Cancer Center and Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA, USA; [2]Department of Radiation Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA

Correspondence to: Joshua D. Palmer, M.D. Department of Radiation Oncology, Jefferson Medical College of Thomas Jefferson University, Kimmel Cancer Center, 111 South 11th Street, Philadelphia, PA 19107, USA. Email: Joshua.palmer@jeffersonhospital.org.

Abstract: The elucidation of driver mutations involved in the molecular pathogenesis of cancer has led to a surge in the application of novel targeted therapeutics in lung cancer. Novel oncologic research continues to lead investigators towards targeting personalized tumor characteristics rather than applying targeted therapy to broad patient populations. Several driver genes, in particular epidermal growth factor receptor (EGFR) and ALK fusions, are the earliest to have made their way into clinical trials. The avant-garde role of genomic profiling has led to important clinical challenges when adapting current standard treatments to personalized oncologic care. This new frontier of medicine requires newer biomarkers for toxicity that will identify patients at risk, as well as, new molecular markers to predict and assess clinical outcomes. Thus far, several signature genes have been developed to predict outcome as well as genetic factors related to inflammation to predict toxicity.

Keywords: Lung cancer; biomarkers; toxicity; novel therapies

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Introduction

In 2013, an estimated 228,000 new cases of lung cancer will be diagnosed in the United States and more than 70,000 will die from the disease. The risk of developing lung cancer for all American men and woman during their lifetimes is between 6-7%. This risk increases with age, genetic susceptibility and toxic exposures (e.g., smoking) (1). Lung cancer is a heterogeneous group of carcinomas comprised of several histologic subtypes: adenocarcinoma, squamous cell carcinoma, and large cell and small cell neuroendocrine tumors. The vast majority of molecular research focuses on the most prevalent histologic subtypes: adenocarcinoma and squamous cell carcinomas.

Since the initial heralding in the last decade of "the six hallmarks of cancer", advances in the study of molecular pathways, identification of biomarkers and novel targeted therapies have made their way to clinical applications and widened the scope of our understanding of the molecular pathogenesis of lung cancer (2,3). The appropriate introduction of targeted therapies into current standards of care remains an open area of clinical investigation.

The current understanding of the mechanisms of transformation from normal physiologic epithelial cells to malignant lung cancer has evolved alongside our increasing knowledge of many other cancer types and falls into a multi-step paradigm (4,5). A series of either chromosomal or nucleotide aberrations and epigenetic events in driver genes lead to immortality and the malignant phenotype of lung cancer (6). It is theorized that during this multi-step transformation, certain driver genes cause "addiction" and are required for tumor maintenance and targeting these biomarkers will lead to the eradication of selective cancer cells.

Various lung cancer biomarkers have been identified, including epidermal growth factor receptor (EGFR) mutations, EML4/ALK fusion genes, p53 mutations, RAS/MAP kinase mutations, Her-2 overexpression and PI3K/mTOR mutations.

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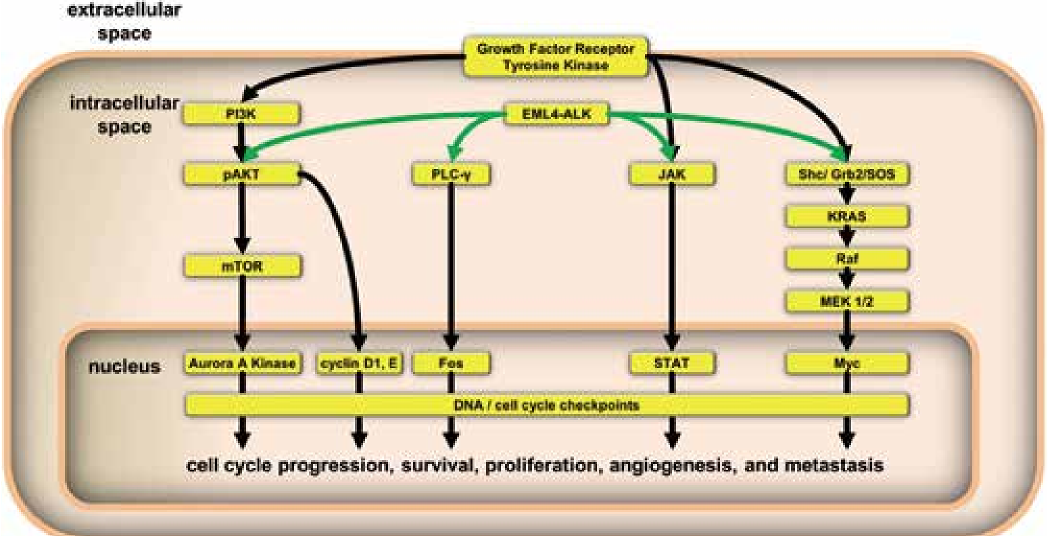

Figure 1 Summary of intracellular signaling pathways containing the crucial driver genes in lung cancer which promote tumor cell proliferation, survival, angiogenesis and metastatic potential.

A consequence of targeted radiotherapy in lung cancer is damage to the surrounding organs at risk which include the lung and heart. The majority of molecular biomarkers of toxicity in lung cancer focus on lung damage or pneumonitis. Attempts have been made to combine dosimetric parameters in lung radiotherapy with various lung biomarkers to define a group of patients most at risk for severe lung toxicity.

Lung cancer molecular markers

The search for a cancer biomarker or targetable genetic aberration requires years of preclinical studies *in vitro* and *in vivo*. Currently there are approximately a dozen biomarkers that have demonstrated clinical benefit and another dozen are currently under investigation (7). Of these, several are considered lung cancer driver genes by the NCI's lung cancer mutation consortium. These include EGFR, KRAS, HER2, PI3K, BRAF and ALK fusions (4). Of these EGFR, KRAS, HER2 and ALK fusions are predictive of response to targeted therapies (5,8-11). These driver genes play an important role in lung cancer tumorigenesis involving alterations in their proliferative potential, apoptotic signaling, angiogenesis and invasion/extravasation. Clinically relevant pathways are depicted in *Figure 1* and include the RAS/MAP kinase, PI3K/AKT/mTOR, JAK/STAT pathways and cell cycle checkpoints. It is known that, in varying degrees, these biomarkers are mutated, amplified or overexpressed in non-small cell lung cancers. *Table 1* outlines the relative frequency with which each driver gene occurs in lung cancer (5,8,12,13).

EGFR

This family of receptor tyrosine kinases (RTKs) include the EGFR or HER1 and HER2-4 (14). They are a group of RTKs with approximately 75% homology that once bound to an extracellular ligand form homo- and heterodimers which leads to their intracellular signaling (5). The vast majority of mutations in this family occurs within the tyrosine kinase domain and correlate with drug sensitivity (15). Therapeutic targets for this family are summarized in *Table 1* and include small molecule inhibitors, gefitinib and erlotinib, and monoclonal antibodies, cetuximab and trastuzumab. Interestingly mutations in EGFR seem to occur more frequently in never-smokers, people of Asian descent, and women with adenocarcinomas (5,15). These groups also seem to be more sensitive to molecular inhibition. Several studies have found both EGFR amplifications and most mutations correlate with improve clinical outcomes (8). There are, however, mutations that predict a negative response to EGFR inhibition which include the T790M mutation, a concomitant KRAS mutation or MET amplification. More recent studies

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Table 1 Lung cancer genetic aberrations and associated targeted therapy

Biomarker gene	Aberration	Targeted therapeutic	Frequency of aberration [%]
EGFR	Mutation or amplification	Gefitinib, erlotinib, cetuximab	[10-25] (35% in Asian patients)
HER2 (ERBB2)	Mutation or amplification	Trastuzumab	[5-10]
BRAF	Mutation	Sorafenib	[2-3]
p53	Mutation or deletion	Advexin a p53 adenoviral vector	[30-50]
VEGF	Overexpression	Bevacizumab, afibercept	
PI3K	Modified and activated	BEZ235, LY294002	[1-3]
mTOR	Activated	Rapamycin, RAD001, CCL-779	[70-75]
RAS	Mutation leading to activation	Tipifarnib, lonafarnib	[10-15] (20-30% in Adenocarcinoma)
MEK	Activated	Trametinib, salumetinib	[1-2]
c-KIT	Overexpressed	Imatinib	[1-2]
EML/ALK	Fusion	Crizotinib	[5-13]

suggest a D761Y mutation in exon 19 and insertion within exon 20 leads to further resistance to targeted therapy (16). HER2 mutations occur much less frequently although mutations seem to correlate with those in EGFR mutated patients. Targeting Her2-4, however, has not led to improved outcomes in unselected patients and large groups of patients harboring these mutations have not been identified (8,9,17,18).

RAS/RAF/MAP kinase pathway

In lung cancer, nearly all clinically relevant mutations in the RAS family occur in KRAS. Once mutated RAS is activated and may lead to cellular transformation and sustained proliferation making this family an ideal candidate for targeting. Several drugs, among them tipifarnib and lonafarnib, are known as farnesyl transferase inhibitors and have been developed to target RAS modification. In order to perform intracellular cell signaling (8), RAS requires modification with a farnesyl group. This allows proper attachment to the cell membrane. Without proper modification and cell membrane localization, RAS becomes ineffective.

BRAF is a part of a family of serine/threonine kinases downstream of RAS. BRAF is mutated in lung cancer but this occurs much less frequently than with melanoma (*Table 1*). Because the mutations in BRAF differ substantially between lung and melanoma, the translational use of vemurafenib for treatment of lung cancer is unlikely. However, the use of oral RAF kinase inhibitors like sorafenib is being studied. Sorafenib is unique in that it is an inhibitor of the RAF/MAP kinase pathway and has activity on multiple tyrosine kinases (VEGF and PDGF) allowing for multiple pathways involved in lung tumorigenesis to be targeted (8,11,19).

Once activated BRAF signals MEK1/2 which goes on to activate the MAP kinase pathway through ERK1/2. These downstream effectors are known to be constitutively activated in human lung cancer cell lines. Oral inhibitors such as Cl-1040 and PD03244901 have been developed and studies are actively being pursued (8,20).

ALK translocations (ALK/EML4 and ROS1)

The echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene (EML4/ALK) is the most common form of translocation. The fusion protein results in a constitutively active tyrosine kinase (21). This fusion product is more common in the young, low volume or never-smokers with adenocarcinoma histology with signet ring features. ALK rearrangements are clinically detected with fluorescence *in situ* hybridization. A dual ALK translocation inhibitor called crizotinib is available to suppress the effects. Both preclinical and clinical testing has demonstrated radiosensitivity and remarkable response rates of EML/ALK positive tumors to therapy with crizotinib (9,22). Several second site mutations L1152R, L1196M and C1156Y have been and confer resistance to crizotinib treatment. ROS1 rearrangements have also been identified recently to remain sensitive to crizotinib (8).

P53

The p53 protein is a transcription factor that is modified

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in various cellular stress situations. It functions to initiate apoptosis or to arrest the cell cycle. P53 is well known, as it is the most frequently mutated gene in human cancers (4). The majority of mutations in p53 are inactivating mutations, or deletions, although some missense mutations result in a gain-of-function phenotype that portends a poor prognosis in lung cancer (8). Classically, cigarette smoking is linked to transversion mutations in lung cancer. Clinical applications to subvert p53 have been made by using adenoviral gene replacement vectors to re-introduce wildtype p53 (4,8,21). This is based on the preclinical work demonstrating that tumors that harbor a mutant p53 undergo apoptosis if wildtype p53 is re-expressed within the cell. Early phase clinical trials have determined this vector to be safe and effective in lung cancer and continued studies are planned (23).

The PI3K/mTOR pathway

Phosphatidylinositide-3 kinase (PI3K) encoded from the oncogene PIK3CA belongs to a family of lipid kinases leading to mammalian target of rapamycin (mTOR) activation that is estimated to be activated in nearly 75% of lung cancers (8). PI3K leads to inhibition of apoptosis and a regulation of growth. PIK3CA is mutated in lung cancer (*Table 1*), leading to high levels of kinase activity and downstream signaling. When combined with radiotherapy, PI3K inhibitors such as LY294002 and wortmannin reduce downstream effects which stall the growth potential and cell killing of human cell lines. These drugs are, however, rather toxic as they are nonspecific and inhibit a broad range of this family of kinases. Most recently, pharmaceutical companies are attempting to isolate isoform specific inhibitors of PI3K for a variety of cancers, IC486068 and IC87114 (8,18,21).

mTOR is a serine/threonine protein kinase. This kinase is the main downstream effector of the pathway that leads to regulation of cell growth. Two complexes, mTORC1 and mTORC2, form a catalytic subunit allowing for both cellular activity and possible therapeutic targeting. Several available therapeutic drugs are available, including Sirolimus and derivatives such as CCI-779, RAD001 and AP23576. Both have shown activity in lung cancer and are under further current clinical study (8,21,22).

JAK/STAT

The Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway has been implicated in preclinical study to increase cell proliferation and inhibit apoptosis through downstream effects like BCL, Cyclin and MYC in lung cancer. JAK localizes toward and is activated by ligand bound receptor tyrosine kinases leading to phosphorylated sites recognized by the SH2 domain of various STATs. They become phosphorylated by JAKs and form homo- and heterodimers which localize to the cell nucleus and regulate gene transcription. Interestingly, several STATs may be phosphorylated directly by EGFR and other kinases. Most notably, STAT3 has been linked to lung cancer oncogenesis within cell lines that carry a mutated EGFR. In fact, in EGFR mutants, STAT3 activation is necessary for cell growth and survival. Downstream of STAT3 is an inhibitor of apoptosis named survivin which functions to increase cell proliferation through the cell cycle and inhibition of apoptosis through caspases. This pathway of signaling is an attractive therapeutic target and preclinical work using TG101209 has demonstrated induced radiosensitivity, likely through inhibition of STAT3 (8,21,22).

TGF-B and angiogenesis

Transforming growth factor beta (TGF-β) is a cytokine that regulates multiple cellular processes, including cell survival, growth and immunomodulation. TGF-β activates downstream effectors in the SMAD family. TGF-β plays a dual role in lung cancer. During early tumorigenesis, TGF-β induces apoptosis and is responsible for growth inhibition. And, as we will see later, it also plays a role in inflammation. However, in late stage lung cancers, TGF-β induces angiogenesis (3,8,22).

Vascular density and angiogenesis correlate with advanced stage lung cancers and poor survival. A critical mediator in angiogenesis is the VEGF family. VEGF receptor inhibitors include the monoclonal antibody bevacizumab and the fusion protein aflibercept which bind circulating VEGF amongst others currently under investigation. Assessing response after treatment with bevacizumab has become a challenge. Pooling available anti-VEGF trials has allowed assessment of possible biomarkers to measure outcome. In fact, recent data suggests biomarkers such as circulating short VEGF-A, as well as modified expression of receptors neuropilin-1 and VEGF receptor 1, are potential candidates to predict outcome (8,24). A prospective biomarker study named MERiDiAN will stratify patients based on their short VEGF isoform and plans to address this issue.

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Biomarkers of radioresistance

The development of radiation resistance relies on innate tumor characteristics. Classically, the most important features in the response of tumors and normal tissues to fractionated radiotherapy are referred to as the “4 Rs”: repair of DNA damage, redistribution of cells within the cell cycle, accelerated repopulation and reoxygenation of hypoxic tumor cells (25). During the accelerated repopulation phase, tumor cells begin to repair their damage and proliferate at a markedly faster rate. During this phase, several cellular mechanisms take place that lead to resistance to radiotherapy: cellular senescence, DNA repair and cell cycle checkpoints regulation. Unfortunately the pathways and mechanisms of resistance are complex, and to date, are poorly elucidated. However, several investigators have shed light on genes likely related to both innate and acquired radioresistance. Innate radioresistance refers to genes present prior to exposure to ionizing therapeutic radiation and the acquired genes are those whose expression is changed after exposure to ionizing radiation. Using various methods of gene expression profiling a series of pathways involved in hypoxia, DNA repair and apoptosis have been studied in human lung cancer cell lines. Eighteen key genes linked to radioresistance were identified but of these genes only three have been validated to date. The three validated genes were MDM2, Livin α and TP54I3 (18,26).

MDM2 involved in innate radiation resistance encodes a protein called E3 ubiquitin-protein ligase which is an important negative regulator of p53 both through ubiquitinylation leading to degradation and inhibition of transcriptional activation (27). It has been demonstrated that up-regulation of MDM2 expression leads to radioresistance and targeted down regulation with siRNA leads to a reversion back to radiosensitivity. The remaining two validated genes are associated with acquired radioresistance where Livin-α is up-regulated and TP53I3 is down-regulated. Livin is a novel inhibitor of apoptosis (IAP) which is normally not expressed at high levels. In 2011, it was found that levels of expression are highly up-regulated after exposure to radiation leading to acquired resistance, especially in isoform α. The tumor protein p53 inducible protein 3 (TP53I3) gene is nearly turned off subsequent to fractionated radiotherapy leading to a depression of p53 cell death signaling (18).

Other potential mechanisms of resistance to radiation include mutations in EGFR and RAS. Preclinical studies have shown low levels of apoptosis in human cell lines with KRAS mutations in codon 12 (12V). It is theorized that this low level of apoptosis is mediated through modification of ERK. This may explain the resistance to radiotherapy. Various investigators have demonstrated a link between high levels of survivin expression and radioresistance (28,29). Radioresistance through mutations in EGFR has been studied and linked to various intracellular pathways yet no clear mechanism has been discovered.

Immunotherapy in lung cancer

Over the past several years, the importance of immune responses in cancer stem from the update of “the hallmarks of cancer” which included several new mechanisms important to cancer cell proliferation and evasion of the body’s innate system of immunosurveillance (30). It was noted that cancer cells require the ability to thrive in a chronically inflamed environment and evade and suppress the immune system. With this knowledge researchers have begun to seek out mechanisms to effectively activate immune reactivity, counteract immune suppression and characterize cancer specific antigens that are present throughout the cell’s lineage.

The basis for immunotherapy lies in mounting an adaptive response to cancer specific antigens. This relies on the tumor microenvironment, myeloid suppressing cells like T-regulatory (Treg) cells and the discovery of conserved cancer cell antigens (30-33).

In fact, Suzuki *et al.* have begun to clarify the importance of the tumor microenvironment on the risk of recurrence (33). The tumor microenvironment was studied by separating eight tumors infiltrating immune cells from the tumor and surrounding stroma and studying the expression of several cytokines in nearly 1,000 early stage lung cancer patients. Several markers were found to be significantly strong predictors of the risk for a recurrence at five years. These markers included an elevated forkhead box P3 (FOXP3): CD3 ratio and high levels of interleukin-7 receptor. The interleukin-7 receptor was also linked to worse overall survival. It was also noted that high levels of interleukin-12 receptor β2 was associated with a lower risk of recurrence. It turns out that FOXP3 is a marker for Treg cells. The expression of FOXP3 was also noted in the tumor stroma emphasizing the necessity of the tumors microenvironment in the relapse potential. IL-12 and its associated receptor acts as a tumor suppressor that is associated with less aggressive tumors. On the other hand, IL-7R has been shown to enhance angiogenesis by upregulating VEGF-D

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and acts through the JAK/STAT pathway. Several therapeutic targets have been suggested to counteract these newly found prognosticators in early lung cancer cells including cyclophosphamide which may deplete Treg cells and alter the FOXP3:CD3 ratio, reintroducing IL-12 or stimulating the IL-12R and blocking angiogenesis and the STAT family (33-35).

Several other mechanisms have been thoroughly studied to manipulate the immune environment including cytotoxic T lymphocyte anigen-4 (CTLA-4), programmed death 1 (PD-1), PD-1 ligands and damage associated molecular-pattern molecules (DAMPs) (33). CTLA-4 is expressed on CD4 cells and inhibits cytotoxic T lymphocytes. Ipilimumab is an antibody which targets CTLA-4. A clinical response relies on nonspecific alterations in immunogenicity through changes in total lymphocyte number and dendritic cells as well as altering expression of indoleamine dioxygenase. Ipilimumab has demonstrated a progression free survival in advanced stage, metastatic lung cancer in combination with chemotherapy. Other inhibitors of T cells include the PD-1 receptor which is a co-inhibitor factor present on T cells that is activated by PD ligands 1 and 2 (PD-L1 and PD-L2). Both PD-1 and PD-L1 have been targeted clinically in metastatic lung cancer demonstrating an objective response in 10-33% of patients with squamous cell carcinoma. Much lower response rates have been noted in adenocarcinomas (34,36). DAMPS such as heat-shock proteins (HSP) and high-mobility group box 1 (HMGB1) enhance autophagy which is down regulated in cancer cells. It is theorized this may play a role in the abscopal effect and manipulation of DAMPS may increase the chances for systemic control of disease (34,35,37).

Lung cancer vaccines have been developed and demonstrated impressive results in several clinical trials. Targets range from conserved proteins, molecular biomarkers to nonspecific targets. Mucin 1 (MUC1) is a cellular adhesion molecule expressed on many epithelial cells and is largely conserved within malignant lung cancer cells. MUC1 targeting vaccines including BLP-25 and TG4010 have demonstrated improvements clinical outcomes in early phase trials. BLP-25 is the only MUC1 vaccine that has thus far demonstrated a significant improvement in overall survival. The phase IIB trial demonstrated a 31% 3-year overall survival compared to 17% with best supportive care (34,38). Although no benefit in survival was demonstrated in metastatic disease. Importantly, the administration of BLP-25 was administered with cyclophosphamide to inhibit T cell suppression. Several phase three trials including the START and INSPIRE trials are currently assessing BLP-25 in the phase III setting. The TG4010 vaccine acts by inducing MUC1 and IL-2 expression through transfection with a recombinant vaccine virus. There have been promising results in early phase studies yet no significant improvement in clinical outcomes. Clinical outcome with this technique relies on the expression and recognition of transfected targets and phase three studies are now excluding patients with increased NK cell activity as these patients tended to have worse outcomes and toxicity. The CIMAvax EGF vaccine has demonstrated an improved median survival through targeting the EGFR receptor but this effect is limited to those patients that produce a good antibody response to the vaccine. MAGE-A3 is another conserved protein that has been targeted for vaccine development which in phase II studies has led to a trend to improved overall survival. This has led to the MAGRIT phase III study. Belagenpumatucel-L is a vaccine targeting TGF-β. The high-dose arm had a significantly improved median survival of nearly one year without significant toxicity. This has led to a phase II trial (NCT00676507) (34,38).

Combining immunotherapy with radiotherapy has been postulated to improve clinical outcome. Commonly after standard fractionated radiotherapy most cells undergo apoptosis as their mechanism for cell death which is non-immunogenic. But it is theorized that with hypofractionated therapy cells in combination with immunomodulaters may make tumor cells more immunogenic. In fact, Shaue *et al.* demonstrated in a murine melanoma model a threshold where doses of 7.5 Gy were immunostimulatory yet less hypofractionated doses were not effective (39). The exact mechanism of enhancement of the innate and adaptive immune systems is unclear but there have been several reports demonstrating marked reduction in systemic disease after local radiotherapy (39,40).

Status of personalized care in lung cancer

Personalized medicine has become a hot topic due to the lower costs of genetic testing and the voluminous research each year that demonstrate new molecular biomarkers. Rather than treating tumors based on stage and anatomical location the ultimate goal of personalized oncology is to identify sub-classes of molecular tumor types, which will lead to improved treatment strategies and prognosis.

Biomarker driven clinical trials utilizing first generation EGFR tyrosine kinase inhibitors (erlotinib and gefitinib), as well as ALK inhibitors such as crizotinib have improved

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clinical outcomes with demonstrated response rates between 50-75% (16,41,42). In fact, these studies have led to a recent change in the National Comprehensive Cancer Network 2013 guidelines for non-small cell lung cancer which recommends molecular testing in the work-up of metastatic lung cancer patients. Now, many clinicians and several multi-disciplinary tumor boards are recommending molecular testing be done earlier and earlier in the clinical presentation of disease.

Although molecular testing is becoming a part of our clinical acumen in lung cancer serious limitations of our current targetable biomarkers exist. The largest limitation in applying these data to the general population lies in the fact that Americans only harbor between 10-30% of ALK and EGFR mutations and between 80-90% of all lung cancer patients do not harbor these mutations at all (8,16,43). In patients that harbor a targetable mutation between 25-50% of them do not respond to therapy. Efforts to determine the mechanisms of resistance amongst patient's harboring these mutations as well as emerging ALK inhibitors and second generation EGFR inhibitors will hopefully address this key issue.

Our understanding of the molecular pathways of driver mutations and their mechanisms of resistance will continue to improve. Many of the aforementioned molecular biomarker subtypes will likely be a part of our growing clinical armamentarium as the fight continues to tailor therapy to each tumor.

Molecular markers: clinical applications and outcomes

The application of novel therapeutics to disrupt driver gene pathways has met with mixed results. Attempts to use these molecular biomarkers earlier in the pathogenesis of lung cancer are under active investigation.

Erlotinib, crizotinib and bevacizumab have played a role in improving clinical outcomes in metastatic lung cancer (11,44-47). Yet, the use of concurrent or adjuvant EGFR inhibitors has led to inferior or equivocal results compared to current standard therapy (47). Also, the use of concurrent bevacizumab remains perilous. Many clinicians believe that the unselected nature of these trials has led to unexpected results. Logically, patients that harbor these mutations should have improved clinical outcomes (45,46,48). This has been noted with the addition of crizotinib in patients harboring the fusion gene with metastatic disease (49). Researchers await the results of the cetuximab data from the RTOG 0617 trial to determine if the addition of targeted therapy will lead to improved clinical outcomes in combined modality therapy. Excitingly, personalized targeted therapy is being explored in an upcoming RTOG trial assessing the efficacy of induction targeted therapy followed by standard therapy. Of course, the drawbacks in this design are that induction therapy will delay local therapy. But the safety of combining these therapies with combined modality therapy remains unclear and adjuvant therapy has demonstrated poor results.

Further genetic testing has been explored to identify sub-groups of patients with improved outcomes. In fact, a 5-gene signature was identified and validated by researchers in Taiwan (50). Using gene expression profiling, risk scores and decision-tree analysis, the researchers found DUSP6, MMD, STAT1, ERBB3 and LCK were independent predictors of relapse free and overall survival. They performed a microarray analysis of 16 genes in 125 patients and grouped patients into high risk and low risk groups. Using their 5-gene signature, the median overall survival in the low risk group was 40 months while the rate for those in the high risk group was 30 months with a $P<0.001$. Relapse free survival was also significant; 29 months in low risk patients and 13 months in high risk patients. Importantly, these genes functions were observed in various realms of tumorigenesis, including apoptosis, cell differentiation and metastatic potential.

Preclinical studies have found other predictive biomarkers, including inhibitors of DNA binding ID1 and ID3. Immunohistochemical staining for ID1/3 was performed in 17 stage III lung cancer patient that received combined modality treatment. Interestingly, a dramatic improvement in progression free and overall survival was demonstrated. In patients without ID1/ID3 co-expression, the median progression free survival was 30 months compared to 1 month in those with co-expression. The median overall survival for patients without ID1/ID3 co-expression was 45 months and for those with co-expression was six months (51). It is theorized that these genes may correlate with the extent of hypoxia leading to resistance to radiotherapy (52).

Recently, there has been a remarkable uptrend of clinical trials addressing the use of targeted therapies earlier in the pathogenesis of disease (53). Importantly, the application of these novel therapeutics is being tailored to individual tumors which will hopefully improve clinical outcomes. The characterization of driver genes and prognostic biomarkers like the 5-gene signature and ID1/3 expression

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is an exciting revelation in lung cancer but we still require further study and validation in large randomized trials to determine if these biomarkers are clinically relevant.

Radiation pneumonitis and novel biomarkers for toxicity

Radiation pneumonitis is characterized by inflammation of the lung after delivering therapeutic doses of radiation to the thorax. Clinically significant pneumonitis is considered any toxicity that will require medical intervention. Clinically significant radiation pneumonitis occurs in approximately 5-50% of patients with lung cancer and is one of the most common clinical toxicities. It is also one of the most dangerous (54). Approximately 80% of clinically significant pneumonitis manifests in the first 10 months following therapy. The frequency of different clinical endpoints varies among patients with radiation pneumonitis: 20-80% will have a radiologic abnormality, 5-50% will have shortness of breath and <3% will develop a bronchial stricture.

Quantitative analysis of normal tissue effects in the clinic (QUANTEC) is the guide radiation oncologists use to interpret dose volume histograms. The recommended dose-volume limits generally used (many caveats exist) in clinical practice include: the volume of lung receiving over 20 Gy (V_{20}) of less than 30-35% and a mean lung dose of less than 20-23 Gy (55). These constraints portend a risk of less than 20% risk of pneumonitis. In patients after a pneumonectomy, more stringent limits include a V_5<60%, V_{20}<10% and a mean lung dose of <8 Gy. There are also factors that affect risk for pneumonitis. Classically, young age groups (<60-70 years old) and active smokers have a lower risk of developing pneumonitis. The use of concurrent chemotherapy increases the risk of radiation pneumonitis.

Acute radiation pneumonitis (within 12 weeks of radiotherapy) and subsequent pulmonary fibrosis which forms within the first 1-2 years results from a cascade of inflammatory cytokines and vasculature changes. Below is a depiction of several key markers of pneumonitis during the pathogenesis of fibrosis (*Figure 2*). The alveolar epithelium of the lung is made up of Type I (>90%) and Type II pneumocytes and upon exposure to radiotherapy there is a large loss of type I pneumocytes through apoptosis. The Type II alveolar cells begin to proliferate and produce surfactant apoproteins to repair the surrounding damage. Cells within the extracellular matrix including macrophages, fibroblasts along with circulating T helper cells begin secreting cytokines including IL-6 and TGF-β recruiting other inflammatory cells and beginning the cascade leading to collagen deposition and fibrosis within the lung parenchyma (56).

Recently, biomarkers and organ interactions have become important predictors of radiation pneumonitis. Inflammatory cytokines are known to participate in the pathogenesis of radiation pneumonitis and they pose a possible serum biomarker for toxicity. An early study linking serum markers to lung toxicity was the ROTG 91-03 trial studying stage II and III lung cancer patients undergoing 60-66 Gy of radiotherapy but were not surgical candidates (57). Some patients in this trial were able to receive concurrent or sequential chemotherapy but during the initial phases of the trial patients received radiotherapy alone. They found that after 10 Gy, elevated serum IL-6 (>0) predicted for acute grade 2 or higher radiation pneumonitis. At the same time, elevated levels of surfactant apoproteins (>797) after 20 Gy were correlated with late radiation pneumonitis. They also noted that a diffusion capacity of <54 and age >60 portends a higher risk of radiation pneumonitis. The remainder of the serum markers studied failed to correlate well with pneumonitis, including TNF and TGF-β.

TGF-β is the most heavily studied and scrutinized inflammatory biomarker for lung toxicity because it has conflicting data regarding its predictive ability for radiation pneumonitis (58,59). Several studies have linked elevations in TGF-β levels to radiation pneumonitis. They reported that levels of TGF-β differ significantly during radiotherapy and that sampling time determines the level of serum concentration. Other studies found that technical factors related to testing blood samples may explain the elevations in TGF-β levels. Still others found that normal tissue production of TGF-β during radiotherapy was influenced by the genetic background of the tumor and the patient (52,59).

Nonetheless, a combined analysis from Michigan and China found that elevation of serum TGF-β1 levels during radiotherapy (at four weeks) compared to pre-treatment TGF-B levels predicted for pneumonitis. The addition of mean lung dose helps stratify patients at the highest risk. Using a TGF-β ratio of >1 and mean lung dose of >20 Gy as risk factors, they categorized patients into three groups: no risk factors (low risk), one risk factor (intermediate risk) and both risk factors (high risk group). The risk of pneumonitis for each group was <5% for low risk, 50% for intermediate risk and 66% for the high risk group. A similar study was

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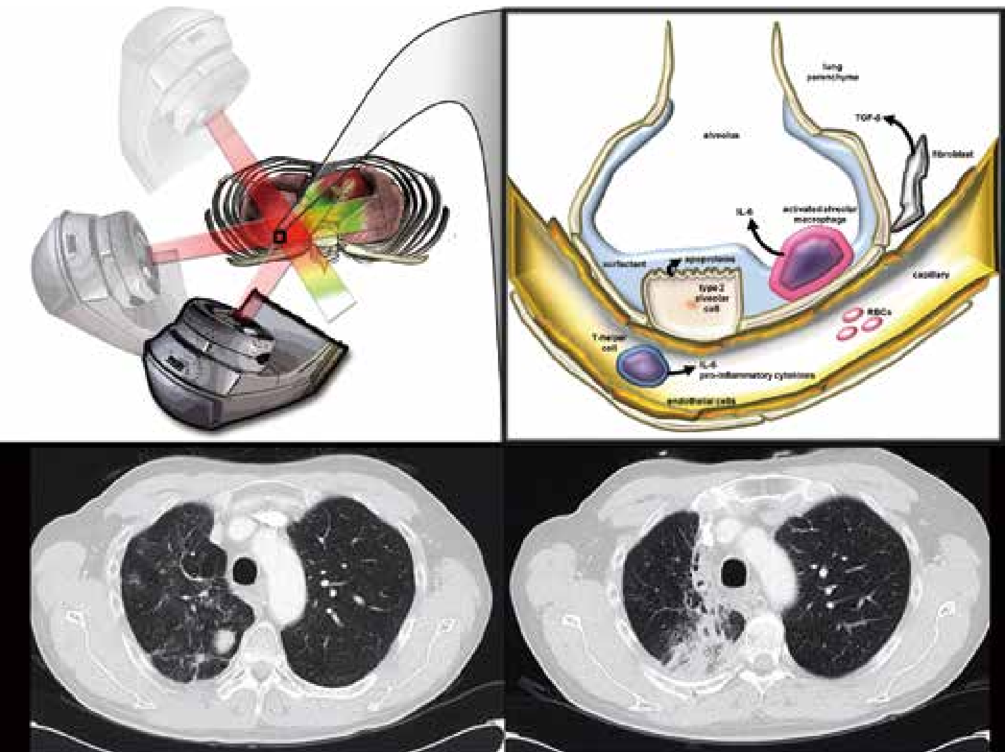

Figure 2 Mechanism of Pulmonary Toxicity. Radiation therapy is targeted at a right lower lobe lung mass (upper left panel). The irradiation of normal tissue during radiotherapy (black box, inset) causes certain patients to develop radiation pneumonitis, which is associated with release of IL-6 from neutrophils, TGF-β from fibroblasts, and apoproteins in surfactant from type II alveolar cells (black box inset, magnification). Pre- and one year post- radiotherapy axial CT slices from a patient that developed radiation pneumonitis in the right lung is displayed (lower panel, left and right, respectively). Illustration created by Nicholas G. Zaorsky, M.D.

performed using TGF-β levels at the end of therapy and V30 (58). They were also able to adequately stratify each set of patients based on these two factors. Several investigators have found the combination of inflammatory markers with dose-volume characteristics seems to be the best predictor for pneumonitis, rather than being compared to any factor alone. Unfortunately, these studies found a marker that must be drawn during therapy and in some cases this was too late to make any significant change in the outcome.

A recent sophisticated study that searched for single nucleotide polymorphisms (SNPs) of TGFβ1 gene found genotypes at lower risk for radiation pneumonitis. This study randomly acquired DNA from 164 lung cancer patient's resected tumor specimen and genotyped each sample to reveal SNPs in the TGF-β gene. The CT/CC genotypes in rs1982073:T869C TGFβ1 allele had a lower risk of developing radiation pneumonitis after radiotherapy independent of dosimetric factors such as mean lung dose and V20 (41). This may allow pre-treatment assessment of pneumonitis risk and further allow personalized radiotherapy treatment planning.

Strikingly, there is data linking parameters of radiation dose administered to the heart to lung toxicity. A single institutional review of hundreds of dose volume parameters found several variables, heart D10, lung D35 and maximum dose of the lung, were significant predictors for radiation pneumonitis in their cohort of patients (60). Due to the confounding variables within this type of analysis, further assessment and generalization to other patient populations are needed prior to using these variables in everyday practice. Additionally, heart toxicity has been linked to several biomarkers including pro-BNP and troponins (61). Though, no studies have linked these biomarkers to heart toxicity after completing radiotherapy to the lungs.

Other mechanism based biomarkers have been developed to determine improved outcomes in patients taking targeted therapies. These mechanism based biomarkers are well known side-effects, such as an acneiform rash

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with EGFR inhibitors, hypertension for VEGR inhibitors, hypothyroidism with multitargeted tyrosine kinase inhibitors and hyperglycemia with mTOR or PI3K inhibitors. Through analysis of the most recent targeted therapy trials in lung cancer, as well as analysis of other anatomic sites, trends were identified linking improved clinical outcomes in those patient's that experienced mechanism based toxicities (62). Conversely, it is postulated that a lack of mechanism based toxicity is a surrogate for lack of effective tumor response. These data are interesting, yet they remain preliminary.

Lately researchers have begun combining targeted therapies in lung cancer with standard chemoradiotherapy. This raises a question: How will the addition of targeted therapies alter the therapeutic window?

Several early phase clinical trials assessing the safety and efficacy of adding bevacizumab to standard chemoradiotherapy in lung cancer have found an alarming rate of tracheoesophageal fistulas. Tracheoesophageal fistulas are normally an exceedingly rare occurrence in the treatment of lung cancer. However, in a small pooled analysis, investigators found more than 10% incidence of tracheoesophageal fistula formation prompting the early termination of these investigations (44,63,64). Another early phase trial assessed the incidence of clinically significant pneumonitis. When combined with chemoradiotherapy in advanced lung cancer, they found a clinically significant pneumonitis rate of 67% (44,63). Although these studies are relatively small, they demonstrate an alarmingly high rate of significant lung and esophageal toxicity occurs with the addition of bevacizumab in standard chemoradiotherapy. This finding has prompted many researchers to abandon the addition of current generation VEGF inhibitors in combined modality lung cancer treatment. Additional studies using next generation anti-angiogenic factors are needed to further characterize the safety and efficacy of this modality of treatment.

The controversial multi-institutional RTOG trial 0617 also assessed whether the addition of targeted therapy to combined modality therapy may improve outcomes. They used a 2×2 factorial design comparing standard dose (60 Gy) versus high dose radiotherapy (74 Gy), with and without the addition of cetuximab. Paradoxically, there were significantly more local failures in the high dose arm, 34% versus 25% in the standard dose arm. Also noted was a startling stratification in survival, with a median survival in the standard dose arm of 28.7 months and 19.5 months in the high dose arm. The only significant difference in toxicity was esophagitis was three times higher (65). Many questions about these results remain unanswered. Some postulate that overall treatment time plays a role. Using tighter treatment margins without using 4D CT scans to determine tumor motion or awaiting the additional dosimetric data.

The appropriate timing of targeted therapies to use in combined modality therapy remains unclear. To address this issue, a trial in the pre-activation stage RTOG 1306 will add targeted therapies as an induction therapy for advanced stage lung cancer. Patients with stage III non-squamous, non-small cell lung cancer with N2 or N3 disease will be enrolled. All patients will have surgical staging and tissue sent for molecular testing that searches for EGFR mutations and ALK translocations. Patients will be randomized based on their mutation analysis to receive either standard chemoradiotherapy or induction therapy with either erlotinib or crizotinib based on their mutation status.

The era of personalized medicine continues to bloom by allowing tailored treatments in addition to standard therapy. However, there are many unknown variables to consider when adding novel therapeutics to other cytotoxic therapies, as we have not completely defined the various therapeutic ratios. We have begun to define newer markers of toxicity. These latest findings will help next generation trials assess and prevent toxicity in lung cancer patients.

Hypofractionated radiotherapy and pneumonitis

Hypofractionated radiotherapy is employed as a means of either dose escalation or shortening overall treatment times for both early and late stage lung cancer (66). However, the optimal dose, fractionation and schedule remain under investigation. There are several early phase clinical trials with data maturing which have combined hypofractionated radiotherapy with targeted agents including erlotinib (NCT00983307) and ZD1839 (NCT00328562). As of November of 2013, there are no active clinical trials assessing targeted therapies and hypofractionated radiotherapy registered to clinicaltrials.gov, which highlights a need for continued investigation. Patient factors and dosimetric information related to pneumonitis in the setting of hypofractionated radiotherapy is derived from early phase clinical trials and large retrospective analysis. A recent phase I study assessing hypofractionated attempting to raise the biologic effective dose (BED) over 100 Gy for patients of all stages revealed 16% grade 2 and no grade 3 radiation

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pneumonitis. However, six patients experienced grade 4 or 5 radiation toxicity including hemoptysis, lung abscess and bronchocavitary fistula. Univariate analysis demonstrated a significant association of high grade toxicity and total irradiation dose over 75 Gy with a 2-year incidence of toxicity of 31% *vs.* 1.8%. The maximal tolerated dose in this trial was 63.25 Gy in 25 fractions. The dose parameters which significantly predicted for 5% toxicity at two years were a D3cc of 75 Gy and a Dmax of 83 Gy (66). The high grade toxicities were attributed, by the investigators, to high doses as mentioned above being delivered to central structures including the proximal bronchial tree. The rate of pneumonitis for stereotactic body radiotherapy (SBRT), a form of ultra-hypofractionated therapy which employs image guidance and smaller treatment margins, has demonstrated rates of pneumonitis between 5-21% (67).

As the use of these techniques has increased, more attention has been paid to the size of the tumor volume treated and the dose to the uninvolved lung. Several studies revealed larger primary tumor volume, mean lung dose, and maximum dose to the tumor predicted for higher rates of pneumonitis (67,68). Reasonable dosimetric guidelines include a mean lung dose less than 6 Gy, a contralateral mean lung dose less than 3.6 Gy, and a V20 <10%. Factors which may predict for increased risk for pneumonitis include concurrent systemic therapy, active smoker, advanced age (>65), central location, and size of treatment volume (>145 cc) (66-69). Since the available toxicity data is more robust in the setting of hypofractionated or SBRT alone, it is prudent that combination targeted therapy and hypofractionated or SBRT be conducted on prospective clinical trials to allow detailed assessment of possible toxicities as available dosimetric and patient factors may underestimate the rates of high-grade toxicity.

Conclusions

Lung cancer is a heterogeneous group of tumor sub-types. Each type carries individualized mutations in multiple driver gene pathways. Classically, cancer therapies have been applied based on anatomic site, stage and other limited prognostic information. With the explosion of data that demonstrates targetable biomarkers in cancer, we are faced with new challenges to balance toxicity with clinical outcomes.

Genetic signatures have been discovered that influence outcome and one day may identify groups of patients that benefit from more aggressive therapy. Novel organ specific toxicity-related biomarkers in combination with radiotherapy derived parameters will improve treatment decisions and allow real-time treatment modifications to prevent long-term toxicity.

New approaches based on tumor and normal tissue characteristics are necessary to continue improving clinical outcomes. New multi-disciplinary tumor boards should be formed based on genetic tumor characteristics rather than tumor sites. Medicine requires an ever-increasing level of sophistication to interpret studies and design clinical trials. Technology, data management and analysis and novel therapies will improve more rapidly than ever before impacting our ability to predict and change clinical outcomes.

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Lung cancer biomarkers, targeted therapies and clinical assays

Jai N. Patel, Jennifer L. Ersek, Edward S. Kim

Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC, USA

Correspondence to: Jai N. Patel, PhD. Levine Cancer Institute, Carolinas HealthCare System, 1021 Morehead Medical Drive, Charlotte, NC 28203, USA. Email: jai.patel@carolinashealthcare.org.

Abstract: Until recently, the majority of genomic cancer research has been in discovery and validation; however, as our knowledge of tumor molecular profiling improves, the idea of genomic application in the clinic becomes increasingly tangible, paralleled with the drug development of newer targeted therapies. A number of profiling methodologies exist to identify biomarkers found within the patient (germ-line DNA) and tumor (somatic DNA). Subsequently, commercially available clinical assays to test for both germ-line and somatic alterations that are prognostic and/or predictive of disease outcome, toxicity or treatment response have significantly increased. This review aims to summarize clinically relevant cancer biomarkers that serve as targets for therapy and their potential relationship to lung cancer. In order to realize the full potential of genomic cancer medicine, it is imperative that clinicians understand these intricate molecular pathways, the therapeutic implication of mutations within these pathways, and the availability of clinical assays to identify such biomarkers.

Keywords: Assay; biomarker; lung cancer; mutation; pharmacogenetic

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Introduction

Given the large heterogeneity in clinical response observed across cancer patients and the narrow therapeutic indices of anticancer drugs, novel methods for individualizing cancer therapy are critical to improve patient outcomes. Our understanding of cancer at the molecular level has resulted in a shift from characterizing tumors solely by anatomical location to consideration of their molecular profile (1). Until recently, the majority of genomic cancer research has been in discovery and validation; however, as our knowledge of tumor molecular profiling improves, genomic cancer medicine in the clinic becomes increasingly tangible (2). As the number of commercially-available clinical assays to test for tumor biomarkers increases, it is critical that clinicians understand the therapeutic implications of mutations occurring within these molecular pathways. This review aims to summarize clinically relevant cancer biomarkers, their potential relationship to lung cancer and the clinical assays available in practice to test for such biomarkers (*Table 1*).

Biomarkers review

Biomarker classification

DNA analysis for pharmacogenetic purposes can be performed with either somatic or germ-line DNA. Somatic mutations are found within the tumor, requiring a tumor biopsy for identification, and are particularly useful in evaluating pharmacodynamic effects of a drug, such as tumor response. Germ-line, or inherited, variations are identified by a peripheral blood sample and help to predict the pharmacokinetic behavior of a drug, and ultimately drug response (3). Cancer biomarkers can be broadly categorized into two classifications: prognostic and predictive. A prognostic biomarker is mainly associated with disease outcome in the absence of treatment (i.e., Oncotype Dx, Mammaprint), while a predictive biomarker is valuable in assessing drug response [i.e., anaplastic lymphoma kinase (*ALK*), epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*)] (4). Biomarkers may also be classified as both prognostic and predictive [i.e., human epidermal growth factor receptor-2

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Table 1 Select cancer biomarkers, targeted therapies, and clinical assay availability

Biomarker	Targeted therapy	Tumor	Clinical assay(s) available	Molecular profiling methodology
ALK/ROS1	Crizotinib, ceritinib	Lung	Vysis ALK Break Apart FISH probe kit[a]	FISH
BRAF (V600E)	Vemurafenib, dabrafenib, trametinib	Lung, melanoma	Cobas 4800 BRAF V600E Mutation Test[a]; THxID BRAF test[a]	Real time PCR
C-KIT	Imatinib mesylate	Lung, GIST	C-KIT pharmDx[a]	IHC
EGFR	Erlotinib, afatinib	Lung, colorectal	EGFR pharmDx[a], Therascreen EGFR RGQ PCR kit[a]; Cobas EGFR Mutation Test[a]	IHC, Sanger Sequencing, PCR
HER2 (ERBB2)	Trastuzumab, lapatinib, pertuzumab, ado-trastuzumab-emtansine, dacomitinib	Lung, breast	HercepTest[a], Pathway[a], Insite[a], PathVysion[a], SPOT-Light[a], HER2 CISH[a]	IHC, FISH, CISH
JAK2	Ruxolitinib	Lung, myelofibrosis and other myeloproliferative disorders	JAK2 V617F Mutation Detection Assay, HTScan JAK2 Kinase Assay Kit	Real time PCR, Kinase activity assay
PD-1	Pembrolizumab, nivolumab	Lung, melanoma	In development	N/A
KRAS	Cetuximab, panitumumab	Lung, colorectal	Therascreen KRAS RGQ PCR Kit[a], DxS KRAS Mutation Test Kit, Genzyme's KRAS Mutation Analysis	Real time PCR

[a], assays that are FDA approved, PMA or 510(k) status. IHC, immunohistochemistry; HER2, human epidermal growth factor receptor-2; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; EGFR, epithelial growth factor receptor; GIST, gastrointestinal stromal tumor; ALK, anaplastic lymphoma kinase; JAK2, janus kinase 2; PD-1, programmed cell death 1; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase.

(*HER2*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)]. Pharmacodynamic biomarkers, a subset of predictive biomarkers, are useful in measuring the treatment effects of a drug on the tumor or on the host and can be used to guide dose selection. Examples include thiopurine-S-methyltransferase (*TPMT*) to guide 6-mercaptopurine dosing and uridine-diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) to guide irinotecan dosing (5).

Lung cancer is the leading cause of cancer-related mortality worldwide. Molecularly targeted therapies have dramatically improved the ability to extend survival in patients with lung cancers positive for *EGFR* mutations and/or *ALK* translocations. Researchers in The Cancer Genome Atlas Network molecularly profiled 230 resected lung adenocarcinomas using messenger RNA, microRNA and DNA sequencing integrated with copy number, methylation and proteomic analyses. Results demonstrated high rates of mutations at a mean of 9 per megabase, while 18 genes were statistically significantly mutated including *RIT1*, *EGFR*, *NF1*, *MET*, *ERBB2*, *RBM10*, and others within the mitogen-activated protein kinase (*MAPK*) and phosphatidylinositol-3-kinase (*PI3K*) pathways (6). Although several genes identified are not currently druggable and their prognostic significance has yet to be elucidated, understanding these molecular pathways and their predictive potential are critical to advancing personalized lung cancer therapy. The remaining article will focus on cancer biomarkers for which targeted therapies are available, their influence on lung cancer therapy, and, lastly, potential new targets for drugs in the pipeline.

Cancer biomarkers and lung cancer

Anaplastic lymphoma kinase (ALK)

Activating translocations of *ALK* resulting in the abnormal fusion gene, *EML4-ALK*, occurs in approximately 2-7% of all non-small cell lung cancer (NSCLC) cases, and encodes

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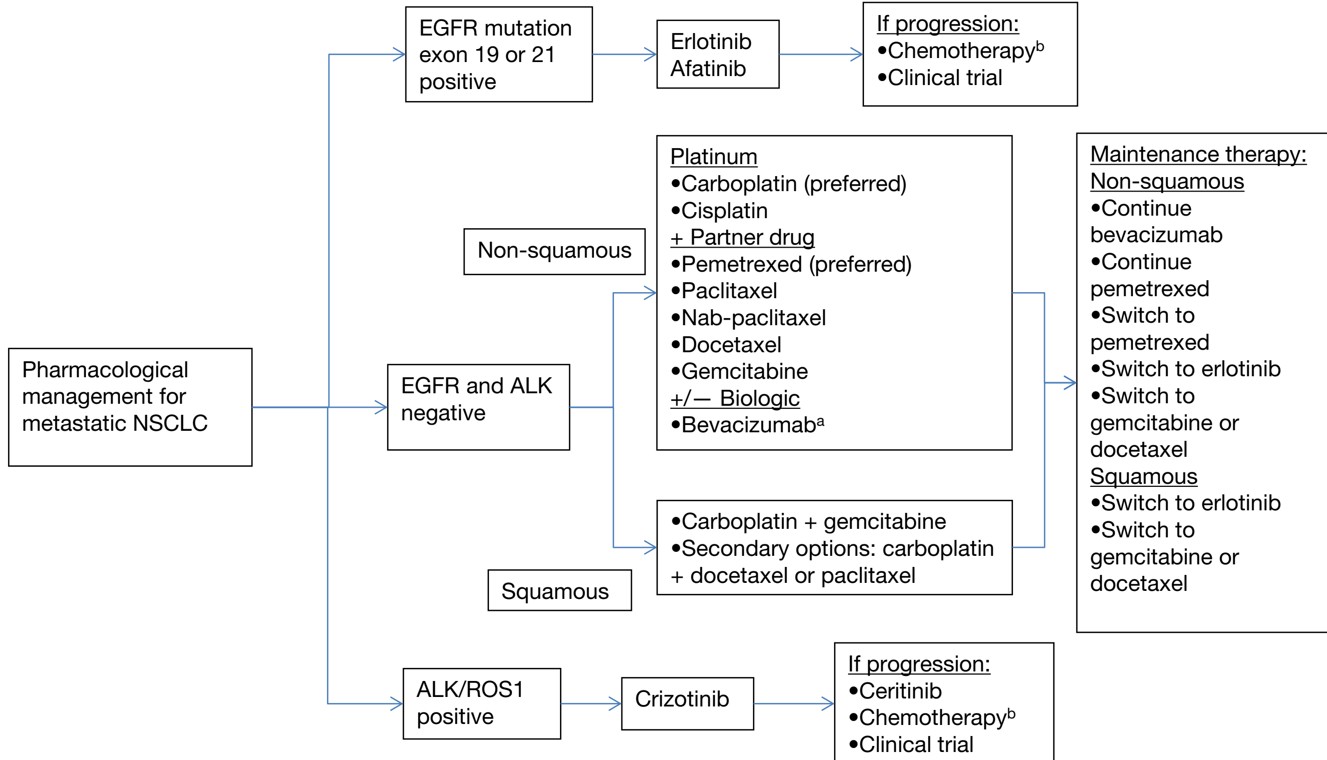

Figure 1 Example of a biomarker-driven treatment pathway for NSCLC, whereby mutations in EGFR or ALK drive targeted therapy selection, while patients with tumors negative for these biomarkers have therapy guided by histology and other clinical factors. NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase.

a cytoplasmic chimeric protein with constitutive kinase activity allowing activation of the *RAS-MEK-ERK*, janus kinase 3 (*JAK3)-STAT3*, and *PI3K-AKT* pathways (7). Similar to *EGFR* mutations, *ALK* rearrangements in NSCLC are associated with clinical and histopathologic features, such as adenocarcinoma histology and nonsmoking history. In contrast to *EGFR* mutations, patients with *ALK* rearrangements tend to be significantly younger and male, with no significant differences in frequency between Asian and Western populations (8). Treatment with crizotinib, a tyrosine kinase inhibitor (TKI) that competitively binds to *ALK*, demonstrated an initial overall response rate (ORR) of 60.8% in *ALK*-positive NSCLC patients treated in a phase I clinical trial, advancing the molecule into an accelerated FDA approval process (7). Results from the randomized phase III trial comparing crizotinib versus docetaxel/pemetrexed in *ALK*-positive NSCLC unequivocally demonstrated that crizotinib results in improved ORR (65% *vs.* 20%; P<0.05) and median progression-free survival (PFS) (7.7 *vs.* 3.0 months; P<0.05) (9). *Figure 1* illustrates a targeted approach to therapy selection in NSCLC based on clinically relevant biomarkers, including *ALK* and *EGFR* (discussed later in the article).

Although the majority of patients with *ALK*-positive NSCLC derive substantial benefit from crizotinib, this benefit is relatively short-lived secondary to acquired resistance. Possible mechanisms of resistance may include novel *EGFR*, *KIT*, *MET*, ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) or secondary *ALK* mutations not previously identified (10). Ceritinib, a second generation *ALK* inhibitor with greater potency compared to crizotinib, received accelerated FDA approval for the treatment of metastatic *ALK*-positive NSCLC in patients who were previously treated with crizotinib. A phase I study

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demonstrated ORRs of 58% and 56% in crizotinib naïve and resistant cases, respectively (11). As evident by crizotinib and ceritinib, the drug development paradigm for highly targeted therapies is changing, allowing earlier, accelerated approval of exceedingly effective therapies, years before phase III randomized studies are completed. Additionally, companion diagnostic test approval will become increasingly common with targeted therapy approval, particularly for newly identified biomarkers [i.e., Vysis *ALK* Break Apart fluorescence in-situ hybridization (FISH) Probe Kit to detect *ALK* rearrangements].

Lastly, evidence suggests that patients with *ALK*-positive NSCLC have improved survival after radiotherapy for brain metastases compared with *EGFR*, *KRAS* or wild-type tumors. The median overall survival (OS) was 13.6, 26.3, 5.7 and 5.5 months in patients with *EGFR*, *ALK*, *KRAS* or wild-type tumors. Subsequent receipt of targeted therapy was also associated with additional improvement in OS (12).

BRAF gene

BRAF mutations have been identified in a wide range of cancers including 50% of malignant melanomas, 45% of papillary thyroid cancers, 10% of colorectal cancers, and 3% of lung cancers (13). Mutations in *BRAF* result in constitutive activation of downstream signaling through the *MAPK* pathway (14). Approximately 50-90% (depending on anatomical location) of these mutations result in the substitution of glutamic acid for valine at codon 600 (*V600E*) (15). In contrast to lung cancer patients with *EGFR* mutations and *ALK* rearrangements who are mostly never smokers, patients with *BRAF* mutations tend to be current or former smokers.

Vemurafenib, a potent and selective *BRAF V600E* inhibitor, and its companion diagnostic test (Cobas 4800 BRAF V600 Mutation Test) received accelerated FDA approval upon demonstrating significant improvements in OS and PFS compared to dacarbazine in metastatic melanoma patients harboring the *BRAF V600E* mutation [hazard ratio (HR) =0.37 for OS, HR =0.26 for PFS; P<0.001 for both] (14). Patients with *BRAF*-mutated colorectal tumors tend to have significantly shorter PFS and OS compared to wild-type patients, and also have the potential to impair the effects of EGFR-inhibitor therapy in *KRAS* wild-type patients (15). However, no benefits with vemurafenib were noted in colorectal cancer, indicating the significance of tumor origin and microenvironment (16). The data for *BRAF* inhibition in lung cancer is scarce, although case reports have demonstrated clinical activity with vemurafenib (complete response after 6 weeks of therapy in a patient with refractory stage IV NSCLC) (17). Another case report demonstrated clinical activity in a metastatic NSCLC patient with brain metastases, with regression of both visceral and intracranial disease (18). Interim results of a phase II study of dabrafenib in *BRAF V600E*-positive NSCLC patients who failed at least one line of chemotherapy showed early antitumor activity with an ORR of 54% (19).

A number of mechanisms have been elucidated for BRAF resistance, including the paradoxical activation of the MAPK pathway through *RAS* mutations (20). Studies have demonstrated significantly improved OS and PFS in metastatic melanoma patients receiving a concomitant mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor, trametinib, in combination with a selective BRAF inhibitor, dabrafenib (21). Both drugs received FDA approvals in 2013 for the treatment of patients with unresectable or metastatic melanoma with *BRAF V600E* or *V600K* mutation who have not already received a *BRAF* inhibitor. Similar mechanisms of resistance may be translated to lung cancer. A randomized phase II trial of docetaxel with and without the *MEK* inhibitor selumetinib revealed that the combination resulted in superior OS, and a statistically significant improvement in PFS and objective response rate (22). Based on promising preclinical data (23), combination of targeted therapies, such as dabrafenib plus trametinib, may ultimately prove useful in treating *BRAF*-positive NSCLC and should be explored further.

C-KIT gene

The *C-KIT* proto-oncogene encodes a receptor tyrosine kinase, which binds to stem cell factor ligand. This interaction allows for the development of melanocytes, erythrocytes, germ cells, and mast cells, ultimately resulting in dimerization, autophosporylation, and signal transduction (24). While gain-of-function *C-KIT* mutations are found in approximately 85% of gastrointestinal stromal tumors (GIST) and are predictive of response to imatinib therapy (25), research suggests approximately 40% of small-cell lung cancers (SCLC) overexpress *C-KIT* (26). However, expression of *C-KIT* in SCLC failed to demonstrate a significant impact as a predictive biomarker of survival, possibly due to tumor microenvironment, resulting in

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futility of target inhibition in this setting (26). Alternatively, evidence suggests *C-KIT* mutations may be a prognostic factor for worse survival (27). Current literature on *C-KIT* inhibition in SCLC is limited and continued researches on its prognostic and predictive value are necessary.

Epidermal growth factor receptor (EGFR)

Activating *EGFR* mutations result in constitutive signaling via the PI3K-AKT and RAS-MEK-ERK pathways (28). Deletions in exon 19 and a missense mutation at exon 21, resulting in an arginine to leucine substitution (L858R), account for 90% of all *EGFR* mutations. Approximately 15-20% of NSCLCs harbor mutated *EGFR*, resulting in significantly improved PFS and OS when treated with small molecule TKIs targeting the *EGFR* domain (erlotinib, gefitinib, afatinib) compared to traditional platinum-based chemotherapy (29). Zhou *et al.* prospectively tested NSCLC patients for mutated *EGFR* and evaluated first-line erlotinib versus chemotherapy (30). Median PFS was significantly longer in erlotinib-treated patients compared to those receiving chemotherapy (13.1 *vs.* 4.6 months, HR 0.16, 95% CI, 0.10-0.26; P<0.0001). The ORR was 83% and 36% for erlotinib and chemotherapy-treated patients, respectively (30). Subgroup analyses from clinical trials revealed that patients with certain clinical and histologic characteristics (female, patients of East Asian descent, non-smokers, and those with adenocarcinomas) are more likely to harbor *EGFR* mutations (31,32).

Currently, screening for *EGFR* mutations is used to select stage IV NSCLC patients that should receive erlotinib in the first-line setting. In 2013, the FDA approved a companion diagnostic test for erlotinib (Cobas *EGFR* Mutation Test) and authorized expanded approval for first-line use in patients with metastatic NSCLC that tests positive for the *EGFR* activating mutation (33). Also in 2013, a second generation *EGFR* inhibitor, afatinib, received FDA approval for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* mutations. Afatinib's irreversible binding mechanism of action allows for enhanced activity in resistant tumors that have progressed after initial *EGFR* inhibitor therapy (34). In a phase III trial, 1,269 NSCLC patients with *EGFR* mutations were randomized to receive afatinib or standard chemotherapy (cisplatin and pemetrexed). The median PFS was 11.1 and 6.9 months in the afatinib and chemotherapy arms, respectively (35).

Two primary mechanisms of resistance to *EGFR* inhibitors include a secondary point mutation in *EGFR* (*T790M*) that blocks the capacity for erlotinib to inhibit the receptor, and the amplification of *MET*, which activates similar downstream signaling pathways (36). Drugs targeting *EGFR T790M* mutations and *MET* amplifications are currently under development.

Human epidermal growth factor receptor-2 (HER2)

HER2 is one of the molecular hallmarks of breast cancer and has resulted in the development of several successful targeted therapies. *HER2* or *ERBB2*, is a member of the ERBB receptor tyrosine kinase family, which includes three additional members: *EGFR* (*HER1/ERBB1*), *HER3* (*ERBB3*) and *HER4* (*ERBB4*). The binding of ligands to the extracellular domain of these receptors results in dimerization, activating a catalytic cascade of events involved in cellular proliferation, differentiation and migration. *HER2* status represents both a prognostic and predictive biomarker as overexpression is associated with higher breast cancer recurrence and mortality rates without consideration of pharmacological therapy; however, *HER2* overexpression also predicts response to anti-*HER2* targeted therapies, which has resulted in drastic improvements in median survival (37). Overexpression of *HER2* may be diagnosed using immunohistochemistry (IHC) analysis (for protein expression) or FISH (for gene expression).

Trastuzumab, the first monoclonal antibody targeting the extracellular domain of *HER2*, was approved in 1998 as first-line treatment in combination with paclitaxel for *HER2*-positive advanced and metastatic breast cancer (38). Lapatinib, a small molecule TKI targeting the intracellular domain of *HER2*, resulted in extended survival in metastatic *HER2* positive breast cancer in combination with capecitabine compared to capecitabine alone (39). Pertuzumab, an anti-*HER2* humanized monoclonal antibody that inhibits receptor dimerization, prolonged PFS in metastatic breast cancer patients when combined with trastuzumab and docetaxel compared to trastuzumab and docetaxel alone (40). Trastuzumab emtansine (T-DM1), an antibody-drug conjugate combining the targeted strategy of trastuzumab with the cytotoxic properties of emtansine, prolonged PFS and OS in patients with *HER2* positive, advanced BC previously treated with trastuzumab and a taxane (41).

Although HER2 overexpression and amplification has been described in 6-35% and in 10-20%, respectively, of NSCLC patients, the first clinical trials including patients

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treated with trastuzumab and gemcitabine-cisplatin or docetaxel, failed to demonstrate an OS benefit in *HER2*-positive patients (42,43). *HER2* mutations have been reported to exist in approximately 1-4% of NSCLC and are more common in Asians, non-smokers, women and those with adenocarcinomas (44). Considering that *HER2*-positive NSCLC may benefit from *HER2* inhibition or dual *EGFR/HER2* inhibitions, TKIs simultaneously targeting *EGFR/HER2* have been investigated. Case reports of afatinib in patients with *HER2*-positive NSCLC have suggested promising outcomes. Of five patients harboring *HER2* mutations, three observed objective responses (45). However, studies with neratinib, an irreversible pan ERBB inhibitor, suggested no benefit in response in *HER2*-positive NSCLC (44). Lastly, dacomitinib, another irreversible ERBB inhibitor, has demonstrated a 14% partial response rate in *HER2*-positive NSCLC (46). Continued research in larger patient populations will provide a better understanding of the clinical utility of *HER2* (or pan-*ERBB*) inhibition in *HER2* positive NSCLC.

Janus kinase 2 (JAK2)

JAKs are non-receptor TKs that mediate the transmission of cytokine and growth-factor-induced intracellular signals. The mutation is a single nucleotide change, resulting in a valine to phenylalanine substitution at codon 617, and occurs in approximately 55% of patients suffering from myeloproliferative disorders (47). The transcription of numerous pro-proliferative and anti-apoptotic genes are up-regulated upon activation of the JAK-STAT pathway. Ruxolitinib is the first *JAK* inhibitor approved by the FDA for treatment of patients with myelofibrosis or myeloproliferative disorders. In the COMFORT-II trial, the proportion of patients achieving at least a 35% reduction in spleen volume at week 48, was 28.5% for ruxolitinib and 0% for best available therapy (P<0.0001) (48).

Although *JAK* mutations in NSCLC are rare, data suggests that the activation of *JAK2* partially accounts for acquired erlotinib resistance. The combination of *JAK2* inhibition with erlotinib in erlotinib-resistant lung cancer cell lines demonstrated restored sensitivity to erlotinib and reduction in tumor size in a murine xenograft model (49). Another study demonstrated a commonly mutated pathway in solid tumors, STAT3, is activated by *JAK2* independent of other key oncogenic drivers in NSCLC; however, treatment with ruxolitinib in STAT3-activated NSCLC cell lines did not result in growth inhibition (50). Clinical trials are currently underway to investigate the influence of *JAK2* inhibition with ruxolitinib in NSCLC patients receiving chemotherapy or erlotinib (ClinicalTrials.gov NCT02119650 and NCT02155465, respectively).

KRAS gene

Mutations of the *KRAS* oncogene have emerged as a powerful negative predictive biomarker to identify patients with metastatic colorectal cancer who do not benefit from *EGFR*-inhibitor therapies, such as panitumumab and cetuximab. Roughly 40% of colorectal tumors harbor a *KRAS* mutation (51). *KRAS* functions as a mediator between the extracellular ligand binding and intracellular signal transduction from the *EGFR* and nucleus (52). The autophosphorylation of the intracellular TK domains at codons 12 and 13 of exon 2 confers constitutive activity of downstream signaling pathways, including RAS-RAF-MAPK and PI3K-AKT pathways (51). Significant improvements in PFS were seen in *KRAS* wild-type colorectal cancer patients receiving *EGFR*-inhibitor therapy in combination with FOLFOX or FOLFIRI, while PFS was reduced in patients harboring *KRAS* mutations (53,54).

A meta-analysis of *KRAS* mutations in NSCLC described a frequency of 26% in tumors of current/former smokers, and 6% in tumors of never smokers (55). *KRAS* mutations have been identified as a predictor of resistance to EGFR-TKIs in NSCLC (56). While patients with *KRAS* mutated tumors experienced a suboptimal response to EGFR-TKIs, *KRAS* mutation status did not appear to affect OS (57). *KRAS* mutations are typically mutually exclusive of *EGFR* mutations and *ALK* translocations. While it has traditionally been extremely difficult to develop drugs to specifically target *KRAS* mutations, recent advances have been made to identify downstream pathways and co-mutations that indirectly affect *KRAS*, such as *STK11* and *TP53*. Early research suggests that a MEK inhibitor plus docetaxel can effectively target these co-mutations. In a preclinical study, *KRAS* mutated mice (also mutated for *STK11* and *TP53*) were treated with docetaxel alone or with an investigational MEK inhibitor, selumetinib (58). Concomitant loss of either *TP53* or *LKB1* markedly impaired the response of *KRAS*-mutant cancers to docetaxel monotherapy. The addition of selumetinib provided substantial benefit for mice with lung cancer caused by *KRAS* and *KRAS*-plus-*TP53* mutations, though mice with co-mutations in *KRAS* and *LKB1* were resistant to the combination. A phase II randomized trial of

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selumetinib plus docetaxel in *KRAS*-mutant NSCLC patients demonstrated a PFS of 5.3 months with the combination versus 2.1 months with docetaxel alone (P<0.05). Response rates were 37% and 0%, and median OS times were 9.4 and 5.3 months, respectively (22). Another oral MEK1/MEK2 inhibitor, trametinib, demonstrated efficacy in combination with docetaxel in *KRAS*-mutant and wild-type NSCLC (59). Confirmatory clinical trials are ongoing to validate the use of these agents in *KRAS*-mutant NSCLC.

Programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1), PD-L2

Cancer immunotherapy rests on the premise that tumors can be recognized as foreign rather than self and can be effectively attacked by an activated immune system. However, during tumor progression, acquisition of traits that allow cancer cells to evade immune surveillance may occur by exploiting checkpoints that control the regulatory immune response (60). PD-1 receptor is an inhibitory receptor that is expressed by T cells with its ligand (PD-L1) found in the tumor microenvironment and a second ligand, PD-L2, expressed by antigen presenting cells (61). PD-L1 and PD-L2 have been shown to down-regulate T-cell activation upon binding to PD-1, especially in cancer, thus interrupting immune response (62).

Pembrolizumab is a highly selective, humanized monoclonal IgG4-kappa isotype antibody that acts against PD-1and blocks the negative immune regulatory signaling of the PD-1 receptor (61,63). Pembrolizumab has been investigated in a number of tumor types, mostly melanoma, but also NSCLC, sarcoma, carcinoid, colorectal, prostate, breast, ovarian, gastric, pancreatic and renal cell cancer (61,63-65). Grade 3 or 4 adverse events have included elevated aminotransferase, renal failure, diarrhea, hypothyroidism, fatigue, abdominal pain, decreased appetite, rash, pruritis (61). Pembrolizumab received accelerated FDA approval in September 2014 for the treatment of melanoma in patients with unresectable or metastatic disease who have disease progression following treatment with ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor. In a phase I study of 450 NSCLC patients who had received prior chemotherapy, 159 patients had tumors with strong PD-L1 expression and received pembrolizumab 10 mg/kg IV every 3 weeks. The response rate was 23% with duration of response of 31 weeks. However, in 35 patients with tumors that were PD-L1 negative, the response rate was 9% (66). Further work is ongoing to determine the predictive nature of PD-L1 expression.

Priority review and breakthrough status was granted for nivolumab (an anti-PD-1 antibody) after investigators demonstrated significantly better response and survival outcomes with nivolumab compared to investigator's chemotherapy in the second line treatment of patients with advanced melanoma. Subsequently, the FDA expanded the approved use to treat metastatic squamous cell NSCLC in patients who have progressed on or after platinum-based chemotherapy. In a phase I trial with expansion cohorts of 129 NSCLC patients receiving nivolumab (1 mg/kg, 3 mg/kg, or 10 mg/kg IV every 2 weeks), the ORR was 17.1% and appeared similar between squamous and non-squamous histologies. A difference in ORR between dose levels was observed: 3% for 1 mg/kg, 24.3% for 3 mg/kg and 20.3% for 10 mg/kg. The median PFS and OS were 2.3 and 9.6 months, respectively. One year after starting therapy, 42% of patients were still alive and durable responses were common with a median duration of response of 74 months (65). CheckMate-017, a phase III randomized study comparing second-line docetaxel to nivolumab (3 mg/kg) in patients with squamous cell NSCLC, was stopped early as the Data Monitoring Committee deemed that the trial had met its primary endpoint, demonstrating superior OS in patients treated with nivolumab (67). Currently, no validated marker exists to identify patients most likely to respond to anti-PD-1 therapy; however, continued investigations into the predictive value of PD-1 and PD-L1 expression is ongoing.

Investigational cancer biomarkers and lung cancer

c-MET

Signaling through the c-MET/human growth factor (HGF) pathway has been shown to trigger a variety of cellular responses, including growth, motility, metastasis, angiogenesis and tissue regeneration (68). High levels of HGF have been associated with more aggressive biology and a worse prognosis in NSCLC and SCLC. *c-MET* is normally expressed by epithelial cells and has been found to be overexpressed and amplified in a variety of human tumor tissues. Furthermore, the *c-MET* pathway is one of the key players in the development of acquired resistance to the vascular endothelial growth factor (VEGF) pathway inhibitors (68). Tumor microarray expression analysis demonstrated 72% *c-MET* expression in human lung cancer tissue and 40% *c-MET* receptor over-expression. Acquired *c-MET* amplification has also been linked to approximately

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22% of non-*T790M* mediated secondary gefitinib resistance in NSCLC patients (69).

A selective *c-MET* inhibitor, tivantinib, has been studied in three phase I trials, either alone or in combination with erlotinib (68). The combination regimen was further studied in a phase II randomized study, which demonstrated a median PFS of 3.8 months in the combination arm versus 2.3 months in the erlotinib arm (HR 0.81, P=0.24), with no significant difference in ORR or OS (70). However, a trend towards greater benefit with the addition of tivantinib was evident in patients with *c-MET* positive tumors. Continued work is ongoing to further assess this agent in NSCLC. Non-selective *c-MET* inhibitors include crizotinib and cabozantinib. Crizotinib was initially synthesized as a *c-MET* inhibitor; however, after observing dramatic response in *ALK*-positive NSCLC, this drug essentially became recognized as an *ALK* inhibitor (68). Early, phase I data suggest adding cabozantinib to erlotinib is safe and effective, and is currently being explored in phase II trials. Lastly, *c-MET* targeted monoclonal antibodies are being studied in this setting, including onartuzumab (MetMab) (68). Phase II data suggests prolonged PFS (3.0 *vs.* 1.5 months; HR 0.47; P=0.01) and OS (12.6 *vs.* 4.6 months; HR 0.37; P=0.002) in patients with *c-MET* positive NSCLC receiving MetMab plus erlotinib versus erlotinib alone (71). As such, a phase III trial is ongoing to validate these findings.

Fibroblast growth factor receptor (FGFR)

The *FGFR* tyrosine kinase family is comprised of four kinases, *FGFR1*, *2*, *3*, and *4*, that play a critical role in cell survival and tumor growth. Genetic alterations of *FGFRs* can lead to deregulated activation in various cancers, including breast, colorectal, bladder, in addition to lung cancer and others. A pan-*FGFR* TKI has been shown to block tumor proliferation in a subset of NSCLC cell lines with activated *FGFR* signaling but has no effect on cells that do not activate the pathway (72). A study demonstrated that *FGFR1* is amplified in 21% of lung squamous cell carcinomas and 3.4% of lung adenocarcinomas (73), suggesting *FGFR1* may be a potential target in mutation-positive lung cancers. In a phase I study, a selective pan-*FGFR* inhibitor demonstrated safety in patients with *FGFR*-positive squamous cell carcinoma of the lung. Early analysis demonstrated partial responses; however, robust efficacy data is not yet published (74). Another phase I trial is ongoing to assess *FGFR* inhibition in patients with a variety of solid tumors, including *FGFR* positive lung cancer (NCT01962532).

PIK3CA

The PI3K pathway is related to tumor growth in a variety of human cancers. PI3K-dependent activity is frequently elevated due to mutations of *PIK3CA*, the gene encoding PI3K, in addition to the loss of phosphatase and tensin homolog (PTEN) protein, a tumor suppressor with a critical role in regulating the PI3K pathway. *PI3KCA* activation initiates events leading to phosphorylation of Akt, which affects additional downstream signaling proteins involved in cell growth, metabolism, proliferation, survival, motility, and invasion (75). In one study, *PIK3CA* mutations in NSCLC were found in 3.9% of squamous cell carcinoma and 2.7% of adenocarcinoma. Furthermore, among *PIK3CA* mutant cases, about 50% of tumors harbored concurrent *EGFR* mutations and 10% had *KRAS* mutations. *PIK3CA* mutation was significantly associated with high expression of PI3K, p-Akt and mTOR, but not correlated with *PIK3CA* amplification. Patients with single *PIK3CA* mutation had shorter OS than those with *PIK3CA-EGFR/KRAS* co-mutation or wild-type *PIK3CA* (P=0.004). A significantly worse survival was also found in patients with *PIK3CA* mutations than those without *PIK3CA* mutations in the *EGFR/KRAS* wild-type subgroup (P=0.043), suggesting that *PIK3CA* mutations confer a worse prognosis (76).

A preclinical study demonstrated that targeted inhibition of *PIK3CA* in SCLC models harboring *PI3KCA* mutations resulted in cell apoptosis, inhibition of cell viability, transformation, and xenograft tumor growth, suggesting a potential role for *PI3KCA* inhibitors in mutated SCLC (77). Ongoing or recently completed trials in lung cancer include single-agent PI3K inhibitors (NCT01501604), as well as combinations with chemotherapy (NCT00974584, NCT00756847) (78).

Conclusions

The implementation of genomic cancer medicine relies on the foundation that genetic aberrations exist in cancer, driver oncogenic events promote mutagenesis, and these aberrations are actionable with highly targeted anticancer agents available to effectively modulate driver mutations (2). Increasing knowledge of tumor molecular profiling has led to more sophisticated treatment guidelines, such as those displayed in *Figure 1*. Understanding the molecular profile of tumors can help clinicians decide on the most appropriate treatment course, assist in therapeutic decision making aimed at preventing or overcoming chemoresistance, and ultimately maximize the number of effective treatment

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options while minimizing patients' exposure to ineffective, yet toxic, therapies. These potential applications have resulted in a large collaboration, called Lung-MAP, among the National Cancer Institute (NCI), Southwest Oncology Group (SWOG), Friends of Cancer Research, the Foundation for the National Institutes of Health (FNIH), five pharmaceutical companies (Amgen, Genentech, Pfizer, AstraZeneca and MedImmune), and Foundation Medicine. Lung-MAP is a multi-drug, multi-arm, biomarker-driven clinical trial for patients with advanced squamous cell lung cancer (https://clinicaltrials.gov/ct2/show/NCT02154490). Real-time biopsies and diagnostic tests will identify whether patients should receive one of five therapies: an FGFR inhibitor, a PIK3CA inhibitor, a CDK4/6 inhibitor, an EGFR inhibitor, or an anti-PD-L1. A single master protocol can be amended as needed as drugs enter or exit the trial based on efficacy. Collaborative, biomarker-driven clinical trials may prove to be more clinically and cost-effective than traditional large, randomized phase III trials.

The number of pharmacogenetic assays available to identify biomarkers is continuously expanding, with several receiving accelerated FDA clearance and/or approval. The decreasing cost of assays and increasing coverage by third party payers will allow wide accessibility of these assays in clinical practice. While next generation sequencing technologies allow for the identification of a multitude of biomarkers, these technologies are not widely available in the community setting and insurance coverage remains a challenge. However, as the costs of genome sequencing continues to decline to less than $1,000, increasing demand from physicians and patients will shift routine testing from research to clinical practice, in addition to a shift from singleplex testing to multiplex sequencing. As the availability of genomic information and our knowledge of cancer at the molecular level continues to progress, clinicians must understand these intricate molecular pathways, the therapeutic implication of mutations within these pathways, and the clinical assays available to identify such biomarkers.

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Footnote

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Angiogenesis inhibition as a therapeutic strategy in non-small cell lung cancer (NSCLC)

Richard D. Hall[1], Tri M. Le[1], Daniel E. Haggstrom[2], Ryan D. Gentzler[1]

[1]Division of Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA; [2]Levine Cancer Institute, Carolinas Healthcare System, Charlotte, NC, USA

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Correspondence to: Ryan D. Gentzler. Division of Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA. Email: ryan.gentzler@virginia.edu.

Abstract: In many cancers, including non-small cell lung cancer (NSCLC), tumor angiogenesis pathways have been identified as important therapeutic targets. Angiogenesis is essential in the process of primary tumor growth, proliferation and metastasis. One of the best characterized group of protein factors for angiogenesis include the members of the vascular endothelial growth factor (VEGF) family, consisting of VEGF-(A-D), and placenta growth factor (PIGF). Targeting tumor angiogenesis has been approached through two primary methods, monoclonal antibodies that block VEGF-vascular endothelial growth factor receptor (VEGFR) binding or small molecule tyrosine kinase inhibitors (TKIs) that inhibit the downstream VEGFR mediated signaling. Many TKIs inhibit multiple pro-angiogenic and pro-proliferative pathways such as the mitogen activated protein (MAP) kinase pathway. Bevacizumab and ramucirumab, monoclonal antibodies targeting VEGF and the VEGFR, respectively, have each led to improvements in overall survival (OS) for NSCLC when added to standard first and second line chemotherapy, respectively. Small incremental gains seen with both bevacizumab and ramucirumab may be further improved upon by incorporating novel agents and treatment strategies, and many additional trials are ongoing.

Keywords: Lung cancer; non-small cell lung cancer (NSCLC); angiogenesis; vascular endothelial growth factor (VEGF); targeted therapy

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Introduction

Lung cancer is the leading cause of cancer related mortality in the United States with more deaths directly attributable to the disease than breast, prostate, and colorectal cancer combined. It is estimated that 158,040 Americans will die from lung cancer in 2015 (1). Despite recent advances in the treatment of non-small cell lung cancer (NSCLC), including the discovery of oncogene driver mutations and subsequent targeting of *EGFR* mutations and *ALK* rearrangements through tyrosine kinase inhibitors (TKIs), significant work remains to reduce morbidity and improve survival for NSCLC patients (2-6).

In many cancers, including NSCLC, tumor angiogenesis pathways have been identified as important therapeutic targets. Angiogenesis is essential in the process of primary tumor growth, proliferation and metastasis (7,8). A key stimulant of intratumoral angiogenesis is tissue hypoxia, which leads to overproduction of pro-angiogenic factors. One of the best characterized and vital groups of protein factors include the members of the vascular endothelial growth factor (VEGF) family, consisting of VEGF-(A-D),

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Table 1 Key clinical trials for bevacizumab and ramucirumab

Trial	Additional agents combined with VEGF monoclonal antibodies	PFS (or TTP)	OS	RR	Notes
Bev					
Johnson *et al*., phase II (19)	Carbo, paclitaxel ± bev	7.4 *vs*. 4.2 months (P=0.023)	17.7 *vs*. 14.9 months (P=0.63)	31.5% *vs*. 18.8%	PFS benefit, not powered for OS
ECOG 4599 (20)	Carbo, paclitaxel ± bev	6.2 *vs*. 4.5 months (P<0.001)	12.3 *vs*. 10.3 months (P=0.003)	35% *vs*. 15% (P<0.001)	OS benefit of 2 months
AVAiL (21,22)	Cisplatin, gemcitabine ± bev	6.7/6.5 *vs*. 6.1 months (P=0.003, 0.03)	13.6/13.4 *vs*. 13.1 months (P=0.420, 0.761)	34%/30.4% *vs*. 20.1% (P<0.0001, 0.0023)	No OS benefit, not powered for OS
AVAPERL (23,24)	Maintenance: pem/bev *vs*. pem (no bev)	7.4 *vs*. 3.7 months (P<0.001)	17.1 *vs*. 13.2 months (P=0.29)	55.5% *vs*. 50.0%	Not powered for OS
POINTBREAK (25)	Carbo/pem *vs*. carbo/paclitaxel	6.0 *vs*. 5.6 months (P=0.012)	12.6 *vs*. 13.4 months (P=0.949)	34.1% *vs*. 33.0%	Maintenance trial included bev in both arms
PRONOUNCE (26)	Carbo/pem (no bev) *vs*. carbo/paclitaxel/bev	4.4 *vs*. 5.49 months (P=0.610)	10.5 *vs*. 11.7 months (P=0.615)	23.6% *vs*. 27.4% (P=0.414)	Not powered for PFS or OS
Ram					
Camidge *et al*., phase II (27)	Carbo, paclitaxel + ram	7.85 months	16.85 months	55%	–
REVEL (28)	Docetaxel (no ram) *vs*. docetaxel/ram		10.5 *vs*. 9.1 months (P<0.0001)		OS benefit of 1.4 months

VEGF, vascular endothelial growth factor; PFS, progression free survival; TTP, time to progression; OS, overall survival; RR, response rate; bev, bevacizumab; carbo, carboplatin; ECOG, Eastern Cooperative Oncology Group; pem, pemetrexed; ram, ramucirumab.

and placenta growth factor (PIGF). Of these, VEGF-A (subsequently referred to as VEGF) is principally responsible for vessel formation in adult tissues (9,10). VEGF binds to a family of transmembrane receptor tyrosine kinases (RTKs) called VEGF receptors (VEGFRs) {VEGFR with three isoforms VEGFR-[1-3]} (11-13). VEGF binds with higher affinity to VEGFR-1, however, its primary effects on angiogenesis are mediated by VEGFR-2, the primary receptor involved in endothelial cell proliferation and migration (10,14). VEGF binding to VEGFR-2 stimulates downstream signal transduction leading to endothelial proliferation, differentiation, permeability, migration and the generation of new blood vessels (15). Tumor angiogenesis is characterized by the formation of abnormal, tortuous, and poorly organized vessels with altered permeability (13,16). These features lead to erratic tumor growth and decreased drug delivery due to changes in the permeability of the tumor vasculature (17).

Targeting tumor angiogenesis has been approached through two primary methods, monoclonal antibodies that block VEGF-VEGFR binding or small molecule TKIs that inhibit the downstream VEGFR mediated signaling. Many TKIs inhibit multiple pro-angiogenic and pro-proliferative pathways such as the mitogen activated protein (MAP) kinase pathway (18). The first anti-angiogenic agent approved for use in NSCLC was bevacizumab (approved in 2006; Avastin®; Genentech Inc., San Francisco, CA, USA). Due to the success of bevacizumab, multiple antibodies and small molecule TKI's targeting angiogenesis have been studied.

In this review, we will provide an overview of the recent advances in the use of anti-angiogenic agents in the treatment of NSCLC. We will review bevacizumab and ramucirumab (*Table 1*), two U.S. Food and Drug Administration (FDA)

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Table 2 Summary of TKIs with anti-angiogenesis properties and their targets

Medication	Molecular targets	Notable clinical response
Sorafenib (29)	VEGFR-2, VEGFR-3, PDGFR, KIT, FLT3, RAF	Improved PFS and TTP
Pazopanib (30)	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, KIT	No difference when added to standard cisplatin/pemetrexed
Sunitinib (31)	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, KIT, FLT3, RET	Improved PFS/ORR, no change in OS
Cediranib (32)	VEGFR-1, VEGFR-2, VEGFR-3	No change in PFS or OS
Motesanib (33)	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, KIT	Improved PFS, no change in OS
Linifanib (34)	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR	Improved PFS and OS
Vandetanib (35)	VEGFR-2, VEGFR-3, EGFR, RET	Improved PFS
Nintedanib (36)	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, FGFR	Improved PFS

TKIs, tyrosine kinase inhibitors; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet derived growth factor receptors; PFS, progression free survival; TTP, time to progression; ORR, overall response rate; OS, overall survival; FGFR, fibroblast growth factor receptor.

approved monoclonal antibodies with specific indications in NSCLC and highlight recent data suggesting new uses for these medications. We will also review data using anti-angiogenic TKI therapy, often in combination with chemotherapy (*Table 2*), a largely unsuccessful endeavor to date due to increased toxicity and lack of meaningful clinical benefit with one recent exception (nintedanib).

Monoclonal antibodies

Bevacizumab

Bevacizumab, the first monoclonal antibody approved by the FDA, is a recombinant, humanized IgG1 monoclonal antibody that binds to VEGF, inhibiting binding to VEGFR-1 and VEGFR-2 (12,37,38). In 2004, a randomized phase II trial was published and compared two doses of bevacizumab combined with a standard chemotherapy doublet (19). Bevacizumab 7.5 mg/kg or 15 mg/kg were added to carboplatin and paclitaxel and compared to chemotherapy alone. Patients who received the higher dose of bevacizumab had a higher response rate (RR) (31.5% *vs*. 18.8%) and longer median time to progression (TTP) (7.4 *vs*. 4.2 months, P=0.023) compared to chemotherapy alone. There were no statistically significant differences in overall survival (OS) between groups. Higher rates of life-threatening hemoptysis were observed in the bevacizumab groups, which in subset analyses were attributed to distinct clinical features including centrally located tumors close to major blood vessels, cavitary tumors, and squamous histology. These clinical features remain contraindications for use and have been excluded from subsequent trials with bevacizumab.

Based on the success of the phase II bevacizumab study, the Eastern Cooperative Oncology Group (ECOG) conducted a large randomized, phase III trial (ECOG 4599) comparing carboplatin and paclitaxel alone or with bevacizumab 15 mg/kg (20). Bevacizumab was continued until progression or intolerance. OS was significantly improved in the bevacizumab group (12.3 *vs*. 10.3 months, P=0.003), and both the response rate (RR) (35% *vs*. 15%, P<0.001) and progression free survival (PFS) (6.2 *vs*. 4.5 months, P<0.001) were significantly improved as well. The experimental regimen was well tolerated overall, but higher rates of Common Terminology Criteria for Adverse Events (CTCAE) grade 3 or greater bleeding events (4.4% *vs*. 0.7%, P<0.001) were observed in the bevacizumab group. Bevacizumab gained FDA approval in combination with first-line chemotherapy for advanced NSCLC in 2006 following publication of ECOG 4599. A second randomized, phase III study (AVAiL) compared another chemotherapy doublet, cisplatin and gemcitabine, with bevacizumab at two different doses, 7.5 mg/kg and 15 mg/kg (21,22). PFS was significantly prolonged with both the high dose of bevacizumab *vs*. chemo alone (6.5 *vs*. 6.1 months, P=0.03) and the low dose bevacizumab *vs*. chemo alone (6.7 *vs*. 6.1, P=0.003). There was no statistically significant improvement in OS in either of the bevacizumab groups, however, the study was not powered to assess for difference in OS because the study was amended after publication of ECOG 4599.

The optimal duration of bevacizumab is unknown.

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Bevacizumab was continued until progression or unacceptable toxicity in ECOG 4599. Several studies, including AVAPERL, POINTBREAK, and PRONOUNCE, have evaluated maintenance chemotherapy in non-squamous NSCLC using pemetrexed combined with bevacizumab (23-26). It is unclear whether the benefit of maintenance therapy in these trials is largely attributed to cytotoxic chemotherapy or whether bevacizumab provides additional benefit. The ECOG 5508 trial, a randomized phase III trial with three arms (carboplatin, paclitaxel, and bevacizumab followed by either bevacizumab alone, pemetrexed alone, or bevacizumab and pemetrexed) recently completed accrual. It is hoped that this trial will provide insight into the additional utility of bevacizumab continuation maintenance beyond 4-6 cycles of chemotherapy. The AvaALL study (NCT01351415) randomized patients with progressive disease after first line chemotherapy and bevacizumab to continued bevacizumab with second line chemotherapy or chemotherapy alone (39). This study completed accrual in early 2015, and results are awaited to determine the benefits of bevacizumab beyond progression. Bevacizumab has also been studied in the adjuvant setting in combination with chemotherapy for patients with stage IB-IIIA NSCLC. The ECOG 1505 study (NCT00324805) randomized patients to chemotherapy alone or chemotherapy plus bevacizumab (40). This trial has completed accrual and results are expected in the near future.

One new area of promise for bevacizumab is among patients with *EGFR* mutant NSCLC. A phase II trial for patients with treatment-naïve metastatic *EGFR*-mutant lung cancer randomized 154 patients to standard erlotinib or erlotinib plus bevacizumab (41). The addition of bevacizumab in this setting resulted in a significantly improved PFS (16.0 *vs.* 9.7 months, HR 0.54, P=0.0015]. Survival data was not mature at the time of publication, but the study was not powered to show a difference in OS. The improvement in PFS was impressive, and it is possible that bevacizumab may have a greater magnitude of benefit is the *EGFR*-mutant population than in the wild-type population. Two ongoing trials, BELIEF (NCT01562028) and ACCRU (NCT01532089) are evaluating erlotinib and bevacizumab in this patient population in Europe and the United States, respectively.

In carefully selected non-squamous NSCLC patients, the addition of bevacizumab to platinum doublet chemotherapy has prolonged OS at the expense of increased rates of clinically significant bleeding. It is important to recognize that adding bevacizumab to platinum doublet chemotherapy should not be used as a standard therapy for all patients with non-squamous NSCLC due to increased risk of complications with relatively modest clinical benefit. There is an ongoing need to identify biomarkers to guide selection of patients who are most likely to benefit from bevacizumab (42). Although baseline VEGF levels have been identified as a potentially useful biomarker that correlates with PFS and OS for patients receiving bevacizumab, this biomarker has not been evaluated prospectively to determine if it is predictive of OS improvement (43).

Ramucirumab

Ramucirumab, a fully human IgG1 monoclonal antibody targeting the extracellular domain of VEGFR-2, gained FDA approval for the second line treatment of NSCLC in 2014. It was first FDA-approved for the treatment of gastric cancer in the second line setting based on results of the REGARD trial resulting in improved OS when compared to best supportive care and placebo (44). Ramucirumab is also approved in the second line setting in combination with paclitaxel for gastric cancer and FOLFIRI for colorectal cancer based on data from the RAINBOW (45) and RAISE (46) studies, respectively. When bound to VEGFR-2, ramucirumab prevents VEGF from binding and activating VEGFR-2, inhibiting formation, proliferation, and migration of new blood vessels (47). This differs from bevacizumab, which targets VEGF. The addition of ramucirumab to standard chemotherapy has been evaluated in both the first-line and second-line settings.

Ramucirumab was first evaluated in NSCLC in an open-label, single-arm phase II trial combined with paclitaxel and carboplatin in 40 patients with untreated, advanced (stage IIIB/IV) NSCLC (27). Ramucirumab (10 mg/kg) was given with paclitaxel and carboplatin in 21-day cycle, and continued for up to 6 cycles. In the absence of withdrawal criteria (disease progression or intolerable toxicity), patients were allowed to continue on ramucirumab monotherapy every 21 days. The 6-month PFS rate was 59.0% and ORR was 55.0%, comparing favorably to historical controls. Another phase II, randomized, open-label trial evaluated the use of ramucirumab in combination with pemetrexed and platinum chemotherapy as first-line therapy in advanced, non-squamous NSCLC (48). Patients were randomized 1:1 to receive pemetrexed and platinum chemotherapy alone or with ramucirumab for 4-6 cycles followed by maintenance therapy with pemetrexed alone or pemetrexed

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plus ramucirumab. This study failed to meet its primary endpoint [PFS, 5.6 months in the pemetrexed-platinum arm *vs*. 7.2 months in the ramucirumab-pemetrexed-platinum arm (P=0.132)]. Subsequent development of ramucirumab has focused on second line therapy as a result of these studies.

The REVEL trial was a multi-center, randomized, phase III trial that compared docetaxel alone to docetaxel plus ramucirumab in patients who progressed after platinum doublet chemotherapy (28). Patients previously treated with bevacizumab (14-15%) and both squamous and non-squamous histology patients were included. A total of 1,253 patients were enrolled and randomized to treatment. Median OS in the docetaxel plus ramucirumab arm was 10.5 *vs*. 9.1 months in the docetaxel plus placebo arm (HR 0.76, P<0.0001). The most common severe (CTCAE grade 3 or greater) adverse events (AEs) were neutropenia, febrile neutropenia, fatigue, leukopenia, and hypertension. Interestingly, rates of grade 3 or greater pulmonary hemorrhage and grade 5 AEs were not different between the two groups, despite inclusion of patients with squamous histology. Based on this study, ramucirumab was approved by the United States FDA in combination with docetaxel for patients with squamous or non-squamous histology after first line platinum-based chemotherapy.

Tyrosine kinase inhibitors (TKIs)

TKIs are attractive treatment options for patients with advanced cancer due to their oral bioavailability and relatively favorable toxicity profile compared to cytotoxic chemotherapy. Numerous TKIs with anti-angiogenic activity (most inhibit VEGFR-1 and/or VEGFR-2) have additional RTKs targets (*Table 2*). Many TKIs have been studied in a variety of combinations and lines of therapy for patients with lung cancer. A number of these drugs are effective as single agents in other advanced cancers, such as renal cell carcinoma and soft tissue sarcomas. Unfortunately, the development of anti-angiogenic TKIs has failed to yield an indication for use in lung cancer due to lack of efficacy or increased cumulative toxicity when combined with chemotherapy. We briefly summarize the more well studied TKIs and highlight challenges with anti-angiogenic TKIs.

One of the first TKIs studied in NSCLC was sorafenib. Unfortunately, in two large, phase III trials evaluating the additional benefit of sorafenib to platinum doublet chemotherapy for the first line treatment of NSCLC did not improve OS when compared to platinum doublet chemotherapy alone (29,49). Sorafenib may have a role in treating advanced KRAS mutant NSCLC following first line therapy and appears to have efficacy in *EGFR* wild-type tumors based on a sorafenib sensitivity signature analysis, but this remains to be tested in a randomized trial (50,51). Pazopanib was studied in a multicenter, randomized, phase II trial combined with cisplatin and pemetrexed chemotherapy. Unfortunately this combination had an unacceptable toxicity profile compared with cisplatin and pemetrexed alone (30). A phase I trial of pazopanib combined with vinorelbine proved to be too toxic as well (52). Sunitinib was studied in combination with erlotinib *vs*. erlotinib alone in a phase III, randomized study in *EGFR* wild-type patients after first line platinum doublet chemotherapy (31). No OS difference was observed but PFS and ORR were improved with the combination (31). A recent randomized, phase II study comparing pemetrexed alone to the combination of pemetrexed with sunitinib (CALGB 30704) failed to show a benefit with statistically superior OS in the pemetrexed only arm compared to the two combination arms (53).

Cediranib is a multi-kinase inhibitor that has been studied in the first-line setting for advanced NSCLC. In a phase II/III trial, cediranib 30 mg daily was compared with placebo in addition to chemotherapy with carboplatin and paclitaxel (54). Interim analysis indicated a trend towards increased PFS, however the study was halted due to safety concerns (increased mortality in the cediranib containing arm). A subsequent phase III study using a 20 mg dose and similar design was conducted (32). This trial was halted at an interim analysis due to significantly higher rates grade 3 or greater hypertension, anorexia, and diarrhea without statistically significant increases in PFS or OS. Motesanib showed promise in an early phase II trial, where two arms of motesanib at low and high doses were compared with bevacizumab in a three-arm trial in combination with carboplatin and paclitaxel for first-line therapy in patients with advanced NSCLC (55). Results from this trial estimated that the efficacy of motesanib 125 mg bid was comparable to bevacizumab. A phase III trial (MONET1) was performed assessing motesanib plus chemotherapy (carboplatin and paclitaxel) *vs*. chemotherapy alone in patients with advanced non-squamous NSCLC (33). While the study found a significant increase in PFS for patients receiving motesanib (5.6 *vs*. 5.4 months, P<0.001), there was no significant improvement in the primary endpoint of OS (13.0 *vs*. 11.0 months, P=0.14). Although there were no specific high grade toxicities attributed to motesanib, the incidence of

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grade 3 or higher AEs and the incidence of grade 5 AEs were significantly higher in the motesanib group.

Linifanib showed modest activity in a phase II study in 139 patients with relapsed/refractory NSCLC setting (56). Patients received linifanib monotherapy at two different doses with an ORR 5%, with PFS of 3.6 months and OS of 9.0 months. A recent phase II study evaluated the efficacy of carboplatin and paclitaxel with or without linifanib in treatment naïve patients (34). Addition of linifanib 7.5 mg to carboplatin and paclitaxel was associated with a significantly improved PFS compared to placebo (8.3 *vs*. 5.4 months, P=0.022). Addition of linifanib 12.5 mg showed no significant increase in OS *vs*. placebo (13.0 *vs*. 11.3 months, P=0.65). Unfortunately both dose arms of linifanib were associated with increased toxicity compared with platinum doublet chemotherapy alone. Vandetanib is an oral multi-kinase inhibitor that has been studied in four phase III trials. The ZODIAC trial assessed docetaxel plus vandetanib *vs*. docetaxel alone following platinum-based therapy and showed that the combination was associated with a significantly increased PFS over docetaxel alone but with increased grade 3 or greater AEs (35). The ZEAL trial assessed pemetrexed plus vandetanib *vs*. pemetrexed alone following platinum-based therapy for advanced NSCLC (57). There was no significant difference in the pemetrexed plus vandetanib *vs*. pemetrexed alone in either PFS or OS, and the addition of vandetanib to pemetrexed increased the incidence of some AEs. A third study by Natale *et al*. compared single agent vandetanib to erlotinib in unselected patients with advanced NSCLC after treatment failure with one to two prior cytotoxic regimens (58). There were no significant differences in either PFS or OS between the vandetanib and erlotinib arms. A fourth study (ZEPHYR) compared vandetanib to placebo in advanced NSCLC after at least one prior cytotoxic regimen and one EGFR TKI line of therapy and detected a small difference in PFS *vs*. placebo but no significant difference in OS (59).

Nintedanib, a potent TKI with anti-VEGFR-2 as well as fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptors (PDGFR) α and β activity has produced promising results in a large trial of NSCLC. The LUME-Lung 1 study, a randomized, phase III, double-blind, placebo controlled trial of 1,314 NSCLC patients compared nintedanib plus docetaxel to placebo plus docetaxel (36). PFS was improved in the nintedanib plus docetaxel arm [3.4 (95% CI, 2.9-3.9) *vs*. 2.7 months (95% CI, 2.6-2.8); HR 0.79 (95% CI, 0.68-0.92), P=0.0019]. In a pre-specified sub-group analysis, patients with adenocarcinoma histology had improved OS with the combination compared to docetaxel alone [12.6 (95% CI, 10.6-15.1) *vs*. 10.3 months (95% CI, 8.6=12.2); HR 0.83 (95% CI, 0.70-0.099), P=0.0359]. The LUME Columbus study (NCT02231164) is an active phase III study that is evaluating the combination of nintedanib plus docetaxel *vs*. docetaxel alone in non-squamous NSCLC after first line platinum doublet chemotherapy.

Conclusions

Angiogenesis inhibition continues to be an attractive therapeutic strategy for patients with NSCLC. To date, small molecule inhibitors of angiogenesis have largely failed to produce meaningful improvements in OS. One exception may be nintedanib, which showed promise in the LUME Lung 1 study and is the subject of an ongoing phase III study (LUME Columbus) (36). If nintedanib ultimately shows a clinically significant benefit in second line therapy, it will have to compete with ramucirumab, which was approved by the FDA in 2014 and is not limited to non-squamous histology.

Bevacizumab and ramucirumab have both led to improvements in OS when added to standard first and second line chemotherapy, respectively. Small incremental gains seen with both bevacizumab and ramucirumab may be further improved upon by incorporating novel agents and treatment strategies. One example of this strategy can be seen with the promising results of adding bevacizumab to erlotinib for *EGFR*-mutant cancers that led to a greater than 6-month improvement in PFS (41). In addition to the BELIEF (NCT01562028) and ACCRU (NCT01532089) studies, the RELAY study (NCT02411448) is studying ramucirumab in combination with erlotinib in first line *EGFR* mutant NSCLC. With the dawn of immunotherapy treatment in lung cancer, it remains to be seen whether angiogenesis inhibitors (either anti-angiogenic TKIs or monoclonal antibodies) when combined with checkpoint inhibitors may have additive effects. Although gains in OS have been small, many other drugs have failed to improve OS in the NSCLC patient population. The improvements in OS seen with both bevacizumab and ramucirumab can be clinically meaningful for patients who have a significantly shortened lifespan.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Management of hyperglycemia from epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) targeting T790M-mediated resistance

Jeryl Villadolid[1], Jennifer L. Ersek[2], Mei Ka Fong[1], Lindsey Sirianno[3], Ellen S. Story[4]

[1]Department of Pharmacy, [2]Department of Solid Tumor Oncology and Investigational Therapeutics, [3]Department of Solid Tumor Oncology, [4]Endocrine Center, Levine Cancer Institute, Carolinas HealthCare System, Charlotte, NC 28204, USA

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Correspondence to: Jeryl Villadolid, PhD, BCPS, BCOP. Department of Pharmacy, Levine Cancer Institute, Carolinas HealthCare System, 1021 Morehead Medical Drive, Charlotte, NC 28204, USA. Email: Jeryl.Villadolid@carolinashealthcare.org.

Abstract: Epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) patients are associated with sensitivity to small molecule tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib, and afatinib. Although studies show an increased progression free survival (PFS) with use of EGFR TKIs in the first-line setting, most patients will develop resistance to therapy after the first 8-16 months. T790M is an acquired resistance mutation reported in 60-70% of patients who initially responded to a prior EGFR TKI. Recently, EGFR TKIs targeting T790M have been developed to overcome resistance with positive results in PFS and objective response rate in patients who have had disease progression on at least one TKI. Two EGFR TKIs targeting T790M, AZD9291 and rociletinib, are new active treatment options for NSCLC but differ in adverse effect profiles. Dose-limiting hyperglycemia has been reported with rociletinib and has required dose reduction, an oral antihyperglycemic, or both, without discontinuation of therapy. This suggests that patients may be effectively treated chronically for hyperglycemia associated with EGFR TKIs targeting T790M, however, guidelines for treatment of hyperglycemia in this setting have not been published. We discuss mechanisms of hyperglycemia associated with TKIs and initial management of hyperglycemia, including benefits and limitations of oral antihyperglycemic options, adjustment of therapy based on grade of hyperglycemia, and recommendations for follow-up glucose monitoring.

Keywords: Hyperglycemia; epidermal growth factor receptor (EGFR); T790M

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Background

The treatment approach to non-small cell lung cancer (NSCLC) has become more individualized based on several biomarkers that have emerged as predictive and prognostic markers for NSCLC. Data show that progression free survival (PFS) is improved with the use of targeted epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in patients with sensitizing EGFR mutations when compared to standard therapy as first-line systemic therapy (1,2). Approximately 45% and 40% of NSCLC patients with a positive EGFR mutation have exon 19 deletion or exon 21 L858R mutations, respectively, which are predictive of treatment benefit to small molecule TKIs such as erlotinib, gefitinib, and afatinib. These sensitizing EGFR mutations are found in approximately 10% of Caucasian patients and up to 50% of Asian patients with NSCLC (3).

Although patients with sensitizing EGFR mutations

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have positive initial responses of 56-74% and a median PFS of 10-14 months, most will become resistant to first-generation TKI therapy (e.g., erlotinib and gefitinib) after about 8-16 months (4). Acquired resistance due to an EGFR T790M mutation occurs in 60-70% of patients with disease progression after an initial response to erlotinib (4). The mutation is due to a replacement of threonine with methionine that interferes with TKI binding by altering the conformation of the tyrosine kinase domain of EGFR, restoring the affinity of the receptor for adenosine triphosphate (ATP), and reducing the ability of TKIs to compete with ATP (4-9). Second generation irreversible EGFR inhibitors such as afatinib inhibit EGFR T790M *in vitro* but are associated with response rates of less than 10% and a PFS of 4 months in patients with NSCLC who have received previous treatment with a first-generation TKI. The clinical activity of afatinib monotherapy is impacted by the inability to achieve the dose required to inhibit T790M due to wild type activity. Vertical pathway suppression with afatinib and cetuximab appears more effective (10). Studies have also shown that the T790M mutation may also occur in patients who have not previously received a TKI (11).

Recently, two newer third-generation EGFR TKIs targeting T790M have been developed to attempt to overcome EGFR TKI resistance. AZD9291 and rociletinib (CO-1686) received breakthrough designation by the U.S. Food and Drug Administration (FDA) in 2014 for the treatment of patients with EGFR T790M mutation-positive NSCLC whose disease has progressed during treatment with a prior TKI. Both agents were active in preclinical models of EGFR-mutated NSCLC with or without T790M, but the clinical adverse effect profiles for the two agents were different. Diarrhea, rash and nausea were the most common for AZD9291, whereas hyperglycemia, nausea and fatigue were the most common for rociletinib. The only dose-limiting toxicity for either agent was hyperglycemia reported with rociletinib, however, a maximum tolerated dose was not identified for either agent (12-14).

AZD9291 is an irreversible inhibitor of EGFR and T790M mutations with a reduced affinity for wild-type EGFR and more antitumor activity in EGFR L858R tumors with a concurrent T790M mutation than afatinib. In a dose-escalation and expansion study, 253 patients with NSCLC who progressed on at least one prior EGFR TKI received at least one dose of AZD9291. The overall objective tumor response rate was 51% (95% CI: 45 to 58) and among 127 patients with centrally confirmed EGFR T790M, the response rate was 61% (95% CI: 52 to 70). The median PFS was 9.6 months (95% CI: 8.3 to not reached) in EGFR T790M mutation-positive patients compared to 2.8 months (95% CI: 2.1 to 4.3) in patients who did not have an EGFR T790M mutation. The most common all-cause adverse events were diarrhea (47%), rash (40%), nausea (22%), and decreased appetite (21%). Six patients (2.4%) reported hyperglycemia, however, there were no dose-limiting adverse effects observed. AZD9291 was effective in the T790M mutation-positive setting with limited skin and gastrointestinal adverse effects (13).

Rociletinib is a covalent inhibitor of mutated forms of EGFR including exon 19 deletions, L858R, and T790M mutations, but not exon 20 insertions. In a dose-escalation and expansion study, 130 patients with NSCLC who progressed following treatment with a first- or second-generation EGFR TKI were enrolled to receive two formulations of rociletinib, the first 57 patients receiving a free-base and the remaining patients receiving a hydrogen bromide salt formulation. The objective response rate among the patients with T790M mutation-positive disease who could be evaluated was 59% (95% CI: 45 to 73) compared to 29% (95% CI: 98 to 51) in 17 patients with T790M mutation-negative disease. Patients received a range of 500 milligrams twice daily to 1,000 milligrams twice daily of the hydrogen bromide formulation being used in all ongoing and future development. Based on the dose relationship with toxicity, it appears that 500 milligrams twice daily has decreased rates of toxicity and preserved response rate. Grade 3 toxicities included QT prolongation and hyperglycemia. Hyperglycemia occurred in 20 of the 92 patients (22%) who received therapeutic doses and 25 of the 92 patients (38%) received glucose-lowering therapy. Hyperglycemia generally occurred within the first 3 weeks of therapy (14).

While the two TKIs targeting T790M are both new active treatment options for EGFR-mutated NSCLC, the adverse effect profile differences may distinguish place in therapy. Patients who had hyperglycemia with rociletinib were most often managed with dose reduction, an oral hypoglycemic agent, or both. No patients in the study discontinued therapy (14), suggesting that hyperglycemia can be managed while on long-term TKI therapy to maintain treatment response and tolerability. Because there have not been published recommendations regarding hyperglycemia induced by EGFR TKIs targeting T790M, this review aims to highlight hyperglycemia management based on previous study protocols, related hyperglycemia guidelines, and reviews in other patient populations and

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anticancer pathways.

Overview of hyperglycemia induced by targeted anticancer agents

Prior to the development of EGFR TKIs targeting T790M, other TKIs have been shown to influence glucose metabolism attributed to various proposed mechanisms and pathways. The molecular mechanism of TKI glucose homeostasis remains unknown and is complicated by the fact that TKIs in the same class can be associated with both hypo- and hyper-glycemia. For example, although imatinib, dasatinib and nilotinib all target the fusion of the breakpoint cluster region gene and Abelson murine leukemia (BCR-ABL) gene for the treatment of chronic myelogenous leukemia, nilotinib causes hyperglycemia in up to 40% of patients and imatinib and dasatinib has been reported to cause hypoglycemia (15). TKIs classified as anaplastic lymphoma kinase (ALK) inhibitors used to treat NSCLC have different effects on glucose within the same drug class. The ALK inhibitor ceritinib causes hyperglycemia in 49% of patients, whereas crizotinib does not cause hyperglycemia (16,17). To date, only hyperglycemia has been reported with EGFR TKIs targeting T790M; hypoglycemia has not been observed in clinical trials of patients receiving AZD9291 or rociletinib (13,14).

Hyperglycemia has been reported with agents inhibiting the phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (PAM) pathway. This pathway affects key insulin signaling pathways downstream by increasing insulin resistance and reducing beta-cell function and mass with an insulin-induced tyrosine phosphorylation pattern mimicking that found in type 2 diabetes (18). A study investigating the mechanism of hyperglycemia for a pan-Akt kinase inhibitor in mice and rats showed increased glucose and insulin levels with hyperglycemia lasting for about 6 hours post dose. Analysis of animal livers showed potential inhibition of glycogen synthesis and/or activation of glycogenolysis, inhibition of peripheral glucose uptake, and lack of response to antihyperglycemic medications such as insulin infusions (19).

The mechanism of action of multikinase ABL inhibitors such as imatinib and dasatinib on glucose metabolism has been demonstrated to occur via human beta cells from chemical-induced apoptosis in vitro through activation of nuclear factor-kappa B (NFκB). The inhibitory effect on platelet-derived growth factor receptor (PDGFR) and tumor necrosis factor alpha (TNF-α) may also affect induction of beta cell apoptosis and insulin resistance in peripheral tissues (15). Imatinib and dasatinib have also been shown to ameliorate hyperglycemia in patients with pre-existing type 2 diabetes. Other multikinase agents such as axitinib, sorafenib, pazopanib, sunitinib, vandetanib, and ponatinib may cause hypoglycemia (20-22). Remission of long-standing type 1 diabetes has also been reported with sunitinib (23). Furthermore, chemical structure analysis has suggested an additional mechanism through modulation of farnesoid X receptor (FXR) involved in glucose and lipid homeostasis (20).

Based on preclinical studies with EGFR TKIs targeting T790M, it is suggested that hyperglycemia or potentially hyperinsulinemia from rociletinib may be caused by a metabolite with targets other than those of the parent molecule. The metabolite inhibits the type I insulin-like growth factor receptor (IGF-IR) and insulin receptor kinases and induces hyperglycemia in rats following an oral glucose tolerance test. The half-life of the parent molecule and the metabolite may allow for reversibility of hyperglycemia in 48-72 hours by withholding EGFR TKI therapy (14). IGF-IR has been proposed as an additional resistance mechanism for EGFR inhibition (24,25).

Initial management of hyperglycemia

Similar to previous reviews for other anticancer agents, the goal of hyperglycemia management of EGFR TKIs targeting T790M should be to maintain quality of life, prevent acute signs and symptoms of hyperglycemia, and avoid complications of sustained hyperglycemia such as infection, diabetic ketoacidosis, and osmotic diuresis. General treatment goals should include: fasting plasma glucose <160 mg/dL, random plasma glucose <200 mg/dL, and HbA1c ≤8%. Modulation of intensity of glucose lowering is a consideration in advanced cancer patients and less aggressive blood glucose goals may be appropriate. Factors to be considered include the risk of hypoglycemia in patients with co-morbid conditions, such as nausea or stomatitis, as well as life expectancy (26). Some reviews suggest home blood glucose monitoring daily for the first week of the first cycle and 2-3 times per week in subsequent cycles for anticancer agents such as PAM pathway inhibitors (18). Based on clinical experience and onset of hyperglycemia with rociletinib, more intensive glucose monitoring during the first several weeks is warranted. In one study protocol, patients receiving rociletinib had fasting blood glucose monitored weekly for 3 weeks during cycle 1, on the first day of each

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subsequent cycle, and at the end of treatment visit (14). Patients with pre-diabetes or diabetes should continue their current monitoring regimens and frequency of home glucose monitoring. Monitoring should be increased if the grade of hyperglycemia advances (*Figure 1*) (18).

All patients should be counseled on signs and symptoms of hypo- and hyper-glycemia, although clinical experience with rociletinib has shown that symptoms more commonly associated with diabetes, such as polydipsia, polyuria, and polyphagia, were less frequent with rociletinib. The symptoms more commonly associated with rociletinib-induced hyperglycemia were nausea, vomiting, diarrhea, and fatigue. The lack of classic hyperglycemic symptoms may reflect the relatively modest elevation in blood glucose encountered in this setting. Conversely, the gastrointestinal effects described in clinical studies with rociletinib use may be in part due to treatment with metformin (14). Providers should be contacted when home glucose values are routinely above 160 mg/dL and any time new symptoms occur. Follow-up laboratory testing should be performed to confirm hyperglycemia (18). We also recommend routine HgA1c testing per American Diabetes Association guidelines (27).

Management of hyperglycemia induced by EGFR TKIs targeting T790M based on grade is shown in *Figure 1*. Because evidence suggests that rociletinib-induced hyperglycemia is due to a mechanism associated with the development of type 2 diabetes, insulin-sensitizing agents are rational first-line agents in this setting in addition to dietary counseling. Of these agents, metformin is the preferred drug for its efficacy, safety profile, and relatively low cost. An initial metformin dose of 500 mg orally twice daily with food is recommended. Recent evidence suggests that the current cut-off values for creatinine in the U.S. labeling should be relaxed. Several studies support the use of metformin in stable mild to moderate renal insufficiency (26,28,29). While several antihyperglycemic medications have been studied for potential antitumor effects, metformin may be particularly promising in this regard (30-32). Potential adverse effects, such as nausea and abdominal cramping, are alleviated in most patients by using the extended release form, initiating at lower doses, taking with food, and coaching through the first 2 weeks of therapy. Extended release metformin has been used in an ongoing study of rociletinib (NCT01526928) with improved tolerability.

If adverse effects persist or hyperglycemia is not controlled after titrating metformin to maximum tolerated doses, another oral agent may be initiated prior to consideration of insulin. Each antihyperglycemic class has strengths and limitations. Dipeptidyl-4 inhibitors may be considered as preferred next-line agents as they are well tolerated and do not result in hypoglycemia, however these agents are not as potent as metformin and have a higher cost. While glitazones and sodium-glucose cotransporter-2 (SGLT2) inhibitors are effective, fluid retention with thiazolidinediones such as pioglitazone and volume depletion with SGLT2 inhibitors, respectively, may limit the use of these classes in patients who may be undergoing toxic oncologic therapies affecting fluid balance. Recent reports have also raised the concern that SGLT2 inhibitors may increase the risk for ketoacidosis (33). If an insulin secretagogue is desired, meglitinides at a lower dose pre-prandially may be preferred because of their rapid onset and short duration of action. Sulfonylureas, particularly long-acting forms, are usually not optimal in patients with unpredictable nutrient intake because of increased risk of hypoglycemia, especially in patients with current or potential renal compromise. The relatively modest efficacy with potential for gastrointestinal adverse effects may render alpha-glucosidase inhibitors less preferred as first or second-line agents. GLP-1 receptor agonists are potent insulin-sensitizers that do not induce hypoglycemia, however, they require injection and may result in significant gastrointestinal effects and undesirable weight loss. For hyperglycemia uncontrolled by oral agents, insulin is the best option for efficacy and flexibility of dosing but requires injection (18). Because of their short half-lives, rapid-acting insulins can be safely used when renal compromise is present and withheld in situations of variable oral intake (26). There is concern that exogenous insulin or medications which increase endogenous insulin levels may promote tumorigenesis and is the subject of ongoing research (34).

In study protocols, TKI therapy was either restarted at the same dose per physician discretion or reduced if glucose levels were difficult to control after initiation of treatment for hyperglycemia. Because of the short half-life of rociletinib, symptomatic patients could hold rociletinib to reverse hyperglycemia and initiate an oral antihyperglycemic agent prior to reaching grade 4 toxicity (14).

Follow-up and monitoring of hyperglycemia

Fasting blood glucose levels of patients on antihyperglycemic medications should be closely monitored throughout therapy with EGFR TKIs targeting T790M. Antihyperglycemic

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Fasting blood glucose (FBG) monitoring:
- Screening/baseline visit; cycle 1: day 1, 8, 15; cycle 2 and beyond: day 1; end of treatment visit

Initial home monitoring:
- Daily (alternate between fasting glucose and pre-dinner glucose)

General treatment goals:
- Fasting plasma glucose <160 mg/dL; random plasma glucose <200 mg/dL; HbA1c ≤8%
- Lifestyle modifications (refer to nutritionist or diabetes specialist if needed)†

Pre-existing diabetes:
- Continue current home glucose monitoring regimen; adjust frequency of monitoring and/or diabetic medication according to standard guidelines and grade of hyperglycemia

Provider should be contacted for:
- FBG >160 mg/dL
- Presence of hyperglycemia symptoms (polydipsia, polyuria, polyphagia, blurry vision)

NOTE: Hyperglycemia generally occurs within the first 3 weeks of treatment

Grade 2 hyperglycemia
(FBG >160 to 250 mg/dL; >8.9 to 13.9 mmol/L)
- EGFR TKI targeting T790M may continue without interruption or dose reduction in asymptomatic patients
- Hold EGFR TKI targeting T790M for 48 to 72 hours if symptomatic
- Twice daily home monitoring (before breakfast and dinner)

→

Asymptomatic
- Repeat FBG within 1 week—if grade 2 results at least twice in 1 week, start antihyperglycemic agent (metformin 500 mg orally twice daily‡)
- Continue home monitoring—if worsens or no improvement, treat according to grade 3 or 4

Symptomatic
- Start antihyperglycemic agent (metformin 500 mg orally twice daily‡)
- Continue home monitoring—if worsens or no improvement, treat according to grade 3 or 4

Grade 3 hyperglycemia§
(FBG >250 to 500 mg/dL; >13.9 to 27.8 mmol/L)
- EGFR TKI targeting T790M may continue without interruption or dose reduction in asymptomatic patients
- Hold EGFR TKI targeting T790M if symptomatic until resolution/improvement in symptoms and FBG <250 mg/dL; <13.9 mmol/L. Dose reduction at restart may be considered if glucose levels prove difficult to control¶
- Twice daily home monitoring (before breakfast and dinner)

→

Asymptomatic
- Start antihyperglycemic agent (metformin 500 mg orally twice daily‡)
- Continue home monitoring—if worsens or no improvement, treat according to grade 4

Symptomatic
- Start antihyperglycemic agent (metformin 500 mg orally twice daily‡)
- Consider addition of fluids due to risk of volume depletion
- Continue home monitoring—may consider increase to before meals three times per day and at bedtime—if worsens or no improvement, treat according to grade 4

Grade 4 hyperglycemia§
(FBG >500 mg/dL; >13.9 to 27.8 mmol/L)
- Hold EGFR TKI targeting T790M until resolution/improvement in symptoms and FBG <250 mg/dL; <13.9 mmol/L. Dose reduction at restart may be considered if glucose levels prove difficult to control¶
- Increase home monitoring to before meals three times per day and at bedtime

→

Start antihyperglycemic agent(s)
- Oral metformin 500 mg orally twice daily‡ with addition of second-line antihyperglycemic to increase glucose uptake or excretion if needed
- Referral to diabetes specialist if hyperglycemia is not controlled by oral agents—insulin may be required (rapid acting insulin preferred)
- Consider addition of fluids due to risk of volume depletion
- Consider post-prandial monitoring or continuous glucose monitor if worsens or no improvement

→

Second-line antihyperglycemics
(If persistent adverse effects or hyperglycemia is not controlled with initial agent at maximum tolerated doses)
- Preferred:
    - Dipeptidyl-4 inhibitors (well-tolerated although less potent and more costly than metformin)
    - Glitazones (use may be limited due to fluid retention)
- Less optimal:
    - Meglitinides (at a lower dose pre-prandially if insulin secretagogue desired)
    - Sulfonylureas (increased risk of hypoglycemia)
    - SGLT2 inhibitors (use may be limited due to potential for volume depletion and isolated reports of ketoacidosis)
    - Alpha-glucosidase inhibitors (modest efficacy and increased gastrointestinal adverse effects)
    - GLP-1 receptor agonists (less hypoglycemia however more gastrointestinal adverse effects and requires injection)
- If insulin initiated, rapid-acting insulin is preferred

Figure 1 Initial management of hyperglycemia induced by EGFR TKIs targeting T790M. †, Some patients may be able to stop therapy with therapeutic lifestyle changes; ‡, U.S. labeling recommends that metformin should be held for computed tomography scans and should not be used if serum creatinine is >1.3 mg/dL in women; >1.4 mg/dL in men or if decreased tissue perfusion/hemodynamic instability. Recent studies suggest that use in mild to moderate renal insufficiency is safe with appropriate monitoring. Using the extended release form, initiating at lower doses, taking with food, and coaching through the first two weeks of therapy may alleviate nausea and abdominal cramping symptoms. May increase to a maximum total daily dose of metformin 2,000 mg orally daily as tolerated prior to starting or adding a second-line antihyperglycemic agent; §, may require hospitalization for more effective glucose control and intravenous fluids; ¶, initial dose reduction recommendation is to decrease rociletinib from 500 to 375 mg twice daily for persistent FBG >200 mg/dL despite anithyperglycemics. Reductions should occur by one dose level (equivalent of 125 mg twice daily). EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

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agents should be discontinued in normo-glycemic patients who are no longer taking EGFR TKIs. Routine monitoring of blood glucose following discontinuation should be performed at subsequent visits to assess need for adjustments or re-initiation of treatment (18).

Discussion

Targeted therapy with TKIs has broadened the scope of treatment in various types of malignancy, including NSCLC. Although there are positive clinical outcomes and additional agents available based on known mechanisms of resistance, agent and target specific adverse effects may limit therapy. The effects of TKIs on glucose metabolism should be considered with close monitoring and initiation of antihyperglycemic therapy based on grade of hyperglycemia.

Current studies investigating EGFR TKIs targeting T790M have included patients with pre-existing diabetes who were treated uneventfully with antihyperglycemic agents (13,14). Metformin is the preferred initial therapy after lifestyle modification, with additional therapy choices dictated in part by individual patient considerations. Anticipated gastrointestinal adverse effects may be prevented or alleviated by simple measures in most patients. It is important that diabetic patients continue to be considered for inclusion in ongoing clinical trials since these patients are a large part of the cancer population. Standard practice recommendations for pre-existing diabetes and consultation with a diabetes specialist is recommended for hyperglycemia management since these patients were not separated into diabetic and non-diabetic cohorts at study initiation and hyperglycemia algorithms in this setting have not been published. Treatment recommendations beyond oral antihyperglycemic agents are unclear as most patients were managed in clinical trials without initiation of insulin. It should be noted that the safety of various antihyperglycemic regimens has not been specifically studied in cancer patients.

Patients who received EGFR TKIs targeting T790M and experienced hyperglycemia more frequently reported adverse events than those that did not (14). The setting of hyperglycemia may also theoretically induce tumor growth since it has been suggested that cells can undergo a signaling switch under hyperglycemic conditions that can lead to alternative mechanisms utilized to activate the mitogenic pathways of the IGF-IR independent from tyrosine phosphorylation of the IGF-IR (35). The effect of hyperglycemia on toxicity and tumor growth *in vivo* remains to be seen, and may not be well studied due to lack of sustained clinical sequelae with appropriate management of toxicity. The outcomes for patients treated with rociletinib who developed hyperglycemia and those without hyperglycemia appear to be similar (14,31). Further study is needed to discern the possible pro- and anti-tumor effects of various antihyperglycemic regimens.

Overall, results have been encouraging with efficacy of EGFR TKIs targeting T790M and the treatment of adverse effects such as hyperglycemia may promote chronic use and tolerability in appropriate patients. Our understanding of the mechanism of hyperglycemia and long-term outcomes following treatment will evolve with follow-up of patients currently receiving EGFR TKIs targeting T790M in ongoing studies.

Conclusions

Understanding the management of potential toxicities of EGFR TKIs targeting T790M such as hyperglycemia may be helpful in clinical-decision making in selection of therapy in an era of new personalized drug development targeting established biomarkers and mechanisms of resistance. Hyperglycemia has been shown to be a dose-limiting toxicity in one agent targeting T790M, however, this can be managed with appropriate antihyperglycemic therapy without EGFR TKI discontinuation in most patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Hippo/YAP pathway for targeted therapy

Emanuela Felley-Bosco, Rolf Stahel

Laboratory of Molecular Oncology, Clinic of Oncology, University Hospital of Zürich, Häldeliweg 4, 8044 Zürich, Switzerland

Correspondence to: Emanuela Felley-Bosco. Laboratory of Molecular Oncology, Clinic of Oncology, University Hospital of Zürich, Häldeliweg 4, 8044 Zürich, Switzerland. Email: emanuela.felley-bosco@usz.ch.

Abstract: Malignant pleural mesothelioma (MPM) is molecularly characterized by loss of function or mutations in the neurofibromin 2 (NF2) and the cyclin-dependent kinase inhibitor 2 genes. NF2 activates a cascade of kinases, called Hippo pathway, which downregulates Yes associated protein (YAP) function as transcription co-activator for TEA domain transcription factors (TEAD). In the absence of functional NF2, the expression of genes essential for cell cycling such as survivin is increased. New therapeutic strategies aimed at interfering with YAP activity include inhibition of hedgehog pathway, which downregulates the YAP protein, verteporfin, which inhibits the assembly of a functional YAP-TEAD transcription factor, and interference with thrombin and lysophosphatidic acid (LPA) receptors downstream signalling, since upon agonist binding they activate YAP.

Keywords: Hippo pathway; Yes associated protein (YAP) oncogene; malignant pleural mesothelioma (MPM)

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Introduction

The Hippo signaling pathway controls organ size through the regulation of cell cycle, proliferation and apoptosis (1,2). It was discovered and linked for the first time to human cancer in 2002 (3). In this review we will briefly introduce this pathway, with an emphasis on components that are altered in human cancers, and then we will focus on its role in malignant pleural mesothelioma (MPM) and finally present potential implication for the therapy of MPM.

The Hippo/Yes-associated protein (YAP) pathway in cancer

The mammalian components of Hippo pathway (*Figure 1*) include Serine/threonine kinase 3 and 4 (STK3 and 4, also called MST2 and 1, orthologs of *Drosophila* Hippo), SAV1, and serine/threonine kinase large tumor suppressor 1 and 2 (LATS1 and 2) (4). Neurofibromin 2 (NF2) (product of *NF2* gene), also called merlin, a member of the Ezrin ezrin/radixin/moesin protein family, promotes plasma membrane association of LATS which results in phosphorylation and activation of LATS1/2 by MST and other not yet known kinases (5). Activation of LATS inhibits the transcriptional co-activator YAP and the co-activator with PDZ-binding motif (TAZ) through their phosphorylation. Indeed, phosphorylated YAP/TAZ cannot accumulate into the nucleus and this hinders their co-transcriptional activity. The dysfunction of Hippo pathway, which leads to increased YAP/TAZ activity with an underphosphorylated form in the nucleus (6), induces oncogenic transformation due to the activation of transcription factors including transcription enhancers activation domain (TEAD) family members (7). In mammals, there are four TEAD family members: TEAD1-4 which have a distinct but not mutually exclusive expression pattern (8). TEAD on its own is unable to induce gene expression and requires additional factors or co-activators for gene expression (8). Upon binding TEADs YAP/TAZ up-regulates the expression of several growth promoting factors, including secretory proteins connective tissue growth factor (CTGF) and Cyr61 (7), AXL receptor tyrosine kinase (9), c-myc and survivin (10,11).

Amplification of YAP-containing chromosome 11q22 amplicon is frequently observed in several human tumors. High levels of YAP are observed in human liver tumors and YAP is a key driver of hepatocellular carcinoma

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Figure 1 In normal tissue repair NF2 activates the Hippo pathway, an essential regulator of cell proliferation. Key components of the Hippo pathway include two kinases: Mst and Lats. The sequential activation of these kinases leads to phosphorylation of the transcription factor YAP. When Hippo signaling is attenuated, e.g., in NF2-deficient cancer cells, YAP phosphorylation is reduced, resulting in its nuclear localization and regulation of target genes such as CTGF, Cyr61, AXL and survivin. Survivin is also controlled by nuclear NF2.

tumorigenesis (12,13). High YAP levels are also seen in about 15% of ovarian cancers where it has been correlated with poor patient prognosis (14). Similarly, a correlation between high YAP expression and poor prognosis has been identified in non-small cell lung cancer and esophageal squamous cell carcinoma (15,16). YAP overexpression is also seen in medulloblastoma (17) intracranial ependymoma (18) and oral squamous cell carcinoma (19). Also, YAP plays a role in human colorectal cancer progression (20). Mutations in Mst1, Mst2, LATS1 and LATS2 are not common but epigenetic silencing of these genes has been reported (21-23).

Hippo pathway activity may also be altered though crosstalk with other signalling pathways, which harbour oncogenic alteration [reviewed in (24)]. The Hippo pathway has been implicated in cell contact inhibition, as YAP/TAZ display a dramatic cell density dependent subcellular localization and phosphorylation (25). In addition, mechanic stress has also been shown to modulate YAP/TAZ activity (26). Recent studies have shown that YAP activity is regulated by G-protein-coupled receptor signaling (27,28). In this context it is of particular interest the activation of YAP/TAZ by thrombin and protease-activated receptor PAR1 (29), since thrombin is generated at sites of tissue injury to promote wound healing. In addition inhibition of mevalonate pathway with simvastatin decreases nuclear YAP (30).

The interaction with sonic hedgehog stem signalling pathway in mesothelioma will be detailed below.

The Hippo/YAP pathway in MPM

MPM is an aggressive human malignancy (31). MPM is mostly associated with asbestos exposure and the latency period after initial exposure is typically longer than 30 years (32). MPM is a rare disease with a 15-year cumulative frequency during 1994-2008 in the 56 countries reporting MPM to be 174,300 (33); however the real incidence of MPM is unknown, since there are countries in which MPM mortality is not reported, including asbestos-producing countries such as Russia, Kazakhstan, China and India (33). MPM mortality rates are estimated to increase by 5-10% per year in most industrialized countries until about 2020 (34). Despite treatment with chemotherapy, radiation therapy or surgery, the disease carries a poor prognosis. The median survival time

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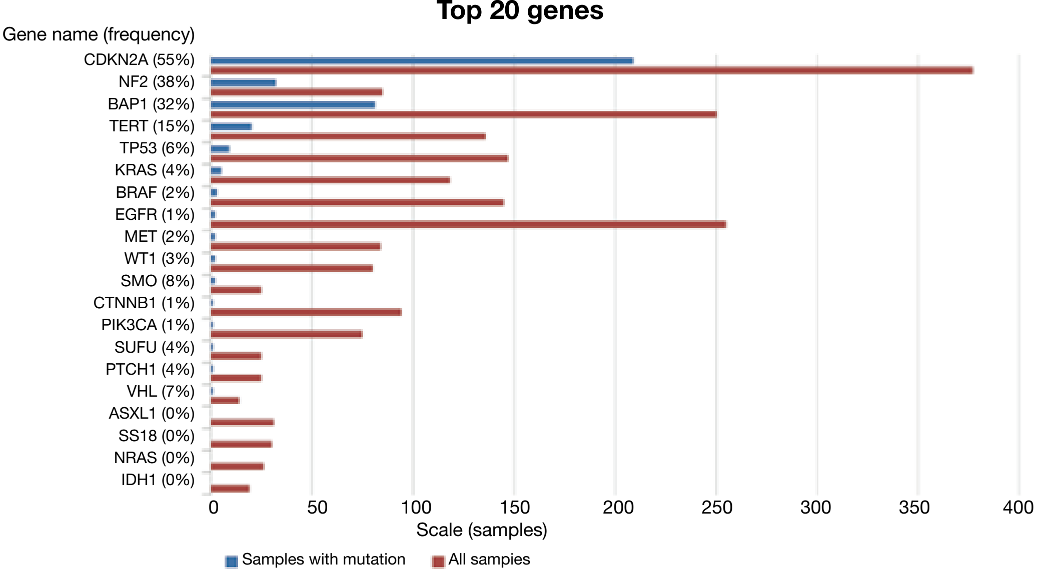

Figure 2 Data mining of version 67 of the catalogue of somatic mutations in cancer (COSMIC, http://www.sanger.ac.uk/cosmic), reveals that the genes that are mostly mutated in malignant pleural mesothelioma are *cyclin-dependent kinase activator inhibitor* (*CDKN2A*), *neurofibromatosis type 2* (*NF2*) and *BRCA-associated protein 1* (*BAP1*).

of patients after diagnosis is only 7-12 months (35).

The mechanism of development of MPM after exposure to asbestos fibres is not well understood. Few hypotheses can be proposed based on experimental data and observation of clinical samples, some of which has been detailed in several reviews (36-42). Chronic tissue repair activates stem cell signalling pathways to regenerate the tissue but, because of persistent system stimulation, oncogenic events occur leading to the formation of a tumor (43). Cells that are stimulated to proliferate upon asbestos fibres exposure may be undifferentiated precursor cells, as it has been shown for mesothelial regeneration after injury (44). Undifferentiated precursor cells have been recently described in normal mesothelial primary cultures (45). The activation of stem cell signalling would normally be kept under control, but, because of persistent system stimulation, oncogenic events occur leading to the formation of a tumor (43). In line with such hypothesis one would expect the oncogenic events to occur within the components responsible for homeostasis in tissue repair and in control of stem cell signalling. Activated stem cell signalling has already been suggested in MPM by the presence of an 11-gene signature, correlated with a stem-cell-like expression profile, which is associated with a poor prognosis in patients with MPM (46). Knowledge about common alterations observed in MPM, which are detailed below, confirms that alteration in NF2 signalling, which is responsible for homeostasis in tissue repair and in the control of stem cell signalling, is the ideal target for an oncogenic event to occur during the development of MPM. This hypothesis is supported by the observation that Hippo pathway restricts the oncogenic potential of intestinal regeneration program induced after injury by dextran sodium sulfate (47).

Data mining of version 68 of the catalogue of somatic mutations in cancer (COSMIC, http://www.sanger.ac.uk/cosmic) (48) reveals that the genes that are mostly mutated in MPM (*Figure 2*) are *cyclin-dependent kinase activator inhibitor* (*CDKN2A*), *neurofibromatosis type 2* (*NF2*) and *BRCA-associated protein 1* (*BAP1*). Although the total number of samples (<500, status February 2014) screened is too low to confidently predict mutation frequencies this estimate is nevertheless useful to infer a general MPM profile. *CDKN2A* and *NF2* are the two most abundantly mutated genes in MPM. Indeed, MPM lack expression of both *CDKN2A* encoded proteins p16 and ARF (49,50) due to gene deletion (51-53) or methylation (54-56). Mutations in *NF2* gene have been found in about 40% of mesothelioma (57-59). In MPM tumors with no detectable genetic

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Figure 3 The function of tumor suppressor NF2 is inactivated by genetic alterations or it is controlled by phosphorylation, which depending on the phosphorylated amino acid, leads to functional inactivation or AKT-dependent degradation. NF2 phosphorylation on Ser518 is favored when levels of protein kinase C-potentiated phosphatase inhibitor of 17 KDa (CPI-17), which inhibits the phosphatase reactivating NF2, are high. On the other hand PTEN deletion results in increased AKT activity and NF2 degradation.

alterations of NF2, its activity is downregulated. Indeed, the activity of NF2 is controlled by phosphorylation (*Figure 3*), which depending on the phosphorylated amino acid, leads to functional inactivation (60,61) or AKT-dependent degradation (62). Experimental animal models indicate that disruption of the NF2 signalling pathway, together with a deficiency in *CDKN2A*, is essential for mesothelioma development (63-65). NF2 is an upstream regulator of the Hippo signaling cascade, which is conserved from *Drosophila* to mammalians (10). The NF2/Hippo connection is supported by mouse genetics, wherein heterozygosity of *Yap* greatly suppresses the *Nf2*-deficient phenotype (66).

It has been proposed that genes which are inactivated in a given tumor type and directly regulate tumor growth by either inhibiting growth or promoting death are "gatekeeper" genes (67). According to the data mentioned above, NF2 does correspond to this definition and should be considered a "gatekeeper" in mesothelioma. Indeed, NF2 mediates contact-dependent inhibition of proliferation by both sensing cell-cell contact and intercepting mitogenic signalling initiated at the plasma membrane (68). In cultured mammalian cells, NF2 inhibits internalization, effector complexing and downstream signalling of activated EGFR upon cell-cell contact. This is consistent with the idea that NF2/merlin normally sequesters EGFR into non-signalling plasma membrane compartments (69). In addition, NF2 is required for the assembly, but not the maintenance, of apico-lateral junctional complexes (70) which means that NF2 loss will be most important when it occurs in dividing cells, for example during tissue repair. Cells that cannot form apico-lateral junctional complexes will be unable to form a well-organized tissue and will be resistant to contact-dependent growth arrest. A role of NF2 in tissue repair is further supported by the observation that the active form of NF2 suppresses tumorigenesis by migrating into the nucleus where it inhibits the E3 ubiquitin ligase CRL4 and through that controls a subset of Hippo pathway target genes (71). This recent observation is consistent with previous evidence of NF2 signaling-dependent activation of the Hippo pathway (72).

Data from the group of Sekido (73) and the group of Ladanyi (74) indicate that NF2/Hippo signalling is disrupted in most MPM. It is important to note that downstream of such a disrupted signalling, TEAD1/YAP1 are activated and YAP/TEAD1 are necessary, but not sufficient (75), for the overexpression of mesothelin. Mesothelin is expressed in normal mesothelium (76) and is a marker of epithelioid mesothelioma (77).

YAP is constitutively active in more than 70% of primary MPM (73), it has been originally described in an inducible transgenic model to be involved in organ size control paralleled by a 30-fold increase in survivin expression (10). A

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recent study has showed that it controls survivin expression in MPM (78). We have observed that Hedgehog signaling is activated in MPM, consistent with the re-activation of a signalling known to be essential during embryonic mesothelium development (79). Treatment with an inhibitor of Hedgehog pathway (HhAntag) of mesothelioma cells, grown in cell culture conditions favoring stemness, was arresting cells growth and this was accompanied by decreased levels of survivin (80). Survivin is not described as a direct target downstream Hedgehog pathway. Hence, we sought for other transcription activators known to be expressed in MPM and to regulate survivin expression and the most obvious was YAP. We confirmed nuclear expression of YAP in MPM and observed that HhAntag reduced YAP protein levels. Transient transfection of a constitutively active YAP (81) rescued HhAntag-dependent survivin decrease, confirming the interaction between Hedgehog and YAP signaling. Tumor bearing mice were randomized in two groups receiving either solvent or HhAntag. The *in vivo* HhAntag treatment dosage (38 mg/kg bw, administered twice daily by oral gavage, 5 d/week) was chosen based on therapeutic range reported in the literature (82). HhAntag led to a significant 35% decrease of the tumor volume after the two weeks of treatment. At the end of dosing regimen, animals were euthanized in order to collect tumor tissue for RNA extraction and immunohistochemical analysis. A different expression profile indicating changes in both tumor and stromal tissue were obtained in the two groups. The effect of HhAntag on tumor volumes was also accompanied by a significant 43% decrease in Ki-67 labelling index. Furthermore, consistent with *in vitro* experiments, we observed a significant 32% decrease in nuclear YAP immunostaining in HhAntag treated tumors. The observation that HhAntag decreases YAP protein is consistent with the role of Hedgehog signaling in maintaining YAP protein stability (17).

More recently, the modulation of YAP by the AJUBA family has been investigated in MPM (83). The mammalian Ajuba family comprises three proteins AJUBA, LIMD1, and WTIP characterized by a so-called, LIM domain. The LIM domain defines a cysteine-rich double zinc finger initially identified in three developmentally important transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* mec-3, from which the acronym LIM is derived (84). Although in Drosophila the unique AJUBA family member ortholog activates YAP by binding LATS (85), in MPM the three different LIM family members seem not to have all the same YAP-activating properties (83).

Implications for therapy

Under the hypothesis that disruption of NF2 function acts as "driver" in MPM, therapeutic intervention on genes that are normally kept under control by NF2 and the Hippo pathway such as, e.g., survivin, would be a reasonable approach. However, this might not be easy to implement. In the context of MPM harboring mutated NF2/Hippo pathway, cancer cells may be addicted to the activity of YAP. Liu-Chittenden *et al.* (86) screened a Johns Hopkins Drug Library, a collection of >3,300 drugs, for compounds that could inhibit the transcriptional activity of YAP *in vitro*. Three compounds related to porphyrin were identified with this assay. One of these, verteporfin, is in clinical use as a photosensitizer in photocoagulation therapy for macular degeneration. Verteporfin was moderately effective at blocking mouse *Yap1*-overexpression- or loss of *Nf2*-driven hepatic tumorigenesis. These data suggest the application of these compounds as anticancer therapies independently of their photosensitizing roles.

Downstream G-coupled receptor signalling we mentioned above YAP activation via thrombin/PAR1 (29) and this activation could be relevant in MPM that depend on PAR1 for growth (87). In these cases one potential option for therapy might be PAR-1 antagonists pepducins such as P1pal-12 (88) which is a cell-penetrating peptide derived from the third intracellular loop of PAR-1. Once inserted into the plasma membrane it is delivered to the PAR-1 intracellular surface, thereby interfering with the receptor/G-protein interaction. Lysophosphatidic acid (LPA) stimulates YAP activity (27) and has also been described to stimulate MPM growth (89), offering another opportunity for intervention.

Simvastatin decreases nuclear YAP and induces growth arrest by interfering with protein geranylgeranylation (30). The same mechanism may participate to lovastatin-mediated protective effect against cisplatin in proliferating normal mesothelial cells (90).

Other druggable targets, which might be relevant in other cancer types, have been summarized in two recent reviews (91,92).

In MPM the strategy may depend on upstream signalling: activated stem signalling, thrombin/PAR1 and LPA/LPA receptor are all potentially interesting targets to inhibit in view of interfering with YAP activation. Nevertheless, for

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the time being, only for Hedgehog stem signalling a direct link with YAP activation has been demonstrated (80).

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Targeted therapy in NSCLC driven by HER2 insertions

Solange Peters, Stefan Zimmermann

Department of Oncology, University Hospital of Vaudois (CHUV), Lausanne, Switzerland
Both authors have contributed equally to this work.
Correspondence to: Dr. Stefan Zimmermann. HFR Fribourg-Hôpital Cantonal, CH-1708 Fribourg, Switzerland. Email: Stefan.zimmermann@h-fr.ch.

Abstract: *HER2* mutations, largely exon 20 in-frame insertions, have been described as an oncogenic driver alteration in 1% to 4% of NSCLC, exclusively in adenocarcinoma histology. The prognostic implication of these alterations is not known. Phase I and II trial data suggest that afatinib, neratinib and dacomitinib have some activity in this molecular subgroup. No comparative data, or any data regarding the activity of pertuzumab or trastuzumab-emtansine is available. HER2 deregulation either by protein overexpression or gene amplification, has little clinical relevance to date, as trials investigating trastuzumab activity merely suggest a benefit in the very small minority of patients whose tumor highly overexpresses HER2, a subpopulation that amounts to 2% to 6% of mostly adenocarcinomas.

Keywords: HER2 mutations; lung cancer; afatinib; dacomitinib; irreversible pan HER-receptor inhibitor

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Introduction

Research into the molecular basis of lung cancer has revealed insights into various critical pathways that are deregulated, and among them, key driver genetic alterations that promote cell survival and proliferation. In the oncogene addiction model, cancer cells harbor gene amplification, rearrangement or mutations that dictate their malignant phenotype, and can thus be referred to as driver alterations (1). Among them, human epidermal growth factor 2 (HER2 erbB-2/neu) is a member of the erbB receptor tyrosine kinase family. The *ERBB2* gene which encodes for HER2 is a major proliferative driver that activates downstream signaling through PI3K-AKT and MEK-ERK pathways (2). Unlike HER1/epidermal growth factor receptor (EGFR), HER2 has no known ligand, and is activated by homo-dimerization or hetero-dimerization with other members of the erbB family. Under resting conditions, these cell-surface receptors are found as monomers folded in a so-called "closed" inactive conformation that prevents dimerization (3). Upon ligand binding to the extracellular domain, conformational rearrangements lead to an "open" state that exposes the dimerization interface. This extracellular dimeric structure results in the transactivation of the intracellular tyrosine kinase portion of each receptor. Three principal mechanisms of oncogenic activation of HER2 have been described: *HER2* gene amplification, gene mutation resulting in molecular alterations of the receptor or HER2 protein overexpression.

HER2 has been found to be amplified in approximately 30% of breast cancers, systematically resulting in protein overexpression. While historically HER2-positive breast cancer had been associated with a poorer prognosis, outcome shave improved significantly through the use of HER2-targeted agents like trastuzumab (4). *HER2* has also been found to be amplified and subsequently overexpressed in a subset of gastric carcinoma and carcinoma of the gastro-esophageal junction, in which it is associated with improved outcomes through the addition of trastuzumab to standard chemotherapy (5). Mutational activation of *HER2* can result from various somatic molecular alterations: small insertions and missense mutations on the kinase domain, missense mutations in the extracellular domain, or large deletions of the extracellular domain that results in a truncated form of HER2 (6).

HER2 alterations in NSCLC

HER2 was shown to be overexpressed in 13% to 20% of

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NSCLC, although 3+ expression is found in only 2% to 6% (7-9) *HER2* gene amplification, as assessed by fluorescent in situ hybridization (FISH) is uncommon, found in 2% to 4% of predominantly adenocarcinoma-type NSCLCs. Similarly to breast cancer, despite the relative lack of large series, concordance between FISH and IHC 3+ has been evidenced (8).

HER2 amplifications have been described as a potential mechanism of resistance to EGFR tyrosine kinase inhibitor (TKI) therapy in mouse models of EGFR-mutant tumor cells, where FISH analysis revealed that HER2 was amplified in 12% of tumors with acquired resistance versus only 1% of untreated lung adenocarcinomas. Notably, HER2 amplification and *EGFR* T790M mutation, the most common mechanism of acquired resistance, were mutually exclusive (10). In a large series of 155 patients with acquired resistance to EGFR TKI that underwent rebiopsy, HER2 amplification was seen in 13%, and no ERBB2 mutation was detected (11).

The identification of *EGFR* mutations, another member of the ERBB-family kinases, in a distinct subset of non-squamous NSCLCs was followed by the identification of *HER2* mutations, which mainly consist of in-frame insertions in exon 20, leading to constitutive activation of the receptor and downstream AKT and MEK pathways. *HER2* mutations fit the definition of genetic driver, and preclinical models have proved the transforming property of this alteration. Transgenic mice expressing the *Her-2* Tyr-Val- Met-Ala mutation develop lung adenosquamous carcinomas. In these models, substantial tumor shrinkage was observed when BIBW2992, a tyrosine kinase inhibitor that inhibits EGFR and Her-2, was combined with temsirolimus, an inhibitor of the downstream effector protein mTOR (12,13). *HER2* mutations have been identified in approximately 1% to 4% of NSCLC. In the initial report, mutations in the HER2 kinase domain were identified in 4.2% of 120 primary NSCLC overall and 9.8% in adenocarcinomas (14). A subsequent study of 671 primary resected NSCLC, HER2 mutations were found in 1.6% of samples overall, but in 3.9% of adenocarcinoma samples, and more frequently in Asian ethnicity (15-17). The largest retrospective series published to date, comprising 65 patients with NSCLC and *HER2* mutations, provides important insights into the clinic-pathological features and correlates: mutations were found exclusively in patients with adenocarcinoma subtype, and predominantly in female patients and non-smokers, a population similar to the EGFR-mutated NSCLC (18). Nevertheless, mutations were found in some men and heavy smokers, suggesting that *HER2* testing could be guided by tumor subtype (adenocarcinoma), but should not be restricted to clinically defined subgroups. All mutations were in-frame insertions of exon 20 within the *HER2* gene coding sequence, with duplication of amino-acids YVMA at codon 775. All *HER2*-mutated tumors were found negative for EGFR-activating mutation in exon 18 to 21, as well as ALK rearrangement and *BRAF* and *PI3KCA* mutations. Of interest, a high frequency of patients with disseminated lung nodules and tumor excavation patterns was observed. Of note, using stringent definition of gene amplification (as opposed to gene copy number gain), *HER2* mutations were not found associated with concurrent *HER2* gene amplification in this series and a previous report (15).

Although oncogenic tyrosine kinase mutations most frequently alter the ATP-binding pocket, as *EGFR* exon 19 and 21 as well as in *HER2* exon 19 or 20 mutations, mutations affecting the extracellular domain have recently been described, resulting in constitutively dimerized and activated HER2 (19). Mutations in the transmembrane domain of HER2 have also been described in familial lung adenocarcinomas (20).

There is scarce data regarding the prognostic impact of *HER2* mutations. In a series of 504 Japanese patients with resected NSCLC, 2.6% were found to harbor a *HER2* mutation. There was no difference in overall survival of patients with *HER2* mutations compared with patients harboring *EGFR* mutations and patients harboring wild types for both *EGFR* and *HER2* (17).

HER2 as a target

In the landscape of lung cancer biomarkers-based precision medicine, HER2 as a target remains poorly described. While in breast cancer HER2 overexpression or gene amplification is widely known to be associated with sensitivity to HER2-targeting drugs like trastuzumab, lapatinib, pertuzumab, and trastuzumab-emtansine, clinical research in lung cancer has been slowed down after the first negative clinical trials of trastuzumab added to chemotherapy in advanced NSCLC. In a phase II trial performed by the Cancer and Leukemia Group B, single-agent trastuzumab did not exhibit significant clinical activity against HER2 2+ or 3+ non-small cell lung carcinoma (21). A randomized phase II trial investigated the addition of trastuzumab to gemcitabine and cisplatin, in 103 previously untreated HER2-positive NSCLC patients. Trastuzumab

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was given both concomitantly to chemotherapy and as a maintenance. Although the combination was well tolerated, it failed to show a survival benefit in all HER2 IHC-positive lung cancer overall. However, 80% of patients with IHC 3+ disease on study treatment were still alive after a follow up of 6months, compared with 64% of the overall population, and a response rate of 83% and median progression free survival (PFS) of 8.5 months was observed in the six trastuzumab-treated patients with HER2 3+ or FISH-positive NSCLC (22). In a phase II trial comprising only 13 patients with HER2-positive tumors (2+ or 3+), the addition of trastuzumab to weekly docetaxel after failure of platinum based-chemotherapy showed limited clinical activity, with a PR rate of 8% (23). The Eastern Cooperative Oncology Group launched a phase II study evaluating the combination of carboplatin, paclitaxel and trastuzumab in patients with HER2-positive (1+ to 3+) NSCLC. Of 139 screened patients, 36% were indeterminate, 5% inconclusive, 27% scored 1+, 22% score 2+, and 13% were 3+. Overall survival was found to be similar to historical data using carboplatin and paclitaxel alone, while patients with 3+ HER2 expression did well in contrast to historical data (24).

These trials are a reminder of the definition of an oncogenic driver alteration, as HER2 overexpression and probably amplification per se are probably only modulators of cancer biology. In addition, as in breast cancer, the need to define-specifically for every cancer type-a threshold of significance for HER2 overexpression becomes obvious. In particular, the biological role of HER2 expression in the absence of gene amplification remains to be defined, potentially explaining the negative results of clinical trials relying on an inaccurate selection of patients.

HER2 mutations may be much more relevant in lung cancer carcinogenesis than HER2 amplification or overexpression, and several kinase inhibitors are being evaluated for the treatment of HER2-dependant lung adenocarcinoma. Lapatinib, an oral reversible dual TKI of EGFR and HER2, has been tested in a phase II trial that included 75 patients with recurrent or metastatic NSCLC; no responses were seen in the 3 patients with *EGFR* mutations. No mutations in *HER2* were found in this population, leaving the question of lapatinib activity in HER2-mutant tumors unanswered (25). In the European retrospective study (18), 2 patients were treated with lapatinib, all experiencing progressive disease. The most promising data to date have been obtained using irreversible TKIs targeting HER2/3 and EGFR, such as afatinib, neratinib, and dacomitinib. Afatinib is a potent irreversible ErbB receptor family blocker. In an exploratory phase II study, 5 patients with *HER2* mutated advanced adenocarcinoma were treated with afatinib, 3 out of which were evaluable for response. Objective response was observed in all three, even after failure of other EGFR- and/or HER2-targeted treatments (26). This series was completed with the treatment of 7 additional *HER2* mutated patients, all 5 evaluable with a stable disease (27).

Neratinib, another irreversible pan ErbB-receptor family blocker, has been evaluated in a phase I trial in combination with temsirolimus on the basis of preclinical data suggesting synergy of HER2 inhibition and mTOR inhibition on lung cancer models. Partial response was observed in 2 out of 6 patients with *HER2*-mutant NSCLC (28). Dacomitinib is an irreversible pan-HER TKI. Tested in a phase II cohort of patients with *HER2*-mutant or amplified lung cancers, dacomitinib demonstrated an overall 13% response rate in the 26 *HER2*-mutant patients, and no response in the 4 patients with *HER2* amplification or the 2 with *HER2* point mutations (29).

Pertuzumab, a first-in-class HER2 dimerization inhibitor, is a humanized monoclonal anti-HER2 antibody that prevents HER2 dimerization and inhibits HER2 signaling. A phase II trial of pertuzumab monotherapy in patients with recurrent NSCLC showed no response in 43 patients, but information on the mutational status of HER2 in these patients is lacking (30).

Ongoing trials

Surprisingly, neither pertuzumab nor trastuzumab-emtansine is presently being studied in *HER2*-mutant lung cancer. A phase II exploratory trial is evaluating neratinib monotherapy and in combination with temsirolimus in patients with *HER2*-mutant NSCLC (NCT1827267). Dacomitinib is being tested in a variety of settings, but its present development remained to date mainly focused on *EGFR*-mutant NSCLC. Its phase I trials in combination with pemetrexed (NCT01918761), or c-MET inhibitor PF-02341066 (NCT01121575) will not improve our understanding of its activity in *HER2*-mutant NSCLC. No late-phase trial targeting this particular subgroup of patients in presently ongoing.

Conclusions

The identification of oncogenic driver mutations in NSCLC has triggered the development of multiple drugs interfering

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with intracellular signaling pathways. HER2 deregulation by overexpression or amplification has been demonstrated to represent an important therapeutic target in breast and gastric cancer, but has to date little clinical relevance in NSCLC, potentially because due to the lack of definition of HER2 positivity in that particular disease. Phase II trial data merely suggests a benefit of trastuzumab therapy in patients with 3+ HER2-positive NSCLC. On the other hand, *HER2* mutations, largely exon 20 in-frame insertions, have been described as an oncogenic driver alteration in 1% to 4% of NSCLC, exclusively in adenocarcinoma histology. The prognostic implication of these alterations is not known. Phase I and II trial data suggest that afatinib, neratinib and dacomitinib have some activity in this molecular subgroup. No comparative data, or any data regarding the activity of pertuzumab or trastuzumab-emtansine is available. In order to improve our understanding of such alterations and aiming at offering new treatment options to our patients, given the high prevalence of lung cancer worldwide and the availability of investigational therapies targeting HER2, routine genotyping of lung adenocarcinoma should include HER2. Patient selection should be based on histology but should not discriminate for other clinic-pathologic features. The few currently ongoing trials are unlikely to foster our understanding of the role of HER2 TKIs in the treatment of this particular subgroup of patients. The sharp contrast between the wealth of investigational activity in other subgroups of NSCLC like *ALK*-rearranged NSCLC, which shares a similar prevalence, and the dearth of clinical research ongoing in *HER2*-mutant NSCLC is striking. Further development of afatinib and possibly of dacomitinib in this setting will be pursued. In addition, assessing the activity of pertuzumab in combination with trastuzumab, as well as trastuzumab-emtansine in patients presenting with NSCLC with 3+ HER2-overexpression would be of great interest.

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Therapeutic integration of new molecule-targeted therapies with radiotherapy in lung cancer

Mariano Provencio, Antonio Sánchez

Department of Medical Oncology of Puerta de Hierro Majadahonda, University Hospital, Madrid, Spain

Correspondence to: Mariano Provencio, MD, PhD. Servicio de Oncología Médica, Unidad de investigación en Onco-hematología, Instituto de Investigación Sanitaria Puerta de Hierro, Hospital Universitario Puerta de Hierro-Majadahonda, Calle Manuel de Falla, 1. Madrid 28222, Spain. Email: mprovenciop@gmail.com.

Abstract: Lung cancer is the most common form of the disease and the leading cause of cancer deaths worldwide. Non-small-cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancers. Forty percent of all cases present with stage III, and many of them are considered inoperable (staged IIIA with mediastinal lymph node involvement) or stage IIIB disease. Concurrent platinum-based chemotherapy and thoracic radiation has demonstrated survival benefits in these patients. We review the role of new target agents in combination with radiotherapy in stage III NSCLC. Antiangiogenics improve tumor oxygenation thereby improving the therapeutic efficacy of irradiation in models. Bevacizumab in combination with thoracic radiation has shown high toxicity. However, other antiangiogenic agents are more promising. Radiation activates epidermal growth factor receptor (EGFR) pathways, inducing radioresistance, cell proliferation and enhanced DNA repair. After promising data from preclinical models and early clinical trials, cetuximab did not show any benefit in a recent phase III trial. Panitumumab and nimotuzumab are under evaluation. Gefitinib has been investigated in combination with radiotherapy for unresectable stage III NSCLC, but results in maintenance treatment after chemoradiotherapy were not encouraging. Erlotinib has also been tested in a phase II trial with chemoradiotherapy. Other new pathways and agents are being studied, such as m-TOR pathway, bortezomib, heat shock protein 90 (Hsp90) inhibition, histone deacetylase inhibitors (HDACS), aurora kinases, mitogen activated protein kinases (MARK) and PARP inhibitors.

Keywords: Non-small cell lung cancer (NSCLC); targeted therapy; chemoradiotherapy; combined modality

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Introduction

Lung cancer is the most common form of this disease and the leading cause of cancer death worldwide. Non-small-cell lung cancer (NSCLC) accounts for approximately 80-85% of all lung cancers. Forty percent of all cases presents with stage III, and many of them will be considered inoperable (staged IIIA with mediastinal lymph node involvement) or stage IIIB disease. Concurrent platinum-based chemotherapy and thoracic radiation has demonstrated survival benefits in these patients (1,2). We review the role of new agents that selectively target tumor-specific pathways used in combination with radiotherapy in stage III NSCLC. Research, which takes into consideration the tumor and toxicity profile, is focused on the identification of new cytotoxic or targeted agents that can be combined and integrate concomitantly with chemoradiotherapy to provide greater efficacy. It is important to identify potential biological targets, the blockade of which would affect multiple downstream signalling cascades. The most promising new agents for use in combination with radiotherapy to treat lung cancer are shown in *Table 1*.

Antiangiogenics

Tumor cells increase their expression of proangiogenic growth factors in response to endothelial damage and

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Table 1 Mayor new agents in combination with radiotherapy

Agents
Antiangiogenics
Vandetanib
Bevacizumab
Thalidomide
Endostatin
EGFR pathway
Cetuximab
Panitumumab
Nimotuzumab
Gefinitib
Erlotinib
m-TOR pathway
Everolimus
Sirolimus
Bortezomib
Heat shock protein 90 inhibition
Celastrol
Histone deacetylase inhibitors
Vorinostat
Aurora kinases
PHA680632
AZ 1152
ZM447439
Mitogen activated protein kinase 1/2 inhibitor
Selumetinib
PARP inhibitors
Veliparib
Olaparib

EGFR, epidermal growth factor receptor.

hypoxia (3,4), and radiation induces cell death as a result of damage to cell membranes, DNA and microvascular endothelial cells within the tumor stroma (5,6). Combined antiangiogenic therapy and radiotherapy may improve tumor control (7) and targeting the VEGFR2 pathway could provide a way to overcome radioresistance. Preclinical data indicate that a hypoxic microenvironment contributes to radioresistance, and suppression of angiogenesis significantly enhances the radiosensitivity of cancer cells.

Vandetanib (ZD 6474), a potent orally available VEGFR2 and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, enhanced the therapeutic efficacy of irradiation in an orthotropic model of human NSCLC (8). Bevacizumab, in a phase II clinical trial study with chemotherapy and radiotherapy (9), showed serious adverse events including tracheobronchial fistulas. When used in combination with erlotinib (10) the principal toxicity was esophagitis but there was a lack of efficacy. Thalidomide showed significant toxicity when combined with chemotherapy and radiation but no additional efficacy (11). Endostatin in concurrent chemoradiotherapy did not show any benefit in overall response (12). Although these agents are often highly active in preclinical studies, the application of antiangiogenic therapy and radiotherapy in the clinical setting requires logical treatment schemes in an appropriate patient group to bring about any potential benefits (13).

Anti-EGFR

EGFR induces receptor homo- or hetero-dimerization and results in the activation of an intracellular tyrosine kinase domain. Receptor activation causes downstream signalling events through activation of the Ras/Raf/MEK/MAPK and PI3K/AKT/mammalian target of rapamycin (mTOR) pathways and has been involved in cellular proliferation, inhibition of apoptosis, angiogenesis, metastasis and chemoradioresistance (14). Radiation activates EGFR autophosphorylation increasing the activity of protein tyrosine kinase, and initiates downstream processes leading to radioresistance. In preclinical studies, NSCLC cells with *EGFR* mutations have increased radiation-induced apoptosis (15).

The monoclonal antibody cetuximab combined with radiotherapy (16) has shown synergistic activity in preclinical models. However, the addition of cetuximab to a combination of pemetrexed, carboplatin, and thoracic radiotherapy did not confer any benefit to NSCLC patients in a phase II randomized study (17). Similarly, no benefits in overall or progression free survival were shown when cetuximab was added to radiotherapy in a phase III trial (18). The safety of the cetuximab combination with radiotherapy was established in the SCRATCH (19) study, where synchronous cetuximab with radical RT were administered to patients with stage III NSCLC, and the results suggest that the early and late toxicities of synchronous cetuximab and radical RT are acceptable. The NEAR trial (20) was designed to evaluate the toxicities and feasibility of combined treatment with cetuximab and intensity-modulated radiation therapy (IMRT) locoregional irradiation in patients unfit for chemoradiation regimens. With an overall response rate of 63% and median

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locoregional, distant, overall progression-free survival of 20.5, 10.9, and 8.5 months, respectively, the median overall survival was 19.5 months and only mild toxicity was reported. Combined radioimmunotherapy with cetuximab is both safe and feasible, especially in elderly patients with multiple comorbidities.

Panitumumab, a fully human monoclonal antibody specific to the EGFR, has been tested in preclinical models. RTOG 0839 is a phase II study of preoperative chemoradiotherapy with or without panitumumab in potentially operable, locally advanced stage IIIA NSCLC (21). Nimotuzumab is a humanised monoclonal antibody specific to the EGFR with similar preclinical and clinical activity to other anti-EGFR monoclonal antibodies, and characterized by a lack of severe skin toxicity. *In vitro* studies have demonstrated that nimotuzumab increases the radiosensitivity of NSCLC cell lines (22). Nimotuzumab in combination with palliative radiotherapy has been studied in two phase I trials which showed low toxicity and absence of rash (23,24). A phase II trial in combination with carboplatin/docetaxel and radiotherapy is awaiting final results (25).

Gefitinib, an EGFR-TKI, has a radiosensitizing effect that was confirmed in cell lines (26). It was studied in combination with radiotherapy in unresectable stage III NSCLC and showed a median overall survival of 16 months with esophagitis (19.5%) being the main toxicity (27). Erlotinib has been shown to enhance radiation response at several levels (cell cycle arrest, apoptosis, induction, accelerated cellular repopulation, and DNA damage repair) (28). In lung cancer cell lines, the radiosensitizing effects of erlotinib differed when the drug was administered using different administration schedules. The highest lethal effect was obtained when radiation was administered after erlotinib, which may be related to PI3K signal transduction (29). A phase II trial (30) investigated concurrent erlotinib, carboplatin, and paclitaxel with radiotherapy in 48 patients, followed by two cycles of chemotherapy. No grade 4 toxicities were reported. Median progression free survival and overall survival were 13.6 and 25.8 months, respectively, and 1-year overall survival was 84%. *EGFR* mutation analysis was performed on 41 tumor samples and only detected in 5; the local control rate was significantly higher among patients with an *EGFR* mutation. In a prospective randomized phase II study (31), RT with or without concurrent erlotinib was administered to unresectable stage I to IIIA NSCLC patients who were not candidates for chemotherapy. The toxicities associated to erlotinib were skin rash (61.5%) and diarrhea (23%), however, erlotinib did not increase the toxicity associated to radiotherapy. The response rate was 55.5% in the radiotherapy arm and 83.3% in the concomitant arm.

m-TOR pathway

The PI3 kinase/AKT pathway is activated by mutation of *Ras* or pathway components, and by deregulated growth factor receptor signalling to *Ras*. The activation of Ras signalling increases the survival of tumor cells exposed to agents that cause DNA damage. mTOR is a critical downstream effector of the PI3K/Akt pathway. In xenograft models of human NSCLC, everolimus plus radiotherapy produces significant tumor growth suppression by increasing the antitumor activity of radiation (32). Sirolimus has been tested with thoracic radiation therapy (60 Gy) and weekly cisplatin in a phase I trial and has demonstrated a safe profile (33).

Bortezomib

Bortezomib, a proteasome inhibitor, disrupts homeostatic mechanisms within the cell and leads to cell death. The ubiquitin-proteasome pathway is essential in the degradation of intracellular proteins and regulates the cell cycle, neoplastic growth, and metastasis. Bortezomib has demonstrated *in vitro* chemotherapy- and RT-sensitizing properties (34), but a phase I (35) trial with carboplatin and paclitaxel with concurrent radiotherapy was halted because of postoperative deaths in patients who underwent right pneumonectomy.

Heat shock protein 90 (Hsp90) inhibition

Hsp90 is a molecular chaperone that mediates the refolding of denatured proteins, such as AKT, HER2, Bcr-Abl, c-KIT, EGFR and PDGFR-α (36). Hsp90 inhibition results in substantial cell death in both chemosensitive and chemoresistant small-cell lung cancer cell lines. Clinically, the geldanamycin compounds are the most mature with manageable toxic effects (37). Celastrol inhibits the ATP-binding activity of Hsp90, and it is considered an effective radiosensitizer acting as a Hsp90 inhibitor and a p53 activator in lung cancer cell lines (38).

Histone deacetylase inhibitors (HDACS)

HDACS play a role in cell motility and are involved in the regulation of many transcription factors. Vorinostat and other HDACs have shown successful results in a wide range

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of cancers, including NSCLC (39).

Aurora kinases

Aurora kinases are a family of serine-threonine kinases that control chromosome assembly and segregation during mitosis and are expressed in a broad range of cancers (40,41). Most Aurora-selective small-molecule inhibitors are currently undergoing preclinical assessment (42-46).

Mitogen activated protein kinase (MARK) 1/2 inhibitor

The MAPK/extracellular signal-regulated kinase (ERK) signalling pathway is involved in proliferation and survival of tumor cells.

Selumetinib, a selective inhibitor of MAPK1/2 (MEK1/2), inhibits tumor hypoxia in human lung and colon carcinoma xenograft models (47) and is currently in an ongoing phase I trial in combination with RT (48).

Parp inhibitors

Poly (ADP-ribose) polymerases are critical in the repair of DNA strand breaks. Ionizing radiation induces DNA strand breaks, and PARP-1 inhibition may sensitize tumor cells to radiation. Veliparib (ABT-888), a PARP-1 inhibitor, with radiation in lung cancer models is effective in enhancing tumor sensitivity to radiation (49), and is being tested in a phase I trial with chemoradiotherapy (50). A trial with another PARP-1 inhibitor, olaparib, in combination with high dose radiotherapy with or without daily dose cisplatin in locally advanced NSCLC, is ongoing (51).

Conclusions

In the development of novel targeted radiation enhancers, some recommendations have to be followed in relation to the determination of agent activity, preclinical testing of radiation enhancement effects, prioritizing agents when biomarker-based patient selection is available, understanding the proper sequencing of combining targeted agents with radiation together with determining early and late safety of the combination in phase I studies as well as regulatory issues. Angiogenic therapies have been shown to enhance radiotherapy in preclinical models. Antiangiogenics reduce vascular density, but improve tumor oxygenation, therefore, it is reasonable to suppose that a combination of antiangiogenic therapy and radiotherapy may improve tumor control. Radiation activates EGFR signalling, leading to radioresistance by inducing cell proliferation and enhanced DNA repair. Numerous clinical trials are currently exploring this combination.

Combining new drugs and concomitant chemoradiation has become an attractive therapeutic option for locally advanced NSCLC, but the addition of targeted therapies to concomitant chemoradiotherapy is still under investigation. Caution has to be exercised with respect to compliance with treatments as this is not always reported in clinical trials. Furthermore, large volume radiotherapy plus targeted drugs should be avoided and especially in hypo-fractionated regimens where high toxicities have been observed (52).

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Predicting resistance by selection of signaling pathways

Niki Karachaliou[1], Rafael Rosell[2,3,4], Miguel Angel Molina[2], Santiago Viteri[1]

[1]Dr Rosell Oncology Institute, Quirón Dexeus University Institute, Barcelona, Spain; [2]Pangaea Biotech, Quirón Dexeus University Institute, Barcelona, Spain; [3]Catalan Institute of Oncology, Hospital Germans Trias i Pujol, Badalona, Spain; [4]Molecular Cancer Research (MORe) Foundation, Barcelona, Spain

Correspondence to: Niki Karachaliou, MD, PhD. Dr Rosell Oncology Institute, Quirón Dexeus University Institute, 08028 Barcelona, Spain. Email: nkarachaliou@oncorosell.com.

Abstract: Epidermal growth factor receptor (EGFR) mutations occur in 17% of non-small-cell lung cancer (NSCLC) patients with notable response to single agent therapy but with low complete remission rate and, eventually, disease progression. Priming BIM, a pro-apoptotic signaling BH3-only protein, induces sensitivity to erlotinib in EGFR-mutant cell lines. Synthetic lethal approaches and preemptive therapies based on the initial expression of BIM may significantly improve the treatment outcome. EGFR mutations result in transient pro-death imbalance of survival and apoptotic signaling in response to EGFR inhibition. SHP2 is essential to the balance between ERK and the phosphoinositide-3-kinase (PI3K)/AKT and signal transducer activator of transcription (STAT) activity, while mTOR can be an additional marker for patients with high BIM expression. Furthermore, stromal hepatocyte growth factor (HGF) confers EGFR tyrosine kinase inhibitor (TKI) resistance and induces interreceptor crosstalk with integrin-b4, Eph2, CUB domain-containing protein-1 (CDCP1), AXL and JAK1. Only by understanding better, and in more depth, complex cancer molecular biology will we have the information that will help us to design strategies to augment efficacy of EGFR TKIs and offer our patients the best, most correct therapeutic option.

Keywords: Lung cancer; epidermal growth factor receptor (EGFR) mutations; biomarkers; signaling pathways

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Introduction

A recent meta-analysis of patients with non-small-cell lung cancer (NSCLC) and epidermal growth factor receptor (EGFR) activating mutations showed that first-generation EGFR tyrosine kinase inhibitors (TKIs) significantly delayed disease progression but had no effect on overall survival (1). Erlotinib, gefitinib and the second-generation, irreversible EGFR TKI afatinib have offered patients with metastatic EGFR positive lung cancer a therapeutic alternative that has proven its superiority over standard platinum-based chemotherapy (2-4). However, primary or acquired resistance limits the therapeutic success of these targeted agents (2). The expression levels of the proapoptotic protein BIM have been found to predict responsiveness to kinase inhibitors in treatment-naïve cancer patients, confirming that this molecule is implicated in modulation of cancer cell dependence on EGFR and other oncogenic models (5,6). The levels of all three major splicing isoforms, BIM extra-long (BIM-EL), BIM long and BIM short, are induced after erlotinib treatment in drug-sensitive PC-9 cells, but not in drug-resistant H1650 [that lacks expression of the phosphatase and tensin homolog (PTEN) protein] and in H1975 cells (that harbor the 'gatekeeper' mutation T790M-EGFR). EGFR signaling influences BIM expression and phosphorylation status mainly via the ERK pathway, and erlotinib appears to induce significant dephosphorylation of BIM-EL which results in an increase in its proapoptotic function (7,8). However, pretreatment BIM expression levels may not be enough to predict outcome to EGFR TKIs. The two primary signaling pathways activated by EGFR are the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase (PI3K) axes. Src tyrosine kinases, activation of the signal

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transducer activator of transcription 3 (STAT3) pathway and downstream signaling have also been well documented (9). EGFR phosphorylation leads to recruitment of multiple effector proteins through recognition and binding of Src-homology 2 domain-containing phosphatase 2 (SHP2) to phosphotyrosine motifs on the receptor (9). SHP2 (encoded by PTPN11), is a ubiquitously expressed SH2 domain-containing protein tyrosine phosphatase (PTP). Despite its direct function in protein dephosphorylation, SHP2 plays an overall positive role in transducing signals initiated from growth factors/cytokines and extracellular matrix proteins and in initiating various downstream signaling cascades, including PI3K, MAPK and STAT3 (9,10).

In this short review we will try to demonstrate, by reviewing the current literature, that "first-line EGFR TKIs monotherapy for patients with mutant EGFR NSCLC is incomplete" and EGFR inhibitors, reversible or irreversible, are unlikely to provide cures in the majority of patients.

BIM expression in treatment naïve cancers predicts responsiveness to EGFR TKIs, but almost 2/3 of patients have low BIM mRNA levels at baseline

We were able to examine BIM mRNA levels in pretreatment tumour samples from 83 patients included in the EURTAC trial (2,5). BIM expression was low or intermediate in 53 (63.96%) and high in 30 (36.14%) patients. PFS to erlotinib was 12.9 months for those with high, and 7.2 months for those with low/intermediate, BIM expression levels, while among chemotherapy-treated patients, it was 5.8 and 5.5 months, respectively (P=0.0003) (5). Overall survival was 28.6 months for patients with high BIM expression and 22.1 months for those with low/intermediate BIM expression (P=0.0364). Multivariate analyses showed that erlotinib was a marker of longer PFS [hazard ratio (HR) =0.35; P=0.0003], while high BIM expression was a marker of longer PFS (HR =0.49; P=0.0122) and overall survival (HR =0.53; P=0.0323) (5). SHP2 plays a fundamental role in NSCLC cells harboring EGFR mutations (11,12). SHP2 is required for the full activation of the MAPK/ERK pathway and its catalytic activity regulates the PI3K/AKT pathway resulting in the positive effect of SHP2 on cell survival (9,12-14). Cragg and colleagues have reported that concurrent treatment of H3255, HCC827, or H1650 cells with gefitinib and a MEK inhibitor does not result in substantially enhanced apoptosis (15). In contrast, SHP2 knockdown reduces ERK phosphorylation and increases cellular sensitivity to gefitinib in cells expressing EGFR mutants, but also in cells expressing wild-type EGFR (11). Activation of receptor tyrosine kinases, including EGFR, results in SHP2 phosphorylation at Y542, which is required for normal SHP2-mediated ERK activation in response to many growth factors (11). Surprisingly, the EGFR L858R mutation leads to decreased ability to activate ERK compared to wild-type EGFR, which correlates with decreased EGFR internalization and reduced phosphorylation of SHP2 and sensitivity to gefitinib (16). Lazzara and colleagues were able to demonstrate that SHP2 Y542 phosphorylation was induced in the EGFR wild type H1666 cells (that carry an uncommon BRAF mutation, G465V) in response to EGF, but not in the H3255 cells which harbor the missense L858R exon 21 mutation, suggesting that SHP2 activity may be less efficiently promoted by EGFR L858R (16). The reduced SHP2 phosphorylation and full ERK activation may partially correlate with decreased EGFR internalization, given that activating mutations of EGFR are endocytosis-impaired (16). However, the mutant-bearing (del19) PTEN-null cell line H1650 did exhibit inducible SHP2 Y542 phosphorylation (16). Therefore, further studies are needed to define the mechanism underlying differential SHP2 involvement beyond the apparent link to receptor internalization.

SHP2 is also required for sustained activation of ERK and epithelial morphogenesis downstream from the MET receptor tyrosine kinase (17,18). Several MET inhibitors have been tested so far that can be classified according to their mechanism of action in selective MET inhibitors (tivantinib, EMD 1204831, SGX523, INCB0280), unselective MET inhibitors (crizotinib, cabozantinib, foretinib, golvatinib, MGC D265 and MK-2461) and antibodies targeting MET (onartuzumab) or hepatocyte growth factor (HGF) (ficlatuzumab, rilotumumab or TAK-701) (19,20). Upon activation of MET by its ligand, HGF, which is provided by stromal cells, EGFR signaling is dramatically altered (21). HGF anticipates the mode of action in EGFR mutant tumours, as EGFR tyrosine kinase activity, as well as the classical downstream signaling, is no longer required for tumour growth (21). Specifically, HGF confers EGFR TKI resistance by inducing two novel cancer-promoting functions: first, it abolishes classical EGFR signaling, which makes cancer cells independent of these signaling mechanisms and neutralizes the point of action for EGFR TK-targeted drugs. Second, it enables the EGFR to interact with proteins, which are known to be markers of a highly metastatic phenotype like the

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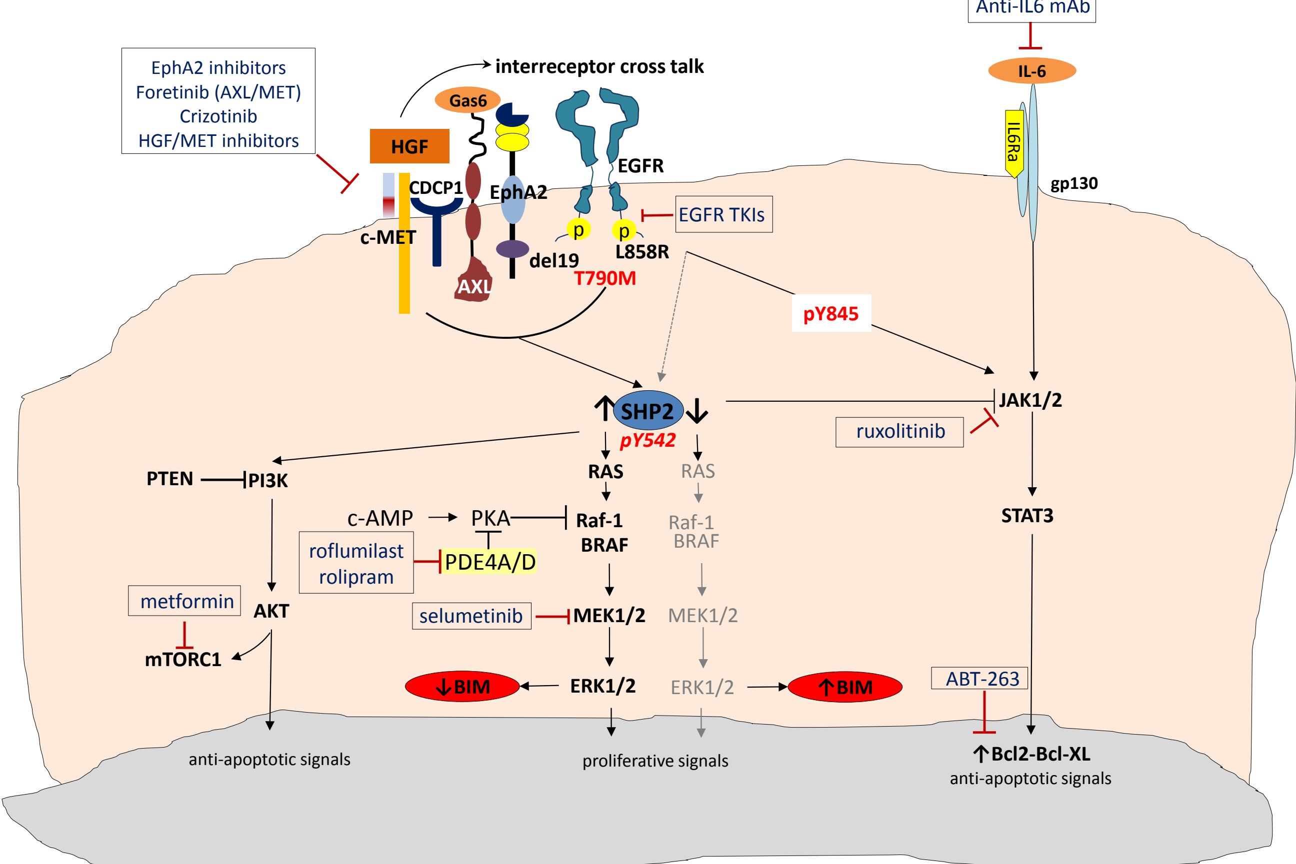

Figure 1 Mechanisms for BIM regulation.

CUB domain-containing protein-1 (CDCP1), EphA2 and AXL, interactions that cannot be affected by EGFR TKI treatment (*Figure 1*) (21). Thus, treatment with HGF/MET inhibitors together with EGFR-targeted therapies, as well as targeting HGF/MET-induced EGFR interactors may both be necessary for elimination of tumour growth (21). At the same time, Gas6/AXL-mediated stimulation of ERK is attributed, in part, to its ability to activate SHP2 (18). Foretinib is an oral multikinase inhibitor targeting MET, RON, AXL, and VEGFR, while YW327.6S2 is the first reported fully humanized AXL blocking antibody that blocks AXL functions by downregulating its expression as well as inhibiting the ligand Gas6 (22,23).

EphA2 is a member of the erythropoietin-producing hepatocellular (Eph) family of receptor tyrosine kinases. Unlike traditional oncogenes that often function only in tumour cells, EphA2 mediates cell-cell interactions both in tumour cells and in the tumour microenvironment, namely the tumor stroma and tumor vasculature. EphA2 is often overexpressed in a variety of malignant cancers, including breast, lung, prostate and colon (17). EphA2 phosphorylates Tyr542 and Tyr580 of SHP2 to enhance and prolong ERK activation downstream of receptor tyrosine kinases in cells stimulated with growth factors, such as EGF, HGF or Gas6 (17). Miura *et al.*, were able to demonstrate that prolonged and enhanced ERK activation in cells stimulated with growth factors were reduced in cells depleted of EphA2 with simultaneous reduction of Tyr542/580 phosphorylation (17). The SHP2-dependent ERK activation signal pathway was hyperactivated promoting cancer cell proliferation in tumors with EphA2 overexpression, measured by mRNA or immunohistochemistry (IHC) (17). Very interestingly, the G391R EphA2 mutation has been identified in a squamous cell cancer cell line (H2170) but also in samples from patients exhibiting NSCLC with squamous histology (24). This mutation activates downstream effectors of EphA2 including mTOR, making this receptor a useful molecular

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therapeutic target even for squamous NSCLC (24). Until now, only a few small molecule inhibitors of EphA2 have been identified (25) but dasatinib has been reported to have potent inhibitory activity against this receptor (26).

Targeting the phospho-peptide binding site in SHP2 seems to be a feasible approach for developing SHP2-selective inhibitors but, despite the great need, little progress has been made. In a recent study, a small-molecule inhibitor (#220-324) was identified that selectively inhibits SHP2 and blocks SHP2-mediated signaling and cellular function (10). Until further studies are performed to optimize this compound and develop new SHP2 inhibitors with increased activity and selectivity suitable for preclinical and clinical studies, combining EGFR TKIs with MET, AXL or EphA2 inhibitors can be a rational and innovative synthetic lethality approach for EGFR mutant NSCLC patients with low baseline BIM expression and high SHP2 activity (*Figure 1*). It seems that IHC staining and mRNA expression of SHP2 are well correlated and can be used as a biomarker for response (27,28).

Additionally, the MAPK pathway can be cross-regulated by the cAMP pathway. This occurs through inhibition of the Raf-1 kinase by PKA, a main effector of cAMP (29). Upregulation of the tumor-promoting factors PDE4A and PDE4D in lung cancer (including the H1975 cell line) impairs cAMP generation through cAMP hydrolysis, activating the MAPK pathway and thus downregulating BIM (29). Whether drugs already approved for nononcologic indications, for example roflumilast which is used to treat asthma and chronic obstructive pulmonary disease, can be safely and effectively repurposed as PDE4 inhibitors in combination with EGFR TKIs, warrants further investigation (*Figure 1*). It is worth mentioning that cAMP is also involved in regulation of mammalian target of rapamycin (mTOR) transcription by diacylglycerol kinase α (DGKα) (30,31). mTOR mRNA levels have been shown to correlate strongly with DGKα mRNA levels in several tumours, and cells treated with PDE4 inhibitors show a significant decrease in mTOR transcription, indicating that DGKα regulates mTOR transcription, probably via modulation of cAMP levels (31).

High BIM at baseline is not enough to offer the maximum benefit to NSCLC EGFR mutant patients treated with EGFR TKIs

Even patients with high BIM levels at baseline (which means that the ERK pathway may not be very active) eventually develop resistance and disease relapse after a median PFS of 12.9 months (5). Binding of EGF to the EGFR induces dimerization, autophosphorylation and transactivation of the receptor's tyrosine kinase activity, providing a variety of binding sites for a series of proteins, thereby initiating activation of downstream signaling pathways (32). For instance, Y845 (pY845) phosphorylation stabilizes the activation loop, maintains the enzyme in an active state and regulates STAT3/5 activity. Phospho-tyrosine 992 (pY992) within EGFR provides a binding motif for phospholipase C-γ (PLC-γ), initiating downstream signaling, including PKC and subsequent ERK activation. Phospho-tyrosine 1068 (pY1068) and 1086 (pY1086) provide a binding motif for Grb2/SH2 domain, which also leads to ERK and AKT activation (32). Phospho-tyrosine 1173 (pY1173) and 1148 (pY1148) represent a motif for PLC-γ and Shc, both of which can initiate activation of the ERK cascade. Interestingly, pY1068, pY1148, and pY1173 are essential for EGFR internalization and degradation, as well as for tyrosine kinase activity (32). In 2004, Sordella and colleagues were able to demonstrate the differential EGF-induced tyrosine phosphorylation pattern seen with wild-type and mutant EGFR receptors (32). EGF-induced phosphorylation of Y1045 and Y1173 is almost indistinguishable between wild-type and mutant EGFRs, whereas phosphorylation of Y992 and Y1068 is substantially increased in both mutants. Y845 is highly phosphorylated in the L858R missense mutant, but not in the wild-type or deletion mutant, and hence appears to be unique in distinguishing between the two types of EGFR mutations (32). Therefore, the effects of EGFR-activating mutations might be most appropriately characterized as "oncogene imbalance", since the ERK pathway is altered in the opposite direction to AKT and STAT (16). In the EURTAC study, patients with deletion 19 had significantly better PFS to erlotinib compared to chemotherapy, in comparison with the smaller group of patients with the L858R missense exon 21 mutation, for whom PFS to erlotinib was not significantly different from the chemotherapy treated group (2). We have previously commented on the findings that the EGFR L858R mutation leads to decreased ability to activate ERK compared to wild-type EGFR which correlates with decreased EGFR internalization, reduced phosphorylation of SHP2 and reduced sensitivity to gefitinib (16). In addition, we can now speculate that these differences in outcome between the two classic mutations can be through full STAT3/5 activation by the missense exon 21 mutation. Inhibition of EGFR

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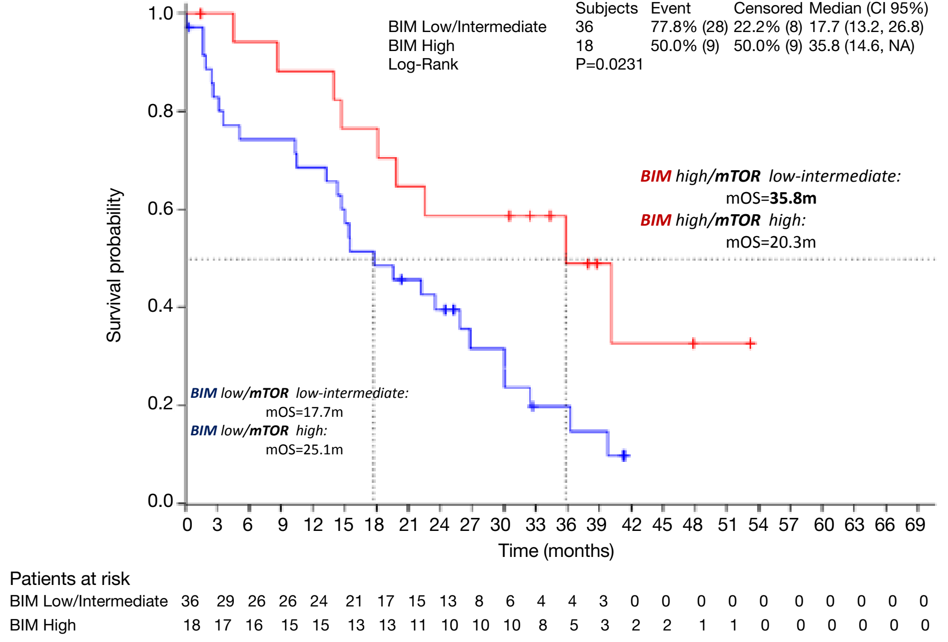

Figure 2 Effect of mTOR on the survival of patients with high BIM.

with EGFR TKIs has no effect on tyrosine phosphorylated STAT3 (33). Therefore combining EGFR TKIs (reversible or irreversible) with a JAK2 inhibitor, like ruxolitinib, can be more efficient in inducing apoptosis regardless of MAPK/ERK abrogation or high levels of BIM in EGFR mutant patients with the L858R mutation (*Figure 1*).

Activating mutations of EGFR may enhance IL-6 production and autocrine stimulation of STAT3 activity, but additional cellular factors are important in modulating this pathway and the response of cells to IL6. The PC9 (del19) cells that harbor activated EGFR have essentially absent STAT3 activity, measured either by immunoblot or DNA binding assay (33). While activating mutations of EGFR may enhance IL-6 production and autocrine stimulation of STAT3 activity, additional cellular factors are important in modulating this pathway and the response of cells to IL6. Indeed, STAT3 activity in lung cancer cells is regulated by IL-6 in conjunction with JAK1/2 activity. SHP2 is a positive regulator of cell growth and migration through stimulation of the MAPK/ERK pathway, but a negative regulator of interferon signaling and the JAK/STAT3 pathway. It has been demonstrated by You and colleagues that SHP2 is involved in protecting cells from the cytotoxic effect of IFNs and that it acts as a negative effector in mediating activation of STATs induced by IFN-α or IFN-γ (34). Therefore, in cells that SHP2 is silent, and BIM or BIM-EL levels remain elevated through the activity of EGFR TKIs, the JAK/STAT3 pathway can be hyperactive inducing anti-apoptotic signals and favoring tumour survival and progression. In these cases the combination of EGFR TKIs with a JAK2 inhibitor like ruxolitinib can abrogate tumor growth (33).

Does mTOR matter more than BIM?

Regardless of BIM status, mTOR is a serine/threonine kinase that is often deregulated during cancer growth. It has been shown that mTOR is important for the oncogenic transformation induced specifically by PI3K and AKT. mTOR integrates cues from nutrients and growth factors, acting as a nexus point for cellular signals to control growth, metabolism, and longevity. Deregulation of either of mTOR's two complexes, mTORC1 or mTORC2, leads to diseases of metabolism, including cancer and diabetes (35). We were able to examine mTOR mRNA levels in 48 tumor samples from the EURTAC study. Eighteen patients (37.5%) had high mRNA expression by terciles and 30 (62.5%) had low/intermediate mTOR mRNA levels. Also, we were able to correlate the mTOR levels with high levels of BIM. For instance, patients with high BIM and low-intermediate mTOR, had a median overall survival of 35.5 months, compared to 20.3 months for the group of patients with high BIM and high mTOR (*Figure 2*) (unpublished data).

mTOR warrants further exploration to determine whether it is a stronger biomarker than BIM to predict outcome

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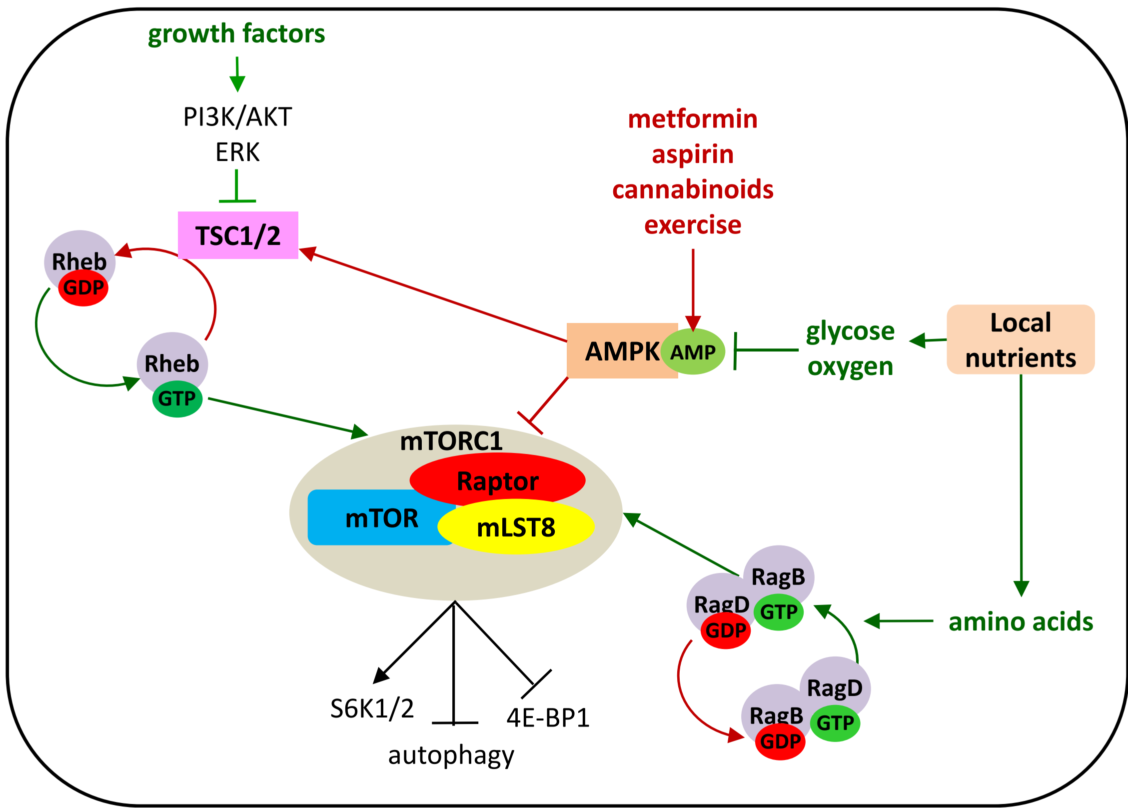

Figure 3 Regulation of mTORC1 activity.

of patients treated with EGFR TKIs or chemotherapy. mTORC1 is essential to the decision process between anabolism and catabolism (36). This complex, which consists of mTOR, Raptor, and mLST8, is activated by amino acids, growth factors and cellular energy to drive nutrient uptake and, subsequently, proliferation (36). The molecular details of these nutrient-sensing processes are not yet fully elucidated. Amino acids activate the Rag GTPases to regulate mTORC1 localization to the lysosomes and growth factors signal through the PI3K-AKT or the ERK pathways to activate mTORC1 by releasing the Ras homolog enriched in brain (Rheb) GTPase from repression by the tumour suppressors tuberous sclerosis 1 and 2 (TSC1, TSC2). Finally, low-energy conditions inhibit mTORC1 by activating AMP-activated protein kinase (AMPK) (36). mTORC1 phosphorylates and activates the ribosomal S6 kinases (S6K1 and S6K2), which are required for translation of a group of mRNAs, and inactivates the binding protein of eukaryotic translation initiation factor 4E (4E/BP), thereby facilitating 4E-mediated translation (37). At the same time, mTORC1 is known to be a major negative regulator of autophagy. Altogether, these effects imply that mTORC1 increases protein synthesis and reduces protein degradation (37). AMPK serves as an energy sensor in all eukaryotic cells and also occupies a central role in linking metabolism and cancer development. It is activated in response to an increase in the AMP:ATP ratio during hypoxia, starvation, glucose deprivation or muscle contraction and regulates aerobic glycolysis (the Warburg effect) in cancer cells and suppresses tumour growth *in vivo* (38). Under starvation conditions, AMPK plays a critical role for cell survival by stimulating energy production and limiting use of energy by active biosynthetic pathways usually operating in proliferating cells (38). Many recent studies have shown that exercise or pharmacologic activators of AMPK, such as metformin, cannabinoids, and aspirin (a synthetic derivative of salicylate), cause AMPK activation and inhibit or delay the onset of tumours in different animal cancer models (38-40). Cannabinoid-mediated metabolism results in strong induction of autophagy and inhibition of cell growth in pancreatic cancer cells (40) (*Figure 3*).

The anticancer mechanism of action of metformin is ambiguous. Although it is an antidiabetic drug, activation of AMPK through phosphorylation of AMPKα at Thr-172 has been widely accepted as a possible mechanism (41).

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However, most studies, which evaluate the antitumor activity of metformin, use concentrations much higher than the recommended therapeutic doses for clinical use. When concentrations are decreased to the same as that found in plasma and tissues of individuals receiving therapeutic doses, inhibition of cell proliferation is not observed (42). A recent study by Gou and colleagues demonstrated that low concentrations of metformin were associated with reduction of ERK and mTOR phosphorylation independent of AKT and AMPK phosphorylation in pancreatic cancer cells (42). These low concentrations of metformin were effective on specific subpopulations of pancreatic cancer cells expressing CD133, a surface marker considered characteristic of cells with extensive proliferative and self-renewal characteristics (cancer stem cells). A similar selective inhibitory effect of metformin was observed on CD133 positive cancer glioblastoma cells (43). In NSCLC, IHC assessment of CD133 expression is correlated with pathological stage and is predictive of unfavorable prognosis for stages II-IV (44). These results provide a basis for combination of metformin with current therapies to improve prognosis of cancer patients and allocate a role to IHC evaluation of CD133 as a biomarker to predict response (43).

Conclusions

If we wish to radically change treatment of EGFR mutant NSCLC to the benefit of our patients, we should start thinking about a different approach based on information derived from additional biomarkers. Patients with low BIM levels at baseline may benefit from the combination of EGFR TKIs with compounds that downregulate or abrogate activity of SHP2, like MET, AXL or EphA2 inhibitors. It should be seriously considered whether, at time of progression, a JAK2 inhibitor should be added in order to overcome loss of the negative impact of SHP2 on the JAK/STAT pathway. Patients with high BIM levels at baseline may have a hyperactive JAK/STAT pathway through either the L858R mutation or loss of SHP2 activity. The combination of EGFR TKIs plus a JAK inhibitor should be seriously considered in these cases. In general, patients with high BIM expression benefit from erlotinib or similar EGFR TKIs, but analysis of mTOR could further improve outcome by selecting patients with high mTOR for combination therapy with EGFR TKIs and mTOR inhibitors. We propose this line of research at the levels of cell lines or xenograft models and at the level of biomarker discovery in tumour samples, in order to verify in the most accurate possible way our assumptions and contribute to the radical transformation of treatment of EGFR mutant lung cancer.

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The role of the immune system in non-small cell lung carcinoma and potential for therapeutic intervention

Joanna Domagala-Kulawik

Department of Internal Diseases, Pneumonology and Allergology, Medical University of Warsaw, Warsaw, Poland

Correspondence to: Joanna Domagala-Kulawik. Department of Pneumonology and Allergology, Medical University of Warsaw, ul. Banacha 1a, 02 097 Warsaw, Poland. Email: domagalakulawik@gmail.com.

Abstract: Over a hundred years after the first description of this disease, lung cancer represents one of the major challenges in oncology. Radical treatment cannot be introduced in more than 70% of cases and overall survival rate does not exceed 15%. The immunosurveillance of lung cancer may be effective in early oncogenesis but is inhibited in the course of developing a clinically detectable tumor. Very low and heterogonous antigenicity of lung cancer cells leads to passive escape from anti-cancer immune defense. The cytotoxic lymphocytes (CTLs) that play a main role in the anticancer response are actively suppressed in the tumor environment and following regulatory mechanisms inhibit the recognition of tumor antigens by antigen presenting cells. The population of regulatory T cells (Tregs) is augmented and the expression of transcription factor—Foxp3 is markedly increased on tumor cells and tumor infiltrating lymphocytes (TIL). It is accomplished by M2 macrophage polarization, the activity of myeloid derived suppressor cells (MDSCs) and a significantly elevated concentration of cytokines: transforming growth factor beta (TGFβ) and IL-10 in the tumor microenvironment. Very active suppression of immune protection is the predominant role of the programmed death 1 (PD-1)-PD-L1 pathway. The blockage of this pathway was found to be an effective treatment approach; therefore the monoclonal antibodies are being intensively investigated in lung cancer patients. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is the molecule capable of inhibiting the activation signal. The antibody anti-CTLA-4 improves CTLs function in solid tumors and lung cancer patients may benefit from use of this agent. The second way in lung cancer immunotherapy is production of anti-cancer vaccines using recognized cancer antigens: MAGE-A3, membrane associated glycoprotein (MUC-1), and EGF. It was recently shown in ongoing clinical trials that combined therapies: immune- and chemotherapy, radiotherapy or targeted therapy seem to be effective. Immunotherapy in lung cancer has an individual character—there is a need to assess the patient's immune status prior to implementation of immunomodulating therapy.

Keywords: Lung cancer; immune response; cytotoxic cells; regulation; immunotherapy; vaccines

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Introduction

Two years ago the 100-year anniversary of lung cancer was "celebrated". 100 years after the first description of 374 cases of lung cancer (1) as high as 1.6 million new cases are diagnosed yearly (2,3). In recent years the clinical and biological patterns of lung cancer have been changed and are varying continuously. The increasing incidence of adenocarcinoma, decrease in the proportion of small cell lung cancer and new observations on lung cancer in nonsmokers are the most striking features of this change and remain a challenge for the progress in thoracic oncology. However, an unchanging fact is that lung cancer is the main oncological problem worldwide and it is a leading cause of cancer death among patients with malignancy. The resection rate that is a treatment of choice in early—stage non-small cell types—is as low as 25-30% (3,4).

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In the advanced stages of non-small cell lung carcinoma (NSCLC) other therapies are used with some effectiveness: chemotherapy, radiotherapy and biological treatment. Recently it became important to perform appropriate cancer molecular characterization and to select patients individually to a treatment strategy with thorough analysis of the histological type, molecular pattern and evaluation of predictive factors. In practice this molecular characterization is performed by analysis of activating mutations of *epidermal growth factor receptor* (*EGFR*) (in clinical practice in exons 19 and 21) and detection of an *anaplastic lymphoma kinase* (*ALK*) rearrangement (5). For tumors with activating *EGFR* mutations, first-line treatment is indicated with an EGFR tyrosine kinase inhibitor (EGFR-TKI, such as gefitinib, erlotinib, and afatinib). Anti-EGFR antibody- cetuximab is accepted in some countries as a biological therapy. The treatment with crizotinib is advised for ALK-positive lung cancer (5-7). However, the prevalence of an *EGFR* mutation in adenocarcinoma of European patients is close to 10%, while in Asian and Japanese patients is up to 30-50% (8). More lung cancer prognostic markers are being published, but without promising effectiveness in practice (5).

Among NSCLC subtypes adenocarcinoma is the most heterogeneous tumor, with known aggressiveness of certain subtypes (i.e., solid tumor with mucus production), and response to anti-EGFR targeted therapy in tumours harbouring *EGFR* mutations (9,10). This direction of targeted therapy has brought some good results, but only in the appropriate selected patients groups (5). Only a relatively small proportion of patients in our country harbor *EGFR* mutations so only small numbers of patients benefit from currently available targeted therapies (11). The current therapeutic approach develops in another direction—with taking into account an advantage of the recognition of the immune response in solid tumors. The goal of such new therapies is to support the host's own anticancer immune response. Here a description of the immune alterations in the course of NSCLC with possible implications for therapy is presented.

Background to the considerations

The morbidity due to lung cancer is strongly correlated to age with the greatest risk in the oldest patients groups of both sexes. Age distribution at lung cancer diagnosis is estimated at approximately 6% in patients below 50 years of age, 29% in patients of 60-69 years old, and 44% in patients over 70 years of age (3). In this context the role of immune system senescence has to be revealed. The following alterations characterize an immune-aging (inflamm-aging): shortening of telomeres, histone acetylation and reduction of antiaging molecules such as histone deacetylases and sirtuins, apoptosis, increased concentration of proinflammatory cytokine- IL-6, and Th2 polarization (12). These disorders are inhibitors of anti-cancer immune response in the course of lung cancer. Immuno-senesce enhances the failure of anti-cancer response.

Cigarette smoking is the main risk factor for lung cancer (2,3). The influence of tobacco smoke on lung homeostasis is complex with a predominant feature being suppression of the immune system (13,14). We have previously reported the noxious influence of tobacco smoke on lung immune status (15-17). Apart from tobacco smoke, many other environmental agents permanently affect the lung milieu: dust, allergens and microbes, with resulting oxidative stress and hypoxia. These factors are capable of causing serious modification of lung immune status. For better understanding of the nature of immune disturbances, the continuous process of self- and down-regulation of the function of immune cells cannot be neglected.

The lung immune system has multiple parts: it consists not only of large numbers of immune cells with a complex cytokine network, but also of structural elements of different function, i.e., epithelial, endothelial and mesenchymal cells. In normal conditions an integration of these elements is fixed and the proportion of immune cells rests within a normal range. In my opinion a valuable way for evaluation of the lung immune status, in steady state and during disease, is bronchoalveolar lavage (BAL) fluid examination. BAL analysis is a low-invasive method and the BAL components reflect the local immune response in a large part of the lung. In clinical practice the main indication for BAL analysis is a diagnosis of diffuse parenchymal lung disorders, interstitial lung diseases and infections. In lung cancer the role of BAL in peripheral tumor diagnosis has also been documented (18). This is a quantitative method, already well standardized (19-21). For the lavage, 200 mL of saline is used; the total cell count and differential cell count are determined in the recovered fluid. The referenced BAL pattern in nonsmokers contains total cell count of about 10 million cells, cell viability is more than 90%, the percentage of macrophages >80%, lymphocytes <15%, neutrophils <5%, eosinophils <1% (19,21,22). The effectiveness of BAL in the evaluation of lung immune status in the course of lung cancer has been described in our earlier works (17,23-25). The elements of the immune response in lung cancer patients may

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serve as biomarkers and predictive factors in regards to immunotherapy, applied in clinical practice. BAL may be performed during bronchofiberoscopy, which is an inherent step in the diagnostic procedure. It should be mentioned that knowledge of defense mechanisms in lung cancer is rather limited to data obtained from peripheral blood samples, reflecting the systemic immune response. As concerns local immune response evaluation it is usually performed by the examination of resected tumors. Since the resection rate of NSCLC does not exceed 30% and small cell types are not-resectable per se, in the majority of lung cancer cases the local immune status cannot be studied. From this perspective the BAL analysis is important as it can be performed at any time of the disease, including advanced lung cancer stages.

Is the histologic type of NSCLC important? Today's classification of NSCLC recommends clear distinction of squamous cell type and adenocarcinoma, which is important in guiding to current treatment with new methods of targeted therapy. In this context it is often important to detect thyroid transcription factor (TTF-1) positive cancer cells as an indicator of glandular differentiation in those cases where it cannot be seen morphologically. TTF-1 is essential for morphogenesis and differentiation of the lungs and is a marker of lung adenocarcinoma. In some studies TTF1 expressing tumors were suggested to be associated with longer survival (26,27). However, the heterogeneity of lung cancer occurs with mixed types entity. Moreover quite often the non-otherwise specified (NOS) type is being diagnosed. In these cases detection of TTF1 positive cells is suggestive for adenocarcinomatous differentiation. In the daily practice we observed that the *EGFR* mutation initially restricted to adenocarcinoma is present also in the squamous cell type. Perhaps in the future the molecular classification of NSCLC may be more relevant than the histological one.

Last but not least, cancer stem cells (CSCs) are a new potential target for solid tumor therapy, including a lung cancer therapy (28-30). The phenotype of CSCs is currently widely investigated, the marker CD133/EPCAM being suggested (31,32).

Cytotoxic attack

Lymphocytes, macrophages and granulocytes are involved in the anti-cancer battle. The niche of lymphocytes is known as tumor infiltrating lymphocytes (TIL) (33,34), of macrophages—the tumor associated macrophages (TAM) (35,36), of neutrophils—the tumor associated neutrophils (TAN) (37), and of eosinophils—the tumor associated tissue eosinophilia (TATE) (38). The main cell population with activity in anti-cancer immune response is the population of cytotoxic T lymphocytes (CTLs) (39). The CTLs population is represented by CD8+ lymphocytes, CD4+ lymphocytes, natural killer cells (NK), natural killer T cells (NKT) and lymphocytes B (40,41). Cancer cells are killed by induction of apoptosis by cytolytic reaction or membrane-receptor induction of programmed death. The successful cytotoxic attack needs an effective antigen presentation by tumor cells and antigen presenting cells (APC). This is achieved mainly by macrophages and dendritic cells (DCs) (42). The latter migrate to lymph nodes after contact with cancer antigens and activate effector cells by presenting the antigen. A crucial role in APC-lymphocyte signal transmission is played by co-stimulating molecules on APC and related receptors on lymphocyte (*Figure 1*) (39,43). As cytotoxic CD8+ lymphocytes and CD4+ cells are "soldiers" of the CTL army, the signal pathway B7-CD28 is widely investigated (39). The blockage of APC-CTL action is observed in malignancy and provides the CTLs inactivation.

Impaired function of the immune system—the mechanisms of immune tolerance

It is well documented that anti-cancer defense is ineffective in clinically detectable cancers and that the greater is the size of a solid tumor mass, the less effective anti-cancer response is observed (44). Lung cancer cells hide against cytotoxic attack by low antigen presentation and low co-stimulatory molecule expression. Moreover, the lung cancer antigens are unstable and badly defined as a result of multiple genetic and epigenetic alterations during oncogenesis (45). Altogether, it leads to a passive cancer cells escape from immunosurveillance. On the other hand, many other elements of this escape relate to active regulation and suppression of the immune anti-cancer response.

There are many mechanisms of CTL inhibition (*Figure 2*). An interaction of programmed death receptors on lymphocytes with their ligands on tumor cells leads to apoptosis of lymphocytes. Recently it was revealed that the expression of the programmed death-1 (PD-1) molecule on T cells plays an important role in the context of cytotoxic effect inhibition (46). PD-1 is present on T helper, T cytotoxic, T regulatory cells, B lymphocytes and NK cells. Tumor cells express high levels of PD-1 ligands: B7-H1 (PD-L1) (CD274) and PD-L2 (CD273, B7-DC). The PD-1-PD-L interaction

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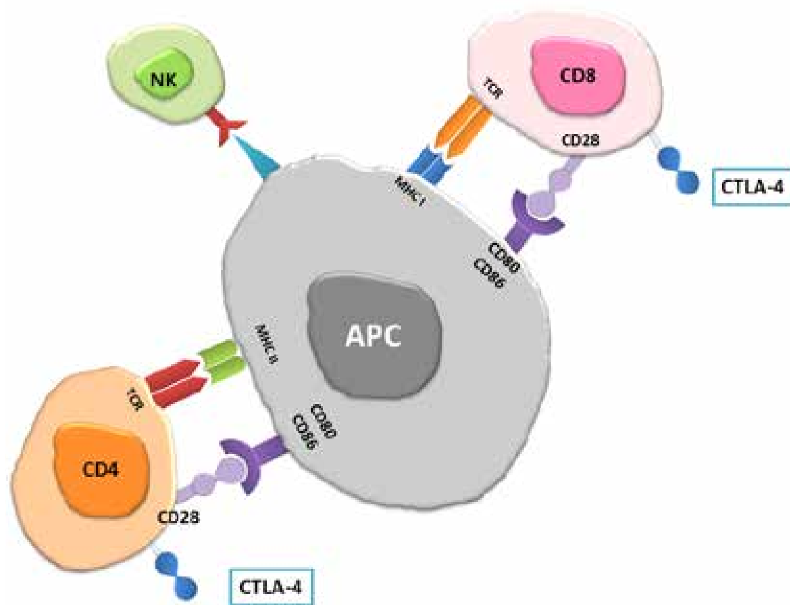

Figure 1 Dendritic cell as an antigen presenting cell triggers priming of tumor specific T cells co-stimulatory pathways. APC, antigen presenting cell; NK, natural killer cell.

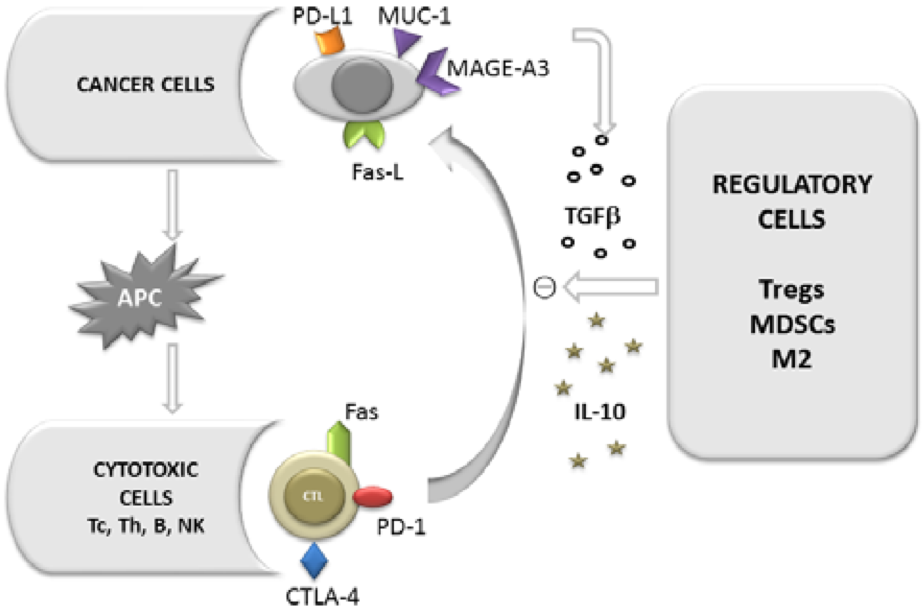

Figure 2 The essentials of tumor cells interaction with immune system. APC, antigen presenting cell; NK, natural killer cell.

has a strong immunosuppressive effect. It has been applied to therapy with the blockade of PD-1/PD-L pathway using a fully humanized PD-1 or PD-L1 antagonistic monoclonal antibodies shown to increase the number and functionality of tumor-specific T cells (39,47-49).

We have previously reported the increased expression of Fas receptor on lymphocytes in the course of lung cancer (50). An interaction of Fas-Fas ligand (Fas-L) causes the death of Fas bearing cells. Apart from an elevated proportion of Fas positive lymphocytes and the high

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expression of Fas receptor on CTLs, the expression of Fas-L on cancer cells is known to be markedly elevated. Alterations of the concentration of soluble forms sFas and sFasL which moderate apoptosis have been also found in NSCLC (51). Thus this receptor pathway plays an important role in the process of reduction of CTL number. To date no therapy targeting this mechanism is currently in clinical evaluation.

Another mechanism of impaired anticancer defense and hiding cancer cells from CTLs attack is a modification of co-stimulatory molecules on cancer cells and on APCs. T cells are the main cytotoxic population that recognizes target cells by interaction with APCs. B7 molecule (CD80/CD86) on the APC and the CD28 receptor on lymphocyte are necessary to activate the cytotoxic effect. However, B7 molecules are also capable of sending a suppressive signal by association with CTLA4 (Cytotoxic T cell antigen 4) (52-54). CTLA4 is a molecule capable of inhibiting the TCR signal on T cells having homology with the CD28-co-stimulatory molecule with strong affinity. CTLA4 leads to inhibition of cell cycle progression, decreased release of IL-2 and increased transforming growth factor beta (TGFβ) production by blocking CD28. By connection with forkhead box P3 (Foxp3), CTLA4 is constitutively expressed on regulatory T cells (Tregs) and promotes their regulatory function (55-57). There are two forms of CTLA-4 expression: on the cell surface after activation, and intracellularly as storage (58,59). Our study (data unpublished) showed a difference in the CTLA-4 expression on T cells deriving from peripheral blood (PB) of lung cancer patients and healthy subjects. The CTLA-4 surface expression in cancer patients was significantly higher, while the intracellular domain was decreased in PB of cancer patients compared to healthy subjects. Our results indicate the importance of cellular traffic of this molecule in malignancy.

Therapeutic approach I

PD-1 and CTLA-4 are considered the main checkpoint molecules for effective immunotherapy in solid tumors. PD-1 antagonists are presented by PD-1 or PD-L1 antibodies: nivolumab, lambrolizumab and pidilizumab. The results of recently ongoing trials with anti-PD-1 antibodies are promising, although the association with detection of PD-L1 on tumor cells before treatment is controversial (39,60).

The anti CTLA-4 IgG1 humanized antibody—ipilimumab binds to CTLA-4 and prevents the inhibition of CD28/B7 signaling. It leads to T cell activation and depletion of Tregs. Similarly to anti-PD-1 agents, the anti-CTLA-4 antibody has shown some benefits, particularly in combination with chemotherapy (48).

Recent studies confirm the importance of regulatory cells in the modification of immune response in malignancy. Regulatory T lymphocytes (Treg) are capable of inhibiting the function of CD4+ and CD8+ lymphocytes, dendritic cells and NK cells (55,61,62). Treg cells play an important role in the immune surveillance and tolerance. The source of natural Tregs (nTregs) is the thymus. The second source is a population activated peripherally (induced Tregs, iTregs). The suppressing cytokines: interleukin-10 (IL-10) and TGFβ are involved in the peripheral activation of Tregs (63). In the lung cancer milieu the concentrations of IL-10 and TGFβ is high, and these cytokines are secreted by cancer cells and immune cells stimulated by cancer (64). They constitute an active regulation of immune response by cancer through induction of Tregs. Tregs are identified by expression of the panel of antigens: a Foxp3, CD25, glucocorticoid-induced TNF-receptor (GITR) (CD357), *lymphocyte-activation gene 3* (LAG3), cytotoxic T lymphocyte antigen-4 (CTLA4) and CD127. The Tregs are defined by expression of CD4, CD25, Foxp3 and low CD127 (65,66). Foxp3 is a transcription factor necessary to keep a proper Treg function. An increased expression of Foxp3 was found in the cancer cells and in TILs and the presence of Foxp3 in breast cancer as well as in lung cancer was a negative prognostic factor (65,67-71).

In addition to type Th1 and Th2 cells, the concurrent polarisation direction of T cells is Th17 differentiation. It is not so pronounced as Tregs, but regarded as significant in regulation of immune response in malignancy. These pluripotent cells are active in antimicrobial defense, albeit their proliferative and cytotoxic effect is low. Th17 cells are defined by production of IL-17A. Other cytokines play a role in Th17 differentiation, i.e., IL-6, IL-1β and IL-23. It is presumed that IL-6 inhibits Tregs development with stimulation of Th17 (72,73). This example of the plasticity of immune system is accomplished by known TGFβ function: TGFβ in low concentration induces Th17 differentiation, while in high concentrations induces Tregs Foxp3+ maturation (73). To our knowledge, there is no direct data on the anticancer effect of Th17. Until now some results indicate that the effect of Th17 is complex as the IL-17 action in cancer milieu is pleiotropic: suppressive and stimulating. The stimulating effect is related to proangiogenic role of IL17A (74-76).

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Therapeutic approach II

The depletion of Tregs by anti-CD25 antibody was proven to be ineffective (77). More rational is putting efforts to change the polarisation of T cell by enzymatic and cytokine profile modification to achieve a re-polarization of Tregs to Th profile. Complex engineering by using the indoleamine 2,3-dioxygenase (IDO) inhibitor plus vaccine provided such a re-polarization (77).

Alveolar macrophages play an important role in lung cancer defense (35). In the solid tumors a population of TAM was widely investigated and their relation with cancer cells is complex. Generally, the function of TAM population is impaired, but their regulatory function in lung cancer immunity is postulated (78). Traditionally, macrophages were considered to be a uniform cell population, but recently have been divided to different phenotypes: M1, M2 and macrophages with regulatory properties (79-81). M1 macrophages as effector cells play an immunostimulating role by secretion of cytokines (IL-12 among others) and reveal phagocytic properties. M2 macrophages with their suppressive function are the main constituents of TAM population, promoting angiogenesis and wound healing (80). They release mainly IL-10. M1 and M2 are activated by different ways: M1 by LPS and IFNγ, while M2 by IL-4, IL-10, IL-13 and TGFβ. Such different polarization of macrophages is detected by diverse phenotype, i.e., M1 cells express mainly CD40, while M2 express CD163, as we have recently confirmed by immunocytochemistry staining (82). For regulatory macrophages no defined surface antigenicity was found, therefore identification is based on cytokine production (TGFβ and IL10). Further subtyping of the M2 population has been recently proposed on the basis of the inductors and mediators balance (83). The presence of M1 in cancer milieu is favorable (84), however M2 vastly predominate among TAM. The potential shift of M1-M2 was confirmed in our experiments by immunocytochemical staining (82).

Myeloid derived suppressor cells (MDSCs) originate as bone marrow derived hematopoietic cells and precursors of immune cells other than lymphocytes. An augmentation of circulating MDSCs in serious diseases and in malignancy has been documented (85). The MDSCs identification can be done by detection of antigens: CD11b, CD14, CD33, HLADR (85). The mediators secreting by cancer cells (i.e., GM-CSF, IL-6 and IL-1) are essential to MDSCs survival in the tumor microenvironment. MDSCs are able to inhibit T cells activation and DC differentiation, and to promote Tregs. Since arginine, cysteine and nitric oxide (NO) are necessary for a proper T cell activation and memory type differentiation, MDSCs inhibit immune response by competitive use of these substrates (86). MDSCs produce a number of radical species and suppressor cytokines, and by this way favour angiogenesis, vasculogenesis and metastases (39,87,88). The process of epithelial-mesenchymal transition (EMT) plays an important role in the context of MDSCs function and inflammatory cell migration. Until now some signaling pathways of cell to cell contact, cell polarity and cell- matrix modulation have been recognized however, the process is complex (89,90).

Therapeutic approach III

Efficacy of 5, 6-dimethylxanthenone-4-acetic acid (DMXAA, Vadimezan) for activation of the antitumor properties of TAM was described in an animal model by Fridlender *et al.* (91,92). Reduction of M2 and MDSCs function may be achieved by blocking the immunosuppressive enzymes and by reversing the hypoxia status in the tumor microenvironment (35,36,93). Nitroaspirin and sindelafil were found to be effective blockers of arginase and NO synthase, enhancing an effectiveness of anticancer vaccines (77). The anti IL-10 and anti-CD40 antibodies combined with chemotherapy were associated with the change of macrophage profile (94). Some unspecific substances are also capable of inhibiting MDSCs (39).

Cancer cells release many suppressor cytokines. In this context TGFβ is the best-recognized compound. An increasing concentration of TGFβ in cancer tissue and in the cancer cells culture as well as in the cancer milieu has been reported (15). The complex TGFβ function and role in tumor progression are presented in *Figure 3*. Several interleukins reveal similar immunosuppressive effect in the lung cancer environment, including IL-10 and IL-2. The latter induces CTLA4 and mediators: vascular endothelial growth factor (VEGF), prostaglandin E2, arginase, reactive oxygen species, sFas, sFasL (39,95).

Regarding the complex role of TGFβ, it is unlikely that a use of a simple anti-TGFβ agent will be effective in cancer immunotherapy. Thus TGFβ is used only as an adjuvant in anti-cancer vaccines production and in combination with other therapies used for CTLs stimulation (39,96,97). Sometimes TGFβ promotes positive immune response and stimulates the CTLs, suggesting a pluripotent function of the cytokine (98). For example the interesting experimental study showed the different effect of TGFβ in relation to the

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	function
TGFβ	inhibition of antigen presentation
	inhibition of leukocyte migration into tumor
	inhibition of T cell proliferation and CTLs function
	supporting the maintenance of Foxp3 expression, Trges function and differentiaition
	modulation of macrophages: shift from M1 to M2
	stimulation of M2 to arinase production
	induction of activated T cells apoptosis
	modification T cell differentation: shift to Th2 profile
	inhibition of CTLs traffic in tumor environment by profibrotic function

Figure 3 Role of transforming growth factor beta (TGFβ) in lung cancer progression. CTLs, cytotoxic lymphocytes; Foxp3, forkhead box P3.

time of tumor development: injection of anti-TGFβ agent before the injection of cancer cells resulted in inhibition of the active CTLs. Thus it may indicate a positive role of TGFβ in anticancer defense in the initial, pre-clinical stage of malignant disease (96).

Tumor antigens and vaccines production

There are two well-known lung cancer antigens that are used for vaccine production (99). The Melanoma Associated Antigen (MAGE-A3), absent on normal cells, is detected on NSCLC cells in about 35-50%, the majority being of squamous histological type (100). The presence of MAGE-A3 is associated with advanced stages of cancer. Some epitopes of this antigen are well recognized by HLA-I restricted lymphocytes Tc and these properties are used for vaccines production. Membrane associated glycoprotein (MUC-1) is associated with epithelial and glandular malignant tissue and is often overexpressed on cancer cells. A high MUC-1 expression is associated with lung cancer cell migration, resistance to apoptosis, and resistance to chemotherapeutic agents (101). The superficial domain of MUC-1 depending on the status of glycosylation is highly immunogenic; it makes possible the use of MUC-1 for T cell response stimulation (102). Recently another transmembrane glycoproteine-epithelial cell adhesion molecule (EpCAM) has been widely investigated in lung cancer; it was found that the detection of circulating lung cancer cells with EpCAM/MUC-1 overexpression was associated with poor prognosis after curative surgery (103).

There are numerous new neo-antigens recognized by genome sequencing of *KRAS*, *EGFR*, and *ALK*. The antigens and proteins encoded by these genes are present on lung cancer cells. The point mutations of these antigens make them immunogenic and useful for vaccine production (44,104).

The anti-cancer vaccines have been extensively investigated since the 1990s. The idea of vaccine production is to enhance antigen presentation by educated DCs. The vaccine formulation comprises the immunogenic tumor-associated antigens formed as peptides, recombinant proteins, gangliosides or whole tumor cells, which are combined with an adjuvant prior to potentiate the immune response (105). This immunoadjuvant is a viral vector, dendritic cell or liposome formulation. The examples of vaccines used in therapeutic approach in lung cancer are presented in the *Table 1*.

The results of recently conducted trials showed that anti-lung cancer vaccines failed to meet expectations with only some benefits in a selected group of patients (106). Therefore an effective direction in studies maybe individualization of immune treatment: the detection of cancer antigen before vaccination (MAGE-A3), enumeration of cytotoxic cells (anti-MUC-1 vaccine was shown to be effective in patients with normal number of activated NK cells) or individual production of dendritic cells with control of patient immune status (107). For evaluation of immunotherapy results the new criteria beyond RECIST WHO are needed and were recently described by Wolchok *et al*. on the basis of melanoma immunomodulating treatment (108). The immune-related response criteria (irRC) were introduced and the main consideration is that immunotherapy could be continued even in the case of radiological pattern of tumor progression.

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Table 1 Summary of immunotherapy trials in lung cancer

Immunotherapy	Immune target	Composition/mechanism of action	Clinical trial/Patients NO/Stage, criteria/Intervention	Results
Ipilimumab	CTLA-4	Monoclonal antibody	(I) Phase II/204/Chemotherapy naïve advanced NSCLC/ Carboplatin–paclitaxel with either placebo, concurrent ipilimumab or phased ipilimumab to maintenance ipilimumab or placebo every 12 weeks (II) Phase III/920/IV squamous type recurrent/Carboplatin–paclitaxel with either placebo, concurrent ipilimumab or phased ipilimumab to maintenance ipilimumab or placebo every 12 weeks (III) Phase III/1100/ Chemotherapy naïve advanced disease/ Carboplatin-paclitaxel with either placebo, concurrent ipilimumab or phased ipilimumab to maintenance ipilimumab or placebo every 12 weeks	Immune related PFS better for ipilimumab + chemotherapy
BMS-936558 nivo-lumab	PD-1	Monoclonal antibody	(I) Phase I with expansion/122/After completion chemotherapy/ Dose-escalation study with nivolumab: 1, 3 or 10 mg/kg (II) Phase III/264/Squamous cell NSCLC recurrent or progressing during/ After platinum-based chemotherapy for stage IIIB/IV nivolumab *vs.* docetahexal (III) Phase III/574/Nonsquamous cell NSCLC recurrent or progressing during/ After platinum-based chemotherapy for stage IIIB/IV nivolumab *vs.* docetahexal	OR in 33% patients with squamous NSCLC
Pembrolizumab	PD-1	Monoclonal antibody	(I) Phase II/III /300/Previously treated PD-L1-positive/NSCLC, pembrolizumab *vs.* docetaxel	Pending
Pidilizumab	PD-L1	Monoclonal antibody	PD-L1-positive locally advanced or metastatic NSCLC, after platinum failure	Pending
Talactoferin	Nonspecific	Recombinant form of lactoferin	IIIB/IV combination with first line chemotherapy	RR 47%, OS—6.1 mos (better than placebo)

Table 1 (*continued*)

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Table 1 (*continued*)

Immunotherapy	Immune target	Composition/mechanism of action	Clinical trial/Patients NO/Stage, criteria/Intervention	Results
Vaccines				
TG4010	MUC1	MUC-1 antigen-specific liposomal vaccine with IL-2 gene	(I) TIME trial, phase IIB/III, randomized, placebo controlled /1000/ Treatment-naive MUC1+, stage IV NSCLC/TG4010 plus chemotherapy *vs*. placebo plus chemotherapy	Some benefit for patients with normal number of activated NK cells
BLP-25	MUC1	MUC-1 antigen-specific liposomal vaccine	(I) Phase II/171/After first line chemotherapy/Vaccine + BSC *vs*. BSC (II) Start phase III randomized/1514/Stage III NSCLC after chemoradiotherapy/Vaccine *vs*. placebo (III) Inspire phase III randomized/420/Asian patients stage III NSCLC after chemoradiotherapy/Vaccine *vs*. placebo	3-y OS 31% for BLP-25 *vs*. 17% BSC
CIMavax	EGF	Recombinant EGF	(I) Phase II/80/After first line chemother/ Cyclophosfamid + vaccine + BSC *vs*. BSC (II) Phase III/438/After first line chemother/ Cyclophosfamid + vaccine + BSC *vs*. BSC	Difference significant in patients <60 years
MAGE-A3	MAGE-A3	Recombinant MAGE-A3 + protein D *Haemophilus influenzae* + adjuvant	(I) Magrit trial, phase III /2270/Completely resected plus stage IB, II or IIIA NSCLC (cohorts with or without adjuvant chemotherapy)/ Vaccine *vs*. placebo	Some benefit, depended on gene signature
Belagenpumatu-cel Lucanix®	TGFβ2	Irradiated NSCLC cell lines transfected with a plasmid containing the *TGF*-b2 antisense transgene	(I) Stop phase III randomized/506/III, IV after platinum based chemotherapy/ BSC + vaccine *vs*. BSC + placebo	OS dose depended-better for higher dose

NO, number; NSCLC, non-small cell lung carcinoma; PFS, progression-free survival; OR, objective response; BSC, best supportive care; OS, overall survival. TGFβ, transforming growth factor beta; MAGE-A3, Melanoma Associated Antigen.

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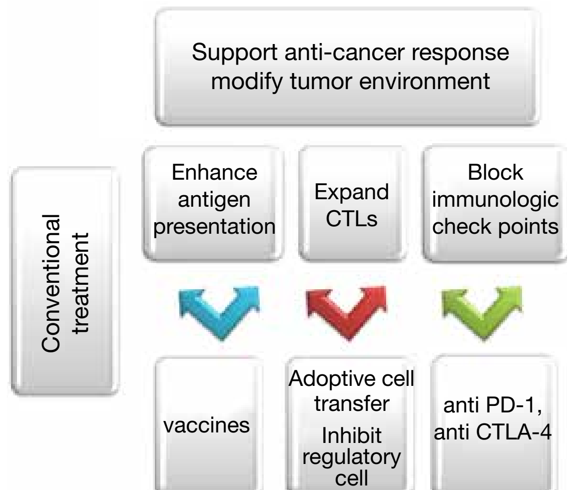

Figure 4 Trends of non-small cell lung carcinoma (NSCLC) immunotherapy in light of the current knowledge. CTLs, cytotoxic lymphocytes.

The immune response in lung cancer is complex, hence the immunotherapy should be multivalent in combination with other therapeutic options. The most current promising direction is to combine immunotherapy with a conventional chemo- and radiotherapy. The rationale for such combination is manifold: by induction of immunogenic cell stress and cell death the cytotoxic agents are capable of enhancing tumor antigenicity, likewise radiotherapy can induce antigen expression and modulate antigenic repertoire (44). The regulatory/suppressor cells (Tregs, M2, MDSCs), an actively multiplied population, seem to be more susceptible to chemotherapy than the less numerous CTLs. Some cytotoxic agents have been shown to kill myeloid suppressor cells and inhibit FoxP3 expression, leading to reduction of the number of Tregs. Radiotherapy favors the release of proinflammatory cytokines, promotes antigen cross- presentation, recruits immune cells, supports DCs migration to lymph nodes and induces death cell receptors on tumor cells (39,44). The immunomodulatory properties of targeted therapy (e.g., cetuximab, crizotinib) have also been described (44,109,110). These observations are currently applied in clinical trials (111).

Figure 4 summarizes today's goals of immunomodulating therapies in lung cancer. Almost every day delivers data on new therapeutical trials providing hopeful results in our battle against this tumor. Some limitation of the potential success of immunotherapy is due to the large number of advanced stages of NSCLC in time of the diagnosis and the fact that this kind of treatment is restricted to these stages in current clinical trials. However, the evidence of some benefit of complex treatment with immunotherapy as an additional arm with chemo- radiotherapy gives us hope for the future.

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Immune checkpoint inhibitors in clinical practice: update on management of immune-related toxicities

Jeryl Villadolid[1], Asim Amin[2]

[1]Department of Pharmacy, [2]Divisions of Immunotherapy and Medical Oncology, Carolinas HealthCare System, Levine Cancer Institute, Charlotte, NC 28204, USA

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Correspondence to: Jeryl Villadolid, PharmD, BCPS, BCOP. Department of Pharmacy, Carolinas HealthCare System, Levine Cancer Institute, 1021 Morehead Medical Drive, Charlotte, NC 28204, USA. Email: Jeryl.Villadolid@carolinashealthcare.org.

Abstract: Immune checkpoint blockade using inhibitors of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) has shown clinically significant antitumor response and has been approved for the treatment of malignant melanoma and squamous non-small cell lung cancer (NSCLC). These immunotherapies are associated with unique set of toxicities termed immune-related adverse events (irAEs) that are very different from toxicities observed with conventional cytotoxic chemotherapy. Prompt recognition and initiation of appropriate management, usually in the form of immunosuppression, usually results in complete reversibility, but failing to do so can lead to severe toxicity or even death. Clinical algorithms describing the management of common irAEs have been published based on clinical trial information and experience in metastatic melanoma with ipilimumab, a human IgG1 monoclonal antibody that binds to CTLA-4 and blocks T cell inhibition. The most common irAEs reported with ipilimumab are dermatologic toxicity, diarrhea/colitis, hepatotoxicity, and endocrinopathies, although other sites can also be affected. Similar irAEs have been observed with agents targeting PD-1. Nivolumab and pembrolizumab are humanized monoclonal antibodies that bind to PD-1 and prevent T cell inactivation. Ipilimumab, pembrolizumab, and nivolumab are approved by the Food and Drug Administration (FDA) for the treatment of advanced melanoma; nivolumab was also recently approved for metastatic squamous NSCLC. This review describes the optimal management of toxicities related to immune checkpoint inhibition from FDA-approved agents targeting CTLA-4 and PD-1.

Keywords: Immunotherapy; toxicity; checkpoint inhibition

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Background

Modulation of the immune response to elicit antitumor activity has been well established in the setting of malignant melanoma and renal cell carcinoma. High-dose interleukin-2 had been the mainstay for management of advanced disease in these clinical settings. Discovery of immune checkpoints that regulate the immune response has led to development of strategies that can be positively exploited to impact T cell activity and generate clinically relevant antitumor activity. Antibodies blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) have been approved for the treatment of advanced malignant melanoma and an anti-PD-1 antibody has been approved for squamous non-small cell lung cancer (NSCLC) (1-10).

Generation of an antitumor immune response is a complex multi-step process—recognition of the tumor antigen in the context of self-human leukocyte antigen

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(HLA) molecules by T cells constitutes the first step. Fine-tuning of the immune response then ensues and involves interactions between molecules expressed on the T cells and antigen presenting cells (APCs). CD28, a stimulatory checkpoint expressed on T cells, binds to the ligands CD80 and CD86 (B7-1 and B7-2) on APCs and results in stimulation of T cells. CTLA-4 is an inhibitory checkpoint protein that is expressed on the surface of activated T cells that also binds to the B7 family of molecules expressed on APCs and inhibits the T cells. CTLA-4 binds to the B7 molecules with a higher affinity than CD28, resulting in loss of co-stimulation through CD28 (11). Ipilimumab, an anti-CTLA-4 antibody, binds to CTLA-4 and blocks its interaction with B7 molecules, preventing T cell inactivation (1). PD-1 is also an immune inhibitory checkpoint expressed on the surface of activated T cells. Interaction between PD-1 and its ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), expressed on APCs on some normal cells and tumor cells, leads to T cell inactivation. Additionally, PD-L1 expressed on T cells can interact with the B7 family of molecules expressed on APCs and results in the T cells switching off. In contrast to CTLA-4-mediated inhibition that is a central event, PD-1-mediated inhibition can occur peripherally in the tumors, providing a potential mechanism for adaptive immune resistance (11). Anti-PD-1 antibodies, nivolumab and pembrolizumab, can bind to the PD-1 receptor, blocking its interaction with PD-L1/L2 to prevent T cell inactivation (2,3). Both checkpoint inhibitor pathways have a mechanism of action that is not limited to one tumor or tissue type (11).

While unrestrained T cell activation with immune checkpoint blockade has been shown to translate into antitumor responses, it can also manifest as toxicity in the form of autoimmune breakthrough or immune-related adverse events (irAEs) (*Table 1*). "Select adverse events" allude to toxicities that have an autoimmune etiology and require careful monitoring and specific management strategies. These toxicities have varying time to onset and include dermatologic adverse events in the form of rash and pruritus, gastrointestinal adverse events in the form of diarrhea and colitis, hepatitis, endocrinopathies, pneumonitis, and renal insufficiency (*Figures 1,2*). Specific treatment algorithms have been developed to guide the treating physician to mitigate these autoimmune toxicities. These toxicities are reversible if treated promptly and appropriately, but can lead to high-grade adverse events, including death, if unrecognized. Treatment consists of immunosuppression using corticosteroids and other agents such as tumor necrosis factor-alpha antagonists and mycophenolate mofetil, depending on severity (13-15).

The use of immune checkpoint blockade so far has been limited to a relatively small fraction of physicians involved in the treatment of malignant melanoma (16). With the proof of principle and efficacy established in this disease process, these agents are being extensively investigated in other malignancies including lung cancer, renal cell carcinoma, gastric cancer, bladder cancer, ovarian cancer, and hematologic malignancies. Early results from some of these investigations are extremely encouraging and will likely lead to more indications in addition to the approved indications for the treatment of malignant melanoma and squamous NSCLC (12). It is therefore essential that the oncology community be aware of the irAEs to recognize them in a timely fashion and be well-versed with their management. We discuss the select adverse events and their management at our institution based on the established algorithms.

Management of common irAEs

Education and communication between patients, caregivers, and the clinical team is vital for timely recognition and successful management of irAEs. The most common adverse events reported in patients receiving ipilimumab are fatigue, diarrhea, pruritus, rash and colitis (1,4,5). Adverse events in >20% of patients receiving PD-1 inhibitors include fatigue, rash, pruritus, cough, diarrhea, decreased appetite, constipation, and arthralgia (2,3,6-10). Treatment-related irAEs with PD-1 inhibitors are predominantly grade 1 or 2 in severity, and can be managed with algorithms developed for irAEs observed with ipilimumab (13-15). Prior ipilimumab exposure does not appear to impact the safety profile of currently approved PD-1 inhibitors (6,8,9). There were no drug-related deaths in a phase I study of 89 patients with advanced melanoma refractory to CTLA-4 inhibition treated with pembrolizumab; four (4.5%) patients discontinued due to immune-related or special interest adverse events, and fatigue was the only grade 3 to 4 event reported in more than one patient (6). In a phase III study of nivolumab that included patients with advanced melanoma who had previously received ipilimumab, no treatment-related deaths were observed (9).

General principles for the optimal management of irAEs include early recognition and judicious use of immunosuppression which based on the severity of the event. Clinical presentations, suggested laboratory and

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Table 1 Summary of checkpoint inhibitor immune-related adverse events (irAEs) reported in selected trials (4-6,8-10,12)

Selected trials	Selected irAEs	All grades, n (%)	Grade 3, n (%)	Grade 4, n (%)
Hodi FS *et al.* 2010 (4): ipilimumab 3 mg/kg monotherapy every 3 weeks, n=131	Dermatologic	57 (43.5)	2 (1.5)	0
	Pruritus	32 (24.4)	0	0
	Rash	25 (19.1)	1 (0.8)	0
	Vitiligo	3 (2.3)	0	0
	Gastrointestinal	38 (29.0)	10 (7.6)	0
	Diarrhea	36 (27.5)	6 (4.6)	0
	Colitis	10 (7.6)	7 (5.3)	0
	Hepatic	5 (3.8)	0	0
	Increased ALT	2 (1.5)	0	0
	Increased AST	1 (0.8)	0	0
	Hepatitis	1 (0.8)	0	0
	Endocrine	10 (7.6)	3 (2.3)	2 (1.5)
	Hypothyroidism	2 (1.5)	0	0
	Hypopituitarism	3 (2.3)	1 (0.8)	1 (0.8)
	Hypophysitis	2 (1.5)	2 (1.5)	0
	Adrenal insufficiency	2 (1.5)	0	0
	Increase thyrotropin	1 (0.8)	0	0
	Decreased corticotropin	2 (1.5)	0	1 (0.8)
Hodi FS *et al.* 2010 (4): ipilimumab 3 mg/kg plus gp 100 every 3 weeks, n=380	Dermatologic	152 (40.0)	8 (2.1)	1 (0.3)
	Pruritus	67 (17.6)	1 (0.3)	0
	Rash	67 (17.6)	5 (1.3)	0
	Vitiligo	14 (3.7)	0	0
	Gastrointestinal	122 (32.1)	20 (5.3)	2 (0.5)
	Diarrhea	115 (30.3)	14 (3.7)	0
	Colitis	20 (5.3)	11 (2.9)	1 (0.3)
	Hepatic	8 (2.1)	4 (1.1)	0
	Increased ALT	3 (0.8)	2 (0.5)	0
	Increased AST	4 (1.1)	1 (0.3)	0
	Hepatitis	2 (0.5)	1 (0.3)	0
	Endocrine	15 (3.9)	4 (1.1)	0
	Hypothyroidism	6 (1.6)	1 (0.3)	0
	Hypopituitarism	3 (0.8)	2 (0.5)	0
	Hypophysitis	2 (0.5)	2 (0.5)	0
	Adrenal insufficiency	3 (0.8)	2 (0.5)	0
	Increased thyrotropin	2 (0.5)	0	0
	Decreased corticotropin	0	0	0

Table 1 (*continued*)

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Table 1 (*continued*)

Selected trials	Selected irAEs	All grades, n (%)	Grade 3, n (%)	Grade 4, n (%)
Robert C *et al.* 2011 (5): ipilimumab 10 mg/kg plus dacarbazine 850 mg/m^2 every 3 weeks, n=247	Dermatologic			
	Pruritus	66 (26.7)	5 (2.0)	0
	Rash	55 (22.3)	3 (1.2)	0
	Gastrointestinal			
	Diarrhea	81 (32.8)	10 (4.0)	0
	Colitis	11 (4.5)	4 (1.6)	1 (0.4)
	Hepatic			
	Increased ALT	72 (29.1)	37 (15.0)	14 (5.7)
	Increased AST	66 (26.7)	34 (13.8)	9 (3.6)
	Hepatitis	4 (1.6)	3 (1.2)	0
Robert C *et al.* 2014 (6): pembrolizumab 2 mg/kg every 3 weeks, n=89	Dermatologic			
	Rash	–	0	
	Rash maculopapular	–	0	
	Gastrointestinal			
	Diarrhea	–	0	
	Hepatic			
	Hepatitis	–	1 (1.1)	
	Respiratory			
	Dyspnea	–	0	
	Pneumonitis	–	1 (1.1)	
	Endocrine			
	Increased amylase	–	1 (1.1)	
	Pancreatitis	–	0	
	General			
	Fatigue	–	5 (5.6)	
Robert C *et al.* 2014 (6): pembrolizumab 10 mg/kg every 3 weeks, n=84	Dermatologic			
	Rash	–	1 (1.2)	
	Rash maculopapular	–	1 (1.2)	
	Gastrointestinal			
	Diarrhea	–	1 (1.2)	
	Hepatic			
	Hepatitis	–	0	
	Respiratory			
	Dyspnea	–	1 (1.2)	
	Pneumonitis	–	0	
	Endocrine			
	Increased amylase	–	0	
	Pancreatitis	–	1 (1.2)	
	General			
	Fatigue	–	0	

Table 1 (*continued*)

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Table 1 (*continued*)

Selected trials	Selected irAEs	All grades, n (%)	Grade 3, n (%)	Grade 4, n (%)
Garon EB *et al.* 2015 (12): all pembrolizumab arms (2 mg/kg or 10 mg/kg every 3 weeks or 10 mg/kg every 2 weeks), n=495	Dermatologic			
	Pruritus	53 (10.7)	0	
	Rash	48 (9.7)	1 (0.2)	
	Dermatitis acneiform	13 (2.6)	0	
	Gastrointestinal			
	Diarrhea	40 (8.1)	3 (0.6)	
	Hepatic			
	Increased ALT	11 (2.2)	2 (0.4)	
	Increased AST	15 (3.0)	3 (0.6)	
	Respiratory			
	Pneumonitis	18 (3.6)	9 (1.8)[†]	
	Endocrine			
	Hypothyroidism	34 (6.9)	1 (0.2)	
	Hyperthyroidism	9 (1.8)	0	
	General			
	Fatigue	96 (19.4)	96 (19.4)	
Robert C *et al.* 2015 (8): nivolumab 3 mg/kg every 2 weeks, n=206	Dermatologic	77 (37.4)	29 (14.1)	
	Pruritus	35 (17.0)	11 (5.3)	
	Rash	31 (15.0)	6 (2.9)	
	Vitiligo	22 (10.7)	1 (0.5)	
	Gastrointestinal	35 (17.0)	3 (1.5)	
	Diarrhea	33 (16.0)	2 (1.0)	
	Colitis	2 (1.0)	1 (0.5)	
	Hepatic	7 (3.4)	3 (1.5)	
	Increased ALT	3.1 (1.5)	2 (1.0)	
	Increased AST	2 (1.0)	1 (0.5)	
	Increased bilirubin	2 (1.0)	0	
	Respiratory	3 (1.5)	0	
	Pneumonitis	3 (1.5)	0	
	Renal	4 (1.9)	1 (0.5)	
	Renal failure	2 (1.0)	0	
	Endocrine	15 (7.3)	1 (0.5)	
	Hypothyroidism	9 (4.4)	1 (0.5)	
	Hyperthyroidism	7 (3.4)	0	
	Diabetes	1 (0.5)	0	
	Hypophysitis	1 (0.5)	0	

Table 1 (*continued*)

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Table 1 (*continued*)

Selected trials	Selected irAEs	All grades, n (%)	Grade 3, n (%)	Grade 4, n (%)
Weber JS *et al.* 2015 (9): nivolumab 3 mg/kg every 2 weeks, n=268	Dermatologic	78 (29.1)	1 (0.4)	
	Pruritus	43 (16.0)	0	
	Rash	25 (9.3)	1 (0.4)	
	Rash maculopapular	14 (5.2)	0	
	Vitiligo	14 (5.2)	0	
	Dermatitis	5 (1.9)	0	
	Rash erythematous	3 (1.1)	0	
	Gastrointestinal	31 (11.6)	3 (1.1)	
	Diarrhea	30 (11.2)	1 (0.4)	
	Colitis	3 (1.1)	2 (0.7)	
	Hepatic	12 (4.5)	2 (0.7)	
	Increased ALT	7 (2.6)	2 (0.7)	
	Increased AST	11 (4.1)	1 (0.4)	
	Respiratory	6 (2.2)	0	
	Pneumonitis	5 (1.9)	0	
	Renal	4 (1.5)	1 (0.4)	
	Increased serum creatinine	2 (0.7)	0	
	Endocrine	21 (7.8)	0	
	Hypothyroidism	15 (5.6)	0	
	Hyperthyroidism	5 (1.9)	0	
	Increased TSH	3 (1.1)	0	
Rizvi NA *et al.* 2015 (10): nivolumab 3 mg/kg every 2 weeks, n=117	Dermatologic			
	Rash	13 (11.1)	1 (0.9)	
	Pruritus	7 (6.0)	1 (0.9)	
	Gastrointestinal			
	Diarrhea	12 (10.3)	12 (10.3)	
	Respiratory			
	Pneumonitis	6 (5.1)	4 (3.4)	
	Endocrine			
	Adrenal insufficiency	1 (0.9)	1 (0.9)	
	General			
	Fatigue	38 (32.5)	5 (4.3)	

†, one patient had grade 5 interstitial lung disease. ALT, alanine transaminase; AST, aspartate aminotransferase; gp, glycoprotein; TSH, thyroid stimulating hormone.

radiologic investigations, and decision considerations for continuation of immunotherapy for selected irAEs are discussed below and summarized in *Table S1*. While not directly related, it is important to note that if prolonged immunosuppression is expected, patients must also receive appropriate antibiotic prophylaxis to prevent opportunistic infections (13-15).

Dermatologic toxicity

Dermatologic toxicities, such as rash and pruritus, occur in approximately 50% of patients treated with ipilimumab. The median time to onset of moderate, severe, or life-threatening immune-mediated dermatitis in patients treated with ipilimumab in one phase III trial was 3.1 weeks and

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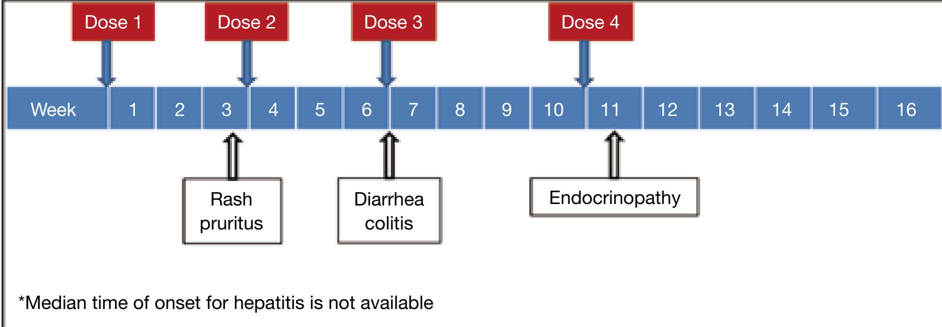

Figure 1 Median time for appearance of immune-related adverse events (irAEs) with ipilimumab based on a phase III study (4).

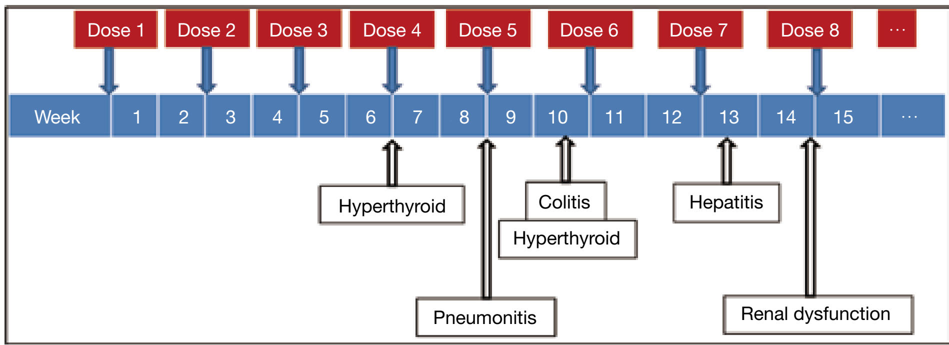

Figure 2 Median time for appearance of immune-related adverse events (irAEs) with nivolumab based on a phase III study (9).

ranged up to 17.3 weeks from treatment initiation (4). Rashes are often mild, appearing after the first or second dose. Rare cases of severe rashes such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are reported in <1% of patients (1,4,5). PD-1 inhibitor data to date shows dermatologic toxicity of all grades in up to 37.4% of patients (2,3,6-10). Workup should include a physical exam for signs and symptoms of reticular, maculopapular, and erythematous rash, usually on the trunk or extremities.

Mild to moderate (grade 1 to 2) dermatologic toxicity, defined by the Common Terminology Criteria for Adverse Events (CTCAE) as a maculopapular rash with or without symptoms (e.g., pruritus, burning, tightness) covering up to 10-30% of body surface area and limiting instrumental activities of daily living (ADL) (17), can usually be treated for symptomatic relief with topical corticosteroid ointments and does not require interruption in immune checkpoint inhibitor therapy (13-15). For grade 2 toxicity, defined in the ipilimumab Risk Evaluation and Mitigation Strategy (REMS) program as a diffuse, non-localized rash that is ≤50% of skin surface (13), it is recommended to withhold ipilimumab and treat with topical corticosteroids with consideration for systemic corticosteroids at 0.5 mg/kg/day prednisone or equivalent if there is no improvement in symptoms within 1 week (13-15). Our institutional recommendation is to continue ipilimumab or PD-1 inhibitor therapy if patients have involvement of <30% of body surface area, are asymptomatic, or toxicity can be managed with topical corticosteroid creams and antipruritics, such as hydroxyzine and diphenhydramine. For patients with 10-30% of body surface area involvement that is symptomatic, ipilimumab or PD-1 inhibitor therapy is held and consideration is given to initiation of steroids at 0.5-1 mg/kg prednisone or equivalent for control of symptoms.

Severe (grade 3 to 4) toxicity may require admission to the hospital and a formal dermatology consultation with consideration of skin biopsy for rashes that show signs of blistering, full thickness dermal ulceration, or necrotic, bullous, or hemorrhagic changes. Immune checkpoint inhibitor therapy should be permanently discontinued and systemic corticosteroids initiated at 1-2 mg/kg/day prednisone or equivalent for these patients. In the pivotal

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phase III melanoma trial, patients who received ipilimumab and developed dermatitis had complete resolution of symptoms with high-dose corticosteroids at a median dose of 60 mg/day prednisone or equivalent administered for up to 14.9 weeks followed by a taper (4). Steroids should be tapered over 1 month following improvement of symptoms to mild severity (13-15).

Vitiligo was reported to occur in both CTLA-4 and PD-1 inhibitor clinical trials. Toxicity can be permanent but does not require interruption of immune checkpoint inhibitor therapy or toxicity treatment (1-10). Oral mucositis and dry mouth are more frequently reported with PD-1 inhibitors. Oral candidiasis may be considered, especially if a patient has been on corticosteroids for the management of other irAEs (13-15).

Diarrhea/colitis

Diarrhea and colitis may present approximately 6 weeks into immune checkpoint inhibitor therapy and appears to be dose-dependent with ipilimumab (1,4,5). Diarrhea at any grade was reported in approximately 30% of 511 patients treated with ipilimumab in a phase III melanoma trial. Less than 10% of patients had severe grade 3 or 4 diarrhea, defined as ≥7 stools above baseline, fever, ileus, or peritoneal signs. Of the 511 patients, five (1%) developed intestinal perforation, four (0.8%) died as a result of complications, and 26 (5%) were hospitalized for severe enterocolitis (4). In PD-1 inhibitor trials, diarrhea and colitis were observed to be less frequent. The incidence of grade 3 or 4 immune-mediated colitis, defined as requiring use of corticosteroids with no clear alternate etiology, occurred in 1-2% of patients (2,3,6-10). Patients who had significant diarrhea/colitis during ipilimumab treatment have subsequently been treated with PD-1 inhibition without developing diarrhea/colitis (6,9).

For mild (grade 1) symptoms, defined as <4 stools above baseline per day, clinical algorithms recommend continuing immune checkpoint inhibitor therapy with symptomatic treatment, without initiation of corticosteroids (13-15). Our institutional recommendations include stool studies testing for *Clostridium difficile* infection, lactoferrin, and ova and parasites in addition to a baseline complete blood count (CBC) with differential, complete metabolic panel (CMP) and magnesium and phosphorus levels for electrolyte repletion in this group of patients. We also recommend avoiding reflex treatment with antidiarrheal agents (e.g., loperamide, diphenoxylate/atropine) that could potentially mask higher-grade toxicity. Our supportive care recommendations include adequate oral hydration, bland diet, closer monitoring, and follow-up depending on the results of stool studies, especially for patients on concomitant medications with potential to mask toxicity (e.g., opioids). Budesonide may be considered in selected cases but is not recommended as standard prophylaxis given no statistically significant difference in the incidence of diarrhea and colitis with or without budesonide in a phase II, double-blind, placebo-controlled study with patients treated with ipilimumab (18).

For moderate (grade 2) symptoms, defined as 4 to 6 stools above baseline per day, abdominal pain, or blood or mucus in stool, infection must be ruled out with a *Clostridium difficile* test, ova and parasites, and a stool culture. Ipilimumab REMS toxicity management recommends withholding immune checkpoint inhibitor therapy and initiating systemic corticosteroids at 0.5 mg/kg/day prednisone or equivalent if symptoms persist for >1 week (13). Our institutional recommendations are in line with the REMS recommendation to hold immune checkpoint inhibitor therapy, but we prefer not to use reflex steroids for stable patients until results from the stool studies are available. We also prefer endoscopic evaluation with flexible sigmoidoscopy to prove autoimmune colitis if symptoms persist >1 week, prior to initiating steroids.

For severe (grade 3 or 4) toxicity, defined as ≥7 stools above baseline per day, peritoneal signs consistent with bowel perforation, ileus or fever, immune checkpoint inhibitor therapy should be permanently discontinued. Ipilimumab REMS toxicity management recommends initiation of systemic corticosteroids at 1-2 mg/kg/day prednisone or equivalent once bowel perforation is ruled out (13). Our institutional recommendations are to admit these patients for observation and intravenous hydration, obtain stool studies, and defer initiation of steroids if the patient is clinically stable until stool studies are available (usually 24 hours). Gastroenterology evaluation with flexible sigmoidoscopy is preferred prior to committing these patients to high-dose steroids. Clinically unstable patients are initiated on high-dose steroids immediately at the time of admission; our preference is methylprednisolone 125 mg intravenously every day for 3 days to evaluate response to steroids, followed by a slow prednisone taper starting at 1-2 mg/kg over at least 1 month. In phase III ipilimumab clinical trials, patients with grade 3 to 5 enterocolitis were treated with high-dose corticosteroids with a median duration of treatment of 2.3 weeks for up to 13.9 weeks (4,5). The duration of high-dose corticosteroid treatment for patients receiving nivolumab and pembrolizumab in

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clinical trials has ranged from 7 days to up to 2.4 months with complete resolution of symptoms in a majority of the patients (2,3,6-10). If there is no improvement in symptoms after 5-7 days of high-dose steroids, our institutional recommendations require consideration for infliximab at a dose of 5 mg/kg in keeping with standard REMS management after ruling out bowel perforation or sepsis (13). Infliximab may be repeated 2 weeks after the first dose if high-grade symptoms persist despite continuing steroids. Mycophenolate mofetil may need to be considered for selected patients. Empiric antibiotics should be considered for patients who present with fever or leukocytosis; prophylactic antibiotics should be administered to patients on long-term immune suppression. Rare cases resulting in bowel perforation may require colostomy (13-15).

Hepatotoxicity

Both CTLA-4 and PD-1 inhibitors can cause autoimmune hepatotoxicity that manifests as increased transaminases and total bilirubin, usually with a median onset approximately 8-12 weeks after initiation of treatment. The incidence of grade 2 hepatotoxicity was 2.5% and grade 3-5 events was 2% in a phase III ipilimumab clinical trial (4). The incidence of immune-mediated hepatitis, defined as a requirement for corticosteroids and no clear alternate etiology, was <5% in PD-1 inhibitor clinical trials (2,3,6-10). In a phase III trial with 268 advanced melanoma patients treated with nivolumab, grade 2 to 3 hepatitis occurred in three (1.1%) patients. Liver function tests returned to grade 1 within 4-15 days of initiation of corticosteroids, however hepatitis did recur in two of the three patients (9). Of the 411 patients treated with pembrolizumab in a clinical trial database, hepatitis occurred in 0.5% of patients with complete resolution following administration of corticosteroids (2,6,7).

Hepatic function should be monitored prior to each dose of ipilimumab, nivolumab or pembrolizumab (1-3,13-15). If an increasing trend in liver function tests is noted, evaluation should be carried out to rule out other infectious, non-infectious, and malignant causes such as progression of disease. We recommend laboratory testing for antinuclear antibodies (ANA), smooth muscle antibody (SMA), CBC with differential, CMP, direct and indirect bilirubin, and gamma-glutamyl transferase (GGT). If hepatotoxicity is suspected, the frequency of liver function test monitoring should increase to every 3 days. Computed tomography (CT) scans and liver biopsy may be considered depending on severity.

For grade 2 hepatotoxicity, defined as an aspartate aminotransferase (AST) or alanine transaminase (ALT) >2.5 times but ≤5 times the upper limit of normal (ULN) or total bilirubin >1.5 times but ≤3 times ULN, further therapy should be held and corticosteroids initiated at 0.5-1 mg/kg/day prednisone or equivalent and continued until improvement in toxicity to grade 0 or 1. Steroids should be tapered over 1 month and immunotherapy may resume (13-15).

For grade ≥3 hepatotoxicity, defined as AST or ALT >5 times ULN or total bilirubin >3 times ULN, immunotherapy should be discontinued permanently, liver function tests should be monitored daily, and a hepatology or gastroenterology consultation and liver biopsy should be considered. Patients should be hospitalized for an AST or ALT >8 times ULN and receive methylprednisolone 125 mg intravenously daily. Additional immunosuppression with mycophenolate mofetil 500 mg orally every 12 hours may need to be initiated if no response is elicited after 3-5 days of steroid therapy. Patients receiving mycophenolate mofetil should also receive appropriate antibacterial and antiviral prophylaxis. Other hepatotoxins such as alcohol or acetaminophen should be avoided (13-15).

Endocrinopathies

Immune checkpoint inhibition can cause autoimmune breakthrough events in the form of endocrinopathies. The incidence of endocrinopathy is reported in <10% of patients treated with CTLA-4 and PD-1 inhibitors in clinical trials (1-10). Given that the presentation for endocrinopathy can be insidious, the true incidence may be underreported due to non-specific symptoms that may mimic other causes such as brain metastasis, sepsis, or progression of disease. Infectious and non-infectious causes should be ruled out in suspected cases; unless an alternate etiology is identified, signs or symptoms of endocrinopathies should be considered immune-mediated.

The most common endocrinopathies reported with immune checkpoint inhibitor therapy are hypophysitis and hypothyroidism (1-10). It is recommended to check thyroid function prior to treatment with immune checkpoint inhibitors. Additional laboratory testing for cortisol, adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), prolactin, and testosterone is indicated for suspected immune-mediated endocrinopathy. Radiographic pituitary gland imaging may be warranted with magnetic resonance imaging (MRI) of the brain with special attention

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to the pituitary gland.

Hypophysitis can present as fatigue, headaches, and visual field defects. Diagnosis is based on levels of pituitary hormones [ACTH, thyroid stimulating hormone (TSH), FSH, LH, GH and prolactin] and radiographic imaging showing an enlarged pituitary, with or without necrosis (12-14). Hypophysitis was reported in 0.5% (2/411) of patients in the initial pembrolizumab clinical trial database with a time to onset of up to 1.7 months, similar to the incidence and onset of hypophysitis reported in ipilimumab clinical trials (2,6,7). The ipilimumab REMS toxicity management recommendation is to withhold immune checkpoint inhibitor therapy and initiate high-dose corticosteroids at 1 mg/kg prednisone or equivalent daily for grade ≥2 toxicity (13). Our institutional recommendation is methylprednisolone 125 mg intravenously daily or dexamethasone 6 mg every 6 hours intravenously for 3 days with a switch to oral prednisone 1-2 mg/kg daily after improvement of symptoms. We prefer a formal endocrinology consultation and follow-up for longitudinal hormone replacement and monitoring. Immune checkpoint inhibitor therapy should be permanently discontinued for severe or life-threatening grade 3 or 4 toxicity (13-15). For patients with existing hypophysitis due to ipilimumab, pembrolizumab may be administered if patients are stable on physiologic hormone replacement therapy (6)

Hypothyroidism was reported in approximately 2% of patients treated with ipilimumab and up to 8.3% of patients with treated with PD-1 inhibitors; the time to onset ranged from 0.7 weeks to 19 months in PD-1 inhibitor trials (2,3,6-10). Hypothyroidism is diagnosed if TSH level is increased with a low free T4 level, whereas hypophysitis presents with a low TSH and low free T4. Immune checkpoint inhibitor therapy may be continued without interruption with appropriate levothyroxine replacement (13-15).

The incidence of primary hyperthyroidism has been lower than hypothyroidism for both CTLA-4 and PD-1inhibition (1-10). In a phase III nivolumab trial with 268 advanced melanoma patients, grade 1 or 2 hypothyroidism and hyperthyroidism occurred in 8% and 3% of patients, respectively, with a time to onset of thyroid dysfunction ranging from 24 days to 11.7 months from initiation of therapy (9). If TSH is decreased, we recommend observation and close monitoring with continuation of immune checkpoint inhibitor therapy. Hyperthyroidism may represent acute thyroiditis secondary to immune activation for which a short period of high-dose steroids (1 mg/kg prednisone or equivalent) may need to be considered for symptomatic patients. Most patients subsequently become hypothyroid and need long-term hormone replacement (13-15).

Other endocrinopathies include severe or life-threatening adrenal insufficiency (usually secondary to hypopituitarism), characterized by hypotension, dehydration, hyponatremia, and hyperkalemia that may mimic sepsis syndrome. The incidence of severe or life-threatening hypopituitarism is reported in <2% of patients treatment with immune checkpoint inhibitor therapy (1-10). Adrenal insufficiency requires immediate hospitalization and management with intravenous corticosteroids after sepsis is ruled out. Corticosteroids should be initiated at 60-80 mg prednisone daily or equivalent and tapered over 1 month. Long-term steroid replacement with hydrocortisone is usually required. If primary or secondary hypoadrenalism is suspected, ACTH and cortisol levels need to be checked and endocrinology consultation considered for interrogation of the pituitary-adrenal axis. Repeat laboratory testing in 1-3 weeks and/or imaging in 1 month should be considered for follow-up of all patients treated for suspected endocrinopathies (13-15).

Management of less frequent irAEs

Other organ systems can be affected after treatment with immune checkpoint inhibitors. Although the incidence of events in other organ systems is low, and the management is in the form of immunosuppression with steroids, some of these events merit mention.

Pneumonitis

Immune-mediated lung injury that manifests as pneumonitis can occur with both CTLA-4 and PD1-inhibitors (1-10). As with other irAEs, the clinical presentation can be deceptive and non-specific, therefore complaints of new cough or dyspnea in patients treated with these agents warrants evaluation with pulmonary function tests and radiographic imaging (e.g., CT scan). Bronchoscopy may be considered to rule out other etiologies including infections prior to treatment with corticosteroids. The overall incidence of grade 3-4 pneumonitis observed with nivolumab and pembrolizumab is <1% (2,3,6-10). In a clinical trial database of 411 patients treated with pembrolizumab, the median time to onset of pneumonitis was 5 months. While most cases can be managed effectively using high-dose corticosteroids with a

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slow taper, fatal events have been reported (2,6,7).

For grade 2 pulmonary symptoms requiring medical intervention or limiting instrumental ADLs, admission to the hospital and pulmonary consultation is warranted. Our institutional recommendations are in keeping with REMS algorithms with initiation of methylprednisolone 1 mg/kg/day intravenously or oral equivalent until improvement to mild severity with a taper over 1 month following treatment (13-15).

For grade 3 or 4 pulmonary symptoms that are severe or life-threatening, including new or worsening hypoxia, limiting self-care ADL, oxygen requirements, and respiratory compromise requiring urgent intervention, immunotherapy should be permanently discontinued and methylprednisolone 2-4 mg/kg/day intravenously should be administered until improvement to mild severity. Steroids should be tapered over at least 6 weeks in this setting with consideration of additional immunosuppressive therapy if symptoms persist after 48 hours, worsen, or recur on steroid taper (13-15).

Asymptomatic elevation of amylase and lipase

Elevation of amylase and lipase has been observed with both CTLA-4 and PD-1 inhibitors (1-10). The phenomenon of asymptomatic increase in amylase and lipase without overt pancreatitis has especially been described with nivolumab and pembrolizumab and does not require holding therapy; grade 3-4 toxicities that are symptomatic require treatment to be held (1-10). New onset diabetes with diabetic ketoacidosis and pancreatic insufficiency has been documented and may warrant endocrinology/gastroenterology consultation as indicated (13-15).

Renal insufficiency

CTLA-4 and PD-1 inhibitors have been associated with renal insufficiency (1-10). Nephritis has been reported in <1% of patients in a pembrolizumab clinical trial database, with one case of grade 2 autoimmune nephritis, and two cases of interstitial nephritis with renal failure confirmed by biopsy (one grade 3 and one grade 4). The onset of nephritis was 11.6 months after the initiation of treatment. All patients recovered with high-dose corticosteroids at a dose of ≥40 mg/day prednisone or equivalent followed by a taper (2,6,7).

The incidence of renal dysfunction with nivolumab is reported to be <1%; elevated creatinine was reported in up to 22% of patients. Grade 2 or 3 immune-mediated nephritis or renal dysfunction occurred in 0.7% (2/268) of patients treated with nivolumab in a phase III study (9). It is important to monitor patients for elevated serum creatinine prior to and periodically during treatment. Management of renal irAEs at our institution is as per clinical algorithms for PD-1-associated renal adverse events in published trials. For grade 1 toxicity, defined as an increased creatinine up to 1.5 times above baseline, creatinine should be monitored at least once a week without interruption of immunotherapy. If serum creatinine worsens to grade 2 or 3, defined as a creatinine above 1.5 times baseline up to 6 times ULN, creatinine should be monitored at least every 2-3 days, immunotherapy should be withheld, and methylprednisolone 0.5-1 mg/kg/day intravenously or equivalent should be initiated until resolution of symptoms to grade 1 or below, followed by a taper over 1 month. Grade 4 toxicity, defined as life-threatening symptoms or creatinine >6 times ULN, warrants daily monitoring of creatinine, permanent discontinuation of therapy, consideration for nephrology consultation and biopsy, and high-dose corticosteroids with methylprednisolone 1-2 mg/kg/day intravenously or equivalent with a taper over at least 1 month (8-10).

Ophthalmologic disorders

Ophthalmologic disorders such as episcleritis, conjunctivitis, and uveitis occur in <1% of patients treated with ipilimumab and may be treated with topical corticosteroids; more severe events require ophthalmologic evaluation and systemic steroids (1,4,5).

Rare irAEs

Rare disorders reported in ≤1% of patients include red cell aplasia, thrombocytopenia, hemophilia A, Guillain-Barre syndrome, myasthenia gravis, posterior reversible encephalopathy syndrome, aseptic meningitis, and transverse myelitis (1-3).

Discussion

Immune checkpoint inhibitors, ipilimumab, pembrolizumab and nivolumab, have shown significant clinical benefit in several malignancies and are already approved for advanced melanoma and squamous NSCLC, marking the advent of immune-oncology. Based on their mechanism of action, these agents can exert toxicities that are unlike conventional cytotoxic chemotherapy. Since immune checkpoint inhibitors are not selective to tumor or tissue type, there is a

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substantial effort underway to explore their efficacy and role in the management of malignancies other than currently approved indications. Preliminary data is encouraging and there are several other immune checkpoint inhibitors in development with impressive clinical activity that are likely to be approved. It is therefore important that the oncology community acclimate to the nuances of immune-oncology therapeutic modalities that may potentially gain acceptance for the treatment of several malignancies.

Although the irAEs profiles of the three approved agents may differ slightly, they share the clinical presentation of symptoms and general principles guiding their management. It is extremely important to make the distinction that immunotherapy is not chemotherapy; the irAEs observed with immunotherapy have a completely different underlying mechanism compared to toxicity observed with chemotherapy. The irAEs can present in an insidious and unpredictable fashion, therefore the clinical team, as well as the patient, have to be educated and aware of the potential toxicities so that they are reported early, generate appropriate level of suspicion and prompt investigation. If identified early, the irAEs are almost always reversible with the initiation of immunosuppression. If they go unrecognized, these events can lead to significant morbidity, organ dysfunction, and even death. As opposed to cytotoxic chemotherapy, the tenet of 'more is better' does not necessarily fit the bill for immunotherapy. The idea is to 'take the brakes off' the immune effector T cells and fine-tune the balance between increased antitumor activity and autoimmunity. While some patients may not incur any toxicity and experience a response, others may have irAEs and not respond. The appearance of irAEs is indicative of the immune status and if no antitumor response is elicited at that heightened level of activation, then 'taking further brakes off' by continuing therapy is unlikely to translate into more benefit justifying the rationale to discontinue. The current recommendations for the management of irAEs are presented and summarized in *Table S1*.

Several questions remain unanswered with the current level of insight into immune checkpoint inhibitors and will likely get resolved as clinical experience evolves with more widespread use in off protocol and clinical trial settings. Based on the mechanism of action, previous clinical trials typically excluded patients with underlying autoimmune disorders (e.g., Crohn's disease, ulcerative colitis, rheumatoid arthritis). It is not clear if the presence of an existing autoimmune disorder constitutes an absolute contraindication. Similarly, it is not known how or if patients on chronic immunosuppression will respond. There is paucity of data regarding the safety and efficacy of these agents in patients with severe organ dysfunction (e.g., patients requiring dialysis for renal insufficiency) since they were also excluded from early studies.

Studies to identify biomarkers and other factors predictive of response and resistance to immunotherapy, and combination trials of immunotherapy with chemotherapy, targeted therapy, or multiple immune-modulators are underway to further define the role of this treatment modality for cancer. Early studies suggest that combination therapy with dual immune checkpoint inhibition (CTLA-4 plus PD-1) may increase efficacy, but at the cost of increased toxicity (19). The addition of granulocyte-macrophage colony-stimulation factor (GM-CSF) to CTLA-4 inhibition has been shown to prolong survival with fewer irAEs in an early study for the treatment of melanoma, however, this needs to be validated (20). Correlation between the expression of PD-L1 on tumors and response to PD-1 inhibitors has not been confirmed and remains an area of active investigation.

Discovery of immune checkpoint inhibitors has afforded an unprecedented opportunity for the development of effective treatment options for some malignancies. It is expected that these agents will be incorporated in the management of other tumor types. It is therefore imperative that the clinical teams including physicians, first responders, nurses, pharmacists as well as the patients become familiar with the irAEs and their management.

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None.

Footnote

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Advances in molecular and immunologic targeted therapies for squamous cell carcinoma of the lung

Michael J. Leukam[1], **Victoria M. Villaflor**[2]

[1]Department of Medicine, University of Chicago, Chicago, USA; [2]Department of Internal Medicine, Section of Hematology/Oncology, University of Chicago, Chicago USA

Contributions: (I) Conception and design: All authors; (II) Manuscript writing: All authors; (III) Final approval of manuscript: All authors.

Correspondence to: Victoria M. Villaflor. Department of Medicine, Section of Hematology/Oncology, University of Chicago, 5841 S. Maryland Ave. MC2113, Chicago, IL 60637, USA. Email: vvillafl@medicine.bsd.uchicago.edu.

Abstract: While targeted therapy has revolutionized the treatment of some forms of non-small cell lung cancer (NSCLC) particularly adenocarcinoma of the lung, the standard treatments for advanced stage squamous cell carcinoma (SqCC) has remained platinum-doublet chemotherapy, and the prognosis remains poor. There remains a great need for breakthroughs in treatments for this common and deadly disease. Recently, a new understanding of the molecular and genetic makeup of SqCC, thanks to large-scale genetic and molecular assays, has resulted in a number of targeted therapies entering clinical investigation for use in SqCC. Treatments targeting common mutations in SqCC have been studied in patient populations not preselected for mutations or overexpression and have had some early success. For example, the vascular endothelial growth factor receptor (VEFGR) inhibitor ramucirumab has been approved by the United States Food and Drug Administration (FDA) for use in certain settings for patients not selected for genetic mutations with NSCLC, including SqCC. Other agents are being investigated in selected populations with demonstrated genetic mutations or amplifications in the targeted pathway based on preclinical and early clinical data suggesting enhanced benefit in those groups. Early results in targeted immunotherapy have been particularly successful in SqCC compared to other histologic subtypes of NSCLC, and the programmed cell death 1 (PD-1) immune checkpoint inhibitor nivolumab has now been approved for second-line therapy in SqCC by the FDA. In this review, several oncogenic signaling pathways will be examined. The recent preclinical and clinical literature establishing those pathways as potential treatment targets, as well as ongoing clinical studies focused on those pathways, will be discussed. Recent and ongoing studies in targeted immune checkpoint inhibition and vaccine immunotherapy will also be reviewed.

Keywords: Immunotherapy; molecular targeted therapy; non-small cell lung cancer (NSCLC); squamous cell carcinoma (SqCC)

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Introduction

Despite advances in risk reduction (primarily smoking cessation), surgical, chemotherapeutic and radiation treatments, lung cancer remains the largest cause of cancer mortality in the US by far (1). Lung cancer is typically divided by histology into small call (15% of diagnoses) and non-small cell (85% of diagnoses) types, the latter comprising of adenocarcinoma, squamous cell carcinoma (SqCC) and large cell carcinoma (2). From the 1950s until the 1980s, SqCC was the most common non-small cell lung cancer (NSCLC) and lung cancer overall (3). In the 1980s, the relative incidence of adenocarcinoma overtook the incidence of SqCC and now remains the most common subtype of lung cancer (4,5). However, the absolute incidence of SqCC has

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been rising in women in recent years, and remains a common malignancy in both men and women (5).

Approximately 60% of all new diagnoses of NSCLC are advanced stage III or stage IV disease and mostly are considered unresectable. Patients with stage IV disease will typically undergo palliative chemotherapy and/or radiotherapy as the only treatment options (6). Until recently, all histologic subtypes of NSCLC were treated similarly with platinum-containing doublets with early data suggesting no differences in progression-free survival (PFS) and overall survival (OS) based on choice of regimen (7). More recent data supports treating histologic subtypes differently, with improvement in OS and PFS in SqCC using platinum and gemcitabine and inferior outcomes in SqCC compared to non-squamous histologies using platinum and pemetrexed (8). A number of specific mutations with approved targeted therapies in adenocarcinoma have further divided the treatment of NSCLC subtypes, including epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) for patients with known mutations in EGRF (9,10), and targeted TKI therapy for anaplastic large-cell lymphoma kinase (ALK) rearrangements (11). Currently there are few such targeted agents for SqCC of the lung, and platinum-containing doublet chemotherapy remains the standard of care.

The current lack of targeted molecular therapies for SqCC may not be long lived. Several whole-genome characterization studies of SqCC of the lung have identified potential actionable targets which differ from those identified in other histologic subtypes. Compared to adenocarcinoma, EGFR and Kirsten rat sarcoma viral oncogene homolog (KRAS) are much less common in SqCC of the lung (12-14). More frequent mutations or amplifications in SqCC are seen in tumor protein 53 (TP53), fibroblast growth factor receptors (FGFR), cyclin-dependent kinase (CDK), the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, and inactivating mutations of human leukocyte antigen (HLA-A) (13,15). Many common mutations in SqCC of the lung are shared with other squamous-type malignancies, suggesting that treatments developed for squamous malignancies of other sites may have activity in SqCC of the lung (12). Additional immunohistochemical and targeted genetic expression studies have contributed to a growing understanding of the unique molecular landscape of SqCC of the lung—the state of which is reviewed herein—with the hope that breakthroughs in targeted therapies will improve the dismal outcomes in this common disease.

Targeted therapy

Epidermal growth factor receptor (EGFR)

EGFR is a cell-surface receptor tyrosine kinase (RTK) involved primarily with regulation of cell proliferation, as well as differentiation and apoptosis. Constitutively activating mutations of EGFR are associated with the development of malignancies, including NSCLC (16,17), though multiple studies have not identified similar EGFR mutations in the SqCC subtype specifically (14,18). The greatest effects of EGFR-targeted TKI therapy in NSCLC have been seen in patients with identified mutations, and EGFR inhibition with TKIs has now become a mainstay of treatment in lung adenocarcinoma in a population defined by identified EGFR mutations (19). Given the lack of such a population in SqCC, studies of small molecule inhibition with TKIs and targeted monoclonal antibody inhibition of EGFR have proceeded in non-selected populations only.

TKIs targeting EGFR that have a proven record of success in selected EGFR mutant adenocarcinoma patients have demonstrated a small benefit in non-selected SqCC populations. A phase III trial of erlotinib as a post-chemotherapeutic maintenance agent in advanced NSCLC showed a significant effect on PFS in EGFR wild-type patients regardless of histologic subtype (20). A recent meta-analysis of 8 randomized controlled of EGFR TKIs in non-selected patients with metastatic SqCC demonstrated a modest but significant benefit in OS and PFS (21).

Monoclonal antibodies specifically disrupting EGFR signaling have also been investigated in SqCC. A recent phase III trial in SqCC comparing cisplatin and gemcitabine alone to the same chemotherapeutic agents with the addition of necitumumab (an anti-EGFR antibody) as first-line therapy found a small but significant improvement in OS (22). Another phase III trial comparing first-line platinum-based chemotherapy to the same chemotherapy plus cetuximab (an anti-EGFR antibody) in NSCLC regardless of mutation status found a marginal benefit in the SqCC subgroup, but not in the other histologic subtypes (23). Similar benefits were not seen in a similar phase III trial in stage IV in non-squamous NSCLC treated with necitumumab along with platinum-based doublet as first-line therapy regardless of mutation status (24). Taken together, these data suggest that the marginal benefit of first-line EGFR inhibition in NSCLC with wild type EGFR is restricted to the SqCC subtype.

Active trials for EGFR inhibition in SqCC include a phase II trial of avelimumab (a monoclonal antibody

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targeting EGFR) in locally advanced or metastatic solid tumors including SqCC of the lung (Clinicaltrials.gov identifier: NCT01772004), as well as a phase II trial of chemotherapy and radiation therapy with or without panitumumab (a monoclonal antibody targeting EGFR) in stage IIIA NSCLC including SqCC (Clinicaltrials.gov identifier: CT00979212).

The overall benefit of EGFR inhibition with both TKIs and monoclonal antibodies in SqCC patients without an identified EGFR mutation is modest compared to the responses seen in EGFR mutation positive adenocarcinoma. There is moderate evidence in the form of the combined weight of multiple supporting studies that EGFR inhibition may be beneficial is some patients with SqCC and is currently a part of treatment options for SqCC in non-EGFR-mutant populations. A better understanding of non-genomic alterations in protein expression unique to SqCC (such as overexpression of wild-type EGFR) may identify the mechanism of the observed benefit, and suggest additional treatment targets not able to be identified through current genetic and molecular screening.

Fibroblast growth factor receptor (FGFR)

FGFRs are a large family of highly conserved receptors for polypeptide growth factors, with 22 members identified in humans. Four (FGFR1-4) are tyrosine kinase receptors, which act upstream from the ras/mitogen-activated protein kinase (MAPK) and PI3K/protein kinase B (AKT) pathways known to be involved in regulation of proliferation in lung cancers (25,26).

Circulating fibroblast growth factors have found to be elevated in multiple types of lung cancer, including SqCC (27). Multiple studies have additionally identified abnormalities in FGFR protein expression or genetic amplifications of FGFR in SqCC. A 2006 study of SqCC cell lines found a significant correlation between EGFR and FGFR3 overexpression (28). In a 2010 study, FGFR1 gene amplification was found in 22% of SqCC samples, and *in vitro*, knockdown of FGFR1 was associated in restriction of cell growth and increased apoptosis in the FGFR1-amplified cell lines only (29). Subsequent studies of FGFR1, summarized in a recent meta-analysis find *FGFR* gene amplifications in 19% of SqCC tumors overall, but do not find a prognostic value for such FGFR1 amplifications in OS or PFS (30).

Despite the presence of a subpopulation of SqCC tumors with known genetic amplifications in FGFR, there are several ongoing trials investigating FGFR inhibition as a treatment mechanism in NSCLC in populations not pre-selected for FGFR amplification. Pazopanib (a multi-targeted TKI whose targets include FGFR1-4) is currently under investigation in non-selected NSCLC patients (including SqCC). An ongoing phase II/III trial is studying pazopanib as maintenance therapy in advanced disease (Clinicaltrials.gov identifier: NCT01208064). A phase II trial is studying pazopanib in combination with erlotinib as second or third line treatment in advanced disease (Clinicaltrials.gov identifier: NCT01027598), and a third phase II trial is investigating pazopanib as a first line therapy in combination with paclitexal for advanced disease (Clinicaltrials.gov identifier NCT01179269). Nintedanib (another multi-targeted TKI initially developed for ILD and NSCLC) is currently being investigated in a phase I/II trial evaluating effectiveness as first line therapy with standard chemotherapy specifically in patients with SqCC regardless of FRFR amplification status (Clinicaltrials.gov identifier NCT01346540). If successes are seen in studies of non-selected populations, subgroup analysis may reveal if the FGFR-amplification-positive group is disproportionately responding, or, as in the EGFR inhibition studies, there is a benefit in FGFR inhibition even the absence of identified amplifications.

Early clinical data is available from studies in groups selected for FGFR amplification. Two studies were completed with pan-FGFR inhibitor experimental medications AZD4547 and BGJ398 in patients with SqCC that had documented amplification of FGFR1. In a phase Ib study of AZD4547 as second-line therapy, an effect was seen (ORR 8%), but the efficacy rate for continuation of the study was not met (31). BGJ398 was studied as second-line therapy in a phase I trial and demonstrated an ORR of 16% (32). Further studies of BGJ398 are planned. Though the early data demonstrate mixed success, given the relatively high frequency of FGFR gene amplifications in SqCC, a successful targeted therapy could represent a major treatment breakthrough. Ongoing studies of FGFR inhibition in FGFR1 amplification positive squamous tumors include a phase II trial of ponatinib—a multi-targeted TKI with activity against FGFR—as second-line therapy in both squamous head and neck cancers and SqCC of the lung with documented FGFR kinase alterations (Clinicaltrials.gov identifier NCT01761747).

Phosphoinositide 3-kinase (PI3K)

The PI3K/AKT signaling pathway is downstream of

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many cell-surface RTKs and is involved with regulation of cell survival, proliferation and metabolism, among other processes. Mutations in this pathway were commonly identified in large-scale genetic screens of NSCLC (nearly half of specimens analyzed), and are particularly associated with the squamous subtype, with a higher frequency of mutations than in adenocarcinoma (14,15,33,34). Specifically, mutations in PI3CA were found in approximately 10% of SqCC in a large genomic analysis, with amplification being the most common alteration (making up approximately 40% of mutations found) (34,35).

Rational therapeutic design focused on this pathway is supported by the finding that inactivation of downstream targets of the PI3K/AKT pathway in mouse models leads to the development of SqCC of the lung (36). In addition, mouse models with SqCC harboring PI3CA mutations have shown response to targeted therapy with PI3K inhibition in preclinical studies (37,38). Based on these early data, trials in human subjects have been launched. Buparlisib (also known as BKM120) is a small-molecule PI3K inhibitor being studied in pretreated metastatic SqCC and nonsquamous patients in a comparative two-stage phase II trial (Clinicaltrials.gov identifier: NCT01297491), and LY3023414, a small molecule inhibitor of PI3K and a downstream target of PI3K [mammalian target of rapamycin (mTor)] is being studied in a phase I trial in patients with advanced malignancies including NSCLC (Clinicaltrials.gov identifier NCT01655225). Effectiveness data in human subjects research has not yet been reported.

Vascular endothelial growth factor (VEGF)

One of the hallmarks of cancer is the ability of a tumor to develop its own blood supply to support further growth (39). Important mediators of angiogenesis include vascular endothelial growth factor and its receptor (VEGF and VEGFR). Bevacizumab is a monoclonal antibody against VEGF that is approved for use in several malignancies, including non-squamous NSCLC, but has been associated with increased hemoptysis in SqCC, and its use is currently contraindicated in that histologic subtype (40-42). Ramucirumab is another monoclonal anti-VGEF antibody, but it has not been associated with the same bleeding risk. In a phase III study of docetaxel with or without ramucirumab for second-line therapy in NSCLC (including SqCC), marginal improvements in OS (10.5 *vs.* 9.1 months) and in PFS (4.5 *vs.* 3.0 months) were seen in patients who received ramucirumab (43). Based on these data, ramucirumab with docetaxel is now FDA approved for second-line therapy for NSCLC, including SqCC subtypes (44).

Ongoing clinical trials involving small molecule inhibitors include a phase III study of vandetanib (an inhibitor of both EGFR and VEGFR) with docetaxel as second-line therapy for NSCLC including SqCC not selected for VEGF or VEGFR mutations (Clinicaltrials.gov identifier: NCT00312377) and a phase II study of lucitanib (an inhibitor of FGFR 1-3, VEGFR 1-3, and PDGFR α/β) as second-line monotherapy in patients with advanced/metastatic disease which has a known amplification or activating mutation in FGFR1, FGFR2, FGFR3, VEGFA, or PDGFRα (Clinicaltrials.gov identifier NCT02109016).

Discoidin domain receptor (DDR)

Discoidin domain receptors 1 and 2 (DDR1 and DDR2) are cell-surface protein RTKs that bind to type I collagen and interact with downstream signaling targets that regulate cell proliferation and survival, including PI3K (45). Mutations in DDR2 were identified in approximately 4% of SqCC samples in one study, and activation of DDR1 was noted in a large survey of oncogenic kinase signaling in NSCSLC (46).

Preclinical studies using xenograft mouse models with tumors made up of SqCC cells containing gain of function mutations in DDR2 demonstrated a strong response to desatinib, a TKI with multiple targets including DDR1 and DDR2 (47). However, both a phase II trial studying desatinib as first line therapy in advanced NSCLC (not selected to DDR2 mutations) and a phase I/II trial in advanced NSCLC (not selected for DDR2 mutation) with a combination of erlotinib and desatinib failed to demonstrate a significant clinical benefit (48,49). Another phase II trial using desatinib in advanced SqCC, with plans to correlate response rates to DDR2 mutation status, was halted early due to excess toxicity (50). Finally, a phase II trial studying desatinib in patients with SqCC and known DDR2 mutations was halted due to slow accrual (Clinicaltrials.gov identifier NCT01514864). While patients with DDR2-mutant SqCC may yet be shown to benefit from inhibition of the DDR2 pathway, the low numbers of DDR2 mutations in the population may make the effect difficult to study. Studies with access to large populations will likely be required to amass enough DDR-mutation positive patients to be adequately powered.

Cyclins and CDKs

Cell division is a highly regulated process that includes several checkpoints (notably the G1-S checkpoint between

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cell growth and DNA replication), which are tightly regulated in part by interactions between checkpoint inhibitors (e.g., retinoblastoma and P53), and checkpoint activators such as complexed cyclins and CDKs (51,52). In a large genetic screening study of NSCLC, cyclins, CDKs, and their regulatory pathways were found to harbor mutations, specifically CCND1 (amplified in 13% of cases), CDK6 (amplified in 4%), and the gene for p16 (which inhibits CDK4 and CDK6), which was mutated or deleted in 45% of tumors (35). CDKN2A and CCND1 are found to be enriched specifically in SqCC, and in one screening study, a subpopulation of tumors containing both a high level of cyclin pathway mutations and a low level of PI3K mutations was identified, suggesting that there are tumors in which cell-cycle directed therapy might be particularly effective (12,15).

Preclinical data gathered involving cell-cycle regulation include the development of two different inhibitors of CDK4/6 (LY2835219 and PD 0332991) that are active against xenograft tumors formed from human cancer cell lines in mice (53,54). Clinical data gathered to date is limited. A phase I study of flavopiridol (a pan-selective CDK inhibitor) in combination with standard chemotherapy as first line therapy was done in 12 patients with NSCLC, in which the drug was well tolerated, and partial responses were seen in 8 patients (55). Further studies will be required to assess the effectiveness of cell cycle targeted therapies in both non-selected and in known CDKN2A or CCND1 mutation positive populations.

Mesenchymal epithelial transition factor and hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) and its RTK mesenchymal epithelial transition factor (c-MET) normally function upstream of multiple pathways involved in proliferation, angiogenesis, survival and migration, and is normally active in adults in times of tissue injury and repair (56). MET receptor amplification has been identified in up to 40% of lung cancer tissues, and both elevation in detectable levels of HGF and overexpression of c-MET are associated with a poor prognosis in NSCLC (57-59). Overexpression of HGF/c-MET has also been linked specifically with progression in NSCLC (60).

Preclinical data supported inhibition of the HGF/c-MET pathway with rilotumumab, an anti-HGF antibody that blocks interaction with the c-MET receptor; in mice with allograft tumors, rilotumumab enhanced the efficacy of both docetaxel and temozolamide (61). Rilotumumab was well tolerated in a phase I study in patients with a variety of solid tumors (62).

Antibodies against c-MET have also been studied in NSCLC populations (including SqCC), such as onartuzumab (anti c-MET monoclonal antibody). In a recent phase II trial of recurrent NSCLC patients regardless of MET expression level status, the intention to treat group demonstrated no PFS or OS advantage, but the subgroup with tumors that overexpressed MET showed an advantage in both PFS and OS, while the subgroup without MET expression showed a decreased OS compared to placebo (63). Based on these results, a phase III trial was begun in NSCLC patients with advanced disease (including squamous histology) whose tumors overexpress MET by immunohistochemistry comparing erlotinib alone to erlotinib with onartuzumab (64). Surprisingly, given the promising phase II data, the METLung phase III trial was halted due to futility given lack of difference in response and progression free survival with the addition of onartuzumab to erlotinib at planned interim analysis (65).

Ongoing studies of c-MET inhibition in NSCLC include a phase II study of an experimental c-MET inhibitor capmatinib as second-line therapy in advanced NSCLC (including SqCC) not selected for c-MET expression level (Clinicaltrials.gov identifier NCT02414139), and a phase I study of experimental c-MET inhibitor PF-02341066 in NSCLC (including SqCC) patients with identified c-MET amplification, proto-oncogene tyrosine-protein kinase ROS (ROS1) mutation or anaplastic lymphoma kinase (ALK) rearrangements (PROFILE 1001, Clinicaltrials.gov identifier NCT00585195)

Immunotherapy

Programmed death receptor and ligands

One mechanism of immune suppression in SqCC is suggested by the relatively high levels of expression of programmed death receptor ligands (PDL) 1 and 2 in SqCC of the lung, which are expressed at levels significantly higher than adenocarcinoma (66,67). Data are mixed on the prognostic significance of elevated PDL1 in NSCLC. A recent meta-analysis found overall decreased OS with increased PDL1 expression (68), though a single study found increased OS in early stage disease only (69). The significance of elevated PDL1 expression is illuminated by its function as part of an immune checkpoint. Evasion of

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immune surveillance or suppression of immune response is considered to be a hallmark of cancer (39), allowing abnormal cells to proliferate without a response from cytotoxic defense mechanisms. When PDL1 and PDL2 bind to the programmed death receptor (PD1) on cytotoxic CD8+ T-cells, activation of PD1 causes anergy and prevents the secretion of pro-inflammatory cytokines (70). PD1 activation on CD4+ T-cells, in part, drives a transformation into immune-suppressing T-regulatory cells (71). These functions normally serve to dampen inappropriate immune responses, but in the case of SqCC, may assist in evasion of the appropriate immune response.

Disrupting the PD1/PDL1 interaction is believed to allow for removal of the immune inhibition of the surrounding T cells, increasing immune anti-tumor activity. Promising results were first seen in hematologic malignancies (pidilizumab, anti-PD1 antibody), followed by melanoma (pembrolizumab, anti-PD1 antibody) (71). Trials of PD1/PDL-1 inhibition have been promising in NSCLC, and the SqCC subtype seems uniquely sensitive to these inhibitors. Early results from a phase I/II clinical trial of MEDI4736, (anti-PDL1 antibody), demonstrated an overall response rate of 21% in SqCC compared to 10% in adenocarcinoma (72). Early results from an ongoing phase I trial of another anti PDL1 antibody (MPDL3280A) evaluated response rates of NSCLC with intensity of pre-treatment infiltrating lymphocyte PDL1 expression and found higher expression correlated with a higher likelihood of response (73). These data suggest it may be possible to identify those patients most likely to benefit from PD1/PDL1 checkpoint inhibition prior to initiating treatment (74).

Clinical trials investigating the effectiveness of nivolumab (an antibody against PD1) in SqCC have demonstrated significant early successes. The Checkmate 017 trial was a phase II study that investigated nivolumab as a salvage therapy in heavily pretreated patients, demonstrating an ORR of 15%, an OS of 8.2 months and a 1-year survival of 41% (75). Nivolumab was also compared to docetaxel in advanced or metastatic in Checkmate 063, a phase III study that was halted early after meeting its primary endpoint of significantly improved OS (9.2 *vs.* 6.0 months) (76). Due to these results, the Food and Drug Administration (FDA) has approved nivolumab in the treatment of SqCC with progression on or after standard chemotherapy (77).

The many ongoing studies of PDL1/PD1 inhibition in NSCLC include a phase III trial of pembrolizumab, an antibody against PD1, versus placebo with or without standard adjuvant chemotherapy for resected stage IB-IIIA NSCLC, including SqCC (Clinicaltrials.gov identifier: NCT02504372) and a phase II trial of nivolumab as second-line therapy specifically in advanced-stage SqCC (Checkmate 171, Clinicaltrials.gov identifier: NCT02409368). A phase III trial of nivolumab as first-line therapy for NSCLC compared to platinum-doublet chemotherapy is now recruiting, and will include an arm specific to SqCC (Checkmate 227, Clinicaltrials.gov identifier: NCT02477826). A large, multi-arm phase I study of nivolumab in advanced NSCLC as monotherapy or in combination with either cytotoxic chemotherapy or with small molecule inhibitors such as bevacizumab and erlotinib is currently underway, and will include separate arms for squamous and non-squamous histologic subtypes (Checkmate 012, Clinicaltrials.gov identifier NCT01454102). Several other early phase trials are underway investigating nivolumab in NSCLC in the maintenance (Clinicaltrials.gov identifier: NCT02434081) and neoadjuvant (Clinicaltrials.gov identifier: NCT02259621) settings, and in combination with c-MET inhibitors (Clinicaltrials.gov identifier: NCT02323126).

Cytotoxic lymphocyte antigen

Cytotoxic T lymphocyte antigen-4 (CTLA4) is expressed on the surface of cytotoxic T-cells, and forms part of a different immune checkpoint by competing with the T-cell costimulatory molecule for their shared ligands CD80 or CD86 (78). T-cell CTLA4 expression is higher in patients with NSCLC, and higher yet in metastatic disease, though the mechanism is unknown (79). Higher levels of expression are found in SqCC compared to adenocarcinoma, and within the patient population with SqCC, higher CTLA4 levels are associated with decreased survival (67).

CTLA4 inhibition is being studied in a range of cancers based on a similar rationale as PD1/PDL1, namely that blocking an immune checkpoint will allow for increased antitumor immune activity. Ipilimumab is an anti-CTLA4 antibody that was studied in a phase II trial of first line therapy of chemotherapy with and without ipilimumab, finding a small but statistically significant improvement in PFS, which was greater in SqCC than in non-squamous subtypes (80). Based on these results, a phase III trial is underway focusing specifically on ipilimumab in SqCC (Clinicaltrials.gov identifier: NCT01285609).

Vaccines

The use of vaccines directed towards malignant cells has

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long been area of active investigation in cancer treatment, with some successes in melanoma and prostate cancer. One early target for vaccine therapy was melanoma-associated antigen A3 (MAGE-A3), a tumor antigen not expressed on noncancer cells but found on approximately 30% of NSCLC tumors. Unfortunately, a large phase III trial of a MAGE-A3 vaccine failed to meet its primary endpoint of increased DFS in NSCLC patients and further investigations of the vaccine in NSCLC are not planned at this time (81). Another potential vaccine target that was considered was mucin-1 gycoprotein (MUC1) which is overexpressed and abnormally glycosylated in NSCLC cells (82). However, in a phase III trial of the anti MUC1 vaccine tecemotide as maintenance therapy after chemoradiation for NSCLC, no difference was found compared to placebo (83).

While tumor associated antigen vaccines for NSCLC have not yet shown hopeful results, there have been some mixed data for whole cell vaccines. Belagenpumatucel-L is a whole-cell vaccine made up of NSCLC cell lines (adenocarcinoma, SqCC, and large cell carcinoma) that were transfected with an antisense plasmid for transforming growth factor beta-2 (TGFβ2) (84). TGFβ2 is a cytokine that suppresses immune cytotoxic function and enhances the development of immune-suppressing T-regulatory cells (85,86). Preclinical studies supported the effectiveness of TGFβ2 antisense oligonucleotides in suppressing or reversing multiple tumor types in animal models (87-89). Despite hopeful results in phase I and II trials, a phase III trial with belagenpumatucel-L in patients with stage IIIB and IV NSCLC did not meet its primary endpoint of improved OS (90,91). However, subgroup analysis found that patients randomized within 12 weeks of completion of chemotherapy had significantly improved OS, particularly noted in patients randomized within 12 weeks with non-adenocarcinoma histology (OS of 19.9 months with belagenpumatucel-L *vs*. 12.3 month with placebo) (91). Based on these subgroup analyses and the overall safety profile, the FDA has supported continued study of belagenpumatucel-L (92).

Lung-MAP

Based on the early data for several molecular targeted therapies and immunotherapies in SqCC of the lung as outlined above, a large, multi-arm phase II/III trial has been developed by the Southwest Oncology Group called Lung-MAP (Clinicaltrials.gov identifier NCT02154490) which will investigate several targeted therapies as second-line therapies simultaneously. In this ambitious study, patients with recurrent stage IIIB/IV SqCC will be tested for a variety of biomarkers and assigned to a targeted arm based on the mutation or amplification their tumor harbors. If none of the study targets are identified in the sample, the patient will be assigned to an immunotherapy arm comparing anti-B7H1 monoclonal antibody MEDI4736 with docetaxel to docetaxel alone. Patients with tumors positive for PI3KCA mutations will be assigned to an arm comparing PI3 kinase inhibitor GDC-0032 with docetaxel to docetaxel alone. Patients with tumors positive for CDK4/6, CCND1, CCND2, and CCND3 will be assigned to an arm comparing palbociclib (a selective small molecule inhibitor of CDK4/6) with docetaxel to docetaxel alone. Patients with tumors positive for FGFR1, FGFR2, and FGFR3 will be assigned to an arm comparing FGFR inhibitor AZD4547 with docetaxel to docetaxel alone. Finally, patients with tumors overexpressing HGF/c-MET will be assigned to an arm investigating anti-HGF antibody rilotumumab in combination with erlotinib versus erlotinib alone. Primary outcome measures are OS and PFS.

Conclusions

Compared to the growing options for targeted therapy in adenocarcinoma, SqCC of the lung continues to rely largely on standard platinum based chemotherapy. The notion of treating SqCC differently than other histologic subtypes has recently been advanced with data supporting superiority of a platinum-based regimen containing gemcitabine compared to other subtypes (8). Many new molecular targets for therapy have been suggested by large-scale genome and phosphorylation studies of SqCC that have identified a molecular fingerprint that is unique among the family of NSCLC.

Some of the molecularly targeted therapies under investigation for SqCC have demonstrated small clinical success when applied to non-selected populations. For example, marginal benefits in OS with the VEGF-inhibitor ramucirumab were demonstrated in a population not selected by genetic mutation or overexpression. These modest benefits were enough for FDA approval for ramucirumab with docetaxel as a second-line therapy for all NSCLC, including SqCC. Additionally, multiple early phase trials of EGFR therapy (both monoclonal antibodies and TKIs) have shown small, but significant, clinical responses in a non-selected population of SqCC, despite

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the rarity of EGFR mutations this histologic subtype. The broad effect of these targeted therapies may reflect a subpopulation that has yet to be identified with dependence on a specific oncogene in the targeted pathway, or may reflect a general principle SqCC proliferation using wild-type signaling pathways. Careful subgroup analysis in clinical studies and advances in the basic molecular science of SqCC may help clarify which patients may realize the greatest benefit for VEGF- and EGFR-targeted therapies. Other targeted therapies have shown their greatest benefit in preclinical and early clinical studies in populations with known amplifications or mutations in the targeted pathway (FGFR, PI3K, DDR2, CDK4/6, and HGF/c-MET). None have yet demonstrated clinical success in a defined subpopulation, but there are many ongoing trials investigating various small molecule inhibitors and monoclonal blocking antibodies. Several targeted therapies are being investigated simultaneously in SqCC subgroups defined by an activating mutation in the phase II/III Lung-MAP trial.

The most exciting recent data has been in the realm of immunotherapy. While vaccine therapy for SqCC has not been proven effective in several phase III trials, encouraging results have been seen in studies of targeted immune checkpoint inhibitors. Both PD1/PDL1 and CTLA4 inhibition have shown greater clinical response rates in SqCC as compared to other histologic NSCLC subtypes, and have demonstrated a favorable safety profile in early phase studies. Based on the clinical response rate and the occurrence of some unusually durable responses in a phase III trial, the targeted PD1 immune checkpoint inhibitor nivolumab was approved as second-line therapy for NSCLC, specifically SqCC (77). Many studies of immune checkpoint inhibition as single-agent and combination therapy in various roles (including first-line therapy) are now underway, and have the potential to rapidly alter the treatment landscape for SqCC.

Even in the midst of a flowering of research in targeted therapies for SqCC, much remains to be learned about the biology and treatment of this difficult disease. Large-scale genomic studies have provided many possible targets for treatment, though the relative importance of each identified mutation to tumorigenesis and the usefulness of each as a treatment target remain largely unknown. Preclinical studies and clinical trials are still working through the many targets identified in screening studies, though high profile failures in targeted therapies such as c-MET inhibition—despite biological plausibility and encouraging early clinical data—suggest that much remains unknown about the signaling interactions upon which SqCC depends to grow and spread. As the science of molecular biology advances alongside clinical medicine, a new generation of basic studies of genetic expression and protein signaling interactions in SqCC over time may be necessary to develop enough treatment targets to control and eventually defeat this dreaded disease.

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Footnote

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Immune checkpoint blockade for lung cancer: state of the art

Sandip Pravin Patel

Division of Hematology and Oncology and Center for Personalized Cancer Therapy, UC San Diego Moores Cancer Center, San Diego, California 92093-0987, USA

Correspondence to: Sandip Pravin Patel. 3855 Health Sciences Drive #0987, La Jolla, CA 92093-0987, USA. Email: sandippatel@ucsd.edu.

Abstract: As the leading cause of cancer-related death worldwide, lung cancer had historically lacked the therapeutic successes seen in other diseases. However, with the advent of targeted therapeutic strategies in non-small cell lung cancer (NSCLC) addressing molecular perturbations in *EGFR*, *ALK*, *ROS1*—and with recent development of second and third-generation inhibitors—there is renewed optimism in lung cancer research for this subgroup of patients. Regrettably, for the vast majority of NSCLC patients without these targetable driver aberrations, the traditional therapeutic backbone has been chemotherapy. Fortunately, an improved understanding of the tumor immune microenvironment and tumor mutanome-related neoantigen generation has revolutionized lung cancer with the promise of durable remissions in the setting of advanced disease. Immune checkpoint blockade targeting T cell inhibitory checkpoints such as CTLA-4, programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1) have shown impressive clinical responses as monotherapy in a subset of patients. The promise of biomarker-driven, personalized immunotherapeutic approaches based on each patient's unique tumor immune microenvironment and mutanome represents a quantum leap in lung cancer therapy. Reviewed here are the recent advances in lung cancer immunotherapy, with a focus on immune checkpoint of inhibitor biomarker development and clinical efficacy data.

Keywords: Lung cancer; non-small cell lung cancer (NSCLC); immunotherapy; immune checkpoint blockade; programmed cell death protein 1 (PD-1); programmed death-ligand 1 (PD-L1)

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Introduction

Molecularly-guided therapy has revolutionized lung cancer with dramatic responses in patients with aberrations in *EGFR*, *ALK*, and *ROS1* (1-3). However, the majority of patients with lung cancer (~70%), lack these targetable driver mutations and have historically been treated with cytotoxic chemotherapy (4). Despite lacking a targetable driver mutation, the majority of patients with lung cancer have an overall higher mutational burden more akin to melanoma, which presents an opportunity to unleash the host immune system against tumor neoantigens (5,6). With an increasing appreciation of the role the adaptive immune system plays in lung cancer, the development of therapies to target maladapted immunological pathways such as CTLA-4 and programmed cell death protein 1 (PD-1; CD279)/ programmed death-ligand 1 (PD-L1; CD274) have ushered in a new era for these patients (7-9).

The central role the immune system plays in cancer has been known for over a century. In the 1890s, Dr. William Coley described a post-operative patient with sarcoma with residual disease at resection, who achieved a complete remission following two severe bacterial skin infections (10). One of the presumed biologic factors in that patient's response is interleukin-2 (IL-2), which has since become a therapy with the potential of durable remissions in a minority (6-10%) of patients with advanced melanoma and renal cell cancer (RCC) (11). Further refinements in immunomodulation would require the discovery of immune checkpoints and their role in facilitating tumor escape, leading to the clinical development of novel immunotherapeutics targeting the cytotoxic T-lymphocyte-

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associated protein 4 (CTLA-4, CD152) and PD-1/PD-L1 immune checkpoints (8,9,12,13). To the surprise of many in the oncology community, early phase trials of immune checkpoint blockade demonstrated efficacy not only in the cancer histologies traditionally thought to be "immunogenic" (melanoma and RCC), but in non-small cell lung cancer (NSCLC) as well (7-9). Reviewed here are recent clinical developments in the field of immunotherapy in NSCLC with a focus on the published clinical efficacy of immune checkpoint blockade, predictive biomarkers for efficacy, and potential future directions utilizing immunotherapeutic combinations.

Immunobiology in NSCLC

Immune checkpoints, such as CTLA-4 and PD-1, are present on activated T cells as a means of immune homeostasis and to minimize the risk of incidental autoimmune attack due to persistent activation of T cells (12). The analogy of T cell activation as akin to starting an automobile is often used. The cognate T cell-antigen presenting cell interaction represents a "key and ignition" paired interaction where only a specific key can activate a particular automobile (Signal 1). Activating (accelerator) and inhibitory (brake) receptors on T cells modulate the strength of the response (Signal 2) based on interactions with the immune microenvironment. Inhibitory checkpoints CTLA-4, PD-1, and PD-L1/PD-L2, among others (13). Overexpression of inhibitory checkpoint ligands by tumors and recruitment of immunosuppressive cells into the tumor microenvironment modifies the tumor microenvironment towards an immunosuppressive state that favors tumor growth, a process termed "immunoediting" (14). Immunoediting is a maladaptive interaction between the host immune system and tumor, which results in the host immune system selecting for a less immunogenic tumor over time, and conversely, a tumor selecting for a less immunologically adept host immune microenvironment.

CTLA-4 is a centrally-acting inhibitory T cell checkpoint which acts on T cells residing in lymphoid organs (15). In contrast, PD-1 is an inhibitory T cell immune checkpoint involved in the peripheral effector phase of T-cell activation, leading to immune tolerance of cells that express PD-L1 and PD-L2 (16). Accordingly, PD-1 knockout mice have a milder autoimmune phenotype relative to CTLA-4 knockout mice (15,17). This finding parallels the clinical severity of observed toxicities to immune checkpoint blockade, which are more pronounced with anti-CTLA-4 therapy relative to anti-PD-1 therapy (18).

PD-L1 is expressed on a variety of somatic cells as well as on B cells, T cells, dendritic cells, macrophages, and mast cells (19). T-cell mediated cytolysis via interferon-gamma release leads to adaptive up-regulation of PD-L1 whereby normal mucosal cells create an immunologic exclusion zone to protect against autoimmune attack in the setting of chronic inflammation. Tumor cells co-opt this immune homeostatic mechanism, design to protect normal mucosa, and express PD-L1 to avoid immunologic surveillance to facilitate cancer growth. While a myriad of PD-L1 immunohistochemistry (IHC) assays with a variety of cutoff thresholds complicate analyses, generally 40-60% of archival NSCLC tumor specimens will have PD-L1 expression (20). Correlative analysis of tumor specimens from multiple clinical trials, utilizing different anti-PD-L1 antibodies with different thresholds of positivity, has generally shown that anywhere from 25-50% of NSCLC specimens are considered PD-L1 positive. The attractive underlying immunobiology of NSCLC has shown merit in clinical practice, with durable responses in select patients with metastatic NSCLC (21-23).

Clinical efficacy

Ipilimumab

Anti-CTLA-4 blockade with ipilimumab in combination with chemotherapy in first-line treatment of metastatic NSCLC provided some of the earliest evidence of the efficacy of immune checkpoint blockade (7). Patients received six cycles of carboplatin/paclitaxel chemotherapy. Those patients who received phased ipilimumab (4 cycles of ipilimumab administered starting with cycle 3 of chemotherapy) had improved progression-free survival (PFS) (5.1 *vs.* 4.1 and 4.2 months, respectively) compared to those patients receiving chemotherapy alone or 4 cycles of ipilimumab starting with cycle 1 of chemotherapy. Immune-related grade 3-4 toxicities, predominantly related to colitis, were seen in 15-20% of patients treated with ipilimumab. Overall, given the modest survival improvement and toxicity with ipilimumab-based therapy in NSCLC, alternative therapeutic strategies would require exploration.

Nivolumab

A phase 1 trial of nivolumab (Bristol-Myers Squibb; BMS-

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936558, ONO-4538), an anti-PD-1 antibody, demonstrated an 18% response rate in 122 patients with NSCLC (9). Of note was the durability of response, in which the majority of responding patients had response duration greater than 6 months (8/14 responding patients), with some responses lasting longer than 1 year (5/14 patients). Additionally, durable stable disease lasting greater than 6 months was observed in 7% of patients on this study. Nivolumab was well-tolerated overall, with grade 3/4 adverse event (AE) rate in 6% of patients in this phase 1 trial.

Published concurrently, a phase 1 clinical trial of BMS-936559, an anti-PD-L1 antibody, resulted in a 10.2% response rate in 75 patients with NSCLC (8). All responding patients sustained their response to at least 6 months, with an additional 8% of patients achieving stabilization of disease lasting greater than 6 months. BMS-936559 was well tolerated with a grade 3/4 toxicity rate of 5%. Given the impressive tolerability and durability of response with monotherapy in select patients with refractory NSCLC, further investigation of nivolumab, in particular, was pursued.

Nivolumab in squamous NSCLC

A multinational, single-arm, phase 2 trial of nivolumab in 117 patients with refractory squamous cell lung cancer (CheckMate 063) demonstrated similarly impressive activity (23). In this study, 15% of patients had an objective response to nivolumab, with a median duration of response of at least 6 months. Time to response (TTR) was 3.3 months, consistent with delayed responses observed in earlier clinical trials. An additional 26% of patients had durable stable disease with a median duration of 6 months. Therapy was generally well tolerated, with 17% grade 3/4 toxicity. Of note, 3% of patients on this study developed immune-related pneumonitis, generally managed with corticosteroids with resolution in 3-4 weeks. However, 4 of 6 patients who developed pneumonitis discontinued therapy permanently, and one patient may have had immune-related pneumonitis as a contributor to death while on study. The presence of durable responses in patients with refractory squamous NSCLC in this study led to a randomized control trial of nivolumab *vs.* docetaxel in this setting (CheckMate 017) (24). In this study, 272 patients were randomized with a primary endpoint of overall survival (OS). Patients treated with nivolumab had a median OS of 9.2 *vs.* 6 months with docetaxel. In the nivolumab cohort, 42% of patients were alive at 1-year *vs.* 24% in the docetaxel arm. The response rate was 20% in patients treated with nivolumab *vs.* 9% with docetaxel (P=0.008). The time to initial response was 2.2 months, and the median duration of response was not reached for the nivolumab group, with 63% of responders with ongoing response. Nivolumab was well tolerated with a 7% grade 3/4 AE rate (no grade 3/4 pneumonitis) with no on-treatment deaths. CheckMate 063 and 017 were the basis for the FDA-approval of nivolumab on March 4, 2015 for refractory squamous NSCLC in patients who had progressed on platinum-based therapy.

Nivolumab in nonsquamous NSCLC

A phase 1/2 trial evaluated nivolumab in 129 patients with refractory NSCLC in both squamous and nonsquamous subtypes (22). The objective response rate (ORR) was similar across histologic subtypes: 17.1% for all NSCLC, 16.7% for squamous NSCLC, and 17.6% for nonsquamous NSCLC across all doses. The OS rate at 3 years in treated patients was an unprecedented 27% in this highly refractory population—54.3% of patients had received three or more prior therapies—with ongoing responses. Of note, patients with *EGFR* mutations (n=12) had similar benefit (ORR =16.7%) relative to the general study population (ORR =17.1%) and patients with *KRAS* mutations (n=21; ORR =14.3%). Nivolumab was generally well-tolerated with a grade 3/4 AE rate of 4.7%, which consisted predominantly of pneumonitis. Twelve patients had immune-related pneumonitis—grade 1/2 in eight patients, and grade 3/4 in three patients (2.3%)—and one patient had fatal pneumonitis, occurring outside the date of formal safety analysis. Overall, this trial demonstrated the impressive durability of response to anti-PD-1 therapy, even in heavily pretreated patients.

Pembrolizumab in NSCLC

Pembrolizumab (Merck, formerly lambrolizumab or MK-3475), an anti-PD-1 antibody, was studied in a large phase 1 trial (KEYNOTE-001) with 495 NSCLC patients (21). All patients received pembrolizumab at either 2 or 10 mg/kg every 3 weeks. The ORR was 19.4% with a median duration of response of 12.5 months. For patients with PD-L1 expression on tumor cells >50%, which represented 23.2% of the study population, the ORR was 45.2%. Responses were durable with 84.4% of patients with sustained response at time of analysis and a median duration of response of 12.5 months in all patients. Patients with PD-L1 >50% had

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a median PFS of 6.3 *vs*. 3.7 months for all patients. Therapy was generally well tolerated with grade 3-5 events reported in 9.5% of patients. Pneumonitis was observed in 1.8% of patients (n=9), with one death. There were no differences in efficacy or AEs between dose levels.

Atezolizumab in NSCLC

Atezolizumab (Roche/Genentech; MPDL3280A) is an anti-PD-L1 antibody that was studied in a phase 1 trial across multiple histologies (25). In this trial, 53 patients with NSCLC (n=41 non-squamous; n=11 squamous) were treated with atezolizumab with an ORR of 23% (21% non-squamous; 27% squamous). PFS at 6 weeks was 45% (44% non-squamous, 46% squamous). PD-L1 expression on tumor infiltrating-immune cells (IC) was associated with response (P=0.015) in NSCLC as well as across tumor types (P=0.007), and had improved performance as a predictive biomarker relative to tumor PD-L1 expression. PD-L1 staining intensity was associated with response in NSCLC, with 83% of patients with the highest PD-L1 immune cell IHC score of 3 (IC3) having a response to therapy. In contrast, patients with IC2 levels of PD-L1 expression had a lower ORR with 43% limited to disease stabilization. Similarly, PFS at 6 months was associated with level of PD-L1 expression on IC. While 83% of NSCLC patients with IC3 levels of PD-L1 expression achieved a 6-month PFS endpoint, only 14.3% of patients with IC2 and 25.6% patients with IC1 reached this endpoint. Of note, no cases of grade 3-5 pneumonitis were observed in this study across histologies.

Summary of clinical experience

Immune checkpoint blockade, particularly with anti-PD-1/anti-PD-L1 directed therapy, has demonstrated impressive activity in select patients with refractory NSCLC. While ORR may range broadly from 16% to 83% based on patient characteristics including PD-L1 expression, the additional presence of durable stable disease associated with impressive survival endpoints (for example, 27% 3-year OS in refractory NSCLC) has set a new standard for NSCLC therapy (*Table 1*) (22,24,25). Immune-related pneumonitis is of particular concern with anti-PD-1 therapy, appearing in approximately 2% of patients. Increased vigilance and early intervention with steroid therapy may improve the outcome of patients with pneumonitis, akin to improvements made in management of immune-related colitis from ipilimumab-based therapy in melanoma (18,26).

There are several unique clinical features of immune checkpoint blockade that are of note, particularly in juxtaposition to cytotoxic chemotherapy and targeted therapy. While responses are durable, radiographic responses can be delayed with TTR ranging from 2-6 months, depending on the study. Limited data suggest that patients with squamous histology (TTR 2-4 months) may achieve a response more rapidly than patients with nonsquamous (TTR 4-6 months) tumors, however further investigation into the molecular mechanisms behind this potential phenomenon (for example, neoantigen burden) will be required (22,24).

Furthermore, some patients may have unconventional immune-related responses (“pseudoprogression”) with initial radiographic progression followed by potential durable stable disease or response (27). However, pseudoprogression is generally rare in NSCLC patients treated with anti-PD-1 directed therapy (3-5% of patients), and patients with clear clinical progression (declining performance status, weight loss, and worsening clinical symptoms) should be switched to alternative therapy. This is unique from melanoma, and in particular with anti-CTLA-4 (ipilimumab, tremelimumab) therapy, which acts to recruit T cells into the tumor microenvironment which may radiographically appear as an enlarging lesion (28). Anti-PD-1/PD-L1 directed therapies predominantly act on immune cells already present within the tumor microenvironment. Moreover, NSCLC, a tumor that is overall less “immunogenic” than melanoma (potentially due to the quantity and quality of neoantigens produced by the tumor), likely results in decreased immune cell recruitment into the NSCLC microenvironment with a reduced rate of radiographic pseudoprogression (5). One common theme across histologies, aside from durability of response, is the ability for patients to recapture responses with retreatment, or to have continued responses off therapy (18,21,22,24). Overall, improved patient selection with the use of predictive biomarkers will maximize benefits, minimize risks, and help clarify treatment decision-making as anti-PD-1/anti-PD-L1 directed therapy use becomes more widespread.

Predictive biomarkers for response in NSCLC

Across histologic subtypes and trials in NSCLC, patients with tumors that are PD-L1 IHC positive seem to preferentially benefit from PD-1/PD-L1 directed therapy (20). While patients with PD-L1 IHC negative tumors may still derive benefit from therapy, patients with

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Table 1 Summary of published clinical trial data in NSCLC with anti-PD-1/anti-PD-L1 blockade

Agent	NSCLC Histology	PD-L1 IHC positivity (NSCLC)	ORR	Survival	Grade 3-4 toxicity (%)	Citation
Ipilimumab (phased with carboplatin, paclitaxel)	Any	N/A	32% *vs*. 18%	irPFS 5.7 *vs*. 4.6 months	15	(7)
Nivolumab	Any	50% positive (limited samples unable to correlate with response)	18%	26% PFS at 24 weeks	18 (1% pneumonitis)	(9)
BMS-936559 (anti-PD-L1)	Any	N/A	10%	31% PFS at 24 weeks	5 (0% pneumonitis)	(8)
Nivolumab	Squamous	29% positive (24% *vs*. 14% partial response rate in 28-8 PD-L1 >5% staining)	15%	20% PFS at 1 year; 40.8% OS at 1 year	17 (3% pneumonitis)	(23)
Nivolumab	Squamous	Assessed at 1%, 5%, 10% cutoffs and not correlated with response	20% (*vs*. 9% docetaxel)	42% OS at 1 year (*vs*. 24% with docetaxel)	7 (0% pneumonitis)	(24)
Nivolumab	Nonsquamous	Assessed with no clear association with response	17%	42% OS at 1 year; 24% OS at 2 years; 18% OS at 3 years	14 (2% pneumonitis)	(22)
Pembrolizumab	Any	23.2% positive with 22C3 PD-L1 >50% staining	19.4% overall (45.2% in patients PD-L1+)	Median PFS 6.3 months (for PD-L1+)	9.5 (1.8% pneumonitis)	(21)
Atezolizumab (MDPL3280A)	Any	26% positive with SP142 >5% staining	23% (83% in PD-L1 IC3 patients)	45% PFS at 24 weeks (83.3% in PD-L1 IC3 patients)	12.6 (0% pneumonitis)	(25)

NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; IHC, immunohistochemistry; ORR, objective response rate.

PD-L1 IHC positive tumors have a higher response rate and survival with anti-PD-1/PD-L1 directed therapy across studies (21,25,29). For example, pembrolizumab has been investigated in NSCLC, utilizing a 50% IHC cutoff for PD-L1 expression on tumor with the 22C3 assay. Based on this cutoff, 23% of tumors were positive for PD-L1 (>50% expression) and these patients had a 45.2% response rate, compared with an ORR of 19.5% in patients with PD-L1 expression 25-50%, an ORR of 12.9% in patients with PD-L1 expression of 1-24%, and an ORR of 6.1% in patients with PD-L1 expression <1% (21). Atezolizumab (MPDL3280A), an anti-PD-L1 antibody, has also been studied in NSCLC utilizing SP142 with 0-3+ grading (3+ for ≥10% cells, 2+ for ≥5 to <10% cells, 1+ for ≥1% to <5% cells; 0+ for <1%) and scoring of both tumor and immune cell PD-L1 expression (25). In this study, PD-L1 expression on IC was found to be more predictive than PD-L1 expression on tumor cells (TC). NSCLC patients with 3+ PD-L1 expression on immune cells (IC3) had an 83% response rate, compared with 14% with IC2 expression, 15% for IC1 expression, and 20% for IC0 expression. A similar trend in response rates associated with immune-cell PD-L1 expression was observed in other solid tumor types with this agent.

Data regarding PD-L1 IHC based on the 28-8 clone for nivolumab are mixed. In squamous NSCLC, PD-L1 IHC was not predictive of response with an ORR in the 15-21% range regardless of tumor PD-L1 expression (23,24). No relationship between PD-L1 IHC and response to nivolumab was observed in another NSCLC trial featuring both squamous and nonsquamous histologies (22). However, CheckMate 057, a phase

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3 randomized control trial of nivolumab *vs*. docetaxel in advanced nonsquamous NSCLC showed a preferential benefit in patients with higher PD-L1 expression on tumor at the 1%, 5%, and 10% cutoffs (30). The reasons behind this disconnect are not clear, but may be related to technical issues related to sample collection and timing of biopsy, or biologic issues such as increased mutational burden in squamous NSCLC relative to nonsquamous tumors, which may overcome the predictive biomarker effect of PD-L1 IHC. Based on this limited data, it appears patients with NSCLC, and particularly patients with nonsquamous NSCLC, with higher levels of PD-L1 by IHC have superior responses to anti-PD-1/PD-L1 directed therapy. However, responses in PD-L1 IHC negative patients can be observed and may be related to biopsy site selection as well as timing of biopsy.

In addition to PD-L1 IHC, other biomarkers may determine which patients derive clinical benefit from anti-PD-1 directed therapy. Immune checkpoint blockade works through activation of existing antigen-specific T cells against the tumor. Thus, tumors with a high mutational burden are more likely to generate a neoantigen for which a cognate antigen-specific T cell exists. This T cell becomes activated in the setting of immune checkpoint blockade, resulting in the efficacy of the immune checkpoint inhibitor. Mechanistically, this has been shown to be the case in microsatellite unstable tumors with high mutational burden and an improved response to PD-1 blockade (6). Furthermore, identification of immunogenic neoantigens based on peptide prediction algorithms analyzing the tumor mutanome has been shown to generate individualized biomarkers for response to anti-CTLA-4 blockade in melanoma (31). A similar approach was taken with anti-PD-1 blockade in NSCLC (32). Of note, ORR and PFS were improved in patients with higher nonsynonymous mutational burden when treated with pembrolizumab. In particular, a molecular smoking signature based on genetic transversions showed that patients with transversion-high (TH) tumors had a higher ORR (56% *vs*. 17% in transversion-low tumors; P=0.03), durable clinical benefit rate (77% *vs*. 22%, P=0.004), and PFS. A molecular smoking signature more significantly correlated with response than clinical smoking history, and never smokers with mutations that resulted in higher mutational burden (e.g., *POLD1*, *POLE*, *MSH2* mutations) had improved responses to pembrolizumab therapy, analogous to microsatellite-unstable gastrointestinal tumors (6).

Future directions

Immunotherapy, and in particular, immune checkpoint blockade, has revolutionized medical oncology and the care of patients with NSCLC. The promise of durable responses in select patients with NSCLC treated with immune checkpoint blockade, in particular anti-PD-1/PD-L1, has set a new bar for cancer therapy. Responses in 15-25% of NSCLC patients treated with anti-PD-1/anti-PD-L1 therapy are durable and can last years in select patients, even those with heavily pre-treated disease. Anti-PD-1/PD-L1 therapy is generally well-tolerated, though vigilance and early intervention on immune-related pneumonitis will be required as these therapies gain greater usage. More importantly, these therapies provide a solid base for combinatorial approaches utilizing targeted therapy, cellular therapy, as well as alternative modes of immunomodulation. Indeed, *EGFR* and *ALK*-aberrant NSCLC tumors can overexpress PD-L1, and combinatorial strategies combining EGFR and ALK inhibitors with anti-PD-1/anti-PD-L1 blockade are currently being tested in clinical trials (33,34). Concomitant inhibition of other immune checkpoints such as LAG3, TIM3, KIR, and BTLA may by synergistic with anti-PD-1 blockade and are under active investigation. Returning to the T cell as automobile analogy, this approach blocks multiple immunologic "brakes" leading to increased acceleration (activation). Additionally, immune costimulation via agonists of the tumor necrosis factor receptor superfamily (TNFRSF) such as OX40, 4-1BB, and GITR may represent an attractive combinatorial approach and are actively being studied (35). This approach is based on the premise that blocking an immunologic "brake" while pressing on an immunologic "accelerator," may result in improved T cell responses against tumor.

Management of immune-related toxicity with combinatorial immunotherapeutics will be crucial, as has been demonstrated by combinations based on CTLA-4 blockade used in the treatment of melanoma (18). However, combinatorial toxicity may be driven by biology (e.g., CTLA-4 as a centrally acting checkpoint) and the development of predictive biomarkers may help guide therapeutic decision-making. For example, patients with metastatic melanoma who had PD-L1 negative tumors derived the greatest benefit from combinatorial ipilimumab plus nivolumab therapy, compared to nivolumab monotherapy (29). Similarly, NSCLC patients with tumors overexpressing PD-L1 tend to have superior responses to anti-PD-1/anti-PD-L1 therapy in most, but not all, clinical

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trials in NSCLC to date. Ultimately, improved predictive biomarkers will help determine which personalized immunotherapeutic combinations will maximize benefit, and minimize toxicity, for each individual cancer patient.

Lung cancer, and in particular NSCLC, has undergone a therapeutic revolution, with the adoption of molecular profiling and early intervention with targeted therapy for patients with driver mutations (36). The embrace of a precision medicine approach to NSCLC has led to the development of novel approaches such as cell-free DNA to assess for mechanisms of therapeutic resistance, and to the rational design of next-generation targeted therapies (37-39). The advent of immune-based therapies requires an expansion of this precision medicine approach to include not only molecular aberrations detected in tumor and in blood, but also to serial assessment of the tumor immune microenvironment. Assays such as PD-L1 IHC, mutational burden, neoantigen prediction, immune transcriptional signatures, T-cell receptor clonality, and others will require further investigation as they become increasingly integrated into clinical testing for therapeutic decision-making (20). Treatment of NSCLC is truly at a crossroads, with multiple potential paths for any particular patient, and the development and utilization of novel biomarkers will be needed in order to best guide patients through increasingly complex treatment decisions. With the advent of immunotherapy in NSCLC, and in particular, immune checkpoint blockade, another promising path has been discovered—and one that has just begun to be explored.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Predicting the prognosis of lung cancer: the evolution of tumor, node and metastasis in the molecular age—challenges and opportunities

Ramón Rami-Porta[1], Hisao Asamura[2], Peter Goldstraw[3]

[1]Department of Thoracic Surgery, Hospital Universitari Mutua Terrassa, University of Barcelona, and CIBERES Lung Cancer Group, Terrassa, Barcelona, Spain; [2]Division of Thoracic Surgery, Keio University School of Medicine, Tokyo, Japan; [3]Academic Department of Thoracic Surgery, Royal Brompton Hospital and National Heart and Lung Institute, Imperial College, London, UK

Correspondence to: Ramón Rami-Porta, MD, FETCS. Department of Thoracic Surgery, Hospital Universitari Mutua Terrassa, Plaza Dr Robert 5, 08221 Terrassa, Barcelona, Spain. Email: rramip@yahoo.es.

Abstract: The tumor, node and metastasis (TNM) classification of malignant tumors was proposed by Pierre Denoit in the mid-20th century to code the anatomic extent of tumors. Soon after, it was accepted by the Union for International Cancer Control and by the American Joint Committee on Cancer, and published in their respective staging manuals. Till 2002, the revisions of the TNM classification were based on the analyses of a database that included over 5,000 patients, and that was managed by Clifton Mountain. These patients originated from North America and almost all of them had undergone surgical treatment. To overcome these limitations, the International Association for the Study of Lung Cancer proposed the creation of an international database of lung cancer patients treated with a wider range of therapeutic modalities. The changes introduced in the 7th edition of the TNM classification of lung cancer, published in 2009, derived from the analysis of an international retrospective database of 81,495 patients. The revisions for the 8th edition, to be published in 2016, will be based on a new retrospective and prospective international database of 77,156 patients, and will mainly concern tumor size, extrathoracic metastatic disease, and stage grouping. These revisions will improve our capacity to indicate prognosis and will make the TNM classification more robust. In the future the TNM classification will be combined with non-anatomic parameters to define prognostic groups to further refine personalized prognosis.

Keywords: Lung cancer; lung cancer staging; prognostic groups; stage grouping; tumor, node and metastasis (TNM) classification

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Introduction

Obvious as it may seem, it is important that the readers of this article keep in mind that the tumor, node and metastasis (TNM) classification of lung cancer is no more and no less than a system to code the anatomic extent of the disease. Therefore, by definition, the TNM classification does not include other elements that, while they can help improve our capacity to prognosticate the disease for a given patient, are unrelated to the anatomy of the tumor, i.e., parameters from blood analysis, tumor markers, genetic signatures, comorbidity index, environmental factors, etc. Prognostic indexes combining the TNM classification and other non-anatomic parameters are called, by consensus between the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC), prognostic groups to differentiate them from the anatomic stage groupings.

The TNM classification of lung cancer is applied to all histopathological subtypes of non-small cell carcinoma, to small cell carcinoma and to typical and atypical carcinoids.

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Table 1 General rules of the TNM classification of malignant tumors

Rule number	Synthetic text
1	Microscopic confirmation of malignancy and histopathological type are required
2	Clinical (c) classification is determined before any treatment; pathological (p) classification is determined after tumor resection
3	TNM groupings of similar prognosis are combined in stages
4	When in doubt, opt for the less advanced T, N, M category and stage
5	Multiple tumors are classified by the highest T followed by m or the number of tumors in parenthesis, i.e., T3[m] or T3[2]
6	Telescoping is allowed to better define categories, i.e., T1a, T1b, etc.

TNM, tumor, node and metastasis.

It is governed by general rules (1-3) (*Table 1*) that apply to all malignancies classified with this system, and by site-specific rules applicable to lung cancer exclusively (4). There also are recommendations and requirements issued with the objective to classify tumors in a uniform way when their particular characteristics do not fit in the basic rules (4).

The three components of the classification have several categories that are defined by different descriptors. For lung cancer, those for the T component are based on tumor size, tumor location and involved structures; those for the N, on the absence, presence and location of lymph node metastasis; and those for the M, on the absence, presence and location of distant metastasis. There are optional descriptors that add information on the local aggressiveness of the tumor (differentiation grade, perineural invasion, vascular invasion and lymphatic permeation) all of which have prognostic relevance (5-8); assess the intensity of the investigation to determine the stage (certainty factor); and assess the residual tumor after therapy (residual tumor).

Origin and evolution of the TNM classification for lung cancer

The TNM classification was developed by Pierre Denoit in a series of articles published from 1943 to 1952. It was soon adopted by the UICC that published brochures covering several anatomical sites, the lung being included in 1966. Two years later, the UICC published the first edition of the TNM Classification of Malignant Tumors and agreements were reached with the AJCC, created in 1959 as the American Joint Committee for Cancer Staging and End Results Reporting, to consult each other to avoid publication of differing classifications. Since then, the UICC and the AJCC have been responsible for updating and revising the TNM classifications of malignant tumors with the participation of national TNM committees of several countries and taking into account the published reports on the topic. The second to sixth editions of the UICC manual on the TNM Classification of Malignant Tumors and the first to sixth editions of the AJCC Staging Manual included classifications for lung cancer that had been informed by a progressively enlarging database initially collected by Mountain, Carr and Anderson, and subsequently managed by Mountain. Their database originally contained a little over 2,000 patients, but it had grown to more than 5,000 by the time the fifth edition of the TNM classification for lung cancer was published in 1997. The sixth edition was published in 2002 with no modifications (9).

While the fifth edition of the classification was being printed, the International Workshop on Intrathoracic Staging took place in London, United Kingdom, in October 1996, sponsored by the International Association for the Study of Lung Cancer (IASLC) (10). At that meeting, in the presence of Dr. Mountain, the limitations of the database that had been used to revise the TNM classification for lung cancer were openly discussed. In essence, it was considered that, while the database consisted of a relatively large number of patients, all of them originated from the United States of America, and, therefore, the staging system could not really be called 'international', as it was called at that time; and, although all tumors had clinical and pathological classifications, the majority had been treated surgically. So, the database was thought not to be representative of the international community, as there were no patients from other countries; or of the current clinical practice, as there were no patients treated with other therapies. Therefore, an agreement was reached to issue a worldwide call to build a really international database of lung cancer patients treated by all therapeutic modalities. This required the constitution of an International Staging Committee that was approved

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and given a small amount of funding, to pump-prime, by the IASLC Board in 1998. Subsequently substantial financial support was secured by an unrestricted grant from Eli-Lilly. Cancer Research And Biostatistics (CRAB), a not-for-profit biosciences statistical center in Seattle, was appointed to collect, manage and analyze the new database. The proprietors and managers of known databases were subsequently summoned to attend a series of preparatory meetings to identify potential contributors to the IASLC international database for the purpose of revising the TNM classification of lung cancer.

The 7th edition of the TNM classification of lung cancer

By 2005, more than 100,000 patients had been registered and more than 80,000 met the established criteria for analysis, the largest database ever collected to revise the TNM classification of lung cancer. All these patients originated in 45 databases of different nature in 20 countries around the world, and had been diagnosed with lung cancer between 1990 and 2000 (11). From 2005 to 2009, the members of the subcommittees for the T, the N, and the M components, and those for stage grouping, validation, small-cell lung cancer, carcinoids, visceral pleura invasion, lymph node map, and non-anatomic prognostic factors analyzed, together with the biostatisticians of CRAB, the specific results, proposed recommendations for changes, and wrote their manuscripts that were eventually published in the *Journal of Thoracic Oncology* (12-23). All recommendations were accepted by the UICC and the AJCC, and included in the lung cancer chapters of the 7th edition of their respective staging manuals (1,2). In addition, the IASLC became the main provider of evidence to the UICC and the AJCC to revise future editions of the TNM classification of lung cancer and other thoracic malignancies, as pleural mesothelioma and thymic tumors had been incorporated into the IASLC Staging Project in 2008 and 2009, respectively. In 2009, the IASLC published its own staging manual and handbook (3,24).

The most important innovations of the 7th edition were the increased relevance of tumor size; the reconciliation of separate tumor nodules in the same lobe, in another ipsilateral lobe and in the contralateral lung with their observed prognosis; the upstaging of malignant pleural and pericardial effusions and nodules to metastatic disease; the relocation of some TNM groups into a different stage; the separation of intrathoracic and extrathoracic metastases; the validation of the TNM classification for bronchopulmonary carcinoid tumors; the recommendation to use the TNM classification for small-cell lung cancer instead of the dichotomous limited and extensive disease classification; and the international and multidisciplinary agreement of a new pulmonary and mediastinal lymph node map. Visceral pleura invasion was defined by the involvement of its elastic layer, and elastic stains were recommended when visceral pleura invasion was not evident with standard stains. These changes were extensively reviewed from the general (25-34), radiological (35,36), clinical (37-39), therapeutic (40-42) and pathological (43,44) points of view; and they were validated, in total or in part, with the series of many institutions (45-63).

The classification of the 7th edition is very useful to indicate prognosis, which is one of the objectives of the classification. The 3-cm cut-point, that had been the only one to separate tumors according to size, was abandoned in favor of five tumor-size groups separated at 2, 3, 5 and 7 cm cut-points, defining groups of tumors with significantly different prognosis (12). The downstaging of separate tumor nodules in the same lobe from T4 (6th edition) to T3 (7th edition), and in another ipsilateral lobe from M1 (6th edition) to T4 (7th edition), increased the awareness of these nodules, that are usually resected, in contradistinction with the contralateral nodules (M1a in 7th edition) that are rarely resected (12). For the N component, the descriptors were unchanged, but the definition of nodal zones, grouping neighboring nodal stations, emphasized the concept of quantification of nodal disease, as it was evident that the more involved zones, the worse the prognosis. Although this information was not used to modify the present N descriptors because it could not be validated clinically, geographically or by T categories, it is practically useful as it helps refine the postoperative prognosis of patients with nodal disease (13). For the M component, the separation of intrathoracic (M1a) from extrathoracic (M1b) metastasis also helps in assessing prognosis as both groups of metastases have different prognosis, but also reconciles common clinical practice as treatment of malignant pleural and pericardial effusions and nodules had been considered palliative, as with metastatic disease, even when these situations were in the T4 category in the previous editions of the TNM classification (14).

The proposed nodal map was the result of a wide international and multidisciplinary consensus (20). It reconciled the differences between the maps proposed by Mountain and Dresler (64) and the Naruke-Japan Lung Cancer Society (65,66), and introduced important

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Table 2 Geographical origin of data used for the 7th and the 8th editions of the TNM classification of lung cancer

Geographical origin	Number*	
	7th edition	8th edition
Europe	58,701	46,560
North America	21,130	4,660
Asia	11,622	41,705
Australia	9,416	1,593
South America	0	190
Total	100,869	94,708

*, total number of submitted patients; TNM, tumor, node and metastasis.

innovations: clear anatomical landmarks for each nodal station, recognizable by the radiologist, the endoscopist and the surgeon; the enlargement of the supraclavicular and subcarinal nodal stations; and the shift of the anatomic midline of the mediastinum to the left paratracheal margin (oncological midline) for the purpose of separating right and left superior and inferior paratracheal lymph nodes (20).

In the new stage grouping, some aggregate TNM combinations moved from one stage to another. Large T2 tumors (T2b N0 M0) were upstaged from stage IB to IIA; T2a N1M0 tumors were downstaged from stage IIB to IIA; and T4 N0-1 M0 tumors were downstaged from stage IIIB to IIIA. The question of how to treat patients with these tumors arose. Were T2b N0 M0 tumors to be treated with adjuvant chemotherapy as the other tumors in stage IIA? The perception was that the changes in classification lead to a change in treatment (41,42), but in principle the answer is that treatment recommendations should derive from properly conducted clinical trials and not from taxonomic changes. The mere change of stage does not provide any evidence on the best treatment. New trials will be necessary to answer this question. In the meantime, the multidisciplinary team will have to decide on the best therapeutic option based on all the available information on the patient, the tumor and the surgical resection.

The application of the TNM classification to small-cell lung cancer provides us with a clear example of its utility in refining prognosis. The traditional limited disease group includes tumors from stages IA to IIIB with a 29% absolute survival difference between them: 5-year postoperative survival rates of 38% and 9%, respectively, with the expected progressive degradation of survival as tumor stage increases (18). This survival difference would be lost if the TNM were not applied to small-cell lung cancer and all tumors were put together in the same category of limited disease.

Towards the 8th edition

The modifications in the T and the M components of the classification, the recognition of the relevance of the quantification of nodal disease, the new stage groupings, and the application of the TNM classification to small-cell lung cancer improved our capacity to indicate prognosis, but the 7th edition of the TNM classification for lung cancer has limitations derived, mainly, from its retrospective nature (67). Not all databases contained the necessary staging details to validate all descriptors, and over half of the registered patients underwent surgical treatment either alone or in combination (11). This high proportion of surgical cases does not reflect common clinical practice and there is the need of a wider representation in the range of therapeutic modalities. To achieve this, the IASLC made a worldwide call to build a new international database to inform the 8th edition of the TNM classification of lung cancer (68). Amazing as it may seem, the call was answered with the submission of more than 90,000 new patients from 35 databases in 16 countries, diagnosed from 1999 to 2010; and 77,156 (70,967 with non-small cell lung cancer and 6,189 with small-cell lung cancer) met the requirements for analysis (69). *Table 2* shows the geographical origin of the data. Europe maintains its leadership in submitting patients, while there was an important drop in contributions from North America and a very relevant increase in cases from Asia, thanks to the massive submission of Japanese registries. Although modest, for the first time there are some patients from South America. Another characteristic of this database is that nearly 4,000 patients were prospectively registered online through the electronic data capture system established by CRAB. These cases have very complete information and have been very useful for certain analyses for which detail matters, such as the number of metastases in patients with M1b disease. *Table 3* shows the types of submitted databases. Clinical trials were in the lead in the database used for the 7th edition, while none was submitted for the 8th. The absence of clinical trials and the surgical cases submitted by Japan account for the relative scarcity of advanced cases in the database used for the 8th edition. *Table 4* shows the types of treatments for each database. In both, there is a predominance of surgical cases, which is more evident in the database for the 8th edition. This fact

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Table 3 Types of databases contributing to the 7th and 8th editions of the TNM classification of lung cancer

Type of database	Number*	
	7th edition	8th edition
Clinical trials	24,239	0
Surgical series	19,172	5,965
Registries	16,660	26,122
Series with all treatments	7,866	0
Consortia	5,912	43,637
Institutional registries	5,492	208
Surgical registries	2,154	0
Institutional series	0	1,185
Unknown	0	39
Total	81,495	77,156

*, number after exclusions. TNM, tumor, node and metastasis.

may question the generalizability of the recommendations for changes derived from the analyses of the database, as it has been shown that some descriptions, for example tumor size, do not have the same prognostic impact in the populations of patients treated with radiotherapy (70).

At the moment of this writing, the members of the IASLC Staging and Prognostic Factors Committee already have analyzed the database and decided on the changes to be recommended in the 8th edition. The original papers describing these analyses and the recommendations for changes already are submitted to the *Journal of Thoracic Oncology* or are in the process of being submitted.

Pending the scrutiny from the international oncological community and the acceptance from the UICC and the AJCC, the most important recommended changes affect tumor size, the relevance of which is greater than it was thought from the analyses of the previous database. Consequently, the recommendation is to define more groups of tumors based on size and to include tumor size as a descriptor in all T categories, from Tis to T4. The recommendation for the N component is to retain the 7th edition descriptors, but to propose the quantification of nodal disease by number of involved nodal stations for prospective registration of data. For the M component, the recommendation is to separate extrathoracic single metastasis from multiple metastases, as they have different prognosis. The stage grouping will be slightly modify, as the suggested changes in the T and the M components lead to the creation of more stages both in early and advanced disease. There will also be recommendations to code the new adenocarcinoma

Table 4 Treatment modalities for submitted patients in the databases used to inform the 7th and the 8th editions of the TNM classification of lung cancer

Treatment modality	7th edition (%)	8th edition (%)
Surgery alone	41	57.7
Chemotherapy alone	23	9.3
Chemotherapy and radiotherapy	12	4.7
Radiotherapy alone	11	1.5
Radiotherapy and surgery	5	1.5
Chemotherapy and surgery	4	21.1
Trimodality	3	4.4

TNM, tumor, node and metastasis.

subtypes, especially adenocarcinoma in situ and minimally invasive adenocarcinoma; the recommendation to apply the TNM classification to small-cell lung cancer will be emphasized; and an attempt will be made to clarify the classification of lung cancers with multiple lesions: second primary tumors, separate tumor nodules, and multiple nodules with ground glass/lepidic features.

The future of the TNM classification

The TNM classification of lung cancer is the most consistent and solid prognosticator of the disease, but it does not explain the whole prognosis because prognosis is multifactorial. In addition to the anatomic extent of the tumor, patient and environmental factors also count. Prognosis also is dynamic, as it may be different at the time of diagnosis, after treatment or at recurrence (71). In the TNM classification, tumor resection plays an important role as it defines pathological staging and may modify the prognostic assessment based on clinical staging. Other than that, the TNM classification does not include blood analyses, tumor markers, genetic characteristic of the tumor or environmental factors that may account for the differences in survival among similar tumors in different geographic areas.

In order to make progress to indicate a more personalized prognosis, instead of a prognosis based on cohorts of patients with tumors of similar anatomic extent, the IASLC Staging and Prognosis Factors Committee decided to expand its activities to the study of non-anatomic prognostic factors. Therefore, in the third phase of the IASLC Lung Cancer Staging Project, the activities of the committee will be

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directed to further refine the TNM classification and to find available factors that can be combined with tumor staging to define prognostic groups. To some extent, this already was done with the analyses of the database used for the 7th edition. Prognostic groups with statistically significant differences were defined by combining anatomic tumor extent and very simple clinical variables, such as performance status, gender, and age. These prognostic groups were defined for clinically and pathologically staged tumors, and for small-cell and non-small cell lung cancers (22,23).

The database used for the 8th edition includes several non-anatomical elements related to the patient, the tumor and the environment that may help refine prognosis at clinical and pathological staging (69). Due to the limitations of the previous databases, future revisions of the TNM classification will need to be more balanced in terms of therapeutic modalities, and better populated with patients from underrepresented geographical areas, such as Africa, India, Indonesia, North, Central and South America, and South East Asia. The data contributed in the future will have to be complete regarding the TNM descriptors, and preferably prospective. The more robust the TNM, the more important its contribution to the prognostic groups.

To achieve all of the above, international collaboration is essential. Those interested in participating in this project should send an email expressing their interest to information@crab.org, stating 'IASLC staging project' in the subject of the email. The IASLC Staging and Prognostic Factors Committee has been very touched by the overwhelming generosity of colleagues around the world who have contributed cases to inform the 7th and the 8th editions of the TNM classification of lung cancer. We continue to count on their collaboration to further revise future editions and to define prognostic groups that will eventually allow a more personalized indication of prognosis.

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Footnote

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Prognostic markers in lung cancer: is it ready for prime time?

Chang-Qi Zhu[1], **Ming-Sound Tsao**[1,2]

[1]Princess Margaret Cancer Centre, University Health Network and [2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada

Correspondence to: Dr. Ming-Sound Tsao. Toronto Medical Discovery Tower 14-401, 101 College Street, Toronto, ON M5G 1L7, Canada. Email: ming.tsao@uhn.ca.

Abstract: Non-small cell lung cancer (NSCLC) is a heterogeneity disease and to date, specific clinical factors and tumor stage are established as prognostic markers. Nevertheless, prognosis within stage may vary significantly. During the last 3 decades, genes/proteins that drive tumor initiation and progression, such as oncogenes and tumor suppressor genes have been studied as additional potential prognostic markers. The protein markers as evaluated by immunohistochemistry (IHC) have previously dominated these studies. However, with the development of high-throughput techniques to interrogate genome wide genetic or gene expression changes, DNA (copy number and mutation) and RNA (mRNA and microRNA) based markers have more recently been studied as prognostic markers. Largely due to the heterogeneity and complexity of NSCLC, single gene markers including *KRAS* mutation has not been validated as strong prognostic markers. In contrast, several gene expression signatures representing mRNA levels of multiple genes have been developed and validated in multiple microarray datasets of independent patient cohorts. The salient features of these gene signatures and their potential value to predict benefit from adjuvant chemotherapy is discussed.

Keywords: Prognostic marker; expression signature; multi-gene markers; immunohistochemistry (IHC); proteomics

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Cancer prognostic markers are patient or tumor characteristics that predict outcome (usually survival) independent of the treatment (1). Thus, they are usually identified and validated in patients who receive no or surgical therapy only. The goal of identifying prognostic markers is to define patient subpopulations with significantly different anticipated outcomes, who might benefit from different therapies. Good prognostic patients may not require additional treatment beyond the primary surgical resection, while poor prognostic patients may derive improved survival benefit from adjuvant therapy. Therefore, prognostic markers could potentially be "drivers" of cancer progression. In turn, these markers could themselves represent therapeutic targets.

Predictive markers, on the other hand, are patient or tumor characteristics that predict benefit from specific treatments (either in terms of tumor shrinkage or survival). In other words, the differences in tumor response or survival benefit between treated versus untreated patients will be significantly different in those with or without the predictive marker (e.g., a mutation). In contrast, the effect of treatment is not expected to be different in patient groups distinguished by a prognostic marker only. The validation of prognostic marker can be established by using data from retrospective series, while the validation of predictive marker should be done in a controlled clinical trial, in which the effect of the marker can be tested in both the treated and placebo groups.

Prognostic markers can be proteins, mRNAs or miRNAs or the gene itself. For the latter, mutations, gene copy number aberrations and single nucleotide variation could potentially also be prognostic. Most markers that have been extensively studied are proteins, which are typically assessed by immunohistochemistry (IHC). However, the high-throughput profiling techniques in cancer genome have led to the identification of mRNA and miRNA prognostic

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signatures. Proteomic signatures generated by mass spectrometry are also emerging (2).

In lung cancer, prognostic markers are most relevant to early-stage (I-IIIA) non-small cell lung cancer (NSCLC) patients, who are potentially curable by complete surgical resection. However, the prognostic significance of a marker should also be assessed during the validation of a predictive marker, as the apparent benefit from a specific therapy could merely reflect the inherently prognostic value of the marker. As an example, VeriStrat (2) is a mass spectrometry-derived proteomic signature, which was initially reported as capable of stratifying advanced NSCLC patients for their responses to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors gefitinib and erlotinib. In two cohorts of patients treated by these TKIs, respectively, the VeriStrat "good" patients demonstrated a significantly longer time to progression and overall survival than the VeriStrat "poor" patients, even after adjustment for other clinical factors. A subsequent retrospective study appeared to validate the independent predictiveness of VeriStrat to erlotinib for progression-free survival (P=0.011) and overall survival (P=0.017) in a randomized phase II trial of first-line therapy with gemcitabine, erlotinib, or the combination in elderly patients (>70 years) (3). When tested in 3 "control" advanced NSCLC patient cohorts (total n=158) who did not receive any TKI treatment, VeriStrat signature was found not to be prognostic. However, all these studies were conducted in patients treated by a single therapy. When VeriStrat was tested in the samples from NCIC CTG BR.21 trial, a randomized placebo-controlled study of erlotinib in previously treated advanced NSCLC patients, erlotinib treatment prolonged survival in both VeriStrat "good" and "poor" patient groups, indicating the lack of predictive value of VeriStrat for erlotinib treatment (4). Importantly the VeriStrat "poor" group had poorer survival in the placebo group patients, consistent with VeriStrat being a prognostic marker (4).

Single gene/protein prognostic markers

Most lung cancer prognostic markers reported are proteins evaluated by IHC. Despite >500 reported studies, not a single protein marker has as yet been validated sufficiently for clinical use (5). For most markers, the results from various studies have been inconsistent. This could largely be accounted for by the lack of standardization in the IHC methods used, including the source and quality of the antibodies used, the staining protocol, scoring algorithm, and statistical approach to analyse the data. Inconsistent results could also be due to the small sample size in some studies, for which cases included are less representative. Institutional and publication biases could also play an important role. As an example, from 1987 to 2005 there were 15 reported studies on the prognostic value of cyclin D1 (CCND1) (6-20). Five studies identified CCND1 overexpression as a negative prognostic marker (6,8,9,14,16), while three other studies associated it with better prognosis (11,18,20); the remaining seven reported no association (*Table 1*). It is noted that the source of antibody varied from laboratory generated to commercial sources, and different antibody dilutions and scoring cut-offs for positive staining were used (*Table 1*). Overall, no conclusive result on the prognostic value of CCND1 could be made from these studies (5).

The most credible prognostic markers reported have been based on samples of patients who were involved in large multi-institutional studies, especially randomized placebo-controlled treatment trials. The advantages of these cohorts include more uniform and better-defined patient characteristics, as well as the ability to test the predictive value of the markers for benefit from adjuvant chemotherapy. The Lung Adjuvant Cisplatin Evaluation-Biology (LACE-Bio) studies are organized by investigators from the four seminal adjuvant chemotherapy trials: the International Adjuvant Lung Cancer (IALT), Adjuvant Navelbine International Trialist Association (ANITA), Cancer and Leukemia Group B (CALGB) 9633, and NCIC Clinical Trials Group (CTG) JBR.10. The goal of LACE-Bio studies include cross validation or pooled analyses of promising prognostic and predictive markers reported by one or more of the member groups. The NCIC CTG group initially reported that high β-tubulin (bTub III) expression by IHC was a poor prognostic marker for recurrence-free survival (RFS) and borderline prognostic for overall survival (OS) in surgery-alone patients, as well as being predictive for survival benefit from adjuvant chemotherapy (21). When the marker was tested in the pooled data set of the other 3 trials (total n=1149), the poor prognostic value of high bTubIII was validated [hazard ratio (HR): 1.27; 95% confidence interval (CI): 1.07-1.51; P=0.008 for OS and HR: 1.30; 95% CI: 1.11- 1.53; P<0.001 for RFS] (22). However, interaction between bTubIII expression and chemotherapy was not significant, which indicates that high bTubIII is not predictive of benefit from adjuvant chemotherapy (22).

One of the most celebrated prognostic and predictive

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Table 1 Immunohistochemistry studies on the prognostic significance of cyclin D1 (CCND1)

Reference	Number of patients	Source of antibody	Antibody type (clone)	Dilution	Univariate significance	Multivariate significance	Cutoff
Esposito, 2005 (6)	105	NA	NA	NA	Poor	Yes	>5% cells stained
Dworakoska, 2005 (7)	111	Dako	MC (DCS-6)	1:100	No	No	Any cell staining
Au, 2004 (18)	284	Dako	MC (DCS-6)	1:300	Good for AD	No	4 tiers system; cutoff for positive not stated
Ikehara, 2003 (8)	72	Nococastra	PC	1:200	Poor	NA	>20% of cells stained
Jin, 2001 (9)	106	BD bioscience	MC (G124-326)	1:50	Poor	Yes	>nuclear background or cytoplasm staining
Dosaka-Akita, 2001 (10)	217	Oncogene science	MC (DCS-6)	1:40	No	NA	Any nuclear staining
Anton, 2000 (11)	467	BD bioscience	MC (G124-326)	1:500	Good for SQ	NA	>10% cells stained
Volm, 2000 (13)	145	Santa cruz biotechnology	MC (Ab-3)	1:10	No	No	Moderate-strong staining
Keum, 1999 (14)	69	Novocastra	MC (P2D11F11)	1:200	Poor	No	>5% cells stained
Brambilla, 1999 (15)	168	Dako	NA	NA	No	No	>5% nuclei stained
Caputi, 1999 (16)	135	Non-commercial	PC	1:100	Poor	NA	0:1-30%; 30-60%; >60%
Kwa, 1996 (17)	96	Non-commercial	PC	1:80	No		>10% nuclei stained
Nguyen, 2000 (12)	89	Dako	MC (DCS-6)	NA	No	NA	Cytoplasmic staining
Gugger, 2001 (20)	92	Novocastra	MC (P2D11F11)	1.6 ug/mL	Good	Yes	Any nuclear staining
Burke, 2005 (19)	106	Oncogene science	MC (DCS-6)	1:40	No	No	Intensity (0-3)+% cells (0-3); positive: 4 or >

MC, monoclonal; PC, polyclonal; AD, adenocarcinoma; SQ, squamous cell carcinoma.

markers for early-stage NSCLC is the Excision Repair Cross-Complementation group (ERCC1) protein, a critical component of nucleotide excision repair mechanism for DNA damage induced by cisplatin. The ERCC1 protein expression was evaluated by IHC in 761 of 1,867 patients involved in the IALT trial (23). High ERCC1 expression was found to be a good prognostic marker (adjusted HR: 0.66; 95% CI: 0.49-0.90; P=0.009) in surgery-alone patients, but adjuvant chemotherapy benefit was seen only in ERCC1-low (negative) patients (23). However, subsequent LACE-Bio cross validation study failed to establish ERCC1 as a predictive marker for adjuvant chemotherapy using the same yet a different batch of ERCC1 antibody (clone 8F1) (24). The group has tested 16 commercially available ERCC1 antibodies and found none of the 16 antibodies could distinguish among the four ERCC1 protein isoforms, whereas only one isoform produced a protein that had full capacities for nucleotide excision repair and cisplatin resistance (24). The result highlights the pitfall of IHC studies using antibodies that have not been characterized rigorously for their properties as well as quality.

Meta-analysis is a cost-effective practice for increasing the sample size and statistical power by combining results of comparable studies or trials. Quite a few meta-analyses have been performed and showed potential prognostic value of HER-2, p53, Ki-67, and Bcl-2, however, with potential institutional and publication biases, caution should be taken to interpret conclusions from meta-analyses. For example, *KRAS* mutation has been reported as a marker of poor prognosis by a meta-analysis (HR: 1.35; 95% CI: 1.16-

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1.56) (25). However, in a recent pooled analysis of 1536 LACE-Bio patients, *KRAS* mutation was not validated as a prognostic marker in NSCLC (HR: 1.18; 95% CI: 0.97-1.44; P= 0.09), nor in adenocarcinoma patient alone (HR: 1.0; 95% CI: 0.78-1.28, P=1.00) (26). Furthermore, contrary to the original finding in the JBR.10 patients, *KRAS* mutation was also not predictive of benefit from adjuvant chemotherapy (26).

Multigene prognostic markers

To date, the large numbers of studies have reported that the prognostic HRs of single marker have reached up to 1.5-1.7. Kwiatkowski *et al*. (27) and D'Amico *et al*. (28) previously demonstrated that multiple cumulative markers may better stratify prognosis compared to a single marker. The invention of microarray technologies has made it possible to explore the prognostic significance of thousands of markers using genome-wide high-throughput and computational approaches. Initial studies were conducted mainly on mRNA expression markers, as the technology was initially developed for this molecule. To date, more than 35 such studies have been reported (29), a large number showing that gene expression signature may stratify early stage NSCLC, or its subtypes (e.g., adenocarcinoma or squamous cell carcinoma), patients with different prognosis or survival outcome.

Since 2005, reports on expression prognostic markers have also included validation in independent cohorts, mostly using published microarray data sets. This was facilitated by the requirement by most high-impact journals that authors make their microarray data publicly available either through their own institute website, such as the Broad Institute (http://www.broadinstitute.org/) or by depositing to publicly repositories, such as the Gene Expression Omnibus (http:// www.ncbi.nlm.nih.gov/geo/) or ArrayExpress (https://www.ebi.ac.uk/arrayexpress/). This requirement has allowed greater level of transparency on gene expression signatures, as independent validation and verification could be conducted. Over the years, as most studies selected to use the platforms developed and commercialized by Affymetrix (Santa Clara, CA), Illumina (San Diego, CA) and Agilent (Santa Clara, CA) and as Bioconductor http://www.bioconductor.org/) was developed based on R, an open source statistical software, to analyze microarray data, significant standardization of microarray analyses has occurred. The Sweave function (http://stat.ethz.ch/R-manual/R-devel/library/utils/html/Sweave.html) and the new development of Knitr function (http://yihui.name/knitr/) in R integrates R code into LaTeX, HTML, Markdown, AsciiDoc, and reStructuredText documents which enables creating dynamic reports and making the data mining process even more transparent and reproducible. As many scientifically rational approaches have been developed and used by investigators to identify gene signatures associated with survival outcome, numerous signatures have been reported. Some are large gene set signatures made up of hundreds of genes, whereas many others are trimmed down to less than 20 genes through optimization process. Although most of these signatures have been validated in one or more independent patient cohort microarray data sets, overlaps between the genes sets have consistently been minimal. This has raised question on the robustness of gene expression signatures as a reliable biomarker. Nevertheless, a permutation study using a common data set has shown that it is statistically possible to identify numerous equally significant prognostic signatures (30). However, validation of prognostic signatures in multiple independent patient cohorts can be extremely challenging, as the signature discovery algorithms that are applied to small data sets (hundreds) containing disproportionately large number (thousands) of data elements may easily introduce data over-fitting, thus difficulty to reproduce in independent data sets (31). Furthermore, independent data sets may also carry institutional biases related to the sample selection, as well as other patient and population demographic features.

Clinically applicable prognostic gene signatures

Several features may facilitate the application of prognostic gene signature in the clinical setting to assist in management of NSCLC patients. Aside from the signatures being validated in multiple independent patient cohorts, the technique to assay the signatures should also be implementable in clinical laboratories, according to the regulatory body approved protocols, such as the Clinical Laboratory Improvement Amendments (CLIA). As the standard pathology practice process tissue into formalin-fixed and paraffin-embedded (FFPE) blocks, technologies that favor the use of FFPE samples would fast-track the adoption of the signature for clinical use. Last but not least, in order for a prognostic signature to assist oncologists in selecting patients for adjuvant chemotherapy, the signature should be predictive, such that the "high risk" patients (as identified by the signature) would likely benefit from the postsurgical chemotherapy, and "low risk" patients

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(who do not benefit and could potentially be harmed by chemotherapy) would be spared the toxicity and cost. In this context, a few signatures are worthy of highlighting.

A 15-gene prognostic signature was established from microarray expression analysis of snap-frozen tumor samples from 133 Canadian patients who participated in the JBR.10 trial (32). These included 62 patients who were treated by surgery alone, and 71 patients who received adjuvant chemotherapy. This stage-independent prognostic signature was developed from the data of surgery-only patients (adjusted HR: 18.00; 95% CI: 5.78-56.05; P<0.001) and was validated in 4 independent published microarray data (total 356 stage IB to II patients without adjuvant treatment), with HR ranging from 1.96 to 3.57 (32). This was more recently further validated in another independent cohort (HR: 1.92; 95% CI: 1.15-3.23; P= 0.012) (33). More importantly, when the signature was applied to JBR.10 patients who received adjuvant chemotherapy, the "high risk" patients demonstrated improved survival (HR: 0.33; 95% CI: 0.17-0.63; P<0.001), whereas low-risk patients did not (HR: 3.67; 95% CI: 1.22-11.06; P=0.013; interaction P<0.001). The predictiveness of the signature was validated by quantitative polymerase chain reaction (qPCR) in 30 JBR.10 patients (19 with surgery only, 11 with adjuvant chemotherapy) who did not have their tumor samples examined by microarray. However, the predictiveness of the signature has not been independently validated, as there are no microarray data sets available from other randomized adjuvant chemotherapy trials for testing. Furthermore, the validation and application of this signature in FFPE samples remain to be demonstrated.

A 14-gene expression was developed using qPCR directly on DNA isolated from FFPE tumor samples of 361 non-squamous NSCLC patients resected at the University of California, San Francisco (UCSF, *Table 2*) (34). The assay was then independently validated in a masked cohort of 433 patients with stage I non-squamous NSCLC resected at Kaiser Permanente Division of Research (KPDOR), and on a cohort of 1006 patients with stage I-III non-squamous NSCLC resected in several leading cancer centers that are part of the China Clinical Trials Consortium (CCTC). The signature reported a 5-year overall survival of 71.4% (95% CI: 60.5-80.0) in low-risk, 58.3% (95% CI: 48.9-66.6) in intermediate-risk, and 49.2% (95% CI: 42.2-55.8) in high-risk patients (P_{trend}=0.0003) at KPDOR. Similar analysis of the CCTC cohort indicated 5-year overall survivals of 74.1% (95% CI: 66.0-80.6) in low-risk, 57.4% (95% CI: 48.3-65.5) in intermediate-risk, and 44.6% (95% CI: 40.2-48.9) in high risk patients (Ptrend<0.0001). Multivariate analysis in both cohorts indicated that no standard clinical risk factors could account for, or provide the prognostic information derived from tumor gene expression. As the signature was developed and tested using qPCR in FFPE samples, its transfer to clinical testing was facilitated and it is already commercially available as the Pervenio Lung RS Test (Life Technologies, Inc, Grand Island, NY). In addition, the assay recently showed prognostic value for small <2-cm node-negative stage IA patients. In this subset of patients, similar to those likely to be identified in emerging computed tomography screening programs for lung cancer, the assay identified in pathologically confirmed stage IA patients, ~25% of patients who had a survival of ~50% versus a >90% survival for low risk patients (39). Importantly, the signature was equally prognostic in patients who did (HR: 2.31; 95% CI: 1.29-4.24) or did not (HR: 2.42; 95% CI: 1.88-3.11) receive adjuvant chemotherapy, suggesting it is primarily a prognostic marker (34). However, to test the predictive value of this assay, a large 1500-patient prospective stage III global trial is now underway to randomize Pervenio Lung RS Test identified "high-risk" stage I patients to receive adjuvant cisplatin based adjuvant chemotherapy versus observation (current standard of care) (40).

The ChipDx is claimed by the author as an "online gene expression based diagnostic system, the creation and delivery of clinically-useful diagnostic and prognostic oncology assays". It published two signatures (35), one is a prognostic signature with 160 genes, identified from 332 stage I-III NSCLC from the Directors' Challenge Consortium cohort (DCC, total n=442) and tested in 264 stage I-II NSCLC, compiling from subsets of 5 NSCLC cohorts [JBR10, total n=133; Duke, total n=89; a data set from the Harvard University (Harvard), total n=139, and a data set from Nagoya University (Nagoya_A), total n=163, *Table 2*] (35). The other is a predictive signature made up of 37 genes, identified from 88 stage I-III NSCLC patients treated with adjuvant chemo- or/and radio-therapy in the DCC cohort, and tested in 109 stage I-II NSCLC from JBR.10 (32,41). The 160-gene prognostic signature was able to stratify 90 high risk patients with significant poorer survival (HR: 2.80; 95% CI: 1.83-4.28, P<0.0001) after adjustment for other prognostic factors. The 37-gene predictive signature was able to separate 70 responders from the other 39 non-responders in the test set. Among the 70 responders, the adjuvant chemotherapy significantly increased survival (HR: 0.23; 95% CI: 0.08-0.61, P=0.0032)

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Table 2 Clinically applicable multigene signature

Signature [year]	Tumor histology	Number of genes in signature	Risk groups	Prognostic	Predictive for ACT	Training set	Test sets	HR in testing set	HR for high risk	FFPE ready
Zhu [2010] (32)	NSCLC	15	Median dichotomized	Yes	Yes	BR10	DCC Duke UM-SQ NLCI	1.96-3.57	0.54	No
Kratz [2012] (34)	Non-Squamous NSCLC	14	Tertile	Yes	NT	UCSF	KPDOR CCTC	1.60-2.37	NA	Yes
Van Laar [2012] (35)	NSCLC	160	< or >60%	Yes	NT	DCC	BR10 Harvard Duke Nagoya_A	2.02-2.23	NA	No
	NSCLC	37	< or >60%	No	Yes	DCC	BR10	NA	0.23	No
Chen [2011] (36)	NSCLC	94	Median dichotomized	Yes	Yes	MSKCC	DCC BR10 Nagoya_B	2.10-2.57	0.48	No
Tang [2013] (37)	Non-Squamous NSCLC	12	Median dichotomized	Yes	Yes	DCC	UTSW Nagoya_A Nagoya_B Samsung Duke UM-SQ BR10	1.55-3.19	0.36	No
Wistuba [2013] (38)	ADC	31	Median dichotomized	Yes	NT	96 prostate Cancer samples	DCC NCCHJ MDACC &IEO	1.95	NA	Yes

BR10, NCIC Clinical Trials BR.10 (GSE14814); DCC, Directors' Challenge Consortium (data at https://array.nci.nih.gov/caarray/project/jacob-00182); Duke, Duke University (GSE3141), UM-SQ, University of Michigan squamous cell carcinoma (GSE4573); NLCI, Netherlands Cancer Institute (data at http://research.agendia.com/); UCSF, University of California, San Francisco; KPDOR, Kaiser Permanente Division of Research; CCTC, China Clinical Trials Consortium; Harvard, Harvard University (data at http://www.broadinstitute.org/mpr/lung/); Nagoya_A, Nagoya University (GSE11969); Nagoya_B, Nagoya University (GSE13213); Samsung, Samsung Medical Centre (GSE8894); MSKCC, Memorial Sloan Kettering Cancer Centre (GSE10780); UTSW, University of Texas South Western (GSE42127); NCCHJ, National Cancer Center Hospital of Japan (GSE31210); MDACC, MD Aderson cancer Center; IEO, European Institute of Oncology; HR, Hazard Ratio.

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after the adjustment of age, gender, stage and histology whereas in the 39 non-responder, no significant difference in survival by adjuvant chemotherapy was observed (HR: 0.55; 95% CI: 0.15-2.04, P=0.38). However, there was no report on the interaction term.

The malignancy-risk gene signature was originally developed for breast cancer and contained a large number of proliferative genes (36,42). The investigators tested their signature in the DCC (31), another data set from Nagoya University (Nagoya_B, n=117) (43) and JBR.10 (32) datasets (*Table 2*). As the signature genes were identified by Affymetrix U133A platform and testing was performed on data obtained using the Agilent platform, cross-platform mapping was used to identify one hundred and sixteen probe sets to represent 87 genes for the validation. The malignancy risk score was the summed products of gene expressions and their weights in the first component, then was median dichotomized to define high and low risk groups, as they were used in the breast cancer. The signature was able to classify NSCLC patients without adjuvant chemotherapy with significant difference in survival (HR: 2.10; 95% CI: 1.26-3.51, $P_{log\text{-}rank}$=0.004 in DCC, HR: 2.17; 95% CI: 1.22-3.68, $P_{log\text{-}rank}$=0.007 in Nagoya_B, and HR: 2.57; 95% CI: 1.17-5.64, $P_{log\text{-}rank}$=0.01 in JBR.10). Furthermore, in the high risk group in JBR.10, the authors observed a significant improvement in survival by adjuvant chemotherapy (HR: 0.48; 95% CI: 0.24-0.96, $P_{log\text{-}rank}$=0.03). In contrast, adjuvant chemotherapy non-significantly decreased patients' survival in the low risk group. Nevertheless, the interaction between risk group and adjuvant chemotherapy was significant ($P_{interaction}$=0.02) indicated that adjuvant chemotherapy might benefit high risk group but not the low risk group.

The University of Texas South Western (UTSW) 12-gene signature (37) was derived from the DCC data set (31). The investigators first identified 797 genes that were univariately associated with patients' 5-year overall survival and then through a partial correlation matrix to obtained 18-hub genes. The 18-hub genes was further trimmed down to a 12-gene signature by incorporating data from synthetical lethality study with paclitaxel and genetic aberrations in Tumorscape. The signature was validated in silico in 5 independent cohorts, UTSW (37), Duke (44), Samsung Medical Center (45), Nagoya_A (43), Nagoya_B (46) but not in squamous cell carcinoma. Additionally, the 12-gene signature was tested in 2 cohorts of NSCLC with adjuvant chemotherapy: UTSW (n=176 NSCLC) (37) and the JBR.10 (n=90, NSCLC) (32). Adjuvant chemotherapy appeared to prolong survival only in the high risk group (HR: 0.34; 95% CI: 0.13-0.86; P=0.017 for the UTSW and HR: 0.36; 95% CI: 0.13-0.97, P=0.038 for the JBR.10) but not in low risk groups (37).

The cell cycle proliferation (CCP) score (https://myriadpro.com/lung-cancer/myriad-myplan-lung-cancer/) was originally derived from FFPE samples of prostate cancer by RT-qPCR (47). The investigators utilized 96 commercially available prostate cancer samples to select signature from 126 cell cycle related genes. Thirty-one genes were selected as a CCP signature based on their correlation with the mean expression of the entire 126 genes (47). Wistuba *et al.* (38) validated the CCP (31-gene) in 3 lung ADC cohorts: DCC (HR: 2.02; 95% CI: 1.29-3.17, P=0.0022, n=442, profiled with Affymetrix U133A, *Table 2*) (31), data set from the National Cancer Center Hospital of Japan (NCCHJ, HR: 2.16; 95% CI: 1.32-3.53, P=0.0026, n=226 profiled with U133 plus2, *Table 2*) (48), and a jointed cohort of a total of 381 FFPE NSCLC patient samples from MD Anderson Cancer Center (MDACC, n=207) and European Institute of Oncology (IEO, n=174) (HR: 1.92; 95% CI: 1.18-3.10, *Table 2*) by qPCR, after adjustments for other prognostic factors (38).

Other molecular prognostic signatures

As mentioned previously, extensive analysis to date has not established the significant prognostic value of *KRAS* or *p53* mutation. Interestingly, several studies have consistently demonstrated that epidermal growth factor receptor (EGFR) tyrosine kinase mutation is a good prognostic marker for both early and advanced-stage patients (49-52). This may potentially account for the generally better prognosis of Asian NSCLC patients. However, a recent large study in early-stage NSCLC patients did not show an independent prognostic value of EGFR mutation in Asian (Korean) patients (53). There are as yet no gene copy changes (e.g., amplification) that have been reported as showing prognostic value. In contrast, many investigators have recently reported the prognostic significance of microRNA (miRNA) or its signatures in NSCLC patients (54-58). These studies remain preliminary, as extensive independent validations to the scale of mRNA signatures have not been performed. The miRNA as a prognostic marker is highly attractive for two reasons: (I) there are less miRNA species and single miRNA may control the expression or function of multiple genes, thus, they are more likely to function as master regulatory elements in gene function, and (II) miRNA assay can easily be

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performed on FFPE samples, as they are of short sequences and are more stable.

Future outlook

During the past decade, we have witnessed the rapid translation of advances in the molecular understanding of lung cancer into clinics, as in the development of targeted therapies and the use of molecular markers to select patients for such treatment. Testing for EGFR mutations and anaplastic lymphoma kinase (ALK) gene rearrangement is now becoming standard for personalizing therapies in advanced NSCLC patients. With the current pace of advances being witnessed, it is almost certain that molecular prognostication would one day be integrated into standard pathologic diagnosis to improve the management, treatment, and survival of early-stage NSCLC patients, just as it has become standard in other solid organ cancers such as breast cancer and colon cancer. Successful practice in this field is the incorporation of molecular markers into the histological classification system of lung cancers (59).

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Integrative oncology drug discovery accompanied by preclinical translational research as prerequisite for clinical development

Jens Hoffmann

EPO Experimentelle Pharmakologie and Onkologie Berlin-Buch GmbH, Berlin, Germany

Correspondence to: Jens Hoffmann, PhD. Experimentelle Pharmakologie & Onkologie Berlin-Buch GmbH, Robert Rössle-Strasse 10, 13125 Berlin, Germany. Email: jens.hoffmann@epo-berlin.com.

Abstract: The molecular heterogeneity of cancer calls for individualized therapies to become the standard of care. It is now generally accepted that target-specific compounds require specific new development programs. But, even for new drugs with general mode of action (i.e., chemotherapy), tailored treatment approaches, such as specific schedules or combinations, have been shown to improve the therapeutic outcome. Therefore, the preclinical development of new therapeutic agents needs, next to the "classical pharmacodynamic studies", the implementation of integrative translational research (TR) as early as possible. New TR approaches, starting already at target identification and validation (TIV) will allow to defining the optimal patient population for clinical development, to tailor individual treatment of the tumor disease and to choose a rational basis among the manifold options for treatment combinations. We will discuss several examples from TR studies, which have initially been started to evaluate the molecular mode of action and to recognize mechanisms which can lead to resistance. Research was extended later to identify predictive response biomarkers and establish a rationale for combination with different therapies. A detailed gene expression analysis of lung cancer cells and apoptotic pathway interference studies in colon cancer cells provided insight in the molecular mechanisms of action. These new findings are correlated with results from other studies performed during the preclinical development program. We discuss pros and cons, successes and failures of our integrative preclinical development program and provide recommendations for future oncology projects.

Keywords: Integrative drug discovery; preclinical translational cancer research; *in vitro*/*in vivo* tumor models; RNAi drug modifier screen

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Recent changes in oncology drug discovery and drug development

Looking back on more than 60 years of drug development for cancer therapy, almost in parallel with the new millennium, processes have changed substantially. This has been driven by increasing costs for the clinical development in contrast to often disappointing improvements for the patients. For more than 50 years, new cancer drugs were characterized in a handful of lowly predictive preclinical tumor models—and all further development work and risks were left to clinicians and patients. Growing insight into the fundamental genetic basics of the disease through analysis of gene expression and mutations and the development of fascinating new technologies in genetic engineering and bioinformatics—key word systems biology—have provided the technical basis for this paradigm shift.

As consequence, primary pharmacology processes in preclinical cancer research have changed (*Figure 1*). Elementary task is the establishment of the right model and access to appropriate tools for each step of the drug discovery process.

Target identification and validation (TIV) process

Before the introduction of target-specific drug discovery, research was driven primarily by phenotypic screening.

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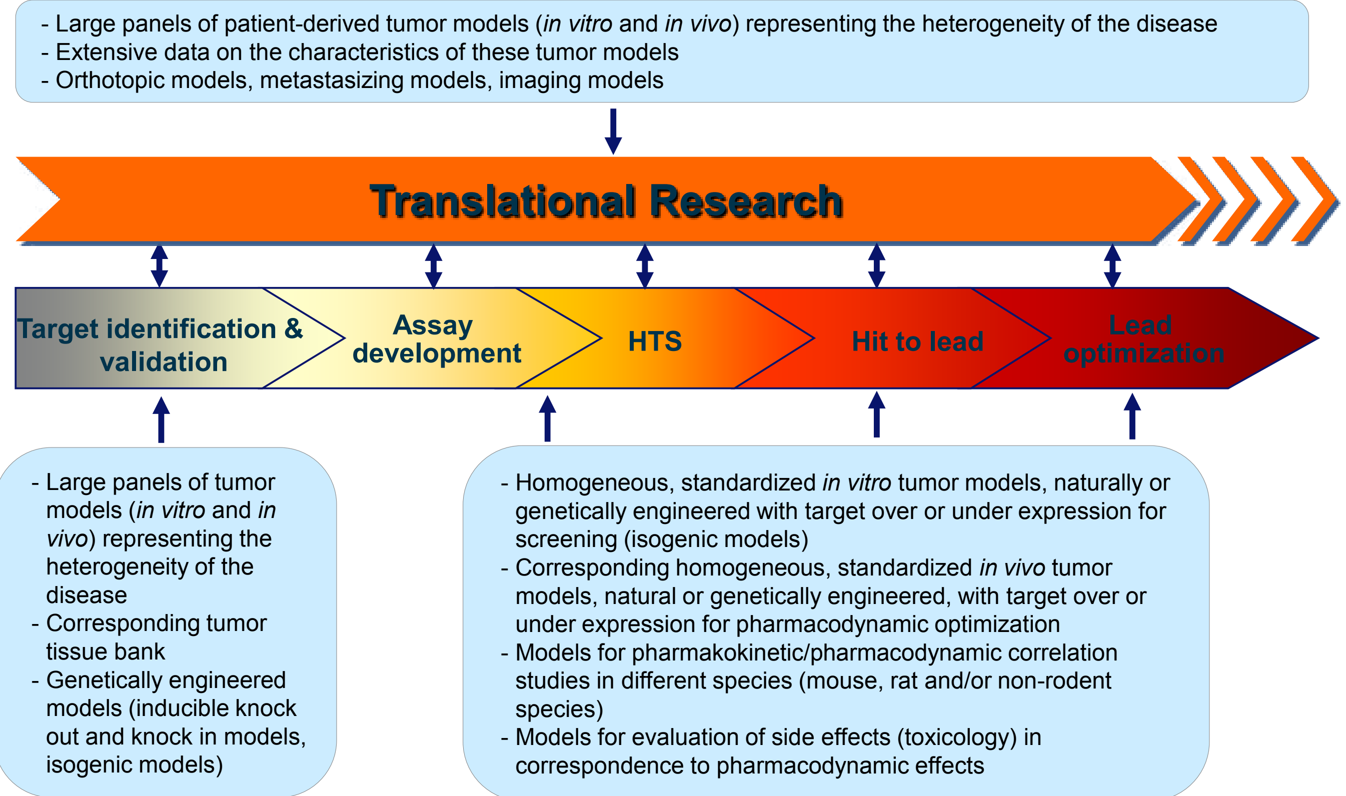

Figure 1 New primary pharmacology processes and models in preclinical cancer research. HTS, high-throughput screen.

However especially in cancer research, the limited knowledge of the molecular mechanisms of disease turned out to be a major disadvantage of the phenotypic screening. The introduction of new technologies to identify targets either in a high throughput setting (i.e., synthetic lethal screens with RNA interference) or by new sequencing techniques, allowing the identification of low frequency disease relevant genetic aberrations, resulted in a tremendous progress and the identification of large numbers of potential targets.

These target-focused approaches provide a specific biological hypothesis which can also be defined as molecular mechanism of action (1). The current challenge is the validation of the hypothesis, especially demonstrating that the specific molecular mechanism is relevant to the disease pathogenesis in a certain population and has a sufficient therapeutic index in the context of the physiological response.

These changes in TIV have also changed the request on the disease models. Have been a handful extensively characterized tumor cell cultures and mouse models been the standard for many decades, the target driven approaches now require models reflecting better the clinical situation (*Figure 2*).

The requirements on new models include among others:

- large panels of tumor models (*in vitro* and *in vivo*) representing the heterogeneity of the disease;
- extensive data about the characteristics of these tumor models (gene and protein expression, gene amplifications, mutations, epigenetics, miRNA expression, histology, reference drug sensitivity);
- corresponding databases containing all these informations and tools allowing bioinformatic analyses;
- tumor tissue banks (frozen and paraffin embedded tissue, tissue micro arrays);
- genetically engineered models (inducible knock out and knock in models, isogenic models).

The target driven drug discovery further requires the definition of strong criteria for the acceptance of the target. The advantage is, that the validation can be supported by first *in vivo* experiments using molecular and chemical knowledge, applying both small-molecule based strategies (selected compounds from available libraries)

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"Models – reflecting better the clinics"

Development of relevant tumor model sytems that are closer to the clinical situation

Common approach

- Screening of established human cancer cell lines
- well characterized systems, used for *in vitro* and *in vivo* experiments
- cultivated long-time as mono-layer on plastic dishes, loss of their cancer "*in vivo*" phenotype
- limited number of models

New approach Phase I: *in vitro* experiments

- Establish primary cell culture models from patient tumors (i.e., 2D or 3D cultures)
- Extensive molecular characterization
- Sensitivity screening with drug libraries
- Prediction (*in vitro* sensitivity and resistance)

New approach Phase II: *in vivo* experiments

- Establish patient derived xenograft models on immunodeficient mice (PDX)
- Validation of molecular characteristics
- Drug testing in predicted models Responder/ Nonresponder (preclinical Phase II)
- Systems biological analysis and modelling

Figure 2 Comparison of tumor models in research & development (R&D).

and biologicals based approaches, such as individually engineered antibodies.

An important part of the preclinical target validation, next to the molecular mechanism of action, is to investigate possible resistance mechanisms, predictors of response, the identification of rational targets for combinations, and further to analyze the physiological mechanism of action.

As one example, we employed the RNAi screening technology, to determine the modifying effects of reduced gene expression on drug activity (2).

To analyze the mechanisms of mitotic arrest induced by targeting microtubules with a new type of microtubule stabilizer (MTS) and to identify additional targets and biomarkers, a siRNA-based RNAi drug modifier screen was performed in four cancer cell lines. The knockdown of more than 300 genes (900 siRNAs) implicated in cell cycle control, apoptosis, chromosomal instability and taxane-resistance was combined with MTS treatment in a high-throughput RNAi drug modifier screen in three breast cancer cell lines MCF7, T47D and MDA-MB435s and, for comparison, the A549 lung cancer cell line.

Defects of the spindle assembly checkpoint (SAC) were identified to cause resistance against drug-induced mitotic arrest and apoptosis. The strongest suppressor effects were observed for the knockdown of components of the SAC (*Figure 3A*). Knockdown of BUB1B, BUB1 and TTK (MPS1) components of the mitotic checkpoint complex, reduced mitotic arrest in MCF7 and A549 cells but had little or no effect on T47D and MDA-MB435s cells. Potential biomarkers for resistance are SAC-defects like mutations in the central SAC-kinase BUB1B.

Chromosomal heterogeneity and polyploidy are also potential biomarkers of resistance since they imply an increased tolerance for aberrant mitosis. RNAi screening showed yet again that the drug is not a substrate of ABC-transporters (2).

The RNAi drug modifier screen demonstrated that the drug-induced mitotic arrest can be enhanced by concomitant inhibition of mitotic kinesins, thus suggesting a potential combination therapy with a KIF2C (MCAK) kinesin inhibitor (*Figure 3B*). However, the combination of the drug and inhibition of the prophase kinesin KIF11 (Eg5) is antagonistic, indicating that the kinesin inhibitor has to be highly specific to bring about the required therapeutic benefit.

Screening results have been validated in single experiments confirming, that the knockdown of BUB1B or CENPE reduced MTS-induced mitotic arrest in all four cell lines whereas KIF2C knockdown enhanced MTS-induced mitotic arrest. In contrast, a significant reduction of MTS-induced aneuploidy without concomitant increase in G2/M-arrest was seen for KIF11 knockdown in all four cell lines.

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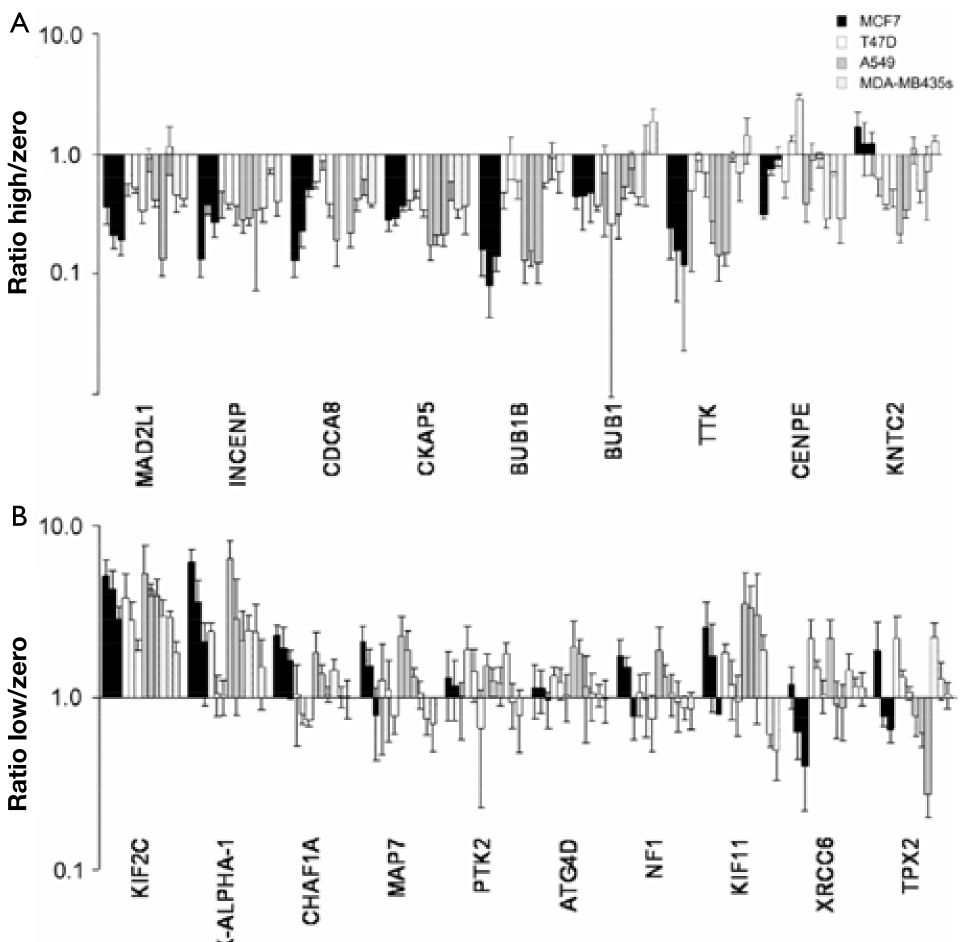

Figure 3 Top modifiers in RNAi MTS modifier screen. The modifier effect of RNAi knockdown on MTS-induced mitotic arrest was analyzed for over 300 genes in MCF7, T47D, A549 and MDA-MB435s cell lines with three different siRNAs per gene. Controls and transfected cells were treated with vehicle, low dose and high dose MTS. Graphical presentation of ratio of means treated *vs.* untreated (ratio >1, enhancement of MTS effects; ratio <1, suppression of MTS effects). (A) Strongest suppressor effects (presented high-dose treatment *vs.* untreated); (B) strongest sensitizer effects (presented low-dose treatment *vs.* untreated). Both panels ranked according to strength of modifier effect. MTS, microtubule stabilizer.

To estimate cell survival, a survival index was calculated as the ratio of remaining cell number after MTS treatment divided by initial number of cells numbers. Survival indices were found to be increased for BUB1B knockdown in all four cell lines and for CENPE knockdown in T47D and SKBR3 but decreased for KIF2C knockdown in MCF7 and A549 (2).

As one example how available small molecules can be involved in the target validation, we have elucidated the influence of KIF11 on the induction of aneuploid cells after MTS treatment by comparing the RNAi-mediated knockdown of KIF11 with the effect of ispinesib treatment, a small molecule inhibitor of KIF11 (3). Similar to the RNAi knockdown of KIF11, ispinesib significantly reduced the MTS-induced aneuploidy without increasing mitotic arrest (*Figure 4A*). The combination of MTS and ispinesib had antagonistic effects in proliferation assays (*Figure 4B*). Both KIF11 knockdown and KIF11 inhibition caused typical monoasters (*Figure 4C,D*). Thus, interference with spindle assembly by KIF11 inhibition specifically antagonizes the MTS-induced aneuploidy but not the MTS-induced mitotic arrest.

To conclude, 1 out of the 300 RNAi-targeted genes had a sensitizing effect on MTS in all four cell lines in the screen,

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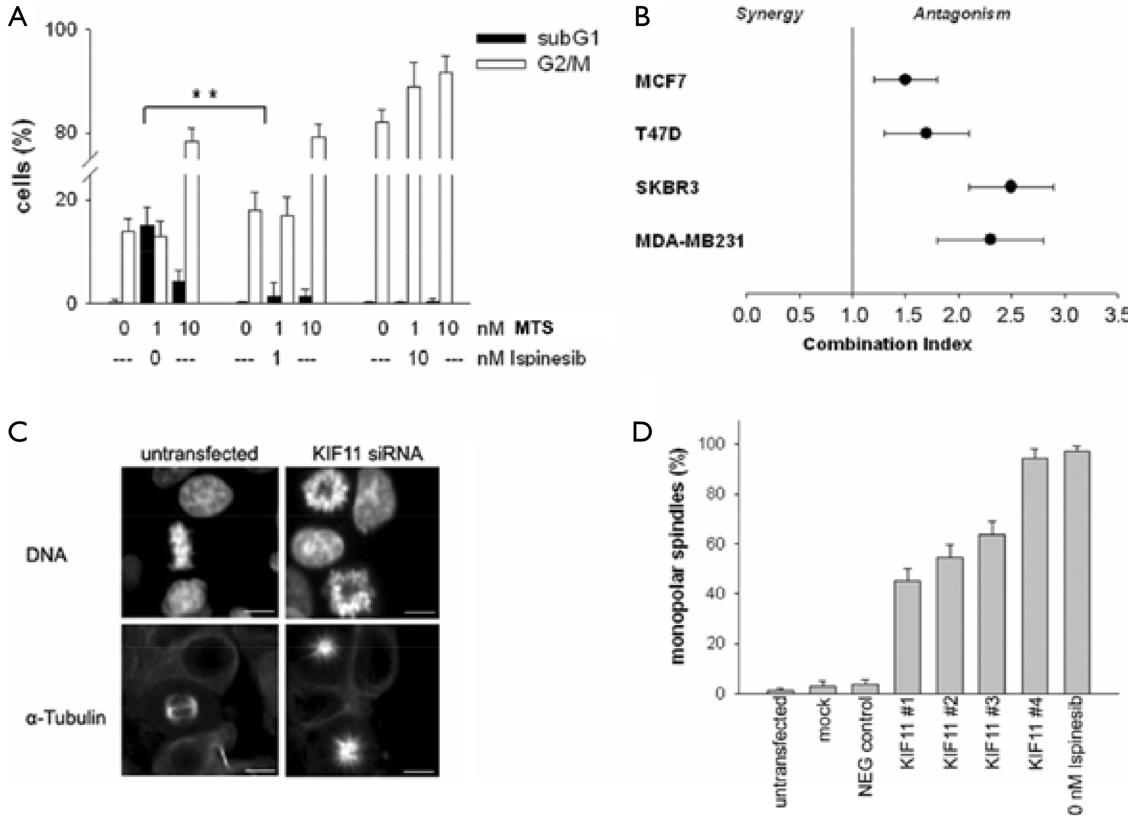

Figure 4 Antagonistic combination of microtubule stabilizer (MTS) and ispinesib. (A) Quantification of subG1 and G2/M cells by FACS analysis of propidium iodide (PI)-stained cells; (B) combination of MTS and ispinesib in proliferation assay. Calculation of combination index (CI) according to Chou (4); (C) induction of monoasters by KIF11-knockdown. Immunofluorescence staining with Hoechst33342 (a) and α-Tubulin-FITC (b). Scale bar =10 µm; (D) quantification of monoaster induction by KIF11-knockdown or KIF11 inhibition with ispinesib. Manual count, means and standard deviations from triplicate experiments.

and 6 out of the 300 RNAi-targeted genes had a sensitizing effect on MTS in at least two cell lines. On the other hand, 5 out of the 300 RNAi-targeted genes had an antagonistic effect on MTS in all four cell lines in the screen, and eleven out of the 300 RNAi-targeted genes had an antagonistic effect on MTS in at least two cell lines. Validation studies were able to confirm modifier effects for four genes. The study also strongly demonstrates that a panel of heterogenous cell lines needs to be included in these types of assays, as results can be diametral from one cell line to another.

Lead identification and optimization (LO) process

The LO is more or less identical with the classical drug development process. The process will be adapted on the validated targets and includes assay and model development, followed by a screening phase of selected compound, peptide, antibody, or RNAi libraries to identify a lead structure (*Figure 1*). Once a lead structure has been identified, optimization processes are started, frequently in parallel for several leads.

As the most difficult part of the targeted drug development, this part can be seen as an extended lead and target discovery phase, addressing the molecular mechanism of action in correlation to optimal pharmacodynamic activity (physiological mechanism of action), optimal pharmacokinetics (PK) [absorption-distribution-metabolism-excretion (ADME)], toxicity, as well as resistance development.

A large number of functions are now involved in this integrated preclinical drug development (IPDD, *Figure 5*), including functions like medicinal and protein

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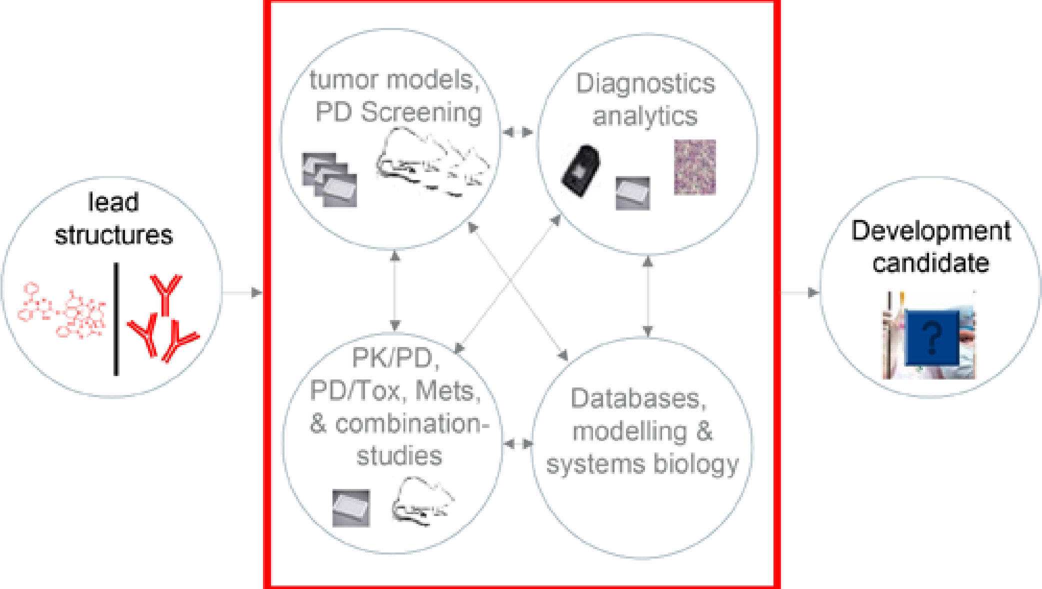

Figure 5 Integrative lead optimization processes involving preclinical pharmacology, PK, Tox, molecular diagnostics, and bioinformatics supports new drug development projects in oncology and provide rational strategies for the selection of clinical development candidates. PK, pharmacokinetics; Tox, toxicology; PD, pharmacodynamics; Mets, Metastases.

chemistry, cell and structural biology, pharmacology, PK and early toxicology (Tox). Data from the screening, now implemented in large data bases, will be further used for computational modelling.

A broad panel of lead optimization tasks and criteria for oncology drug development has been established, which should address:

Predictive pharmacology:

- Demonstrate the extent of target inhibition in correlation to pharmacological effects (i.e., inhibition of tumor growth, -blood flow, -metabolism);
- Identification of main indications [primary tumors, metastases (Mets)];
- Biomarker identification & validation with preclinical models (i.e., by comparison of gene expression profiles from primary tumors);
- Drug sensitivity modifiers screen [i.e., high-throughput screen (HTS) proliferation assays or siRNA technology];
- Combination studies in tumor models;

Resistance:

- Target of drug transporters (ABC transporters), cellular uptake and intracellular distribution;
- Gene regulation by the drug in sensitive and resistant models;
- Mechanisms of apoptosis, mitotic catastrophe and immunomodulation;

Toxicity/PK/imaging:

- Modulation of adverse effects;
- Questions of PK/pharmacodynamics (PD) modeling, scheduling;
- Imaging of response;

Similar to the TIV process, increased demands on the lead optimization have changed the requests on the disease models. The target driven approaches now require models with defined levels of target expression which will be mainly generated by genetic modifications and cloning:

- Homogeneous, standardized *in vitro* tumor models, naturally or genetically engineered with target over- or under-expression for screening (isogenic models), models for classical drug resistance;
- Homogeneous, standardized *in vivo* tumor models, natural or genetically engineered with target over- or under-expression for pharmacodynamic optimization (transgenic mice);
- Models for pharmakokinetic/pharmacodynamic correlation studies in different species (mouse, rat and/ or non-rodent species) models for evaluation of side effects (Tox) in correspondence to pharmacodynamic effects.

For example, several studies, performed during the

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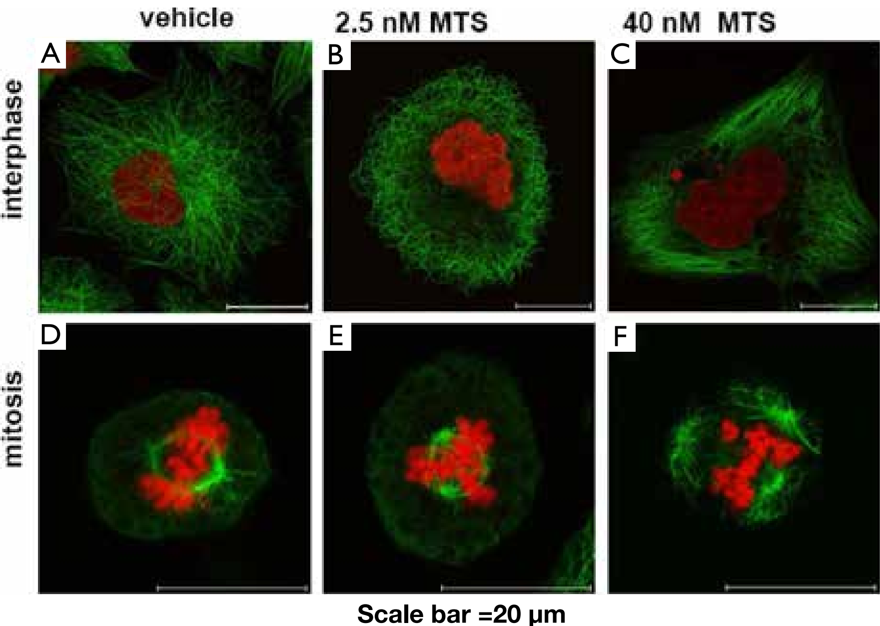

Figure 6 Effect of MTS on tubulin cytoskeleton of lung cancer cells. Immunofluorescence staining of α-tubulin (green) and DNA (red) in A549 lung cancer cells after incubation with either vehicle (0.1% ethanol), 2.5 nM, or 40 nM MTS. Scale bar =20 μm. Representative pictures of interphase and mitotic cells are shown. MTS, microtubule stabilizer.

development of the already mentioned new MTS, will be discussed. Microtubules are considered as important target for cancer treatments because disruption of microtubule dynamics interferes with cell functions and mitosis, leading ultimately to a G2/M arrest and apoptosis, and several microtubule stabilizing taxane derivatives have been developed as anti-cancer drugs (5). To overcome limitations associated with the established drugs, compounds from different structural classes have been synthesized and tested for activity (6). Extensive preclinical *in vitro* studies have been set up to demonstrate improved target activity for these new compounds (7).

A defined panel of tumor cell lines (sensitive and multi-drug resistant) was tested in comparison to the available standard (paclitaxel) and found to be strongly sensitive to the new MTS with only moderately variations in response (IC50 between 0.3 and 5.5 nM) (7). So far, no natural resistant cell line was identified and even treatment for more than one year with the new MTS did not result in development of resistance (unpublished own results).

Further mechanistical investigations in tumor cell lines demonstrated, that the new MTS induces a more rapid and potent tubulin polymerization than paclitaxel. A rapid and effective influx into cells, combined with the evasion of P-glycoprotein efflux pumps, have been identified as key qualities resulting in consistently more potent activity than microtubule-stabilizing taxanes (8). However, in line with other MTSs, it causes mitotic arrest, followed by activation of the mitochondrial apoptotic pathway. Profiling of the pro-apoptotic signal transduction pathway using a panel of small interfering RNAs revealed that it acts in a fashion comparable to paclitaxel. In HCT-116 colon cancer cells, the MTS induced apoptosis was partially antagonized by the knockdown of pro-apoptotic members of the Bcl-2 family, including Bax, Bak and Puma, whereas knockdown of Bcl-2, Bcl-X_L or Chk1 sensitized cells to cell death (8).

Further mechanistic studies in lung cancer cells (9) revealed a concentration-dependent disturbance of cellular organization with two apparent phenotypes. At low concentrations, an aneuploid phenotype occurred, whereas the classical "mitotic arrest" phenotype was induced only at higher concentrations (*Figure 6*). Interestingly, the treatment with low doses effectively inhibited cell proliferation, but—compared to high concentrations—induced apoptosis only marginally. Analysis of differential gene expression in tumor cells treated either with high and low drug concentration demonstrated a non-overlapping set of regulated genes:

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High dose investigational drug	Low dose investigational drug
• up-regulation of genes which are involved in G2/M phase transition and mitosis: cyclin B, cyclin A, Bub1, Aurora A, Aurora B → Mitotic arrest and induction of mitochondrial apoptosis, mediated by members of the Bcl-2 family proteins	• up-regulation of genes which are downstream targets of p53 and $p21^{CIP}$ • p53 upregulation arrests cell cycle and allows repair processes to take place → p53 upregulation reduces apoptosis and may be involved in development of resistance

Figure 7 Dose dependent differential gene regulation in lung cancer cells results in diverse molecular response. Up-regulation of TP53 and its downstream effectors by low concentrations of microtubule stabilizer (MTS) is responsible for the relative apoptosis resistance of A549 lung cancer cells and might represent a new mechanism of resistance. A different phenotype appears to be induced at higher MTS concentrations, with progressively more perturbed microtubule dynamics, formation of microtubule bundles and activation of the spindle assembly checkpoint (SAC) leading to an arrest in mitosis and induction of mitochondrial apoptosis, mediated by members of the Bcl-2 family proteins.

Genes involved in G2/M phase transition and the SAC, like *cyclin B1* and *bub1b* were up-regulated by treatment with high dose MTS. In contrast, treatment with the low concentration revealed an up-regulation of direct transcriptional target genes of TP53, like *cdkn1a*, *mdm2*, *gadd45a* and *fas*. This resembles an activation pattern which is caused in response to mild, repairable damage, and induces cell cycle arrest, rather than strong damages which promote apoptosis. This allows repair processes to take place and the cells to survive. Knockdown of TP53 led to a significant increase in apoptosis induction (9).

These mechanistic data confirmed, that up-regulation of TP53 and its downstream effectors by low concentrations of MTS is responsible for the relative apoptosis resistance of A549 lung cancer cells and might represent a new mechanism of resistance (*Figure* 7).

A different phenotype appears to be induced at higher MTS concentrations, with progressively more perturbed microtubule dynamics, formation of microtubule bundles and activation of the SAC leading to an arrest in mitosis. Mainly, this result in an induction of mitochondrial apoptosis, mediated by members of the Bcl-2 family proteins, and is substantially similar to that seen with paclitaxel and other epothilones (8). But, mitotically arrested cells may also undergo aberrant mitosis or mitotic slippage and endo-reduplication. The variations in the extent of apoptosis among breast cancer cells after MTS treatment could be explained by differences in the apoptotic signalling rather than by differences in mitotic arrest.

Translational research (TR) process

TR in oncology from the perspective of the drug developer should provide the simple answer: "who is the right patient for my new drug", whereas the oncologist is interested in: "which is the right drug for my patient". This means that in the later stages of cancer drug development and in the management of patients with cancer, "predictive biomarkers" are urgently needed which can be used to identify optimal target populations of patients; predict the efficacy of the drug and patient's response, resistance and toxicity; and rapidly distinguish between non-responders and patients who respond to therapeutic intervention (10).

The U.S. Food and Drug Administration (FDA)'s Center for Drug Evaluation and Research (CDER) has provided a guidance document on the qualification process for biomarker (titled "Draft Guidance for Industry: Qualification Process for Drug Development Tools"). Requirements set in this document make clear, that the qualification process for a biomarker has many parallels to drug discovery and development, starting with biomarker identification and validation, followed by assay development and optimization, and finally followed by validation in clinical trials. In the preclinical oncology research departments from most pharmaceutical and biotech companies, the TR has now become an integrative

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part of the development. Considering the heterogeneity of cancer, it has become clear that this research requires new approaches.

As TR needs:

- large panels of patient-derived tumor models (*in vitro* and *in vivo*) representing the heterogeneity of the disease;
- extensive data on the characteristics of these tumor models (gene and protein expression, gene amplifications, mutations, epigenetics, miRNA expression, histology, reference drug sensitivity, and corresponding databases containing all this information and tools allowing bioinformatic analyses);
- orthotopic models, metastasizing models, imaging models.

This type of research is now frequently performed in academia-industry partnership.

During the development of our previously mentioned MTS, we have addressed the questions for a predictive biomarker in lung cancer patients with a new type of preclinical study. This was based on the observation, that interestingly, some tumor models, i.e., the NCI-H460 lung cancer cells, which are highly sensitive to MTS in cell culture, developed treatment resistant tumors on nude mice (unpublished own results). Human tumors accumulate genetic and molecular abnormalities, leading to broad heterogeneity. Large panels of tumor models reflecting tumor heterogeneity might have increased value for predicting the response to new therapeutic agents in the clinic. Consequently, it is important to use a large panel of clinically relevant tumor models for translational studies. However, from the *in vitro* studies with 20 breast cancer cell lines and in more than 30 other cell lines, we have not been able to identify natural resistance mechanisms to MTS. This led us to work with extended panels of *in vivo* models.

To address this discrepancy between *in vitro* and *in vivo* activity, further studies across a panel of human lung cancer xenograft models were performed (*Figure 8A*). In this heterogeneous panel response to MTS—treatment was determined in an integrative preclinical phase II design—further resistant tumors were identified (*Figure 8B*). We have observed 64% overall responses [response analysis according to the response evaluation criteria in solid tumors (RECIST) clinical trial criteria] with MTS in the 22 non-small cell lung cancer (NSCLC) xenograft models (11). Genome-wide gene expression and mutational analysis were used to identify predictive markers for response and to explore the mechanism of MTS's anti-tumor activity *in vivo*. Tumors with wild-type TP53 as well as high expression of genes involved in cell adhesion, hypoxia or angiogenesis were more likely to be resistant to MTS treatment (11). For validation, combination experiments were performed with drugs or siRNA is, targeting some of the identified resistance mechanisms, i.e., tumor angiogenesis, hypoxia or TP53. Indeed, when combined with MTS treatment, combination therapy resulted in restored anti-tumor activity in resistant tumor models [(9,11) , unpublished own results)].

Hypoxia triggers pathways that drive angiogenesis and tumor progression, and the presence of genes associated with these pathways has previously been associated with a negative prognosis and resistance to therapy (12). Up-regulation of CA9 and CA12 gene expression, in particular, has been detected in a large number of common malignancies and is implicated in tumor development (13). The data presented in the NSCLC study show that the combination of MTS with an inhibitor of angiogenesis such as bevacizumab or sorafenib results in an enhanced antitumor effect in tumor models with an activated HIF1a/hypoxia pathway (11). No correlations were found between MTS activity and overexpression or mutations of *egfr* and *k-ras* genes suggesting that MTS may be active in patients with NSCLC tumors with these changes (*Table 1*).

In our NSCLC xenograft study, response to MTS correlated with low expression or expression of mutant TP53 (*Table 1*). In cell culture studies, we performed earlier, treatment of A549 cells with low concentrations of MTS resulted in stabilization of TP53 and induction of TP53 target genes, potentially resulting from consistent translation of the long-lived TP53 mRNA during prolonged mitosis (14). TP53 check point induction by low MTS concentrations targets genes such as *cdkn1a* or *gadd45a* and induces cell cycle G1 arrest, rather than promoting apoptosis (15-17). This may allow for repair processes cell survival. It might be possible that in tumors, harboring areas with low vascularization, only very low amounts of MTS will actually reach the tumor cells. In terms of chemotherapy, this would indicate an unfavorable condition, because cells might start re-growing after the cell cycle arrest. *In vitro*, as we have demonstrated here, the MTS-induced aneuploid cells may arrest permanently or enter senescence. Yet, it is an open question whether *in vivo* these cells undergo apoptosis, enter senescence or start re-growing eventually. Previously, we have shown that the knockdown of TP53 increased the rate of apoptosis after MTS treatment in A549 lung cancer cells (9). Additionally,

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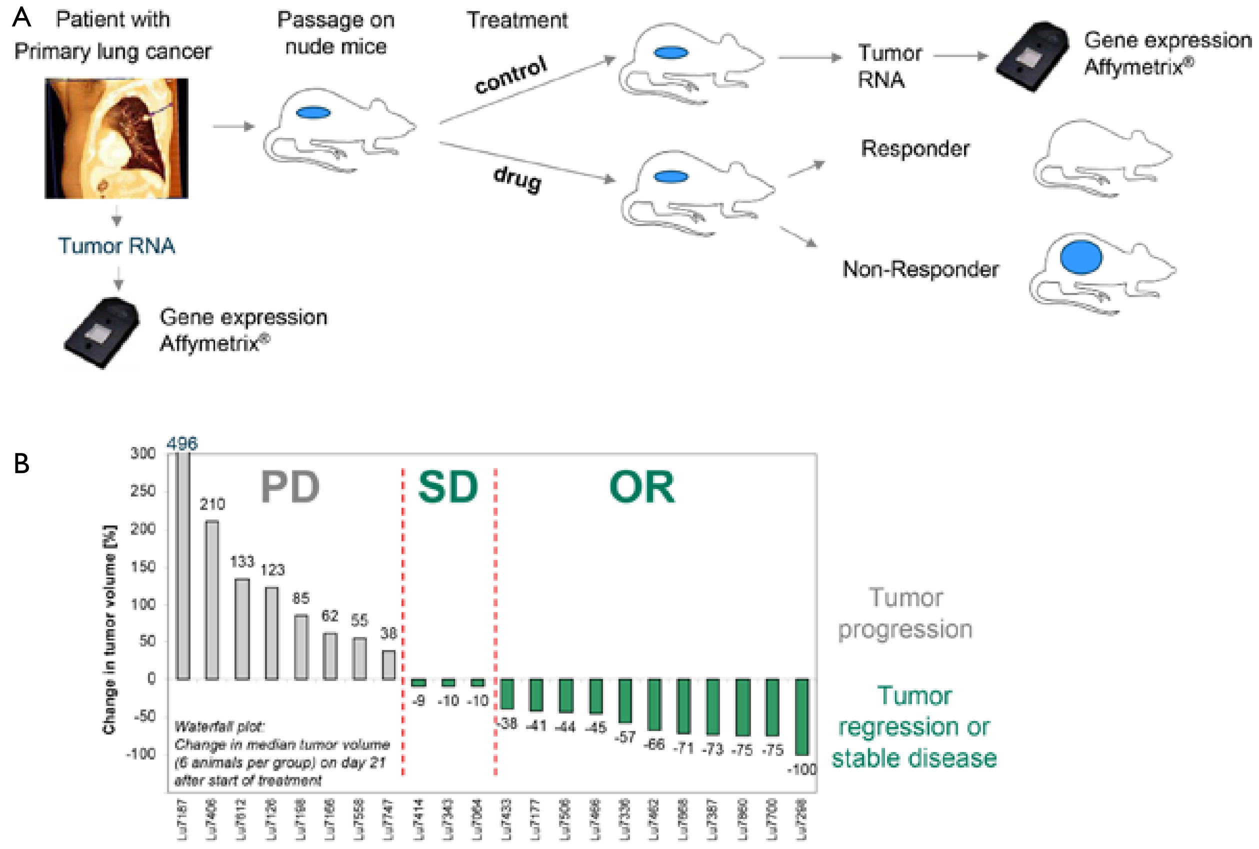

Figure 8 Design of a preclinical phase II study (A) and results summarized in a waterfall plot (B) showing the change in median tumor volume of all 22 patient-derived NSCLC xenograft models 21 days after the initiation of MTS treatment. Analysis by clinical criteria; median change in tumor volume of >+20% is considered tumor progression (P); change in tumor volume of >–30% to <+20% is considered SD; change in tumor volume of <–30% is considered PR or CR. NSCLC, non-small cell lung cancer; MTS, microtubule stabilizer; SD, stable disease; PR, partial regression; CR, complete regression; RECIST, response evaluation criteria in solid tumors.

in our studies on patient- derived NSCLC xenografts, a pronounced long-term response to MTS was seen when TP53 was mutated. The question remains whether these tumors might have a higher probability to respond to MTS, therefore investigations, whether mutational status of TP53 could serve as predictive biomarker in clinical trials, warrants further investigation. Additionally, it could be of clinical relevance if patients with TP53 wild type tumors benefit from combination therapy with drugs inhibiting TP53 or only certain specific functions of TP53, i.e., blocking TP53-dependent transactivation with no effect on p53-mediated apoptosis.

In conclusion, results have been generated from a large set of patient-derived xenograft models via genome-wide gene expression analysis, and mutation analysis of selected genes to identify potential markers of response and refractoriness to MTS in NSCLC. Our data suggest that MTS may be active where other chemotherapies are not. Clinical investigations of the marker genes (e.g., *CA9*, *CA12*, *EPHA4*, *ITGA6*) together with *TP53* gene expression and mutation analysis could be used as predictive marker.

Besides these mechanistic molecular biology driven studies, more classical pharmacology studies have been performed to demonstrate effects of MTS on brain and bone metastases. Taxanes are unable to cross an intact blood-brain barrier, which can result in the lack of activity against brain metastases (18). We investigated the activity of MTS in new models for brain metastasis of breast and lung

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Table 1 P53 mutations shows strong correlation (P<0.05) with response to MTS whereas EGFR and K-RAS mutations do not

Lung cancer PDX	Response	EGFR	K-RAS	p53
Lu7298	Responder	wt	wt	Y234C
Lu7700		wt	wt	H193Y
Lu7860		Q787Q	wt	V153F
Lu7387		wt	wt	wt
Lu7668		wt	wt	wt
Lu7462		wt	G12C	G245V
Lu7336		Q787Q, A836R	G12D	P190L
Lu7466		wt	wt	R196P
Lu7506		wt	wt	190:del1bp (frshift)
Lu7177		wt	wt	M246V
Lu7433		R836R	wt	258E >STOP
Lu7064	Stable disease	wt	wt	162B:del13bp (frshift >STOP)
Lu7343		wt	wt	wt
Lu7414		wt	wt	wt
Lu7747	Non responder	wt	wt	wt
Lu7558		wt	wt	I232F
Lu7166		wt	wt	wt
Lu7198		IVS18+19; IVS18+73	G12C	wt
Lu7126		wt	wt	wt
Lu7612		wt	wt	wt
Lu7406		wt	wt	P278T
Lu7187		wt	G12C	wt
Frequency	64%	18%	18%	55%

MTS, microtubule stabilizer; EGFR, epidermal growth factor receptor; wt, wild-type.

cancer, respectively.

Our studies aimed to determine whether MTS could cross the blood-brain barrier and reduce brain tumor/metastases growth more effectively than other anticancer agents in clinically relevant human tumor models (19). The preclinical studies provided direct evidence that MTS has free access to the brain, leading to highly effective levels of the drug in the brain tissue, which maintained for several days. *In vivo* studies demonstrated that MTS resulted in significant inhibition of tumor growth in both the subcutaneous and intracerebral glioblastoma xenograft models, whereas paclitaxel showed consistent activity in the subcutaneous models only. Similarly, in models of brain metastases, including patient-derived models of NSCLC, MTS showed superior antitumor activity against brain tumors compared with paclitaxel or temozolomide (19).

Bones are a preferred site for metastases in patients with breast cancers. We showed that MTS inhibited tumor burden and bone destruction, in addition to reducing tumor-induced cachexia and paraplegia. MTS treatment significantly lowered the number of activated osteoclasts and significantly reduced the osteolytic lesion area, bone volume loss, and bone resorption, inhibiting the vicious cycle of both tumor growth and bone resorption, suggesting a substantial benefit in the treatment of patients with breast cancer at risk from bone metastases (20).

Summary and outlook

What have been the "lessons learned" from the preclinical development of MTS? Depending on the stage of the drug discovery program, different models are required. For primary *in vitro* screening, cell lines can be utilized easily from the available large panels or generated by genetic engineering. They can be selected based on the target or the question to be answered. For example, we have used a pair

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of cell lines with high and low P-glycoprotein expression to optimize our MTS against drug efflux pumps causing multidrug resistance (7). For secondary *in vitro* screening, larger panels of tumor cell lines with known sensitivity or resistance to available standard drugs are used for further profiling.

However, as we have learned from our mechanistic studies with HCT-116 cells (8), A549 cells (9) and from the drug sensitivity modifier screen reported here using MCF7, T47D, A549 and MDA-MB435s cells, it is of utmost importance to perform these studies in a panel of three or more different tumor models. If we have performed the RNAi drug modifier screen in only one cell line, we would on the one hand have missed important targets which we have seen only in the other three cell lines (e.g., KIF11, CENPE), and on the other hand, we would have identified many modifying genes which turned out to be not relevant in other cell lines. The *in vitro* mechanistic studies revealed rather general mechanisms involved in apoptosis induction (Bcl-2 family and Bax) or cell cycle arrest (tumor suppressor TP53 or SAC kinases) to be involved in the sensitivity to MTS. However, the identification of KIF2C (MCAK) knockdown, synergizing with MTS effects, has impressively shown the potential of this technology. Thus, KIF2C inhibition seems to be a valuable combination strategy for MTS.

Looking at *in vivo* anti-tumor models, a differential pattern of sensitivity can be observed. Broad activity was also seen in most of these models, however most interestingly, some tumor models, i.e., the NCI-H460 lung cancer cells, which are highly sensitive to MTS in cell culture, developed treatment resistant tumors on nude mice. To address this gap between *in vitro* and *in vivo* activity, further studies need to be performed. This gap also reminds us that *in vivo* experiments are still crucial and remain an integral part to evaluate tumor response in the near future.

Although mouse xenograft models derived from established human cancer cell lines have undoubtedly enhanced the understanding of the anti-tumor activity of novel anti-cancer agents, these models have several disadvantages. Depending on the number of cell passages, xenografts can behave very differently to the primary tumor (21), and combined with other deficiencies in pre-clinical approaches [reviewed in (22)], this can reduce the relevance of established xenograft models for predicting the probability of success of anti-cancer drugs in clinical studies for some tumor localizations. Analysis of antitumor activity in patient-derived xenograft models has provided a more accurate selection process for the identification of agents which have activity in clinical trials, suggesting that some of these models may provide a useful hint for activity in the clinic (23). Genome-wide analyses of gene expression using oligonucleotide microarrays have allowed the determination of molecular characteristics present in xenograft models that mirror tumor behavior and relate to disease progression and survival (24). Furthermore, correlations between the growth of xenograft models derived directly from patient tumors and the clinical prognosis of donor patients have been reported (25,26). In the future, the use of patient-derived human tumor xenografts will therefore play a key role in the search for more efficacious cancer treatments (27-31). The ability to identify and assess anti-tumor activity in well-characterized xenografts in correlation with particular genetic or molecular characteristics may aid the development of new therapeutic regimens. In our studies, increased basal expression of genes involved in cell adhesion, angiogenesis and the hypoxia pathway was observed in lung cancer xenograft models that do not respond to MTS. In these models, the combination of MTS with drugs targeting VEGF signaling led to an enhanced anti-tumor activity compared with either agent alone.

Conclusions from what we discussed here are:

- Drug discovery, systems biology, and TR are moving together to address all the new hallmarks of cancer increasing the success rate of drug discovery;
- *In vitro* versus *in vivo* models or vice versa—as we have shown both models have limitations and advantages, however, when used critically, all generate important and reliable results;
- Panels of patient derived xenograft models represent an important tool for TR;
- Predictive value of the preclinical models is increasing steadily, however, even genetically engineered "humanized" mice are still not men.

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Functional and molecular imaging in cancer drug development

Vikram Rao Bollineni[1], Sandra Collette[2], Yan Liu[1]

[1]Department of Translational Research, Radiotherapy and Imaging, [2]Statistical Department, European Organisation for Research and Treatment of Cancer, Brussels, Belgium

Correspondence to: Yan Liu, MD, PhD. Department of Translational Research, Radiotherapy and Imaging (TRI), European Organisation for Research and Treatment of Cancer (EORTC), Avenue E. Mounier 83, 1200 Brussels, Belgium. Email: yan.liu@eortc.be.

Abstract: Imaging biomarkers have a potential to identify key metabolic pathways that are up-regulated in cancer cells compared to normal cells. In early drug development, they can provide valuable information on the dissemination of the drug and estimate whether the drug reaches the target and, consequently, to determine the appropriate clinical benefit. The use of imaging as an early surrogate biomarker of response is also appealing, since it allows to tailor treatment regimens in individual patients. The aim of this review is to describe various imaging biomarkers covering most important cancer hallmarks such as cell death, proliferation, metabolism, vascularity, and hypoxia. We highlight the current status of using molecular imaging such as fluorodeoxyglucose (FDG), fluorothymidine (FLT), fluoromisonidazole (FMISO), and fluoroazomycin arabinoside (FAZA) positron emission tomography (PET) as well as advanced magnetic resonance imaging (MRI) techniques such as dynamic contrast enhancing (DCE) and diffusion weighted (DW)-MRI, and their potential roles in cancer drug development.

Keywords: Imaging biomarkers; molecular imaging (FDG-, FLT-, FMISO-, FAZA-PET); advanced magnetic resonance imaging (MRI) techniques (DCE-, DW-MRI); cancer drug development

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Introduction

Biomarkers are becoming an indispensable part of drug development. A biomarker is a representative that serves as an indicator of a patho-physiological process, or as a response to treatment which affects such a process (1). A more ideal use of imaging biomarkers for drug development can serve multi-purposes, such as disease staging, patient stratification, risk assessment, pharmacokinetics/pharmacodynamics (PK/PD), drug safety and efficacy. The use of non-invasive imaging biomarkers to assess drug therapies has become more common during the last decades. From December 11, 1992, to July 1, 2010, the U.S. Food and Drug Administration (FDA) granted accelerated approval of 47 new indications for 35 anticancer drugs using surrogate endpoints, and most of them were objective response rate and progression-free survival (PFS), typically measured by magnetic resonance imaging (MRI) or computer tomography (CT) (2). However, vigorous debate has challenged the use of anatomic assessments alone, as it may take two or three months to detect any shrinkage, thus only morphological information can be obtained. But, it may not be a suitable tool to assess response when agents targeting signaling pathways are involved, most notably in patients with gastrointestinal stromal tumor (GIST) treated by cytostatic targeted agents (3). To better understand tumor microenvironment (TME), and thereby select specific agents targeting metabolic key pathways, morphological information is not enough. Therefore, the addition of functional information on TME through imaging biomarkers would aid principle investigators to design personalized treatment planning by using specific targeted drugs (4).

Functional imaging of TME has several advantages: (I) it is a non-invasive procedure; (II) various sites of the tumors can be visualized and quantified simultaneously; (III) functional imaging using biomarkers can generate

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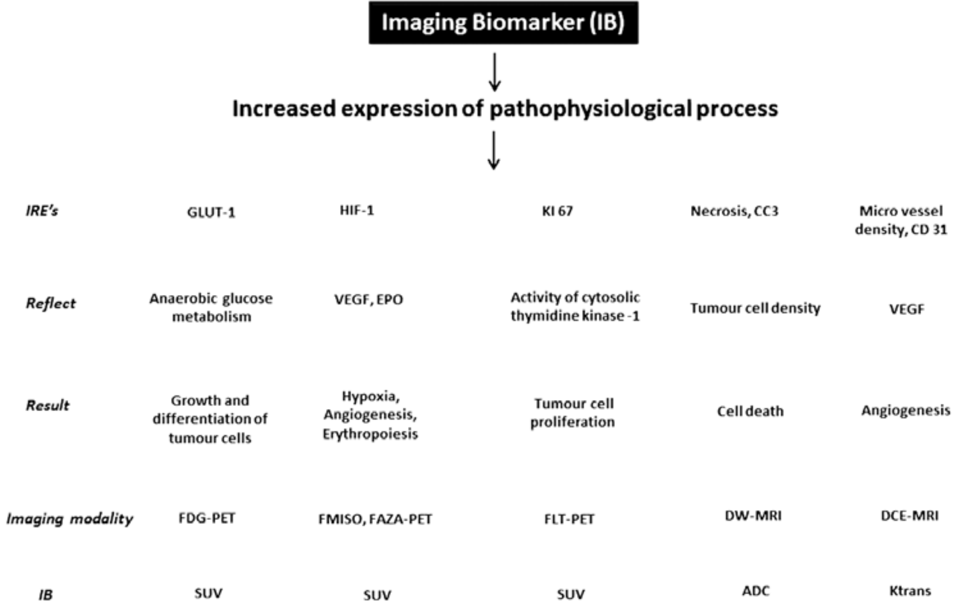

Figure 1 Schematic presentation showing how imaging biomarkers identify overexpressed pathophysiological processes in cancer cells. IB, imaging biomarker; IRE's, imaging responsive elements; GLUT-1, glucose transporter-1; HIF-1, hypoxia-inducible factor-1; CC3, cleaved caspase 3; CD 31, platelet-endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor; EPO, erythropoietin; FDG-PET, fluorodeoxyglucose-positron emission tomography; FMISO, FAZA-PET, fluoromisonidazole, fluoroazomycin arabinoside-PET; FLT-PET, fluorothymidine-PET; DW-MRI, diffusion-weighted-magnetic resonance imaging; DCE-MRI, dynamic contrast enhancing-MRI; ADC, apparent diffusion coefficient; SUV, standardized uptake value; Ktrans, volume transfer constant; PET, positron emission tomography; MRI, magnetic resonance imaging.

three dimensional images of the tumor which allows better quantification; (IV) moreover, functional imaging is capable of visualizing heterogeneous metabolic processes, such as glucose metabolism or tumor hypoxia, which are important contributors to tumor resistance and progression.

Molecular imaging using various labelled radioactive tracers such as fluorodeoxyglucose (FDG), fluoromisonidazole (FMISO), fluorothymidine (FLT), and functional imaging using advanced techniques such as dynamic contrast enhancing (DCE)-MRI and diffusion weighted (DW)-MRI, gain an increasing importance in cancer drug development (*Figure 1*). Quantitative measurements of imaging biomarkers compared to mere visual evaluation allow for more objective evaluation of disease, and more accurate monitoring through time (1). Therefore, the purposes of this review are (I) to summarize the basic principle and qualification of various imaging biomarkers; (II) to investigate key metabolic pathways up-regulated in cancer cells by using imaging biomarkers and to facilitate targeted cancer drug development; and (III) to describe pitfalls and recommendations when imaging biomarkers are implemented in multicenter trials.

Imaging measurements and qualification

FDG (glucose metabolism)

The most frequently used positron emission tomography (PET) tracer in oncology is FDG for measuring glucose metabolism of the cell (5). However, FDG is not a substrate for metabolism in the glycolytic pathway. Therefore, the degree of trapped FDG uptake in the cells reflects the level of glucose metabolism and could be potentially used as imaging biomarker for early treatment response assessment in cancer patients (6). Maximum standardized uptake value (SUVmax) is a quantitative index to characterize FDG biomarker uptake, hence approximating the glucose metabolism; high SUVmax is associated with aggressive tumor metabolism and poor survival (7,8).

The transport of FDG, a glucose analogue, into cells is mediated by glucose transporters (GLUT-1 and 2) through the plasma membrane (9). Several published studies support

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significant positive correlation between FDG-PET uptake and the expression of GLUT examined by immunohistochemical staining (10-12). Primarily, the overexpression of GLUT characterizes enhanced tumor glucose metabolism and thereby increased FDG uptake is noticed on PET scan.

Demetri *et al.* (13) showed that, in all GIST patients with a response, the FDG-PET uptake in the tumor had decreased from baseline as early as 24 hours after a single dose of imatinib administration. In addition to that, in all patients, increased FDG-PET uptake from baseline is associated with disease progression. Also, FDG-PET uptake results were correlated with progression on CT or MRI.

Multiple studies have evaluated the role of FDG-PET and showed it promising in assessing response to treatment in solid tumors (14-16). However, the interpretation of SUV is not straightforward, with many factors affecting the values that can be derived. It was shown that a reliable drop in SUV, indicating a tumor response, is only seen in patients with high initial SUV (17). Caution should, therefore, be exercised when we interpret quantitative molecular imaging.

FAZA, FMISO (tumor hypoxia)

Tumor hypoxia is an important adverse prognostic factor and contributes to resistance for both chemotherapy and radiotherapy in several tumor types (18). Under hypoxic cell conditions, tumor hypoxia biomarkers undergo definite reductive metabolic pathways, resulting in reactive tumor metabolite markers which selectively bind to macromolecular cell components that can be detected by the PET signal, but which are washed out from normoxic cells (19,20).

FMISO was the first tracer tested clinically for tumor hypoxia, and it is still widely used (21-23). The novel hypoxia specific tracer, fluoroazomycin arabinoside (FAZA), has generated higher tumor-to-background ratios compared to FMISO in preclinical studies (24,25). FAZA also becomes a more attractive tracer for clinical use due to its more rapid clearance of unbound tracer from non-hypoxic tissues (24).

A clinically relevant exogenous hypoxic biomarker is pimonidazole. With this biomarker, high resolution image of hypoxia distribution at micro-regional level can be obtained using immunohistochemistry. The tumor hypoxia determined by pimonidazole binding assay is consistent with radiobiologically relevant hypoxic volume (26). Dubois *et al.* (27) found significant correlation between the hypoxic area derived from pimonidazole stained tumor section with the FMISO-PET defined hypoxic volume in an experimental rat tumor model ($r=0.9066$; $P<0.0001$).

FLT (tumor cell proliferation)

FLT was introduced by Shields *et al.* (28) as a PET proliferation imaging biomarker. FLT is monophosphorylated by thymidine kinase 1 (TK1), which leads to intracellular trapping. Since the concentration of TK1 is upregulated during the S phase of the cell cycle, the uptake of FLT reflects proliferation.

Tsuyoshi *et al.* (29) evaluated the effect of gemcitabine-based secondary chemotherapy with FLT- and FDG-PET imaging biomarkers in patients with stage IIIc recurrent ovarian cancer. FLT SUVmax decreased earlier than FDG SUVmax. Interestingly, FLT SUVmax correlated better with a reduction in size as measured by CT. Given the good imaging properties and strong correlation between functional imaging parameter (proliferation) FLT uptake and CT morphological parameters, SUVmax of FLT appears to be a promising biomarker for monitoring response to gemcitabine-based secondary chemotherapy treatment in recurrent ovarian cancer patients.

The rationale behind the FLT-PET uptake in tumors is based on TK1 activity and Ki-67 index dependence on proliferation. Since the concentration of TK1 and Ki-67 is overexpressed during the active proliferation phase of the cell cycle (S phase), the uptake of FLT is supposed to depend on TK1 and Ki-67 concentration. In a preclinical study, Rasey *et al.* (30) showed strong correlation between FLT and cell growth, TK1 activity and also with the percentage of cells in S phase of cell cycle (28,31). Recently, Yamamoto *et al.* (31) demonstrated a significant positive correlation between the proliferation index derived from Ki-67 immunohistochemistry with the FLT-PET uptake ($r=0.81$, $P<0.01$) in patients with newly diagnosed and recurrent gliomas ($n=56$). Given the strong correlation between the FLT uptake and TK1 and Ki-67, FLT appears to be a promising tracer for imaging proliferation.

DW-MRI (cell density)

DW-MRI is an advanced MR technique widely used for the detection and characterization of cancer as well as for monitoring the response to therapy. DW-MRI depends on the microscopic mobility of water in tissues, and it provides a unique imaging biomarker of water interaction with cellular, subcellular and macromolecular entities that impede free water movement (32). In oncologic imaging,

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DW-MRI has been used to evaluate tumor microstructure, e.g., cell membrane integrity and cellularity, which reflects lesion aggressiveness and tumor response. The acquisition of DW-MRI is non-invasive, does not require any exogenous contrast agents, does not use ionizing radiation, can be obtained relatively rapidly, and is easily incorporated into routine patient evaluation. The apparent diffusion coefficient (ADC) is the quantitative parameter of DW-MRI, and has been shown to be of high potential value for assessing treatment response (33,34). A low ADC reflects restricted diffusion and can be found in hypercellular tissues such as tumors, lymph nodes or in areas of fibrosis. A high ADC reflects less restriction of extracellular water motion and can be found in tissues with high glandular components or distinct necrosis. Cell kill due to efficient drug treatment leads to a loss of cell membrane integrity and reduction in tumor cell density with increase in the interstitial space, and hence it changes ADC measurement in the tumor tissue.

Foroutan *et al.* (34) evaluated the correlation between ADC and cell death in an osteosarcoma xenotransplant model at pre-treatment and at early time points following treatment. Pixel-by-pixel histograms were produced for each mouse prior to and following the treatment to quantify ADC. Cleaved caspase 3 (CC3) was used as an immunohistochemical marker to quantify cell death. Statistically significant differences in ADC maps were observed between control mice and treated mice, which demonstrates an increase in ADCs towards higher values in treated animals compared to controls. CC3 activity was also significantly higher in the treated animals compared to controls. Overall, a positive correlation was observed between increase in ADC values and cell death depicted by CC3 staining.

DCE imaging (blood flow and vascular permeability)

DCE imaging (MRI, CT and ultrasound) allows non-invasive quantification of TME and its vascular structure and function. The degree of DCE signal intensity reflects the pathophysiological factors, which include tissue perfusion and capillary permeability (35). Serial images are acquired dynamically before, during and after administration of a contrast agent: gadolinium for MRI, iodinated contrast for CT and microbubbles for ultrasound. The acquired data are fitted to mathematical models to obtain quantitative parameters through regions of interest. The volume transfer constant (Ktrans) is often used as a marker for the permeability of tumor vasculature. Other measures used are the rate constant Kep and the initial area under the gadolinium concentration curve (IAUGC).

Understanding the dynamics of tissue parameters is crucial for developing anti-angiogenic drugs. Vascular targeting agents such as bevacizumab or vandetanib are developed to reduce vascular permeability and promote tumor necrosis. Kummar *et al.* (35) investigated the effect of the anti-angiogenic drug vandetanib in patients with lymphomas. They observed a positive correlation between DCE-MRI parameters and plasma vascular endothelial growth factor (VEGF) levels. Similar results were reported by Donaldson *et al.* (36) who showed that tumors with poor permeability significantly correlated with the expression of plasma VEGF and the hypoxia marker pimonidazole. High expression of VEGF is associated with tumor angiogenesis and hypoxia, and thereby promotes tumor growth.

Imaging in cancer drug development

Stratifying patients

Molecular and functional imaging provides additional information on tumor characterization, which could help to “pre-select” and “enrich” a patient population. For example, in patients treated with gefitinib, a low baseline SUV of ^{18}F-FDG has been shown to have prognostic value and to be associated with a higher response rate and a prolonged PFS (37).

Identification of tumor hypoxia could facilitate the use of hypoxia stimulated pro-drugs, which selectively kill hypoxic cells. Tirapazamine (TPZ) is such an example. The relatively limited benefit obtained in a trial reported by the CATAPULT I study group was likely due to poor patient stratification with inclusion of patients with better-oxygenated tumors (38). Recently, Rischin *et al.* (39) compared the cisplatin/5-FU *vs.* cisplatin/TPZ regimen in patients with head and neck squamous-cell cancer, in which FMISO-PET hypoxic imaging was used to stratify the tumors into hypoxic and non-hypoxic ones. The authors have shown that TPZ improved local tumor control in hypoxic but not in non-hypoxic tumors.

Imaging-guided therapy could promote personalizing treatment, for example by adjusting the treatment for non-responders at an initial phase of treatment. Within the drug development, this sort of response monitoring could be used for selecting a homogeneous patient group for further studies by choosing only those patients who show early

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metabolic response. Several trials are currently investigating the use of FDG-PET/CT for early response-adapted therapy in lymphoma, with therapeutic stratification based on interim FDG-PET/CT results (40-42). The PET-response-guided treatment has also been investigated in adenocarcinoma of the oesophagogastric junction, and the MUNICON phase II trial showed the feasibility of imaging-guided stratification by using the early metabolic response assessment from FDG-PET to clinical decision making in the treatment of solid tumors (40).

Verifying biological target engagement

The downstream effects of vascular endothelial growth factor receptor (VEGFR) inhibition on DCE-MRI have been documented in more than 30 phase I and II trials with a significant reduction in Ktrans and/or IAUGC being reported with multiple agents (43).

The more direct approach of using PET in cancer drug development is by labelling the drug itself. The anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab was used to treat breast cancer patients with HER2 expressing tumors and showed improved survival (44). Radionuclide labelled trastuzumab can visualize the affinity of the targeted agent *in vivo*, which allows us to collect vital information about the pharmacokinetic properties of the drug such as injected dose versus accumulated drug concentration in the organs and its regional bio-distribution. In this case, the use of radionuclide imaging may overcome problems associated with biopsies, including sampling errors and discordance of expression between primary tumors and metastases. Moreover, the drug uptake by the target tissue can be quantified at sequential imaging scans, and it might give us insight into drug's action at the target tissue and its association with the tumor response.

Defining dose setting

In phase I trials, dose-escalation is usually undertaken to define the maximum tolerated dose (MTD), under the assumption that the most pronounced changes are likely to be detected at the highest dose. But, target saturation may already be reached at lower dose levels. Through direct visualization of target inhibition, imaging changes are likely to be apparent at lower doses than the MTD, and imaging may be used in choosing the optimal biological dose. In a study of brivanib, a dual VEGFR and fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitor, Jonker *et al*. (45) evaluated DCE-MRI responses in several dose schedules in selected patients, known to respond to anti-VEGFR therapies, and then selected the optimal schedule for a phase II trial. Despite this experience, imaging is not commonly used for selecting dose or schedule, and such data are limited, so the use of imaging to determine the optimal schedule of a targeted agent or to monitor drug activity has to be further explored for cancer drug development.

Novel surrogate endpoint for early evaluation of drug activity

A growing understanding of the underlying molecular pathways active in cancer has led to the development of novel therapies targeting VEGFR, EGFR, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), protein kinase B (Akt) and other pathways. Unlike the cytotoxic chemotherapy, many of these molecular targeted agents are cytostatic, causing inhibition of tumor growth rather than tumor regression. In this context, using tumor shrinkage as a surrogate endpoint may not be the most adequate mean to measure therapeutic response, as the response rates only based on change of tumor size are low, despite a high percentage of patients having prolonged stable disease and sometimes even improvements in survival. Therefore, functional imaging provides a unique potential opportunity to assess antitumoral activity at early stage.

Many have stimulated the FDA to accept novel surrogate endpoints, such as novel imaging endpoints that can be measured earlier than tumor shrinkage and are likely to predict clinical benefit. A qualified biomarker accepted by the FDA as a surrogate endpoint needs to match several important criteria: (I) the endpoint must have an accepted, standardized definition; (II) data from multiple clinical studies must demonstrate a strong correlation of the surrogate endpoint with clinical outcome; (III) well-powered prospective studies must have been performed to validate the surrogate endpoint (i.e., truly predictive of clinical benefit with meaningful improvement in patient outcome) (46). The strength of evidence will vary, depending on whether the surrogate is intended for use in accelerated approval or definite regulatory approval.

FDG uptake (SUV) has been proposed as an appropriate novel surrogate endpoint for early evaluation of drug activity in clinical trials. There have been many retrospective and some prospective studies in a variety of cancer types that have demonstrated a promising correlation

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between SUV decrease and survival (41,47). To date, these studies have been primarily performed in single institutions with small numbers of patients. To our knowledge, there are two ongoing multicenter trials prospectively designed to validate FDG-PET as a surrogate endpoint in lymphoma (CALGR-53030) and non-small cell lung cancer (RTOG-0235/ACRIN6668). Large prospective multi-center clinical trials are needed to assess the degree of correlation by comparing a pre-defined threshold in SUV change to clinical outcome.

Imaging in multicenter clinical trials

Standardization

Although many imaging biomarkers have been described for cancer research, few of them are widely considered adequate to provide unambiguous assessment of response, and enough for making decisions to stop or continue drug development processes. Implementing molecular and functional imaging to assess response requires that an observed change of the imaging biomarker due to treatments must be greater than the intrinsic and extrinsic variability of the biomarker in the absence of treatment. High reproducibility of molecular and functional imaging techniques relies on good quality data and standardized procedures. Standardization is the first and crucial step when imaging is implemented in multicenter trials. In this context, the EORTC-PET study group issued recommendations for the measurement of [^{18}F]FDG uptake in monitoring treatment response in 1999 (48). These recommendations included suggestions for patient preparation, pre-therapy and post-therapy imaging delays, and techniques for measuring SUV. Following that, guidelines of the National Cancer Institute (NCI) and the European Association of Nuclear Medicine (EANM) for tumor PET imaging enriched the standardized procedures (49,50), making it more feasible to include PET in large multicenter trials. Regarding advanced MRI techniques, such as DCE, the techniques are relatively simple but require strict protocols, careful acquisition, accurate dosing of contrast agent and suitable selection of injection rate, image timing, and image analysis for quantification. In US, the Quantitative Imaging Biomarker Alliance (QIBA) DCE-MRI technical committee provided guidelines and defined basic standards for DCE-MRI measurement and quality control that enable consistent, reliable and fit-for-purpose quantitative measurements when DCE MRI is implemented in multicenter trials (51). In Europe, the Quantitative Imaging in Oncology: Connecting Cellular Processes to Therapy (QuIC-ConCePT) consortium was created and resourced by the Innovative Medicines Initiative (IMI), Europe's largest public-private initiative (4). It aims to qualify three specific imaging biomarkers of tumor cell proliferation, apoptosis, and necrosis, to allow drug developers to demonstrate reliably the modulation of these pathologic processes in tumors of patients in future trials (4). The precompetitive research and public-private partnerships may reduce the duplication, and develop imaging biomarkers in a most robust, consistent and cost-effective way, so as to accelerate drug development.

Recommendation

Providing a benchmark, based on a set of common principles of implementing functional and molecular imaging in multicenter trials, is important to facilitate exchange of data, promote quality, accelerate research and reduce attrition rate for drug developers. In addition to the summary on the utility of imaging biomarkers based on literature review, we provide general recommendations for principal investigators designing and conducting multicenter clinical trials that include functional and moleculare imaging biomarkers (*Table 1*).

Conclusions

In the past decade, advances in biology and genomics have led to the development of targeted agents against cancer. This paradigm shift emphasizes the need for specific imaging biomarkers to identify key metabolic changes within the TME and thereby selecting a specific drug of choice. Non-invasive *in vivo* imaging offers unique, sensitive and clinically transformable information for cancer drug development, notably via efficient patient selection, imaging-guided therapeutic stratification, verification of biological target modulation and dose adaptation. In addition, functional and molecular imaging may potentially allow us to depict accurate changes in tumors, particularly before anatomic changes are evident, and to predict long-term clinical benefit. However, large prospective multicenter studies are needed to further qualify and validate the potential functional imaging biomarkers by demonstrating a strong correlation with clinical outcome. When imaging is implemented in multicenter clinical trials, we highly recommend designing studies with sound methodology and conducting studies

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Table 1 Recommendations when imaging is integrated in multicenter clinical trials

Protocol design	Before site activation	During accrual	After accrual
• Early engagement with experts from relevant disciplines; • Rational discussion on why the selected imaging biomarker is appropriate, including feasibility cost-effectiveness; • Preliminary imaging biomarker quantification data (e.g., reproducibility, accuracy); • Understanding of biological mechanism; • Selection of appropriate criteria; • Early definition of statistical power calculation with simulation and adaption	• Development of imaging guidelines; • Requirement of Dummy run, i.e., test before accruing patient; • Evidence of scanner calibration (e.g., scanner accreditation); • Elaboration of standard operating procedures, quality assurance & quality control program; • Organization of imaging central review (e.g., review panel, procedures, and turn-around time); • Evidence of proper site training	• Ensuring of imaging data protection; • Compliance of all electronic processes; • Quality assurance and quality control of imaging data; • Appropriate data management and tracking; • Documentation of all processes; • Interim analysis to reassess feasibility of approach and whether statistical power will still be reached (optional)	• Closure of database; • Data analysis according protocol and guidelines; • Exploration of potential routes to integrate imaging biomarker in future clincial trials, or usage in clinical routine , and of necessary methodological improvements

with adequate standardization of data acquisition and analysis techniques.

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New challenge of developing combined radio-drug therapy

Philippe Maingon[1], Anne-Sophie Govaerts[2], Sofia Rivera[3], Conchita Vens[4], Emad Shash[2], Vincent Grégoire[5]

[1]Radiotherapy Department, Centre Georges-François Leclerc, Dijon, France; [2]EORTC Headquarters, Brussels, Belgium; [3]Radiotherapy Department, Gustave Roussy, Paris, France; [4]Radiobiology Department, Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands; [5]Radiation Oncology Department, Université Catholique de Louvain, Brussels, Belgium

Correspondence to: Professor Philippe Maingon, MD. European Organisation for Research and Treatment of Cancer (EORTC)-Radiation Oncology Group (ROG), Radiotherapy Department-Centre Georges-François Leclerc, 1 Rue du Professeur Marion, 21000 Dijon, France.
Email: pmaingon@cgfl.fr.

Abstract: Combined modality treatment can be used to improve control of the local disease at the expense of increased toxicity. Several randomized trials have demonstrated that this combined modality therapy is better than radiotherapy alone or chemotherapy alone in the treatment of locally advanced diseases. Several new targets as well as potential new radio-sensitizers have been identified. To speed-up the process of developing new combined modality treatments, good preclinical models for optimization of the ratio between efficacy and toxicity and a well established methodology within a network of advanced high-tech laboratories and clinical departments devoted to early phase trials, are mandatory. The Synergy of Targeted Agents and Radiation Therapy (STAR) platform of the European Organisation for Research and Treatment of Cancer (EORTC) is gathering these tools.

Keywords: Radiotherapy; chemo-radiation; early phase trials; radiobiology; targeted agents

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Introduction

Radiation therapy (RT) is one of the three therapeutic pillars among cancer treatment regimens. Numerous approaches have been tested to improve the therapeutic ratio of RT, and these include increasing the dose delivered to the tumor, altered fractionation schemes, combined modality treatment with chemotherapy, and, more recently, novel targeted agents (1). This last approach is based on a mechanistic understanding suggesting that a combined approach with RT could enhance the killing of tumor cells through inhibition of DNA repair processes and the inhibition of tumor repopulation between RT fractions.

Combined modality treatment can be used to improve control of the local disease at the expense of increased toxicity. Several randomized trials have demonstrated that this combined modality therapy is better than RT alone or chemotherapy alone in the treatment of locally advanced cancer of the head and neck (HNC), lung, esophagus, and rectum, as well as high grade glioma. Following the introduction of 5-fluorouracil (5-FU) associated with RT in advanced HNC in the early 1980's, different combinations using classical cytotoxic agents have been studied (2).

The Radiation Oncology Group (ROG) of the European Organisation for Research and Treatment of Cancer (EORTC), formally known as the Radiotherapy Group, has conducted more than 80 clinical trials, three quarters of which have been randomized phase III trials. The ROG has played a major role in changing clinical practice having shown the impact on outcome of combined chemo-RT in the pre-operative treatment of locally advanced rectal cancer (3), of the combination of temozolomide and RT in glioblastoma (4), and of chemo-RT in the post-operative setting of HNC (5). It did so for locally advanced prostate carcinoma with the combination of hormonal and radiation therapies (6). Furthermore, this success story has been associated with a dramatic improvement in quality of life of patients receiving chemo-radiation for advanced laryngeal

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carcinoma in voice preservation trials as well as for rectal and anal cancer in sphincter preservation trials (7-14).

Rationale

The first trial to use a monoclonal antibody (MoAb) was one in which patients with HNC were treated with cetuximab combined with RT. This phase III trial comparing RT alone to concomitant treatment with RT and cetuximab showed that the outcome of patients receiving RT in combination with this chimeric MoAb directed against the epidermal growth factor receptor (EGFR) is improved compared to patients who underwent RT alone (15). Furthermore, this molecule has been proven efficacious in locally advanced or metastatic HNC in combination with 5-FU and cisplatin (16). Preclinical and clinical studies have demonstrated the efficacy of cetuximab in combination with RT. However, increased skin toxicity, mainly acneiform rash, has been reported underlining the need for better early assessment of potential toxicity in various preclinical models. In a comparison between RT combined with platinum based chemotherapy versus RT with cetuximab, a significantly higher number of grade 3 oral mucositis and dermatitis were observed in the cetuximab containing arm; this must be outweighed by the higher risk of hematological toxicity by cisplatin based radio-chemotherapy. So far, these adverse events have been reported only for HNCs; in trials on thoracic or pelvic RT with cetuximab, increased rates of skin toxicity have not been observed. Unfortunately, following this preliminary experience, other antibodies combined with RT have shown neither an increased rate of local tumor control nor an increase in overall survival as compared to radio-chemotherapy. Panitumumab, a MoAb directed against EGFR, has a higher affinity and fewer hypersensitivity reactions due to its non-chimeric character, but it failed to demonstrate a significant therapeutic impact when combined with RT (17). Similar results have been obtained with nimotuzumab (18).

There are many factors that determine tumor cell sensitivity to radiation. Three important biological processes have been shown to affect tumor response and outcome after RT: hypoxia, the ability of the surviving cells to repopulate during the course of treatment, and the intrinsic radio-resistance of the tumor cells. In addition, micro-environmental host factors such as tumor infiltration of inflammatory cells and other bone marrow-derived cells (BMDCs) have been shown to play a role. A complementary approach associated with the development and the implementation of new technologies is to contribute to the reduction of normal tissue injury induced by ionizing radiation. This is especially important in dose escalation studies, when the aim is to increase the probability of tumor control. Both approaches, increasing tumor cell kill and decreasing morbidity, even in the context of combined modality treatment, can improve cure rates and quality of life of cancer patients undergoing RT.

It is obvious that the development of a combined modality strategy is of key importance for about half of the patients suffering from cancer, considering that local control of the primary tumor should first be obtained. RT is the main actor along with surgery for this goal. Therefore, the traditional research on RT focusing on improving technical delivery has to be associated with improvement in combined modality treatment to optimize the acute tolerance and late toxicity of associated treatments on normal tissues.

Combining novel targeted approaches with radiation therapy (RT)

Several processes have been identified as potential targets for radio-sensitization, and perhaps the most famous one is modulating DNA repair (19). Owing to genetic instability, tumors are often defective in one aspect of DNA repair, but usually have backup pathways for accomplishing repair. Attacking these backup pathways can render the tumor radio-sensitive while leaving the normal tissue relatively resistant. Since tumors are often defective in one of the cell cycle checkpoints (such as the G1/S checkpoint), the modulating of cell cycle checkpoints is another important potential approach. Inhibiting remaining checkpoints can leave tumors with less repair time, resulting in greater cell kill than in normal tissues. The PI3K-AKT, nuclear factor-κB (NF-κB), MAPK pathways and others can mediate radio-resistance and are often aberrantly activated in tumors. Attacking these pathways with specific inhibitors of signal transduction is a promising avenue for increasing the radio-sensitivity of tumors (20).

More recently, efforts have been made to better understand the role of the microenvironment: tumors often contain radio-resistant and chemo-resistant hypoxic cells. Several methods are available to attack or exploit tumor hypoxia, leading to tumor-specific effects. Tumor vasculature can also be attacked in ways that increase the response to ionizing radiation. Moreover, a variety of strategies for modulating normal tissue damage has shown promise in ameliorating ionizing radiation

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damage to normal tissues. These include protection with radical scavengers, stimulating recovery with cytokines, modifying the p53 response, reducing the negative effects of inflammatory cascades and oxidative stress, and stem cell therapy.

Radio-sensitization

DNA damage response (DDR)

DNA damage induced by ionizing radiation triggers the DDR which comprises molecular events that mostly involve post-translational modification of proteins that activate intracellular signaling pathways. Repair of double strand breaks (DSBs) requires arrest of cell cycle progression to avoid further damage before commitment to S-phase or mitosis. Oncogene-driven DNA replication stress has been implicated as a cause of constitutive activation of DDR and tumor progression. Defects in both DNA repair and checkpoint responses in tumor cells affect the response to ionizing radiation and can be exploited for targeted radio-sensitization strategies. Inhibitors of important molecules in DSB repair, such as ataxia telangiectasia mutated protein (ATM) or DNA-dependent protein kinase (DNA-PK), have been shown to sensitize cancer cells and xenografted tumors to RT. New agents have been developed in recent years and tested in phases I, II, and III trials concomitantly with RT or chemo-RT to sensitize cancer cells and xenografted tumors to RT. One class of such drugs, the poly (ADP-ribose) polymerase (PARP) inhibitors, has shown activity in conjunction with RT in several cancer cell lines (21). Clinical trials assessing the toxicity and potential benefit of combining RT with PARP inhibition are now ongoing.

Cell cycle checkpoints

Besides DNA repair, cell cycle checkpoints constitute another important component of DDR. Induction of DNA damage by ionizing radiation in normal cells halts their progression through the cell cycle and prevents further accumulation of damage and its serious consequences. In contrast, in cancer cells with an impaired G1 checkpoint, cell cycle progression will continue unabated, and therefore removing the G2 block will increase damage and its transmission to progeny (22). This will ultimately lead to the loss of clonogenicity, and this is the rationale of checkpoint inhibition strategies. ATM and downstream proteins such as the cell division cycle 25 (CDC25) phosphatase represent cell cycle checkpoints in response to ionizing radiation that would otherwise prevent the propagation of DNA damage. CDC25 phosphatase and ATM inhibitors have been used as single agents but have also been demonstrated to enhance cell kill in combination with DNA-damaging drugs and RT.

Signal transduction pathways: the EGFR-PI3K-AKT axis

The importance of PI3K-AKT as a survival pathway has led to the development of a multitude of blocking antibodies and small-molecule inhibitors. The most successful to date is the EGFR-specific antibody cetuximab, which in combination with RT significantly increased local-regional tumor control and overall survival in a phase III trial in HNC. Receptor tyrosine kinase inhibitors (TKIs) can also abrogate the EGFR signaling cascade, and this leads to increased radio-sensitivity. In some preclinical models, antibodies seem to be more effective at modifying the ionizing radiation response than TKIs. Four pathways demonstrate a clear role in the response and sensitivity to ionizing RT: PI3K-AKT, nuclear NF-κB, MAPK, and TGFβ. The activation of AKT by phosphorylation occurs through growth factor receptor pathways, e.g., receptor overexpression such as EGFR, loss of PTEN, oncogenic mutations in NRAS or KRAS, all of which have been shown to be associated with increased resistance to RT in tumor cell lines. EGFR-directed MoAbs and TKIs most likely increase radio-sensitivity by inhibiting DNA repair.

Histone deacetylase (HDAC) inhibitors

HDAC-I acts predominantly by inducing differentiation, apoptosis, and cell cycle arrest with a preferential cytotoxicity for tumor cells (apoptosis induction). HDAC-I induces cell death (via an unclear mechanism involving mitochondrial apoptosis, autophagy, regulation of reactive oxygen species, etc.) and cell cycle arrest mainly in G1 but also demonstrates anti-angiogenic, anti-invasive, and immune-modulatory activities (23). The exact mechanism by which HDAC inhibitor-induced radio-sensitization occurs is currently unclear, but may be, at least in part, due to the preventing the repair of damaged DNA. Thus, HDAC inhibitors appear to inhibit DNA double strand breaks (DSB) repair leading to enhanced tumor cell death. Radio-sensitization has also been explained by modulation of cell cycle regulation and down-regulation of surviving signals. One of the major advantages of HDAC inhibitors as radio-sensitizing agents for cancer therapy is the fact

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that they are relatively specific for malignant cells and spare normal tissues. Several *in vitro* studies have demonstrated this finding of no increased radio-sensitivity in normal tissue cell lines when exposed to HDAC inhibitors, and some HDACs may actually play a role in protecting normal tissue from radiation-induced side effects (inhibition of TNF-α and TGF-β). Even though cutaneous T-cell lymphoma (CTCL) is the only cancer for which HDAC inhibitors are currently FDA-approved, many clinical trials are assessing the efficacy and safety of other HDAC inhibitors when used alone or in combination therapies in both solid and hematologic malignancies. Clinical trials, in conjunction with new insights from basic scientific research, will help elucidate which treatment combinations and dosing regimens are optimal for various types of cancers in the context of varied patient characteristics and biomarker profiles.

Heat shock protein 90 (HSP90) inhibitors

Hsp90, a 90 kDa heat shock protein, is a highly expressed molecular chaperone that mediates maturation and activation of client proteins, and plays a critical role in establishing resistance to RT. Among Hsp90 clients are a number of proteins which contribute in a cell type-dependent manner to tumor cell radio-resistance. Exposure of a variety of solid tumor cell lines to clinically relevant Hsp90 inhibitors results in tumor growth suppression and increased induction of therapeutic cell death. Whereas an increase in radio-sensitivity of tumor cells was consistently reported, the radio-sensitivity of normal fibroblasts was not affected by Hsp90 inhibition. This suggests that there might be a potential for tumor-selective radio-sensitization. The molecular chaperone Hsp90 has been the focus of a number of investigations as a multi-target approach to radio-sensitization. The Hsp90 inhibitors evaluated to date enhance the *in vitro* radio-sensitivities of cell lines initiated from prostate and lung tumors (24,25).

Hypoxia/angiogenesis: modulating the microenvironment

Both, hypoxia and vascularization, can exert a considerable influence on the response to ionizing radiation, and both are rewarding processes to intervene for improving the response to therapy. Hypoxia leads to the activation of the hypoxia-inducible factor (HIF) and unfolded protein response (UPR) pathways, both of which determine survival under this stress. High expression of hypoxia-inducible genes is often associated with poor prognosis. More continuous exposure can lead to down-regulation of the DNA repair gene RAD51 and others as well as an increase sensitivity to crosslinking agents. A recent systematic review of published and unpublished data identified 4,805 patients with HNC undergoing curative intended primary RT alone. These data from 32 randomized clinical trials were analyzed with regard to the following endpoints: loco-regional control (32 trials), disease specific survival (30 trials), overall survival (29 trials), distant metastases (12 trials) and complications to RT (23 trials). Overall, hypoxic modification of RT in HNC resulted in a significantly increased therapeutic benefit (26).

The problem of hypoxia can be tackled in several ways. The first is to increase blood oxygen. Oxygen delivery can be increased by using drugs such as efaproxiral which reduce oxygen-hemoglobin binding. Using a different approach, Ogawa *et al*. (27) developed a radio-sensitizing treatment that directly oxygenated the tumor by intratumoral administration of hydrogen peroxide and sodium hyaluronate. Second, many oxygen-mimetic/electron-affinic drugs have been developed that specifically radio-sensitize hypoxic, but not normoxic cells. Several drugs underwent clinical testing, but only the 5-nitroimadazole nimorazole showed efficacy in phase III trials. A randomized phase III trial will test the added value of this drug to cisplatin combined with RT in an EORTC trial dedicated to locally advanced human papilloma virus (HPV) negative HNC. The third is by mimicking the redox systems of nitroimidazoles, and fourth, the chemistry of transition metal complexes has been exploited for use in radio-sensitization, the best example of which is cisplatin. Fifth, hypoxic cytotoxins have been developed that kill hypoxic cells with far greater efficiency than normoxic cells. This is an alternative to radio-sensitizing hypoxic cells, and modelling studies indicate that it is the more effective strategy to combine with RT. Tirapazamine is the archetypical drug, although it did not show efficacy in a recent phase III trial in combination with RT and cisplatin. But, we are well aware of the impact that compliance by the centers to the RT protocol can have on the results of a randomized phase III trial dedicated to locally advanced HNC (28). Poor compliance jeopardized the outcome and created a demonstrated bias in the final analysis of the trial.

Hypoxia-induced up-regulation of HIF1α leads to angiogenesis through the up-regulation of vascular endothelial growth factor A (VEGFA) and other growth factors. In addition, vasculogenesis (vascular formation from circulating BMDCs) has been shown to be crucial

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for the growth of tumors that recur after RT. VEGF is the key pro-angiogenic growth factor that is secreted by almost all solid tumors and acts through VEGFR1, VEGFR2 or VEGFR3 receptors on endothelial cells. Vasculogenesis also depends on hypoxia, which is more extensive in recurrent tumors and leads to up-regulation of cytokines which in turn recruit and activate the BMDCs necessary for vascularization. In preclinical models, inhibiting vasculogenesis by various interventions, both genetic and pharmacological, dramatically increases tumor responses after RT and was more effective than inhibiting angiogenesis. This represents a fairly new and promising way to increase the response to RT.

Apoptosis modulation

Modulation of apoptosis sensitivity, mainly linked to caspase activation, has emerged as a promising strategy to increase radiation-induced cell kill and improve clinical outcome. There are two major pathways for the activation of inducer caspases: the mitochondrial-dependent intrinsic pathway and the extrinsic pathway involving ligand-binding death receptors at the cell surface. Most apoptotic stimuli, including radiation and chemotherapy, depend on the intrinsic mitochondrial pathway in which induced permeability of the mitochondrial outer membrane permits the release of pro-apoptotic factors into the cytosol. The activation of the extrinsic pathway depends on ligand binding to cell surface death receptors, such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors and CD95 which are members of the TNF receptor superfamily and activate inducer caspases at the death domain in their cytoplasmic tail (29). Interventional strategies that target apoptotic pathways can be distinguished as those that promote pro-apoptotic signaling and those that inhibit anti-apoptotic signaling. While of interest as a therapeutic modality in itself, TRAIL is also an excellent candidate for combination therapy, since TRAIL and radiation activate partially distinct apoptosis signaling pathways, and a molecular basis for synergy may lie in the up-regulation or sensitization of the TRAIL receptor complex by radiation.

Prerequisite for the development of novel combined radio-molecularly targeted agents

To speed-up the process of developing new combined modality treatments, good preclinical models for optimization of the ratio between efficacy and toxicity and a well established methodology gathering quality controls within a network of advanced high-tech laboratories and clinical departments devoted to early phase trials, as well as trained in implementing new imaging modalities including fluorodeoxyglucose (FDG)-positron emission tomography (PET) and new tracers, useful for monitoring or predicting response to RT, are indispensable (30). Even if it has been well demonstrated that FDG PET is useful to study the glucose metabolism of tumor cells, proliferation could be studied by fluorothymidine (FLT)-PET and hypoxia by fluoromisonidazole (F-miso) and fluoroazomycin arabinoside (FAZA) (31). Receptor-based imaging has also been introduced with tracers targeting EGFR, HER2 and the somatostatin receptor. Efficacy induced by RT combined with chemotherapeutics or targeted agents induce changes in a tumor's physiology, metabolism and proliferation which often precede volumetric changes. Therefore, reliable biomarkers and imaging modalities that could assess treatment response more rapidly or even predict tumor response to treatment at an early phase, would be very useful in identifying the responders and/or avoiding toxic therapies in non-responders. The currently available assays to detect the most prominent types of radiation induced cell death (apoptosis, necrosis, mitotic catastrophy, autophagy and senescence) *in vitro* and *in vivo*, have been described comprehensively by Verheij (32).

In the framework of the EORTC, a strategic initiative called Synergy of Targeted Agents and Radiation Therapy (STAR), offers industry and pharmaceutical companies an efficient and robust preclinical evaluation of the combination of new molecular targeted therapy and RT. The duration of phase I RT combination studies is perceived as a major challenge by the pharmaceutical industry (33), and there is a lack of acceptable endpoints for such RT studies and of defined regulatory pathways to consecutive approval. In order to convince the pharmaceutical industry of a dedicated drug development program, the EORTC ROG is providing a network of experienced radiotherapy departments, a radiation therapy quality assurance (RTQA) program dedicated to clinical trials, disease oriented group working parties, strong connections with the EORTC Imaging and Pathobiology Groups, and working in close connection with a headquarter platform. It could facilitate the rational selection of agents and provide a straightforward methodology for conducting early clinical trials. In the future, multi-study agreements between pharmaceutical companies with such academic and independent clinical trial groups should be favored so as to

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incorporate pre-clinical research into agreements for drug development.

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Statistical principles for omics-based clinical trials

Michael C. Sachs

National Cancer Institute, Biometric Research Branch, MSC 9735, Bethesda, MD 20892-9735, USA

Correspondence to: Michael C. Sachs. National Cancer Institute, Biometric Research Branch, 9609 Medical Center Drive, Room 5W114, MSC 9735, Bethesda, MD 20892-9735, USA. Email: michael.sachs@nih.gov.

Abstract: High-throughput technologies enable the measurement of a large number of molecular characteristics from a small tissue specimen. High-dimensional molecular information (referred to as omics data) offers the possibility of predicting the future outcome of a patient (prognosis) and predicting the likely response to a specific treatment (prediction). Embedded in the vast amount of data is the hope that there exists some signal that will enable practitioners to deliver therapy personalized to the molecular profile of a tumor, thereby improving health outcomes. The challenges are to determine that the omics assays are valid and reproducible in a clinical setting, to develop a valid and optimal omics-based test that algorithmically determines the optimal treatment regime, to evaluate that test in a powerful and unbiased manner, and finally to demonstrate clinical utility: that the test under study improves clinical outcome as compared to not using the test. We review the statistical considerations involved in each of these stages, specifically dealing with the challenges of high-dimensional, omics data.

Keywords: Genomics; personalized medicine; predictive biomarker; statistics

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Introduction

Omics technologies that generate a large amount of molecular data about biospecimens have the potential to provide accurate predictions of a patient's prognosis and predictions of their response to a specific treatment regime. The idea of omics-based tests is that distinct subgroups of patients can be identified using multi-dimensional molecular data and therefore treatment decisions can be personalized to that subgroup. An omics-based test can guide the decisions to treat or not to treat and help identify the particular therapy most likely to work. The challenge is to identify and demonstrate definitively that the use of an omics-based test improves clinical outcomes in a patient population.

An omics-based test can be used to predict a patient's prognosis, which is their expected clinical outcome. A test that provides accurate predictions of prognosis, regardless of treatment, is referred to as prognostic. A predictive omics-based test is one that accurately predicts disease outcomes with the application of specific interventions. Predictive markers are therefore useful for the selection among two or more treatment options. Statistically, a prognostic omics-based test is strongly associated with clinical outcome and a predictive omics-based test modifies the association between treatment and clinical outcome (interaction). High dimensional omics data can be used to identify specific molecular targets as potential mechanisms for drug development; however the use of omics technologies for drug development is beyond the scope of this review.

The path from development to definitively evaluating an omics-based test for prognosis or prediction of treatment response is long and arduous. Often, the end goal is to develop a test suitable for use in a clinical trial for guiding treatment. The oncology literature is full of reports that develop and/or evaluate omics-based tools for prognosis and prediction. Developing a simple test based on high-dimensional omics data can be complex and requires careful application and interpretation of statistical methods. Definitive evaluation of a prognostic or predictive omics-based test is costly and rife with methodological pitfalls. We aim to review the relevant issues, providing the resources

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to ask the right questions when critically weighing the evidence presented in a report of an omics-based study. *Figure 1* gives an overview of the omics test development process. Ultimately, for a practicing oncologist the question is: "Is this omics-based test something I want to use to improve outcomes of my patients?"

The long road to implementing a test in a practice starts with analytical validation of the assay involved, that is, demonstrating that the omics-based assay accurately and reproducibly measures the molecular quantities. After the assay performance is established, development of the test and preliminary evaluation are necessary. Those involve reducing the high-dimensional data into a one-dimensional quantity that will be used to make a decision. This one-dimensional quantity is often a risk score: an estimate of the probability of a specific clinical outcome. It is necessary to establish the clinical validity of this risk score, that is, to demonstrate that the risk score is independently and strongly associated with clinical outcome. Care must be taken to completely separate the development of the risk score from the evaluation, otherwise estimates can be optimistically biased. Finally, the risk score must be translated into a binary decision, often using a threshold. It remains to demonstrate that the use of the test to make this decision improves patient outcomes. *Figure 2* illustrates the types of studies that are involved in the omics test development and evaluation process.

The following sections specify questions to be considered while reading a report of an omics-based clinical study. We review the importance of such questions, and common pitfalls to watch for. In the planning or reporting of an omics-based trial, answers to these questions should be made clear to the reader. Formal efforts to guide reporting have been developed, such as the REMARK checklist (1), the GRIPS statement (2), and an omics checklist (3).

Terminology

An omics-based test, or simply an omics test, is a mapping from the set of features on the omics assay to a single number. This number can be a binary value, such as good or poor prognosis, or it can provide a continuous scale, such as a risk score. It must be feasible to perform the test on an individual patient basis, by measuring the omics assay on the individual's tissue. The assay generates a multitude of measurements, which we will refer to as features, and then fixed mathematical calculations are done to transform the many features into the single test value. Examples of such features are gene expression values, protein expression measurements, or genetic mutations. We use the term specimens to refer to individual patient tissues or fluids on which the assay would be run. We use the term sample in the statistical sense, meaning a group of individuals randomly selected from a population.

Investigators determine the way that the mathematical calculations are done in the development phase. Often, there is a complete sample which is randomly allocated into development and validation sub-samples. These are also sometimes referred to as training and test sets of samples. At the end of the development phase, the model for the mathematical calculations is fixed and the algorithm is locked down.

That model is evaluated definitively in the validation phase in a completely independent sample. In order for the validation to be unbiased and definitive, it is imperative that no information from the validation sample leaks into the development phase. The validation should mimic realistic clinical use as much as possible, and that means that no further refinement to the test is allowed based on the observed results.

A given study may cover only one of the many steps and the entire process may be reported across multiple peer-reviewed publications. For example, at least four key publications were devoted to the development and validation of Oncotype DX, which is a commercially available omics-based prognostic test used in breast cancer (4-7).

What is the intended clinical use?

As with all clinical studies, the end goal is to improve patient care. Omics studies are no different, and a clear statement of the intended clinical use of the omics-test should be prominent. Carefully describing the context for the use of the assay determines the type of study needed to develop and validate it. The intended use of the assay also provides an overarching context in which to interpret the population under study, the assay measurements, and the statistical methods.

Omics-based tests in oncology generally are used for one of two clinical purposes: prognosis or prediction of treatment response. A prognostic test is used to predict the likely clinical outcome of a patient. Often a prognosis is used to guide management of the disease. Patients with a very good prognosis may opt not to receive any treatment, while patients with a poor prognosis may opt for more aggressive treatment. An omics-based prognostic test that is currently

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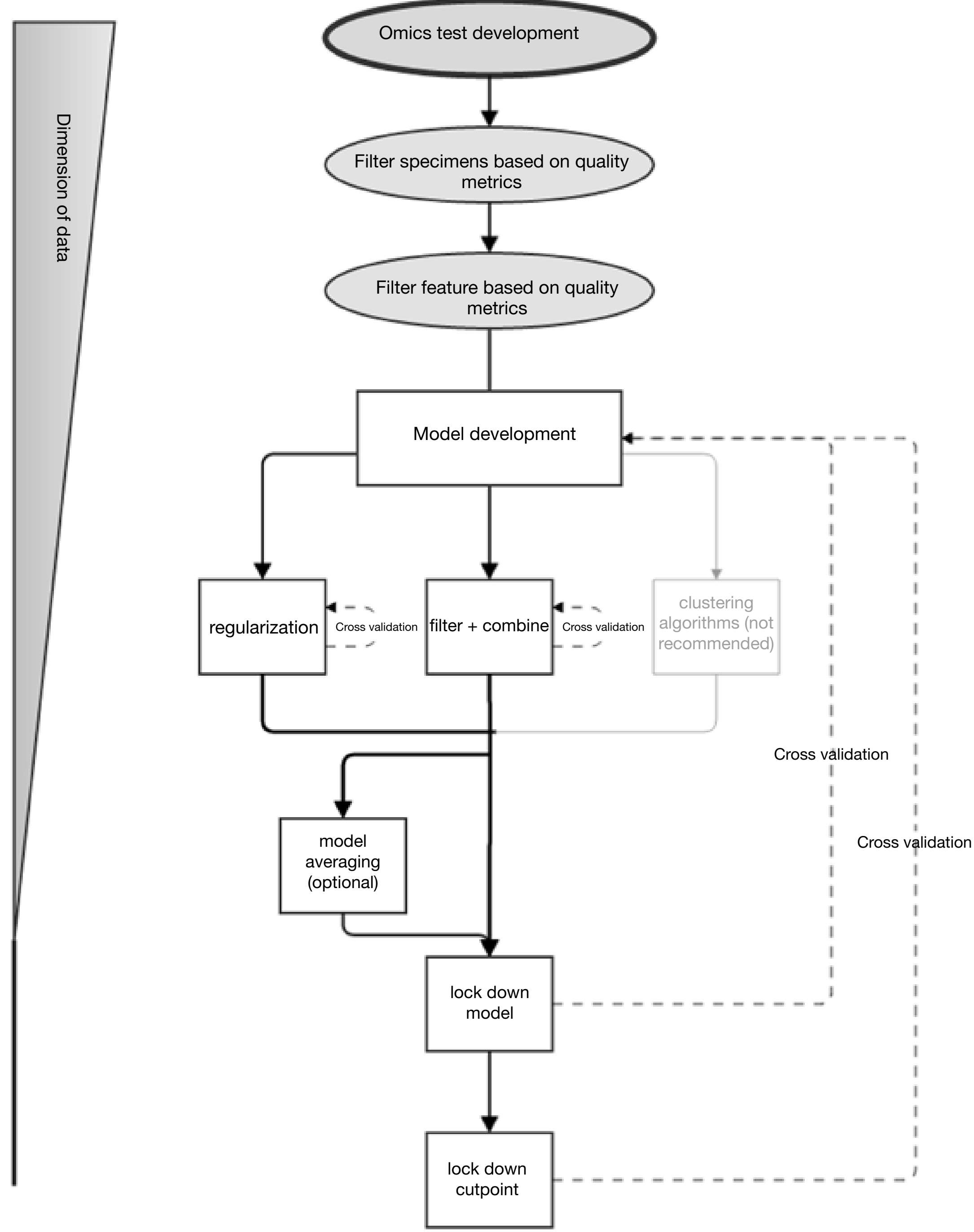

Figure 1 Schematic illustrating the omics test development process.

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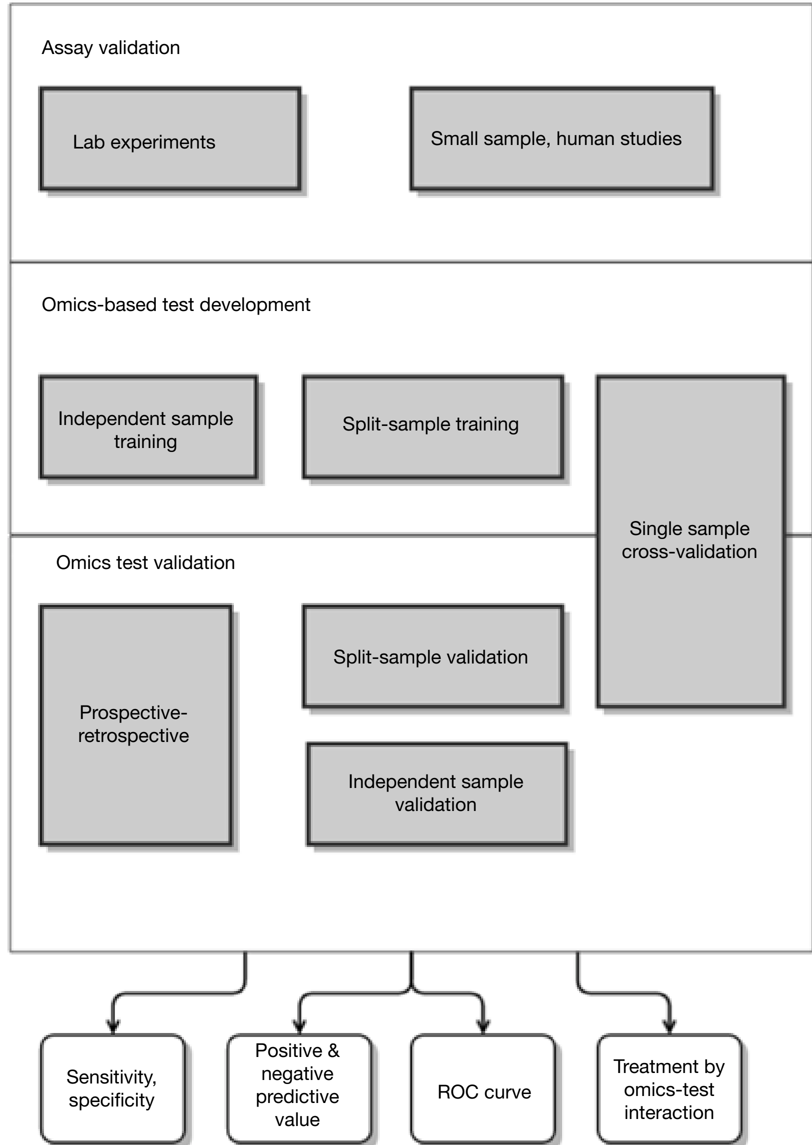

Figure 2 Schematic illustrating the types of studies involved in omics test assay validation, test development, and validation.

used in practice is EndoPredict, which is used to predict the risk of recurrence in ER-positive, HER2-negative breast cancer (8). For patients with a low risk of recurrence, it has been demonstrated that the risks of chemotherapy do not outweigh the benefits. Prognostic tests are clinically useful for guiding general disease management.

Predictive tests are most useful for selecting patient populations for treatment with specific targeted therapies. This presumes the existence of a particular molecular targeted therapy. The predictive test is used to identify patients who will benefit from the targeted therapy. Predictive tests are generally based on only one or a few molecular characteristics that the therapy targets. For example, HER-2 is a gene that is associated with a more aggressive form of breast cancer. Trastuzumab is a drug that specifically targets HER-2 and has been shown to be effective in HER-2 positive breast cancer (9). While targeted therapies generally target only one molecular characteristic, omics assays can be used to identify molecular targets for less well-understood drugs. However, most successful targeted therapies have associated predictive tests that were developed based on the underlying biology

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rather than a broad search over a large number of molecular features (10).

What is the patient population of interest?

Along with the intended clinical use, a report should have a clear statement of the intended population in which the test is being evaluated. This could be broad or quite specific. For the omics test to be useful, it must provide sufficient information above and beyond the standard of care in the target patient population. The distribution of the omics test and the expected benefit in the population should be clearly specified in advance.

The expected benefit of a new omics-based test could differ greatly by patient population. For instance, a prognostic test has more potential for benefit in stage 2 breast cancer than it does in stage 1 breast cancer, as the prognosis for stage 1 is already very good. Evaluating an omics-based test in a broad population that encompasses multiple stages or multiple disease types can be difficult, as the test must provide more information beyond that provided by standard clinical and pathological factors.

Are the assay methods and laboratory procedures valid?

Analytical validation of an assay involves evaluating the performance of the measurement in terms of accuracy, bias, and precision under a variety of conditions. Conditions refer to pre-analytic factors such as specimen quality, specimen collection, storage, and processing procedures, and technical aspects such as laboratory technician and batch effects from reagent lots or other assay materials. The high-dimensional nature of omics data makes it very difficult to assess each of the hundreds or thousands of outputs from a single assay. In developing an omics-based signature that only uses a subset of the components of a high-dimensional assay, one can analytically validate the final signature alone. However, prior to developing the signature, one must develop detailed standard operating procedures for specimen handling and processing to ensure a baseline level of validity.

Study reports must state what type of specimens are used and whether the test is applied to formalin-fixed paraffin embedded (FFPE) or only fresh-frozen tissue. Most omics-based assays require a minimum percentage of tumors to be successful. A report should clearly state what criteria were used to screen tissue specimens prior to running the assay. Generally this involves criteria for the rejection of poor-quality specimens on the basis of percent tumor, percent necrosis, or some other marker of tissue quality.

Molecular assays can successfully be run on decades of old FFPE tissue (11). However, factors involved in the tissue processing and storage can impact the analyte extraction and quality (12-14). Relatively little attention has been given to studying the downstream effects of pre-analytic factors on the individual omics features. In one study, the authors observe that older FFPE specimens tended to have lower expression levels and that this effect was different for different genes. The investigators modified their assay to account for this differential effect (15). Due to the high dimensionality of omics assays, a small amount of bias on each feature can translate into large errors when incorporating data from hundreds or thousands of features into a single continuous measurement. Therefore it is important to assess the impact of processing on the individual features in addition to the overall test.

In addition to processing and storage, technical aspects of an assay can impact the final results in a predictable way (16,17). There could be technical effects, differences due to reagent lots, and other batch effects. Such batch effects are commonly recognized yet often ignored in high-dimensional assays (18). Efforts should be made to measure the impact of these technical aspects and minimize them to the greatest extent possible. The way in which specimens are assayed should be randomized to prevent confounding batch effects with the clinical outcome. Development and validation samples are sometimes run in the same batch or with the same lot of technical aspects. This does minimize batch effects; however, it can provide an overly optimistic assessment of the test, because in clinical use, running specimens all in the same batch is not always an option.

Similar to developing criteria for rejection of tissue specimens, in omics settings, criteria should be developed for the rejection of individual features (e.g., genes, proteins) prior to the development of the test, if problems cannot be resolved through improved assay procedures. Features that do not pass the pre-specified quality metrics should be removed from consideration from the final test. Note that this feature processing step does not involve any clinical outcome measurements. As a concrete example, in the development of the gene expression based test EndoPredict, investigators chose to exclude probe locations that have a dynamic range less than 2, probes for which fewer than 1% of the specimens had calls, and probes whose 90th percentile was less than 350 units (8). Quality control steps of this nature can ensure a more robust and reproducible

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development of the test.

Even with careful quality control and a locked down standard operating procedure, it is difficult to completely eliminate the effects of technical factors on assay results. Therefore, when designing the development phase, the investigator must be mindful not to confound technical factors with the clinical outcomes. The problem of batch effects is widespread in omics research and can lead to spurious or irreproducible results (18,19). As an extreme example, consider developing an omics-based test to predict a binary clinical response. In the development phase, all of the assays for the clinical responders were run using reagent A, while all of the assays for the clinical non-responders were run using reagent B. If it were the case that the reagent has a significant effect on the assays, then the development phase would then lead to what seems like an excellent predictor, except it is predicting the batch effect rather than the clinical outcome.

Are the statistical methods for test development appropriate?

Once the analytical validity of the omics assay is established, the features are translated into a binary classification, a multi-category classification, or a continuous risk score. The methods used to perform this translation must be carefully evaluated to ensure that the features of the omics assay have been properly translated into a clinically meaningful quantity.

Unfortunately, a common approach to developing prediction models is to use cluster analysis of omics features, ignoring the clinical outcome among the development samples. Cluster analysis is a class of methods that is used to partition individuals into groups based on the similarities or differences among the omics features (20). The number of groups or clusters is not known in advance, but rather it is data dependent. Clustering is unsupervised in the sense that discovery of the groups is done without regard to the clinical outcome. The resulting clusters are not designed to provide valid information regarding a prognosis or prediction of response to therapy (21). A common argument in favor of clustering is that it identifies biologically distinct groups. However, the groups are identified using a statistical algorithm and the biological relevance is only considered post hoc. For developing omics-based prognostic or predictive tests, it is better to use supervised statistical methods which are designed to address those aims, outlined below.

Often, there are more features measured than there are patients in the sample. In such high-dimensional settings, it is required to identify a subset of the features that will be used in the final multivariable mathematical model. There are two broad statistical approaches to this problem: filtering and regularization.

Filtering is a statistical approach where univariate methods are applied to each of the many omics features in turn. Typically, the univariate method involves estimating the association of the feature with the clinical outcome. Then, a criterion, chosen in advance or selected using cross-validation, is applied to the statistic to select a subset of features. For example, suppose an investigator is interested in developing a gene expression based test to predict clinical response to a new therapy. For each of the 1,000 gene expression features that are available, one could compute a *t*-statistic comparing the expression levels for responders versus non-responders. Genes with *t*-test P values greater than 0.0001 could be filtered out, and the remaining ones used in a multivariable logistic regression model to predict response (22) describes a novel approach to filtering that is applied successfully to predict B-cell lymphoma subtypes using gene expression microarrays.

Regularization is an approach in which all of the features in consideration are entered into a special multivariable statistical model for prediction of the clinical outcome, even if there are more features than study participants. The special model includes a penalty component which encourages the model to remove completely or downplay the impact of features that are not relevant. There are various types of penalty functions each with different properties, such as the lasso (23), the ridge penalty (24), the elastic net (25), and others (20). Each type of penalty term contains at least one tuning parameter, which may be pre-specified or selected using cross-validation.

Each type of approach has its merits, and within each class there are a variety of specific models to choose from. It is difficult to determine what method will work best in advance. Instead of selecting a single model to use, multiple models can be averaged to improve prediction (26). This approach, called Bayesian model averaging, has proven successful in different applications, including prediction of cancer subtypes (27). It is more common, however, to try several different methods then select the one that performs the best on a small subset of the development sample. This is appropriate as long as the model selection is done entirely separately from the final validation sample. Leaking of information from the validation data into the model selection process can cause bias in insidious ways.

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In many oncology settings, such as pediatric cancers, patients and specimens may be very sparse. It may be difficult to enroll sufficient subjects to develop an omics test and then perform preliminary validation on an independent sample. In that case, cross-validation can provide an unbiased estimate of prediction error, if done properly (20). Cross-validation refers to the idea that a model can be evaluated in the same sample in which it is developed. Similar to a split sample approach, in cross-validation only a small portion of the sample is left out at a time. The model is estimated on the remaining samples, and the performance is evaluated on the left out independent portion. This process is repeated many times to get a more precise estimate of the performance (28) describe a cross validated trial design tailored for sparse data settings.

In doing cross-validation, it is important to validate the entire model estimation process, not only part of it. For that reason it is often best to avoid complex test development procedures involving multiple, data-driven selection steps and/or tuning parameters so as not to mistakenly leak information from the validation data (29). More complex procedures can also lead to overfitting, in which the model identifies random noise in the data, rather than a true signal of clinical use.

How is the validation study designed?

Once the mathematical model is estimated and completely locked down based on the development sample, a study to definitively evaluate the locked-down test should be designed to address the clinical use in the population of interest. The key characteristic of the evaluation study or sample is that it is completely independent of the sample on which the test was developed. Once the test is defined and locked down, no information from the evaluation sample can be used to change the features of the test. The evaluation sample could be a randomly selected subgroup from the same parent study as the development set, or it could be from a separate study altogether conducted in the same population. As long as the population and the intended clinical use are clearly defined, the evaluation can be done definitively.

A definitive evaluation can be done retrospectively, meaning that stored specimens are selected from a study that has completed. The omics assay is then run on the archived specimens and the locked down test is associated with the clinical outcomes, which have already been observed at the time of the assay measurement. This retrospective design can yield high quality evidence of the test's characteristics, if it is done carefully. It is imperative to develop a protocol for the study in which the omics test is clearly and completely defined, the main hypotheses are specified, and the assay standard operating procedures are detailed. The archived specimens need to come from a study or trial with a well-defined population under study, not a convenience sample. Sample size and power calculations should be done with the same rigor as they are in a clinical trial. Such a study, called "prospective-retrospective", can yield a high degree of evidence in the evaluation of an omics-test, and with great efficiency (30).

Alternatively, prospective studies can be used to evaluate an omics-based test by performing the assay at the start of the study and then following patients for clinical outcomes. Again, all of the key details need to be specified up front in the protocol. The details of the study design should be tailored to appropriately answer the clinical question definitively. Several review articles are available that describe the potential study designs for the evaluation of prognostic and predictive tests (31-34). Details of specific designs and statistical approaches are available for Bayesian approaches (35,36), adaptive or sequential approaches (37,38), and standard frequentist approaches (39-41). This has been and continues to be an active research area in statistics, which means that designs are continually evolving to appropriately address the clinical question in the population of interest.

In the design, careful consideration should be given to the study power and sample size. A prospective study in which patients may be undergoing painful biopsies or unnecessary treatment should not be done unless there is a high probability of definitively answering the scientific question. Likewise, precious archived specimens should not be wasted on a retrospective study that is under-powered. Most standard statistical tools for power analysis apply to prognostic tests, however predictive or therapy-guiding omics-based tests require a different approach. Many protocols for predictive tests are powered to detect the interaction effect between the treatment and the test (42). The existence of a treatment-by-test interaction is necessary but not sufficient for the test to be useful in guiding therapy (43). Tools for power and sample size analysis have been designed to specifically address the question of a qualitative interaction (44-46).

Are the development and validation samples strictly separated?

This issue has been discussed in previous sections, yet

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this error occurs so frequently that it requires an in-depth discussion. The evaluation sample for the assessment of a prognostic or predictive test needs to be completely independent from the development sample. This is especially true for omics-based tests, whose development is often complex. Any information from the evaluation sample that leaks into the development sample can bias the results, making tests appear better than they truly are.

Leaking information between samples can happen in subtle ways. Sometimes, part of the model development process is repeated on the validation data. This is called partial resubstitution (21). For example, a common model development approach is to first filter a subset of 50 genes from a larger set of 450,000 based on their observed association with the outcome. Then, the 50 genes are put into a regression model to develop a single risk score. Occasionally, investigators will perform the filtering on the development sample and then re-estimate the regression model using the combined development and validation samples. This gives overly optimistic estimates of the performance of the algorithm. Partial resubstitution can be difficult to detect when the model development is more complex, and if cross-validation is used to estimate the performance.

In settings where relatively few samples are available, cross-validation is an efficient and valid approach to estimating performance (47). The key point whether using the split sample approach or cross validation is that the entire model building process must be validated. Even informal checks of the model on the validation sample, such as viewing survival curve plots, prior to locking down the model can unknowingly cause bias.

Are the statistical methods appropriate for test validation?

To assess the value of an omics-based test for prognosis or prediction we need to estimate the association between the test and the clinical outcome on an independent sample (the validation sample). Appropriate statistics are essential to measure this association. Often, investigators will report only the odds ratio (in the case of a binary clinical outcome) or the hazard ratio (for a time-to-event clinical outcome) for the omics test. The odds ratio or hazard ratio is insufficient to determine the clinical utility of an omics-based test (48). Ideally, a statistical method or set of statistical measures should be chosen to address the intended clinical use of the test.

For a prognostic test, how often does the test correctly predict recurrence (true positives) and how often does it correctly predict non-recurrence (true negatives)? It is imperative to report both of these measures, also known as the sensitivity and specificity; because one can correctly predict all true positives simply by predicting that all cases are positive. Is the performance good enough to change clinical practice? Patients want to know the likelihood of recurrence given their test results; this is called the positive predictive value. If the likelihood of recurrence is very low overall in the population, as it is in stage 1 breast cancer, then a new test must be highly informative for it to be practice changing. For continuous-valued tests, extensions to these measures exist and can be visualized with the receiver operating characteristic (ROC) curve. Furthermore, extensions also exist for time-to-event clinical outcomes such as overall or progression free survival (49) provides an excellent reference for statistical measures for the evaluation of diagnostic and prognostic tests.

In recent years, a number of potentially misleading statistical methods have crept into common usage. The net reclassification index (NRI) and its sibling, the integrated discrimination improvement (IDI), were designed to assess the added value of a new test to existing criteria: the incremental value. For example, it is often of interest to determine whether a novel omics-based test adds value to standard clinical and pathological features. The NRI and IDI specifically evaluate whether the novel component enhances the differentiation of patients into risk groups. This does not address the question whether the novel component correctly classifies patients (50,51). Others have noted additional problems with the statistical operating characteristics of the method, most importantly, that it is not a valid measure (52-54). More fundamentally, it is not clear what clinical question this measure addresses; does it matter if patients are classified differently if we don't know whether they are classified correctly?

A proper evaluation of an omics-based test takes a comprehensive and pre-specified approach to address the intended clinical use. For predictive omics-based tests to guide therapy, a rigorous approach to evaluation has been described, along with statistical software for general use (55). This continues to be an active area of biostatistical research.

Concluding remarks

The use of omics-based tests for prognosis, predicting, and therapy selection is steadily increasing in oncology. Careful evaluation of the quality of studies by consumers of the

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clinical oncology literature is imperative to provide a high level of patient care. Formal sets of reporting criteria exist for the producers of such literature (1-3,56) and these are also useful for readers to be aware of. We hope that the discussion here has brought attention to the issues from the readers' perspective and will help promote critical evaluation of the relevant literature.

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Footnote

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An overview of the NCI precision medicine trials—NCI MATCH and MPACT

Khanh Do[1], **Geraldine O'Sullivan Coyne**[2], **Alice P. Chen**[2]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; [2]Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD, USA

Correspondence to: Alice P. Chen, MD. Division of Cancer Treatment and Diagnosis, National Cancer Institute, 31 Center Drive, Bldg 31, Rm 3A44, Bethesda, MD 20892, USA. Email: chenali@mail.nih.gov.

Abstract: The concept of oncogene addiction was first proposed by Weinstein in 2002, postulating that tumors rely on a single dominant mutation, the oncogenic "driver", for growth and survival. We have since come to realize that the genomic landscape of tumors is heterogeneous and more complex than previously thought. Advances in biotechnology and bioinformatics over the past decade have shifted treatment paradigms with regard to the development of molecular targeted therapeutics to identify and target the presumptive dominant lesion. As such, the decision of choosing targeted treatment strategies has become increasingly more reliant on the reporting of genomic screens of patients' tumor tissue. Whether this change in treatment paradigm will translate into improved clinical benefit, remains to be seen. To this end, the United States National Cancer Institute (NCI) has launched precision-based medicine trials to address this question. NCI Molecular Analysis for Therapy Choice (MATCH), a genomic pre-screening study, was designed to explore the efficacy of using targeted agents to target specific molecular aberrations and whether these same therapies have comparable activity across different tumor subtypes. Molecular Profiling-based Assignment of Cancer Therapy (MPACT), is a smaller, provocative trial designed to address whether targeting an oncogenic "driver" would be more efficacious than one not. The Exceptional Responders' initiative further aims to evaluate patients who have derived an unexpected durable benefit to these therapies, with retrospective analysis of their tumors to delineate potential predictive biomarkers which could predict response. The results of these trials will serve to help guide the field of precision medicine and personalized care.

Keywords: Precision-based medicine; targeted therapy; NCI MATCH; Molecular Profiling-based Assignment of Cancer Therapy (MPACT); Exceptional Responders

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Background

The concept of oncogene addiction was first proposed by Weinstein (1), and has led to a whole new approach to cancer treatment. The discovery of imatinib, the Bcr-Abl tyrosine kinase inhibitor, in the treatment of chronic myelogenous leukemia, revolutionized treatment paradigms with regard to targeted therapies, as this was the first targeted agent to illustrate the concept that treating the principal driving oncogene can have a powerful impact on response (2). More recent efforts to catalog driver mutations across the entire cancer population have led to the development of a plethora of targeted agents. Subsequent generations of molecularly targeted agents have effectively subcategorized tumors into smaller molecular subsets, such as EGFR and ALK inhibitors in non-small cell lung cancer and BRAF inhibitors in melanoma, in an effort to duplicate this success. As a further example, trastuzumab has received approval for gastro-esophageal and gastric cancers in addition to HER2 overexpressing breast cancers

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(3,4). These efforts have led to the realization that the targeting of these mutations has the potential to transcend tumor histologies, effectively categorizing tumors based on the molecular signature.

Recent advances in biotechnology and bioinformatics over the past decade have led to a greater appreciation for the heterogeneity of tumors and the complex signaling pathways involved in the resistance to treatment. This complexity requires a network-based streamlined approach to the interpretation of data generated from a profile of the tumor. The current challenge of clinical trial design is focused upon the identification of molecular alterations in tumors and the selection for those patients who would be most likely to benefit from a particular targeted therapy. The Division of Cancer Diagnosis and Treatment of the United States National Cancer Institute (NCI) has accepted this challenge and is presently engaged in several trials dedicated to precision-based medicine (http://dctd.cancer.gov/MajorInitiatives/NCI-sponsored_trials_in_precision_medicine.htm). The NCI Molecular Analysis for Therapy Choice (MATCH), Molecular Profiling-based Assignment of Cancer Therapy (MPACT), and Exceptional Responders study are among these trials.

NCI MATCH trial

The NCI MATCH trial was initiated as a broad-based genomic pre-screening study to assign patients whose tumors harbor specific molecular aberrations to relevant targeted treatments, without regards to tumor histology type. This trial aims to establish whether patients with tumor mutations, amplifications or translocations of interest are likely to derive clinical benefit if treated with agents targeting that specific molecular change in a one stage single-arm design. To provide the greatest opportunity to patients, this trial will cover a large range of mutations with matching options. In order to design such a complex trial, a panel of experts in developmental therapeutics, clinical trial design, genetic sequencing, molecular oncology, informatics, and statistics were consulted to develop an algorithm that would define clinical action based on genetic variants reported in the genes of interest. The structure of the study involves a master protocol to ensure the common elements of the subprotocols remain consistent across the arms. This study is additionally designed with the flexibility to open and close arms under the umbrella of the master protocol, with each arm treated as a separate phase 2 trial.

To ensure for adequate patient enrollment, the trial will be run through the NCI National Clinical Trials Network (NCTN) and NCI Community Oncology Research Program (NCORP). NCORP will help bring this nationwide study to patients treated in the community setting and increase accessibility to patients. The ECOG-ACRIN group will coordinate the trial for the NCTN, with broad representation through having separate principal investigators for each of the sub-protocols, each representing the different groups within the NCTN. The large portfolio of agents needed for the success of this trial required the participation of a multitude of pharmaceutical partners. The Cancer Therapy Evaluation Program (CTEP) of the NCI assisted in the coordination and contracting of these agents. The NCI Center for Biomedical Informatics and Information Technology (CBIIT) along with members of the NCI MATCH team generated the informatics structure for this trial. Multiple committees, including Agents and Genes Working Groups, Sample and Sequencing Network Working Group, and Protocol Logistics Working Group, among others, were established to concurrently develop the multitude of components for this massive endeavor.

NCI MATCH will accrue patients with solid tumors, with disease that has progressed following at least one line of standard systemic therapy, or for whom no standard therapy exists. As this is an exploratory trial, histologies for which there is already an FDA approved indication with that agent, or that have been shown to not respond to a particular agent, will accordingly be excluded from the corresponding agent. The study is designed to assign targeted treatment based on a biopsy obtained after enrollment. Molecular changes will be the selection criterion for entry to a particular arm. The study drugs included in this trial include single agents and combinations that have either received FDA approval or are investigational agents that have achieved at least a recommended phase 2 dose.

The NCI MATCH trial will collect somatic (tumor) genomic data from all patients enrolled through a screening biopsy. As tumors sometimes accumulate additional mutations after various treatments or with continued growth and metastasis, a biopsy closest to the time of initiating treatment will be pursued in order to obtain the most reflective state of the tumor. A biopsy after progression will also be pursued, with special interest in those patients who initially responded to treatment, to assist in understanding the mechanism of resistance. The Molecular Characterization (MoCha) Laboratory of

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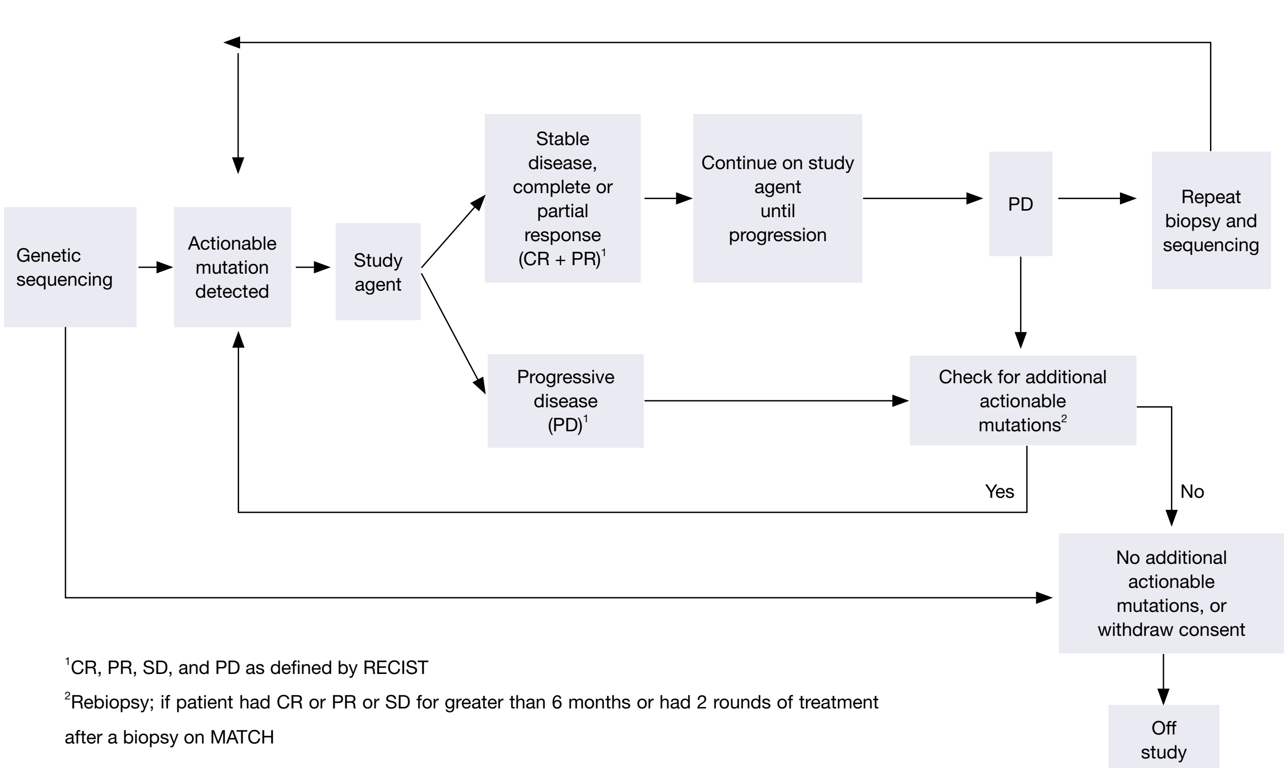

Figure 1 NCI MATCH study design (5). MATCH, Molecular Analysis for Therapy Choice; NCI, National Cancer Institute; SD, stable disease; RECIST, Response Evaluation Criteria in Solid Tumors.

the NCI was charged with development of the assay to identify these actionable mutations. The patient's tumor biopsy will be screened for pre-defined variations in genes within a NCI MATCH CLIA-certified laboratory. The molecular profiling assays will include large-scale parallel tumor sequencing (next generation sequencing) strategies, including a targeted Ampliseq panel as well as other molecular assays such as immunohistochemistry (IHC). The selection of treatment will be rule-based and will be applied by a rigorously validated informatics system to derive a tentative treatment assignment. If a patient is ineligible for the original assigned treatment arm because of a pre-defined clinical ineligibility criterion, and patient's tumor harbors additional abnormalities for which treatments are available on the study, the system algorithm will continue to provide assignments until all available options are exhausted (*Figure 1*).

On this trial only malignant tissue will be screened. As such, definitive abnormalities in germline tissues (heritable diseases) cannot be identified with any certainty. Due to the concern that some of the genes tested may be of germline origin, a committee of multidisciplinary experts (genetics, oncology, bioethicists, patient advocates) was formed to address this ethical concern. Currently, findings will be communicated to the treating clinician with the recommendation to consider germline testing if clinical and/or family history is consistent with the presence of such an inheritable germline mutation. In many cases the medical significance of genetic variants are unknown (6,7). With the changing field of genomics, a steering committee has been tasked with monitoring the changing landscape.

This study affords a unique opportunity to collect information about the prevalence of mutations, translocations and amplifications in genes associated with cancer, and how these tumors respond to targeted therapy in the treatment-refractory tumor setting. DNA variants and changes in RNA expression from tumors collected at the point of progression on treatment is anticipated to illuminate resistance mechanisms that will inform subsequent studies and improve upon patient outcomes.

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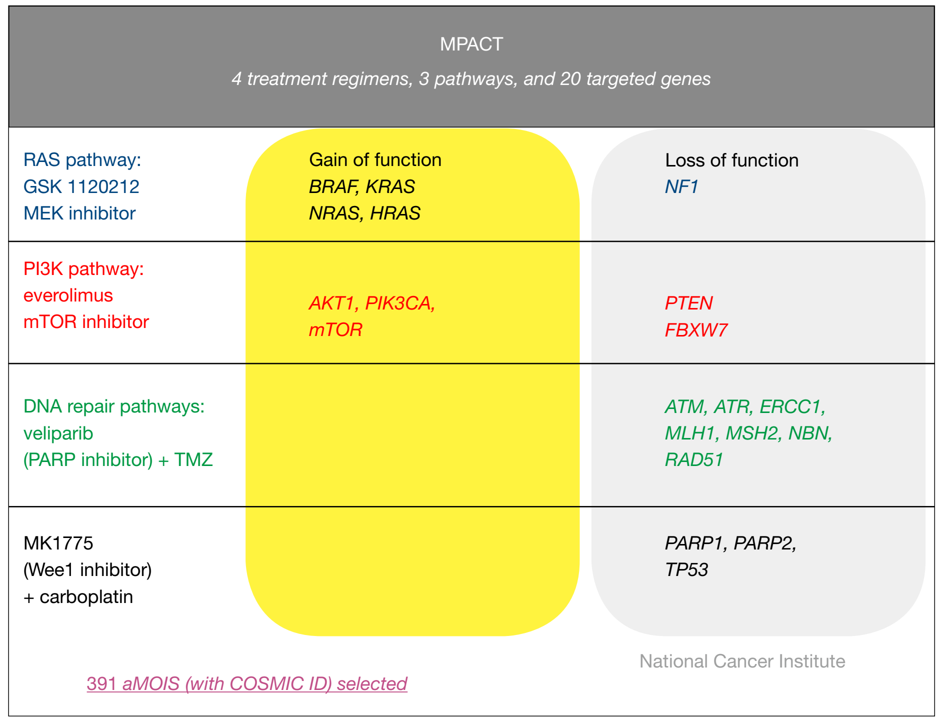

Figure 2 MPACT pathway (5). MPACT, Molecular Profiling-based Assignment of Cancer Therapy; COSMIC, the Catalogue of Somatic Mutations in Cancer.

NCI MATCH has opened in August 2015.

MPACT trial

MPACT was designed to address the question of whether targeting an oncogenic driver would be more efficacious than not targeting the mutation. This pilot trial aims to establish whether advanced cancer patients who have exhausted all standard treatment options with proven benefit and have tumor harboring mutations in one of three main genetic pathways (DNA repair, PI3K, or RAS/RAF/MEK) are more likely to derive clinical benefit if treated with agents targeting that pathway than if treated with agents targeting one of the other pathways not identified to be dysregulated within the tumor. The agents administered in this trial are at recommended phase 2 dosing schedules. Currently the trial involves three pathways and four treatment arms (*Figure 2*): (I) veliparib (PARP inhibitor) with temozolomide for defects in the DNA repair pathway; (II) AZD-1775 (Wee1 inhibitor) plus carboplatin for defects in DNA repair pathway; (III) everolimus (mTOR inhibitor) for mutations in the PI3K pathway; or (IV) trametinib DMSO (MEK inhibitor) for mutations in the RAS/RAF/MEK pathway. Because of known benefits of BRAF inhibitor in melanoma and PARP inhibitors in *BRCA* ovarian cancer patients, these selected exclusions were built into the trial. The patients may remain eligible to be screened but will only be eligible to receive any of the study treatments if they have other actionable mutations.

Similar to NCI MATCH, patients undergo tumor biopsies at the time of enrollment with the tumor sequenced in a CLIA-certified lab for actionable mutations. Distinct from NCI MATCH, patients for whom an actionable mutation is detected undergo a 2:1 randomization to one of two arms based on results of molecular profiling analysis (*Figure 3*) where the investigator and patients are blinded to the molecular target. Patients randomized to the treatment arm would receive drug or drug combinations designed to target the identified genetic mutation. Patients randomized to Arm B would receive drug or drug combinations not prospectively identified to target the identified mutation. Patients in whom no actionable mutations are identified in one of the three pathways (DNA repair, PI3K, or RAS/RAF/MEK) would be deemed ineligible for further treatment. Patients who have been treated and subsequently progress on their respective treatment arm will have their

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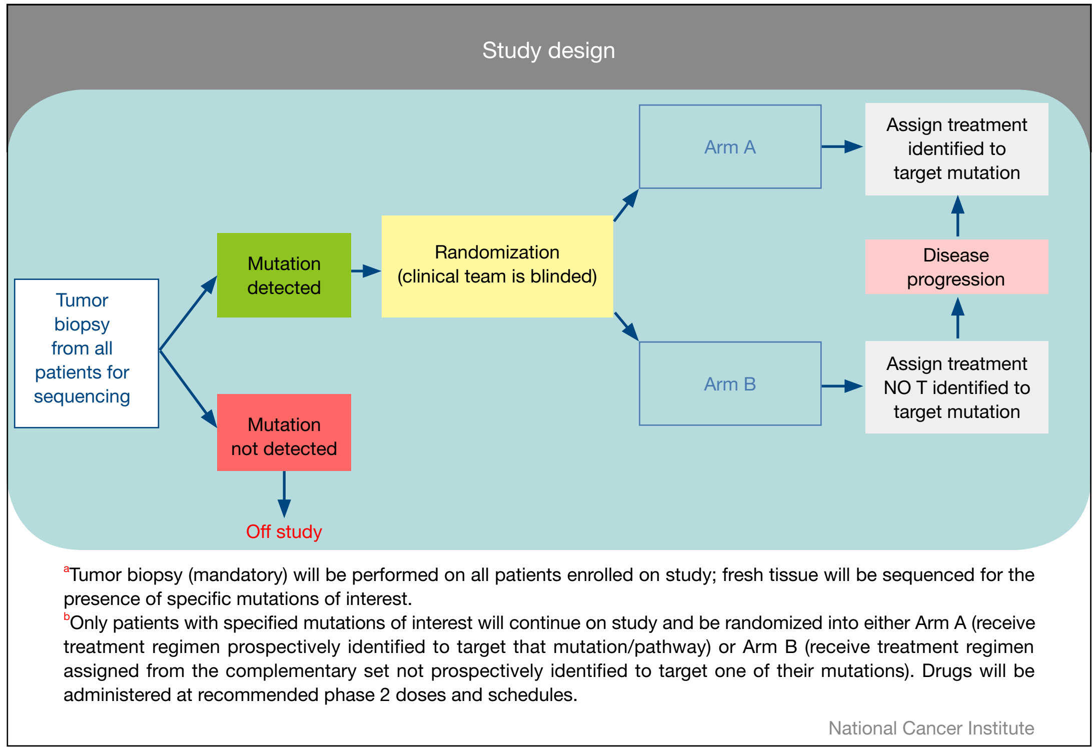

Figure 3 MPACT study design (5). MPACT, Molecular Profiling-based Assignment of Cancer Therapy.

molecular profiling analysis unblinded and are permitted to crossover to the treatment arm if originally assigned to the control arm. Similar to NCI MATCH, emphasis is placed on repeat biopsy at time of progression to further understand the resistance mechanisms and whether exposure to targeted agents may have created a selection pressure for the acquisition of new lesions. Given the relative frequencies of mutations in the pathways of interest in this study, approximately 700 patients will be enrolled to acquire 180 evaluable patients with the initial four arms, assuming the population screened is similar between the treatment arm and the control arm. This trial is also designed to have flexibility with regard to the addition of new pathways/treatment arms. The endpoint of the study will compare the response rate [complete response (CR) + partial response (PR)] and/or 4-month progression-free-survival of the treatment arm versus the control arm. MPACT is currently open in the Developmental Therapeutics Clinic, NCI but will be available at other sites through the NCI-sponsored Experimental Therapeutics Clinical Trials Network (ETCTN) in the near future.

The backbone of both these precision-based medicine trials is heavily dependent upon having an accurate, reliable, and rapid molecular assay for the identification of actionable mutations. For MPACT, genetic sequencing will be performed in the CLIA-certified MoCha at the Frederick National Laboratory for Cancer Research (FNLCR). The genetic variants to be assessed and treatment algorithms have been prospectively defined to allow for assignment of specific treatment arms on study. For MPACT, 20 genes were selected for the initial analysis panel based on several criteria: (I) the biological pathway(s) affected by the targeted therapy were examined (pathways: RAS/RAF/MEK signaling pathway, PI3K/AKT pathway and DNA repair pathways; (II) genes within these pathways were selected based on demonstrating a minimum frequency (5%) of somatic variants as listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database; (III) genes known to modulate the targets of the study drugs; (IV) a Molecular Tumor Board review of the preclinical and clinical literature for the selection. A variety of specimen and assay quality checks are built into the assay process.

As the selection of treatment arm is rule-based, an informatics system, called GeneMed was designed to streamline the annotation of sequencing data, facilitate the review of variant mutations, and aid the identification

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Table 1 Definition of exceptional response

Definition of exceptional response
Exceptional Responders are patients who meet the following criteria
CR to a regimen in which CR is expected in <10% of similarly treated patients
PR >6 months in a regimen in which PRs >6 months are expected in <10% of patients with similar disease treated with same or similar regimen
CR or PR of unusual duration, such that the internal review committee considers it to be an exceptional response
Examples below
PR of duration >3x the median expected PR duration (in cases where PR is expected in >10% of patients with the same disease treated with the same regimen)
CR of duration >3x the median expected CR duration (in cases where CR may be seen in >10% of patients with same disease treated with same regimen)
The observed duration of CR (or PR) is longer than expected for 90% of patients with same disease treated with same regimen

CR, complete response; PR, partial response.

of the actionable mutation. The results from the assay are processed based on predefined rules, and a treatment selection is assigned. If patient has two actionable mutations, the decision of which mutation will determine treatment selection is also rule-based.

Exceptional Responder

Despite best efforts, the majority of single agent anti-neoplastic drugs that enter phase 1 and 2 clinical trials ultimately fail to demonstrate sufficient efficacy to support further development. In rare exceptions, however, one or two patients achieve a significant response to the therapy or derive unexpected long-term benefit on these trials. These small subsets of responders from these otherwise "failed" trials/treatments may hold the key to insight in tumor biology and the identification of the molecular markers that predict for response to treatment. In support of this approach, several case reports have highlighted these "Exceptional Responders". As an example, a urothelial cancer patient with a TSC1 and NF2 mutation achieved a durable CR to everolimus in a phase 2 trial that had failed to meet its phase 2 endpoint (8). Alterations in these genes were known to be associated with mTORC dependence in preclinical studies. The authors sequenced 13 additional patients with bladder cancer who had received everolimus, and found that 4 of 5 patients with TSC1 mutations had tumor shrinkage, whereas those without the mutation did not. A second patient with urothelial cancer, identified to have a novel mTOR mutation by whole exome sequencing, also had a CR to the combination of everolimus and pazopanib (9). These reports, as well as others in the literature, suggest that a search for elusive molecular targets in responders as a means to enrich studies for those patients most likely to benefit from any particular treatment holds promise for a more successful drug trial. The ability to identify molecular markers that are able to predict a clinical response in any particular subsets of patients will provide the tools necessary to conduct further studies consistent with the principles of precision medicine and allow for more rapid development of novel strategies.

The Exceptional Responders initiative aims to establish a repository of information on tumor biology based on data collected from these unique responders. The success of this endeavor depends upon having accurate and reliable demographics, clinical history, and response data for patients who have been treated, adequate tissue for analysis, robust analytical techniques/platforms, and appropriate bioinformatics/biostatistical tools. The Exceptional Responders project will collect tissues from patients who fit the definition of Exceptional Responders (*Table 1*) and use whole exome sequencing, and/or targeted NGS assay deep sequencing for full genomic analysis of patient tumors. If sufficient material is available, further exploration with additional analyses [e.g., whole genome sequencing, messenger RNA (mRNA)-sequencing, micro RNA (miRNA) sequencing, promoter methylation analysis, single nucleotide polymorphism (SNP) analysis, etc] will be performed. All data will be de-identified and placed in a controlled-access database to serve as a repository of information to allow investigators to mine data and design and build clinical trials around this information, based on molecular features predictive of benefit to a particular drug or drug class.

Factors to consider

The essential factors for the success of these NCI precision-based medicine trials bring to light fundamental issues inherent in transformative clinical trial design. The data

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generated from these trials have to be interpreted with certain assumptions: (I) target engagement by the selected agent has been confirmed either preclinically or clinically—even in best of circumstances, errors have been made especially in early development. One glaring example is that of iniparib, which failed to inhibit poly (ADP-ribose) polymerase *in vitro*, though this was not discovered until it had gone through phase 3 clinical development (10); (II) the assay used has been validated, confirmed to be reliable for the target, and transferable across sites—guidelines and standard operating procedures must be in place to ensure that biospecimen collection, storage, and processing meet quality standards for further sequencing and that regardless of where the specimen is handled, the same result can be expected. As an example, the lack of these institutional standards delayed initiation of the Cancer Genome Atlas initiative (11); (III) biopsy material obtained is representative of the entire tumor and metastatic site(s)—while driver mutations likely represent a significant portion of the existing tumor, tumor heterogeneity is a well-known challenge in the design of molecular targeted clinical trials. Even strategies such as serial tumor biopsies cannot completely eliminate this as a factor in the interpretation of data. Future development of circulating tumor cells or circulating DNA may help to further delineate driver mutations from those of bystander mutations. Radiographic record of biopsy sites may improve understanding of tumor heterogeneity; (IV) there is an available therapy for the target of interest—selection of the most reasonable agent for a specific target can sometimes be limited due to factors such as drug availability, ease of administration, or proven ability to combine with established agents in a particular clinical setting. Limitations in therapeutic options can further be complicated in situations of variants of unknown significance where benefit has not yet been confirmed.

The results of both NCI MATCH and MPACT will be informative and provide opportunities for further investigation. Though NCI MATCH's primary endpoint is response rate, it is exploratory in nature. With a mix of histologies, including those of rare tumors, any response could provide interesting leads. How these leads will be explored and confirmed is not currently established. The strength of MPACT is heavily dependent upon accurate selection of the driver mutation in order to focus exploration of a particular pathway. Both trials contain multiple arms with small number of patients designed not as definitive trials but more as exploratory trials in order to guide further exploration of both tumor and pathways.

Conclusions

Advances in biotechnology and bioinformatics over the past decade have allowed for molecular characterization of patient tumors, opening opportunities for the development of tailored therapeutics based on characterization of a patient's tumor. The Precision Medicine Initiative is a priority for the NCI, and was recently noted during President Obama's 2014 State of the Union address (12). Currently the NCI is sponsoring several trials strategized to test the benefit of targeted therapy. NCI MATCH, MPACT and Exceptional Responder initiative are among these trials. Others including lung MAP and ALCHEMIST will be discussed by others in this journal. The results of the Exceptional Responders initiative in particular will be central to the identification of molecular features of tumors that would predict for response to a particular drug or class of drugs. For many currently standard chemotherapy drugs or regimens, the exact mechanism of action may not be known, and thus Exceptional Responders to such regimens may provide critical new data. The information obtained from this trial will be made available to investigators in a database that can be shared, built upon, and further mined. The NCI MATCH trial will evaluate these targets and whether they behave similarly across histologic subtypes. The provocative MPACT trial further seeks to address the larger question of the importance of targeting "actionable mutations" with targeted agents, and whether this will translate into meaningful clinical benefit above that achieved by current treatments.

The premise of both the NCI MATCH and MPACT trials relies heavily on the precision and accuracy of molecular tumor characterization techniques to find the target. Predefined rules allow for the elimination of bias from the selection process and allow for rapid decision-making once molecular targets are identified. Both trials also require a reliable informatics system to process the results of the assay and output of treatment selection. From a patient aspect, this process must additionally be sufficiently rapid to provide meaningful treatment options for patients willing to undergo biopsy and remain untreated while awaiting results. Additionally, with the continuing explosion of genomic data being generated, this cannot be a static process. The structure of these trials allows for flexibility with these changing data, allowing for addition of new variants and targets, and the removal of ineffective ones. By building in biopsies at the point of progression, these trials will also allow for a broader understanding of

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resistance mechanisms invoked with exposure to subsequent therapies and the intricate interplay between these molecular pathways.

Additional factors which need to be considered in implementation of these transformative trials involve the management of reporting of genomic data. Reporting of incidental findings to patients requires forethought, especially in situations of mutations of unknown significance. Results from these trials will provide a strong structure to build new and better treatment options for oncology patients in the twenty-first century. As a consequence, oncologists will increasingly be called upon to deal with assisting patients to understand the vast amount of genetic data generated from these studies and how best to use the information to assist in management of their cancer. With the evolution of vast amounts of information and the identification of smaller and smaller subpopulations of patients who would benefit from any one particular therapy, the question of how these particular treatments will garner regulatory approval remains to be seen.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Design and statistical principles of the SHIVA trial

Xavier Paoletti[1,2], Bernard Asselain[1,2], Maud Kamal[3], Nicolas Servant[2], Philippe Huppé[2], Ivan Bieche[4], Christophe Le Tourneau[2,3]

[1]Department of Biostatistics, Institut Curie, Paris, France; [2]Unité INSERM, Institut Curie, Mines ParisTech U900, Paris, France; [3]Department of Medical Oncology, Clinical Trial Unit, Institut Curie, Paris and Saint-Cloud, France; [4]Unité de Pharmacogénomique, Service de Génétique, Institut Curie, Paris, France

Correspondence to: Xavier Paoletti, PhD. Department of Biostatistics and Epidemiology, Gustave Roussy Paris Campus, 114 Rue Ed Vaillant, 94805 Villejuif Cedex, France. Email: xavier.paoletti@gustaveroussy.fr.

Abstract: Most molecularly targeted agents (MTAs) are expected to work in subgroups of cancer patients characterized by the presence of molecular alterations in the tumor cells. However, clinical development is generally carried out according to tumor type. The SHIVA randomized trial on the contrary has been set up to investigate which of tumor biology or tumor location and histology is the most important to select treatment in patients with cancer refractory to standard of care. Statistical principles, specificities, strengths and limitations of this trial that evaluates an omic-based algorithm to select the targeted agent are reviewed. In particular, the need for a randomized trial where the various steps to build the algorithm are explicitly described and standardized is emphasized. The impact of an algorithm that would be partly misspecified (i.e., that would lead to correct treatment selection for some tumor molecular profile but not for all) is quantified.

Keywords: Heterogeneity; omic-based algorithm; precision medicine; randomization

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Introduction

During the last two decades, most new agents have been designed to target molecular alterations involved in carcinogenesis. For instance, trastuzumab, a monoclonal antibody targeting HER2, has been approved for HER2-overexpressing breast cancer treatment in 1998 (1) and vemurafenib, a BRAF kinase inhibitor, has been approved in melanoma patients harboring the V600E BRAF mutation (2) to cite only two agents among numerous striking examples. Most of these agents are expected to produce anti-tumor activity only in the presence of the matching molecular alteration or companion biomarker. Even though the complete characterization of the target is an area of research for many agents such as mTOR inhibitors and antiangiogenic agents, the objective of treating patients based on the molecular profile of their tumor is claimed by most of the sponsors and investigators. Nevertheless, molecularly targeted agents (MTAs) have been assessed to date according to tumor location and histology. Investigations in other tumor types are then pursued on a case by case basis. For instance, trastuzumab eventually also demonstrated anti-tumor activity in advanced/metastatic stomach cancers overexpressing HER2 (3). However, this approach is rapidly limited by the sample sizes required for clinical trials: the combination of the (low) prevalence of some alterations as well as some specific tumor types transforms several subgroups into rare diseases. The sequential development of a MTA in multiple tumor types with the same molecular abnormality in most cases is thus unrealistic.

Recent advances in high-throughput technologies allow for the screening of a large panel of molecular alterations in a reasonable timeframe for clinical practice, which opens the possibility to select and personalize treatment based on the molecular profile of the tumors. The National Cancer Institute (USA) has recently defined personalized medicine as "a form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease" (4). The question of whether personalized medicine based on the molecular profiling of

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the tumor of cancer patients improves their outcome has arisen. In a prospective cohort study, von Hoff and colleagues investigated the benefit of selecting treatment for refractory cancer based solely on the tumor biology (5). They found that 27% of the patients had a progression-free survival (PFS) increased by 30% as compared to the time to progression (TTP) obtained with the previous line of treatment, assessed retrospectively. In a comparative non randomized trial, Tsimberidou and colleagues reported that in patients with at least one druggable molecular alteration identified in their tumor, matched MTA compared with treatment without matching was associated with a higher objective response rate, longer PFS, and longer survival (6). However, the lack of randomization *vs.* standard of care in these studies did not allow for drawing robust conclusions (7).

Genesis of the SHIVA trial

The SHIVA trial was designed within the Institut Curie to evaluate whether tumor biology is a more important driver for treating cancer patients than tumor location and histology. For ethical reasons, only patients with cancers refractory to approved treatments for their disease were selected, similar to the two above mentioned studies (8). Furthermore, the concept appeared particularly attractive for less common or rare tumor types for which dedicated randomized trials of MTAs are usually not carried out. This supported the idea to include all solid tumor types that can be evaluated for efficacy using the same criteria. A very large set of MTAs is under development, with various levels of evidence of activity depending on the stage of development. We decided to use only approved drugs in order to control for this source of heterogeneity. Use of combinations that have often strong biological rationale was limited by safety issues as few phase I trials combining two approved MTAs have been published.

We initiated the SHIVA trial (NCT01771458), a randomized proof-of-concept phase II trial comparing molecularly targeted therapy approved at the time of the trial (outside of their approved indications) based on metastasis molecular profiling *vs.* conventional chemotherapy (or best supportive care) in patients with any kind of cancer refractory to standard of care. The intervention evaluated in this trial can be described as a complex algorithm that determines the association of a treatment with a putative adequate target.

We introduce here the rationale for the design, the choice of endpoints, the type of conclusions we can expect and specificities due to this type of clinical question. We emphasize the necessity of randomized trials, and explore the power of the trial in case only part of the algorithm would be efficient, that is if only some MTAs actually work in the presence of the selected target while others do not.

Design of the SHIVA trial

The primary objective of the SHIVA trial is to compare the efficacy in terms of PFS of molecularly targeted therapy based on molecular profiling versus conventional therapy in patients with solid tumors refractory to standard treatments. PFS is defined as the delay between randomization and progression according to RECIST 1.1 (9) or death, whatever the cause. Secondary efficacy objectives are to investigate the tumor growth according to the treatment arm, to explore the possible variation in treatment effect according to the altered pathway (interaction test), and to compare the tumor growth obtained with the MTA and the standard treatments for patients who cross over. Tumor growth is defined quantitatively as the sum of the size of the targeted lesions identified using RECIST 1.1 standardized by the delay between measurements. In this secondary analysis, patients with clinical progression and no evidence of radiological progression and patients with new lesions will be analyzed based on the radiological measurements only. Additional analyses including clinical progression and the occurrence of new lesions will be included in a sensitivity investigation.

The flowchart of the study is provided in *Figure 1*. The SHIVA trial includes an observation cohort study as well as a randomized trial. In brief, the molecular profile of a patient tumor is performed on a mandatory biopsy/resection of a metastasis and analyzed by a molecular biology board made of biologists, physicians and bioinformaticians. If no molecular alteration for which an approved matched MTA exists in the frame of the SHIVA trial was identified, the patient is not eligible for the randomization and is entered into a prospective observational cohort. If one or several molecular alterations are identified, the molecular biology board applies a pre-defined algorithm to select the best MTA (see *Table 1*). Patients are then randomized between receiving the selected MTA or receiving a conventional treatment according to the investigators' choice (that is based on tumor type, histopathological characteristics etc.). The investigator and the patient are blinded to the molecular profile. More details can be found in the paper by Le Tourneau and colleagues (10). The protocol for the research project has been approved by an Ethics Committee

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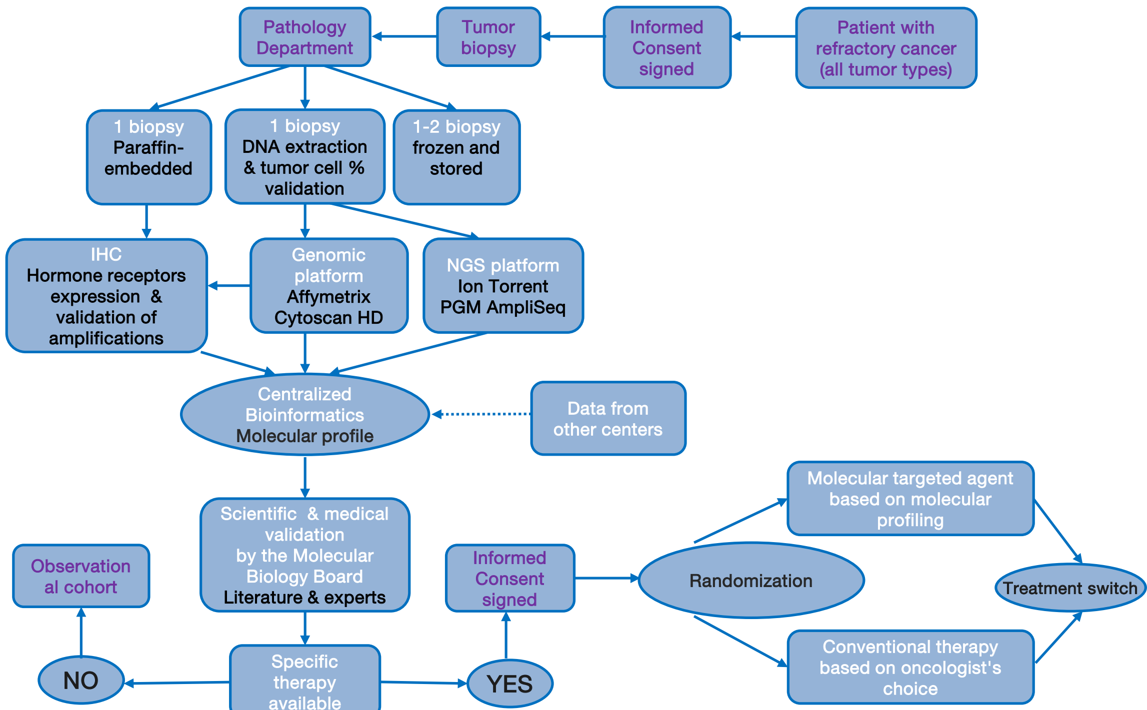

Figure 1 Flow chart of the SHIVA trial with a two-step information process leading to enroll part of the patients in a randomized controlled trial and a prospective cohort study.

and the trial conforms to the provisions of the Declaration of Helsinki. The remainder of this communication focuses on the randomized trial of the SHIVA program.

It is well known that prognosis differs depending on the tumor type, although patients with the same cancer in terms of location and histology might also display different prognosis (11). In order to control for patient heterogeneity from differences in prognosis, randomization is stratified according to the signaling pathway relevant for the choice of the MTA and the patient prognosis based on the two categories of the Royal Marsden Hospital (RMH) score for oncology phase I trials (12). Although molecular alterations may be prognostic for PFS, it was not possible to stratify the design for all possible molecular alterations. Three main signaling pathways have been arbitrarily identified: (I) the hormone receptors pathway; (II) the PI3K/AKT/mTOR pathway; and (III) the MAP kinase pathway (see *Table 1*). Therefore, combining the two levels of prognosis from the RMH score with the three molecular pathways, the randomization and the planned primary analysis are stratified on six strata.

A cross-over is allowed at disease progression for patients in both treatment arms (patients who received conventional chemotherapy were proposed the MTA and vice-versa). Last, quotas were introduced so that no more than 20% of the randomized patients had the same tumor type.

A feasibility evaluation was planned after the first 100 patients to check the availability of the molecular profile within four weeks after the biopsy, to review the different steps to draw the molecular profile and the algorithm to guide treatment's selection (10). No modification of the process and the algorithm was required after this interim feasibility analysis.

The population of interest included various tumor types and various number of previous lines of treatment, similar to the population enrolled in phase I trials. The expected PFS of this population in the control arm could be derived from the one reported in phase I clinical trials of cytotoxic agents that have been eventually approved: 6-month PFS in this patient population was around 15% (13). Under the hypothesis that doubling the 6-month PFS rate from 15% to 30% was clinically relevant (i.e., HR =0.63), a total of 142 events is required to detect a statistically significant difference in PFS between the randomized arms with a type I error of 5% and a power of 80% in a bilateral setting. To observe these events after an accrual time of 18 months and

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Table 1 SHIVA treatment algorithm established to select molecularly targeted agents based on the molecular profile

Targets	Targeted therapies	Molecular alterations
KIT, ABL1/2, RET	Imatinib	Activating mutations/amplification
PI3KCA, AKT1	Everolimus	Activating mutation/amplification
AKT2/3, mTOR, RICTOR, RAPTOR	Everolimus	Amplification
PTEN	Everolimus	Homozygous deletion
		Heterozygous deletion + inactivating mutation
		Heterozygous deletion + loss of expression using IHC
STK11	Everolimus	Homozygous deletion
		Heterozygous deletion + inactivating mutation
INPP4B	Everolimus	Homozygous deletion
BRAF	Vemurafenib	Activating mutation/amplification
PDGFRA/B, FLT3	Sorafenib	Activating mutation/amplification
EGFR	Erlotinib	Activating mutation/amplification
HER-2	Lapatinib + trastuzumab	Activating mutation/amplification
SRC	Dasatinib	Activating mutation/amplification
EPHA2, LCK, YES1	Dasatinib	Amplification
ER, PR	Tamoxifen or letrozole	Protein expression ≥10% IHC
AR	Abiraterone	Protein expression ≥10% IHC

Comments for oncogenes: (I) known activating mutations in the littérature or in databases like COSMIC; (II) amplification is defined by an amplicon size ≤10 Mb and a gene copy number ≥6 for diploid tumors and ≥7 for tetraploid tumors; (III) only focal amplification with an amplicon size of maximum 1 Mb were directly validated by the MBB. If amplicon size >1 and <10 Mb, IHC is required. Comments for tumor suppressor genes, inactivation of tumor suppressor genes implies that the 2 alleles that code for a particular protein are affected: (I) homozygous deletion (loss of 2 alleles); (II) heterozygous deletion: Loss of one allele if the second hold an inactivation mutation or can be validated by loss of expression using IHC; (III) loss is defined by 1 copy for diploid tumors and 1 or 2 copies for tetraploid tumors; (IV) deletion corresponds to 0 copy.

a minimum individual follow-up of six months, about 200 patients would need to be randomized onto this trial.

Design specificities relating to the use of high throughput technologies

The evaluation of a complex intervention such as the SHIVA algorithm to select the MTA raises specific issues that not only impact the design, but also the statistical analysis and the final interpretation. First, this complex intervention combines two aspects: the treatment effect and the choice of the putative matching target. Therefore, the resulting efficacy can be related to either of the two and the final interpretation is the evaluation of the whole strategy compared to another strategy (physician choice) which uses different treatments and a different modality to select the treatment. Second, several sources of variability related to the complexity of the intervention may contribute to the final results of the experiment. Eleven different targeted treatments have been administered based on 22 targets characterized by several dozen molecular alterations (see *Table 1*). A fundamental assumption behind the design is that the intervention has similar effects (or absence of effects) in all six strata, whatever the allocated treatment and whatever the molecular alteration used to select the treatment. This is the homogeneity assumption. In case the algorithm is only partly efficient, the power of the study is impacted. The magnitude of the impact is investigated in the following section. Third, as in any scientific experiment, the algorithm to select patients must be duly described, reproducible and applicable to all participants. Defining the treatment algorithm was challenging as the knowledge regarding the biology of the tumors and the high-throughput platforms evolve quickly with time and initial biological assumptions might become outdated.

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Strengths of the selected design

Randomization

A randomized clinical trial is mandatory to evaluate the added value of omic-based classifiers to guide patient's treatment compared to standard approaches (14,15). Although the tumor biology, the mechanisms of drug resistance, and the role of the tumor environment are known to be crucial to accurately predict patient outcomes, they remain largely unknown, making it necessary to have a comparator. Furthermore, the prognosis of the highly selected patients (those whose tumors have a set of pre-defined molecular alterations) enrolled in such trials is not well-known, and only randomized experiments can disentangle the benefit of the intervention from the benefit obtained with supportive care or conventional chemotherapy outside of standard of care. Randomization is the only way to control for known and unknown confounding factors and to evaluate the causality in such a complex intervention. The more complex the intervention, the more numerous the unknown confounding factors. Likewise, only an intent-to-treat analysis that makes full use of the randomization is appropriate. However, as shown in the next section, this is necessary but this may not be sufficient to provide a clear picture of the benefit of the complex intervention.

Blinded design

Blinding to the molecular profile is a crucial component to evaluate the benefit of the intervention (16). The expectations of the physicians and of the patients in omic-based algorithms to select MTAs are high, and there is a risk of bias in the interpretation of treatment efficacy that would favor the intervention arm. Ideally a double blind trial should be designed; however this was impossible in the SHIVA trial due to the numerous treatments administered to the patients in both arms with various formulations (oral or intravenous).

Algorithm reproducibility

The treatment algorithm to select the best MTA based on a molecular profile was defined by the biologists and the physicians. It includes molecular alterations (in particular oncogene activations and gene suppressor inactivation) that had been demonstrated to have a predictive value of the effect of some treatments in the clinic, such as HER2 amplification and BRAF mutations. Others were based on a strong biological rationale that had not been validated in the clinic, such as PIK3CA mutations. The complete treatment algorithm was defined and secured before initiating the trial. Based on the knowledge of targetable signaling pathways, it makes explicit the definition of what should be considered a druggable molecular alteration (activating and inactivating mutations, focal amplifications, heterozygous and homozygous deletions, etc.), the thresholds for quantitatively measured molecular alterations (fold change and maximal size of focal DNA amplification for instance), validation of some protein expression measures using IHC, the priorization between molecular alterations when several of them were relevant and the correspondence between molecular alterations and MTAs. Each of these aspects is a potential source of variability. Extensive theoretical work has been performed in the SHIVA trial to enable strong control of the underlying heterogeneity, in line with the recommendations of McShane and colleagues (17). Amplifications, gene losses and deletions were clearly defined as a function of copy number alterations corrected to the tumor cell content and the size of the amplification. Similarly, for mutations analyses, thresholds for variant calling were set according to the frequency, strand ratio and reads' coverage (10). The molecular alterations included in algorithm are precisely documented in terms of techniques used to assess these alterations (18). Furthermore, the molecular technologies are evolving rapidly and in the SHIVA trial two different sequencing panels (Ion Ampliseq Panel version 1 and version 2) were used. Therefore, before updating the sequencing protocol several samples were analyzed in parallel with both panels to ensure the reproducibility and the homogeneity of the results. In the same way, all bioinformatics analyses defined during the feasibility part of the project were centralized and applied to all patients regardless of recruitment center. No modification of the bioinformatics workflows were accepted after the feasibility part of the project. Finally, all patients enrolled in the trial are analyzed in the same way. This is crucial as any research must be self-explanatory and reproducible. A treatment algorithm that relies only on understated experts' opinion would not be applicable outside of the center and conclusions would not be applicable and generalizable to other samples.

Cross over

Cross-over is allowed in the SHIVA trial to patients at

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disease progression. Patients initially randomized in the intervention group may then receive conventional chemotherapy based on their tumor type, and patients in the control arm may receive the MTA matching the molecular alteration identified on the biopsy performed at inclusion, provided all eligibility criteria are still fulfilled at the time of progression. The analysis plan included a comparative analysis of the TTP after each of the two treatments using the patient as his (her) own control for the subset of patients who could receive second treatment. The randomization between the two arms of treatment can also be seen as a randomization between the two sequences of treatment, fulfilling one requirement of cross-over designs. The statistical power of this analysis might theoretically be higher than the one comparing the treatment efficacy between the two groups as it enables control for the various sources of patients-related heterogeneity such as the natural history of the disease (the tumor location and histology), the history of previous treatments etc. if TTP for the two lines of treatment are correlated (14). Furthermore, in this planned cross-over, all tumor evaluations are performed using the same criteria, the same set of target lesions identified prospectively. This gives a better and more robust assessment of the two consecutive TTP compared to retrospective assessment. However, cross-over was not mandatory and in the likely case that a large fraction of patients cannot receive both arms (i.e., no crossover) due to clinical deterioration for instance, the power would be lower and the conclusions may be biased. Accordingly, the primary analysis relied on the first period only.

Tumor diversity

Quotas for tumor types were set up in the protocol to avoid over-representation of more frequent tumor types such as breast, lung or colorectal cancers. No more than 20% of the randomized patients are allowed to be enrolled for a given tumor type. A wide diversity of tumors has been enrolled. Differences between the treatment arms would then be unlikely related to a given tumor type. This would reinforce the interest of developing new treatments based on biology first, possibly across multiple diseases.

Biopsy of a metastatic site

In the SHIVA study all patients must undergo a biopsy of a metastatic site before being treated, so that we are sure that the molecular profile established reflects what will be treated as controversial results have been reported on the agreement between molecular profiles measured on the metastasis and on the primary (19,20). However, patients were allowed to receive chemotherapy (but no MTA or hormone therapy) between the time of biopsy and randomization. Establishing a molecular profile on the primary tumor may not accurately reflect the molecular profile of the tumor at the time of treatment, especially if patients have been previously treated with MTAs that can act as a selection pressure in some malignancies, driving clonal evolution and selecting for certain resistant subclones or developed de novo on treatment (21).

In summary, the randomized design for the SHIVA trial allows for comparing two complex strategies on a valid endpoint, while controlling for numerous confounding factors. A statistically significant difference between the two arms would be appropriately interpreted as the superiority of treating patients with MTAs based on molecular alterations and a pre-defined treatment algorithm compared to the conventional approach based on tumor location and histology. In other words, do we perform better than what we usually do for these patients?

Limitations of the selected design

Interpretation

An important question that will not be addressed in the SHIVA trial is the independent effect of the treatment algorithm. The design will not enable the disentanglement of the treatment effect from the algorithm effect. If a given MTA is active irrespective of the measure of the target (that is of the algorithm), we would draw the same conclusions as if the treatment worked thanks to the adequate selection of the patients. The US NCI sponsored M-PACT trial (NCT01827384) presented in the same issue of the journal has been designed to specifically address the question of the added value of the algorithm. Conversely, the control arm used in the M-PACT trial does not correspond to any standard of care and the trial will not be able to conclude whether the global strategy is superior to the usual practice. Both trials are therefore quite complementary.

Population heterogeneity

If randomization guarantees that the two groups of patients have comparable characteristics and the same overall prognosis, heterogeneity may dilute the expected benefit.

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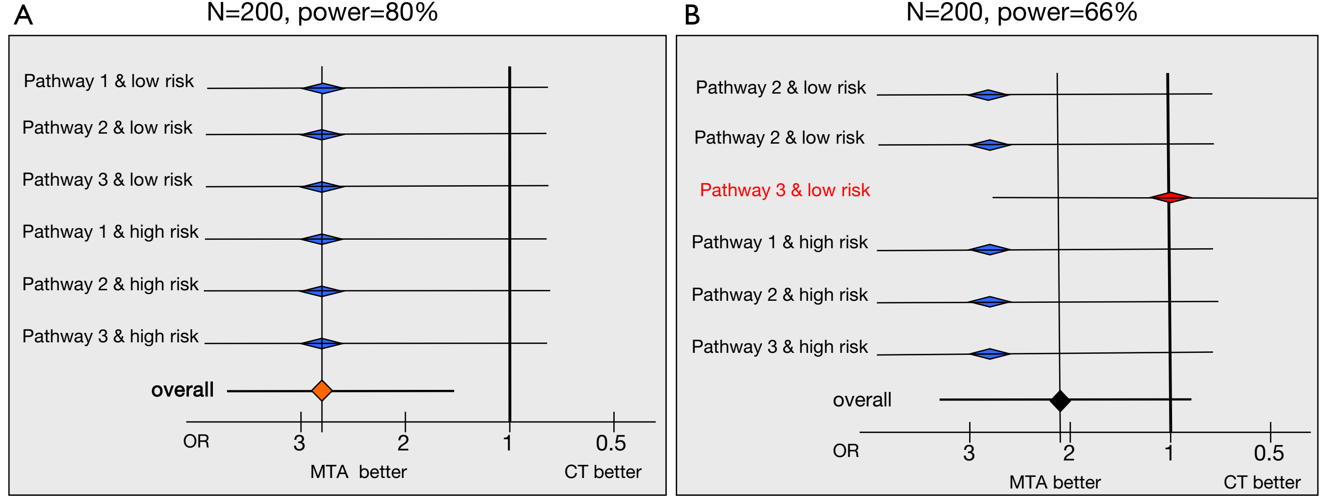

Figure 2 Impact of heterogeneity in the treatment effect related to the SHIVA algorithm assuming balanced prevalence for the different strata (pathways and RMH risk score) and the same follow-up for all patients censored at the cut-off date. High and low risk denote the RMH risk group; pathway 1, 2, 3 correspond to the grouping of the different targets; MTA stands for MTA selected on the target; CT stands for standard chemotherapy; N is the total sample size; OR stands for odds ratio; point estimates and 95% confidence intervals (horizontal lines) are provided. (A) Homogeneous benefit of the targeted treatment selected based on molecular alterations in all strata; (B) benefit of the targeted treatment selected based on molecular alterations in all but one stratum. RMH, Royal Marsden Hospital; MTA, molecularly targeted agent; CT, standard chemotherapy.

Heterogeneity impacts any clinical research, but several sources of potential heterogeneity across patients are specific for (or more likely with) this kind of trial: the location and histology of the tumor, the molecular alterations, the assays used to identify the molecular alterations, and the diversity of treatments under study. Stratification of the randomization and of the analysis on the RMH prognostic score and on the signaling pathway is an efficient mean to control part of this heterogeneity, assuming no interaction between the strata and the treatment effect. It was impossible to stratify on the numerous tumor types. On the contrary, as noted in the previous section we tried to increase the diversity of the tumor types to be able to draw conclusions that would be broadly applicable. This source of heterogeneity is intrinsic to the question addressed by the SHIVA trial and we tried to build on it, while controlling for the other identified sources.

Homogeneity and power

Beyond the expected heterogeneity in the population' prognosis, there is a risk of heterogeneity in the effect of the MTA selected based on the molecular alteration. Statistically, this would mean an interaction between the MTA effect and patient's characteristics. In other words, the algorithm to select the right treatment would be efficient for some molecular alterations (or equivalently for some treatments) and not for others. For instance, suppose that the treatment selected in case of an alteration on the PI3K/AKT/mTOR pathway is not active in this subset of patients, this would reduce the power of the primary analysis. Our ability to detect a 50%-reduction in the rate of progression or death at six months would be lower than the planned 80%. This is illustrated by the forest plots in *Figure 2*. Each line represents the MTA effect in a different stratum. In panel A, we have homogeneity of the treatment effect across all strata: whatever the signaling pathway and the prognostic group, the PFS rate is increased by 50%. Conversely, in panel B, no treatment effect is observed in one of the strata and the overall power of the primary analysis is reduced from 80% to 66%. The magnitude of the power loss depends on the number of strata where the MTA is not active, as shown in *Table 2*. The size of each stratum is also directly related to the power (results not shown). Homogeneity tests (or interaction tests) are part of the statistical analysis plan in order to detect this pattern of results. However, interaction tests are notoriously underpowered as shown in *Table 2* and a strong heterogeneity may remain statistically undetected at the 5% level.

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Table 2 Power of the randomized comparative trial to detect an overall increase in the progression free survival rate at 6 month from 15% to 30% in case of heterogeneity assuming balanced prevalence of signaling pathways and RMH risk groups

Number of strata with MTA better	Power for the comparative test (%)	Power for heterogeneity test (%)
6	80	–
5	66	25
4	49	36
3	32	38
2	17	34

In strata where MTA selected on the target is not better than CT, we assumed the same rate of progression at 6 months. Homogeneity is tested using Woolf's test. RMH, Royal Marsden Hospital; MTA, molecularly targeted agent; CT, standard chemotherapy.

Endpoints

The primary endpoint for SHIVA is PFS, which is used in many clinical trials to evaluate treatment benefit in advanced disease. As secondary endpoint, the quantitative measure of the tumor growth is analyzed (22). This endpoint has been increasingly investigated in recent years due to the potential increased information carried in continuous outcomes (23). In particular, an improved ability to detect interactions between the treatment effect and baseline characteristics such as the signaling pathway is expected. However, recent works have demonstrated that none of the endpoints based on the tumor growth proposed to date were a good surrogate of the patient's survival (24), and this is not clear whether a treatment effect measured on the tumor growth would be strongly predictive of a treatment effect on the PFS; furthermore, the best way to combine information from tumor growth and the occurrence of new lesions or clinical symptoms is still an area of research. In the SHIVA trial, this endpoint may help to provide a better understanding of the data, but it could not be used as a primary endpoint instead of PFS.

Perspectives

More than 900 MTAs are under development (25). However the large majority (95%) are tyrosine kinase inhibitors, 4% target the cell cycle, while less than 1% target alternative pathways, which limits the range of eligible targets. The prevalence of molecular alterations varies strongly according to the tumor type (26), also by the stage of the tumor (27), and the exposure to previous MTAs. Many subgroups represent less than 15% of the cancer patients with a tumor type. The SHIVA randomized trial has been set up to investigate which of tumor biology or tumor location and histology is the most important to select treatment in patients with cancer refractory to the standard of care. Interpretation of the results of such trials are complicated by the complexity of the algorithm, but only randomized trials can disentangle the consequence of prognostic factors in these highly selected patients from the intervention effect and enable to control for confounding factors to allow reliable conclusions (28).

The statistical principles for the SHIVA trial integrate various aspects to reduce the variability related to the potential heterogeneity of the population. This heterogeneity will be balanced between the two treatment arms and thus should not induce spurious association, but it may dilute the effect of the intervention. Standardization of the process to identify druggable molecular alterations and the matching MTA, as well as the blinding of the results are key elements in such trials. The same principles as those applied for the development of diagnostic tools should be implemented (29).

There is clearly a need for more sensible endpoints to evaluate such complex interventions. PFS is mildly sensitive to treatment variations and interaction tests to identify differential effects according to the matching between treatment and target are not powerful with 200 patients. Pharmacodynamic endpoints such as functional imaging or biomarkers are promising to detect early treatment failure but have none yet validated.

Overall, cancer biology is at the heart of this type of histology-agnostic trial. Current knowledge of tumor biology does not enable us to systematically predict the final outcome as shown by the disappointing efficacy obtained with vemurafenib in BRAF mutated colon cancer (30), or those obtained with crizotinib in neuroblastoma with ALK-translocation (31). Taking into account the presence or the absence of several molecular alterations might improve the

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accuracy of the treatment algorithms using systems biology approaches. However, any treatment algorithm should be clearly defined and rigorously evaluated in randomized trials. In addition, the tumor environment is likely an important factor of success of a therapeutic approach, as illustrated with the recent approval of immunotherapeutics. Nevertheless, the question of what is the strongest predictor of the treatment effect and whether matched MTA to molecular profile compared to conventional chemotherapy is more effective for cancer patients is crucial for the scientific community as well as for the patients. A total of 741 patients have been enrolled in 18 months and 197 have been randomized to date. Final efficacy results of the SHIVA trial are expected in 2015.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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An overview of the design and conduct of the BATTLE trials

Suyu Liu, J. Jack Lee

Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Correspondence to: J. Jack Lee, PhD. Department of Biostatistics, The University of Texas MD Anderson Cancer Center, 1400 Pressler Street, Houston, Texas 77030, USA. Email: jjlee@mdanderson.org.

Abstract: Our increasing knowledge of biomedicine and genomics for human malignancies has placed us within reach of achieving personalized cancer medicine. The Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE)-1 trial was the first completed, prospective biopsy-mandated, biomarker-based, adaptive randomized clinical trial for patients with advanced non-small cell lung cancer (NSCLC). The ongoing BATTLE-2 trial continues to search for effective targeted therapies by further refining the clinical trial design. The BATTLE program has demonstrated the feasibility and promise of novel biomarker-based clinical trial platforms, which has moved us one step closer to personalized medicine. In this paper, we describe the design and conduct of the BATTLE trials, summarize the main findings, and report the experiences and lessons learned from our pursuit of developing targeted therapies in cancer.

Keywords: Bayesian adaptive designs; personalized medicine; predictive biomarkers; targeted therapies

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Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States and the world. It accounts for more deaths each year than the combined deaths resulting from breast, colon, prostate, liver, and kidney cancers (1). Non-small cell lung cancer (NSCLC), one of the two major forms of lung cancer, accounts for about 85% of all lung cancers. It is often diagnosed at an advanced stage. Systemic chemotherapy is currently the mainstay for treating metastatic lung cancer. In recent years, targeted agents have been developed for selected patient populations that are more effective and less toxic than conventional chemotherapy. Examples of such agents are erlotinib and gefitinib, which are tyrosine kinase inhibitors (TKIs) of the epidermal growth factor receptor (EGFR). These agents are used in patients who have NSCLC and mutated EGFR (2-5).

Recent advances in biomedicine and genomics have brought better understanding of cancer-causing mechanisms and the ability to identify the corresponding therapeutic targets. Pharmaceutical companies and research institutions are working diligently to screen a myriad of compounds and their combinations that have the potential to address these therapeutic targets and achieve clinical benefits (6). The time and resources devoted to drug development are enormous. However, specific targeted agents may not benefit the general population of patients but work for only a small proportion of patients, and some agents may not work well at all. Therefore, modern drug development involves not only testing the targeted agents for their treatment benefit, but also requires the identification of the target patient population with the corresponding predictive markers.

Challenges exist in the discovery, testing, validation, and functional investigation of the co-development of targeted therapies and their corresponding predictive markers. First, the predictive markers that correspond to the targeted therapies are often unknown at the beginning of a trial. Hence, methods need to be developed to select markers by carefully sieving through a large number of candidate biomarkers for discovery and validation. Second, finding the optimal strategy for testing the treatment effect is not a trivial matter: investigators must determine whether the targeted treatment should be tested first in the unselected

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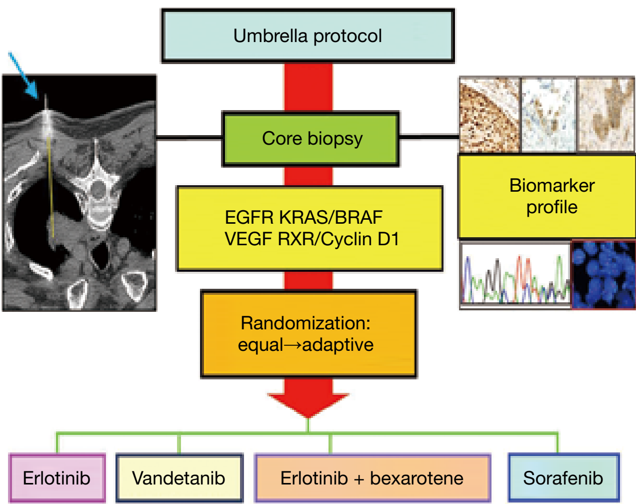

Figure 1 BATTLE-1 schema. EGFR, epidermal growth factor receptor; BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination.

population or in the selected population. That task is even more complicated when there are multiple agents with multiple putative markers to be developed. It can be difficult to efficiently pair the agents and biomarkers in a clinical trial when the properties of neither are well understood. Third, in order to match biomarkers and treatments, the biomarker assay has to be done in real time in a reproducible environment. Furthermore, as biomarker analyses are often based on the original tissues removed at the time of diagnosis because that is the only tissue available, they may not accurately reflect the current status of the disease. For example, when patients experience cancer recurrence, they have likely received several lines of therapy; therefore, any biomarker findings for such patients that are based on tissues removed prior to those treatments may or may not reflect the biomarker status of their recurrent tumor.

In view of these challenges, it is desirable to use a trial design that is adaptive so the conduct of the trial can be modified on the basis of cumulative information learned from the trial. For example, adaptive randomization allows for a higher probability that more patients will be assigned to better treatments based on the cumulative outcome and biomarker data. Assigning more patients to more effective treatments based on the corresponding predictive markers not only enhances the individual ethics of the trial, but also improves the accuracy in estimating the treatment effects in such a setting because of the increased sample size in the matched groups. Interim monitoring of the trial with an early stopping rule can stop patient enrollment for clear findings of efficacy, lack of efficacy, and/or unacceptable toxicity. Seamless phase I/II or phase II/III trials can shorten the development time by removing the "white space" between trial phases. Adaptive trial designs are promising in identifying useful predictive markers and effective therapeutic agents in an efficient way while providing the best available treatments to patients during the study (7-10).

The novel phase II Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) program described in this article consists of the BATTLE-1 trial—the first completed, prospective biopsy-mandated, biomarker-based, adaptively randomized phase II clinical trial in patients with previously treated NSCLC (11,12)—as well as the subsequent BATTLE-2 trial (13). We have demonstrated the feasibility of what was previously thought to be an impossible task: acquiring tumor tissues in patients with recurrent lung cancer and subjecting them to real-time biomarker analysis (14). The success of this program has opened a new era of targeted agent testing that is integrated with discovering and validating novel markers and offering better treatments for patients enrolled in the trials. This program sets an excellent example for the design and conduct of clinical trials that implement Bayesian adaptive designs in the development of targeted therapy. It is a step toward achieving personalized medicine.

The rest of this paper is organized as follows. In sections 2 to 5, we describe the design, conduct, and results of the BATTLE-1 trial, as well as the lessons we learned from that process. In section 6, we present additional publications and work related to the BATTLE-1 trial. In section 7, we describe the BATTLE-2 trial. In section 8, we summarize the impact of the BATTLE trials and conclude with a brief discussion on the future direction of related research.

Design of the BATTLE-1 trial

The concept of the BATTLE-1 trial was initially discussed in 2005. The BATTLE-1 program consisted of one umbrella trial and four parallel phase II studies with biomarker-based, targeted therapies in patients with advanced NSCLC who had been previously treated with chemotherapy and subsequently experienced disease relapse. *Figure 1* shows the BATTLE-1 schema. The four treatment

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Table 1 Marker group definitions in BATTLE-1

Marker group	Biomarkers			
	EGFR	*KRAS/BRAF*	*VEGF/VEGFR*	*RXR*/cyclin D1
1	+	x	x	x
2	–	+	x	x
3	–	–	+	x
4	–	–	–	+
5	–	–	–	–

"+" is positive; "–" is negative; "x" is either positive, negative, or unknown; EGFR, epidermal growth factor receptor; BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination.

arms were erlotinib, sorafenib, vandetanib, and the combination of erlotinib and bexarotene. The treatments were chosen to target each of the four selected gene pathways in NSCLC that were of the highest scientific and clinical interest at the time when the trial was designed. It was assumed that each treatment could be more efficacious in patients with a certain biomarker profile that matched the agent's mechanism of action.

BATTLE-1 was a biopsy-mandated study. Eligible patients gave their consent to undergo a tissue biopsy before they were treated. A core-needle biopsy, guided by computed tomography or ultrasound, was used to collect tissues for the required biomarker analysis and the additional gene expression, mutation, and proteomic biomarker analysis. A patient's treatment assignment was based on his or her biomarker profile, which was defined by eleven pre-specified biomarkers: *EGFR* mutation, *EGFR* overexpression/amplification, *EGFR* increased copy number (in the *EGFR* pathway), *KRAS* mutation, *BRAF* mutation (in the *KRAS/BRAF* pathway), *VEGFR* expression, *VEGFR-2* expression (in the *VEGFR* pathway), *RXRα* expression, *RXRβ* expression, *RXRγ* expression, and cyclin D1 expression (in the *RXR*/cyclin D1 pathway). The screening of these eleven individual markers meant that we could have had 2,048 possible marker combinations, even from simply dichotomizing each marker as positive or negative. To reduce the number of parameters, we sequentially examined the presence or absence of certain biomarkers to classify each patient into one of the five marker groups listed in *Table 1*. For example, if any of the biomarkers related to the *EGFR* pathway were positive for a given patient, the patient was classified into the *EGFR* marker group (marker group 1) regardless of the status of the other biomarkers for that patient. Otherwise, if the patient's tumor sample showed *KRAS* or *BRAF* mutations, the patient was classified into the *KRAS/BRAF* marker group (marker group 2) regardless of the status of the remaining biomarkers for that patient, and so forth. If none of the pre-specified biomarkers were positive for a given patient, the patient was classified into the fifth marker group, which included patients for whom the biomarker information was missing or incomplete.

The goal of the BATTLE-1 trial was to establish a clinical trial platform that advanced trial design in the development of targeted therapies, and to use the biomarker data to assess the clinical benefit of targeted molecular agents in patients with advanced NSCLC. Specifically, we aimed to provide an accurate estimate of the true disease control rate (DCR) for each of the treatment arms in each of the marker groups. In addition, the trial design was adaptive so that it assigned more patients to the more promising treatment arms based on data accumulated in the trial up until that time according to each patient's biomarker profile. Conversely, the trial suspended patient enrollment in the ineffective treatment arms early based on the patient's biomarker profile.

The 8-week DCR was chosen as the primary endpoint to use in evaluating the treatment effect. It was an easily and quickly assessable endpoint that had been shown to be a reasonable surrogate for the overall survival time in patients with advanced lung cancer (15). In order to simultaneously evaluate the four treatments and five marker groups and to identify the most efficacious treatment in each marker group, a Bayesian hierarchical probit model was applied (11). This model allowed for borrowing statistical strength among the five marker groups within the same treatment arm, which can improve the accuracy of estimation if patients from different marker groups who receive the same treatment show similar treatment responses.

An outcome-adaptive randomization scheme was employed in the BATTLE-1 trial. Eligible patients were first equally randomized into each of the four treatment arms based on their marker group membership. Once at least one patient had been treated in each of the 20 treatment-by-marker subgroups, adaptive randomization began. Patients were adaptively randomized into the treatment arms in proportion to the estimated posterior DCRs within each biomarker group. Adaptive randomization allowed us to learn the performance of each treatment arm in each marker group during the trial, and to use the updated knowledge to

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guide the assignment of patients to treatment arms as the trial continued. As a result, more patients received the more efficacious treatments as the study progressed.

In the BATTLE-1 trial, we applied the Bayesian adaptive design to continuously update and learn from the information and to perform interim monitoring for futility. The Bayesian framework allows for the natural implementation of an early stopping rule such that the assignment of patients in a particular marker group to a given treatment arm can be suspended if the treatment is found not to be promising for that marker group. A not-promising treatment was defined as one that had a likelihood of its estimated DCR being higher than 50% (and the targeted DCR or the DCR under the alternative hypothesis) was lower than 10%. At the end of the study, we declared a treatment as successful in a given marker group if the probability of the estimated DCR exceeding the historical threshold of 30% (the DCR under the null hypothesis) is greater than 80%. Before we applied the design in the BATTLE-1 trial, we conducted extensive simulation studies to evaluate the performance of the design under various scenarios. The probability cutoffs were calibrated so that the type I and type II error rates were well controlled. When a treatment-by-marker subgroup had a true DCR of 30%, the probability of it being declared a success was 20% or less (type I error). If a treatment-by-marker subgroup had a true DCR of 60%, the probability of declaring a treatment a success was at least 80% (statistical power). The probability of declaring a treatment a success can be as high as 95% when the DCR is 80%. A high type I error rate was selected in order to increase the statistical power such that we would have a high probability of selecting a potentially efficacious treatment and a low probability of overlooking a potentially efficacious treatment.

In contrast to the traditional single-arm design for phase II studies, which would have involved 20 separate parallel studies to evaluate the efficacy of four treatments in five biomarker groups, the Bayesian adaptive design allowed us to enroll patients under one study. The use of a hierarchical design and early stopping rules for futility improved the efficiency of the study. The outcome-adaptive randomization scheme enhanced the individual ethics of the trial and patient comparability across the different treatments. The adaptive design which puts all patients under one roof also enhances the patient comparability in contrast to the sequentially conducted multiple single-arm phase II trials.

Conduct of the BATTLE-1 trial

With four treatments, five marker groups, real-time biomarker analysis, and a Bayesian adaptive design, it was a logistically challenging task to conduct the BATTLE-1 trial efficiently and effectively. To facilitate the conduct of the trial, we built an integrated web-interfaced database application. *Figure 2* illustrates the trial conduct and the associated web application. All of the information about the patients and the study was stored in an electronic database. The information for each patient was carefully recorded from the day the patient registered for the study to the day the patient completed the study. An eligible patient was registered and then evaluated with a baseline physical exam, lab test, mandated biopsy, molecular pathology assessment, and biomarker analysis to determine the appropriate marker group. By design, the goal was to perform molecular testing within 2 weeks of the tissue biopsy. Then, adaptive randomization was performed by calling an R computer code through the web services to generate an assignment for the patient to a treatment arm. Regular clinical visits took place during the initial 8-week treatment period and patient compliance and any adverse events related to the treatment were recorded. The primary endpoint of disease control was evaluated at 8 weeks after randomization. The radiographic measurement of tumor size and the clinical outcomes were recorded to determine the tumor response. Information obtained at subsequent follow-up visits was also recorded until the patient went off study due to either disease progression, experiencing toxicity, or loss to follow-up. All data were stored within CORe, the MD Anderson regulatory environment, and the study-specific SQL 2005 database. Reports were generated periodically to monitor the study progress.

A requirement of the Bayesian adaptive trial design is timely measuring and reporting of the study outcomes such that the randomization probability and the posterior probability for futility monitoring can be calculated accurately on the basis of the most recent data. Whenever a patient’s disease control status was updated, the posterior distribution of the estimated DCR was calculated and updated accordingly. The updated information was used to compute the randomization probability and check whether the early stopping boundary for futility had been reached for certain treatments in certain marker groups. If the early stopping boundary for futility were reached, patient randomization would be suspended for that treatment in that marker group. All these computationally intensive calculations were performed in R code automatically and

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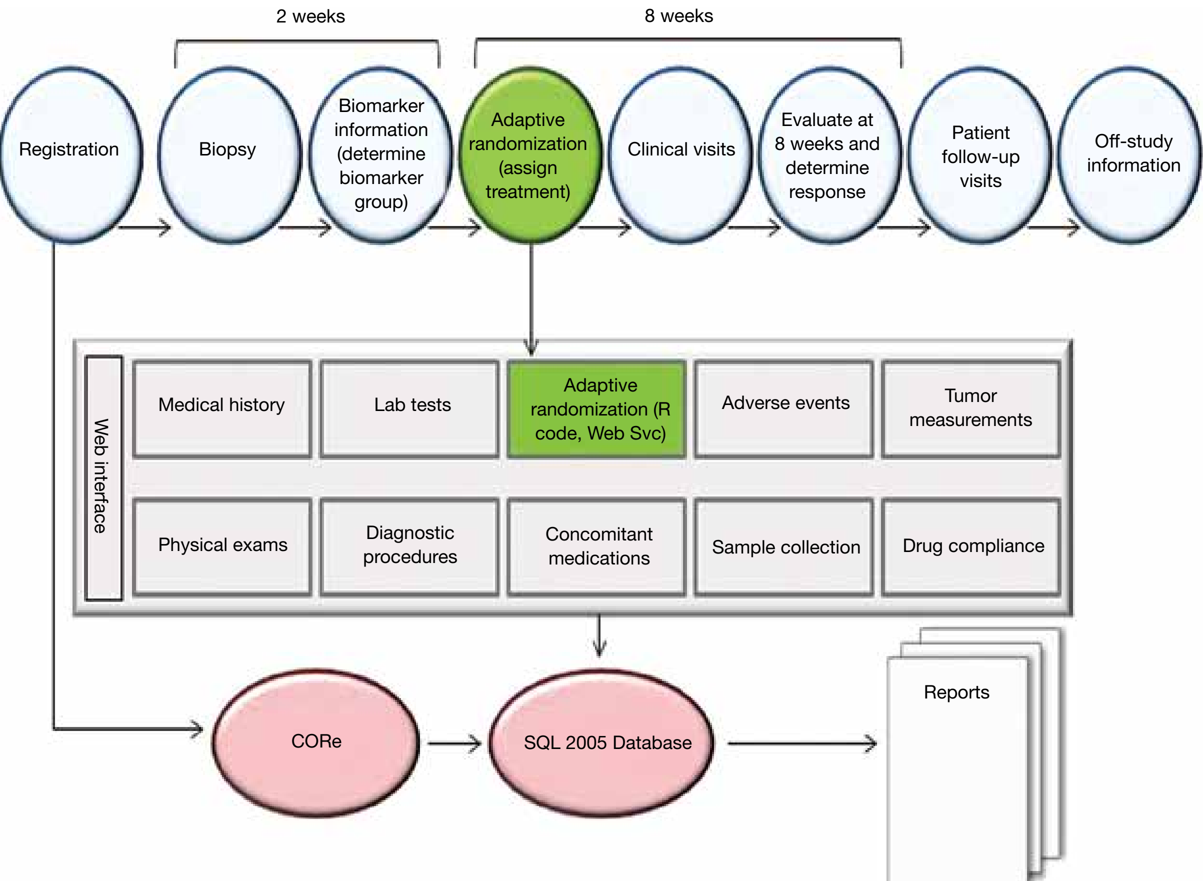

Figure 2 Schematic diagram of BATTLE-1 trial conduct via web-interfaced database application. BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination.

assessed through the web services. This adaptive learning and dynamic treatment allocation very nicely illustrated the motto of Bayesian adaptive design: "We learn as we go". To meet the timeliness requirement of measuring and entering the 8-week disease control status, an automatic e-mail notification system was developed. It was programmed to send an e-mail to the designated research coordinator to remind the coordinator to schedule a patient visit when 6 weeks had passed since the patient had been randomized. The system also kept track of the time when the 8-week endpoint was recorded and automatically sent e-mail alerts when an endpoint evaluation was overdue for more than 2 weeks.

To accurately evaluate the treatment outcome, an endpoint review committee was formed that included clinicians, radiologists, and research nurses. The committee reviewed the treatment outcomes during and at the end of the study to ensure consistent criteria were followed while blinded to the patient's treatment assignment. During the trial, automatic alerts were sent to the appropriate personnel to alert them to a delayed response entry, suspension of patient accrual to certain treatments for a subgroup, or other unexpected or adverse events.

The conduct of the BATTLE-1 trial required substantial teamwork and collaboration. It involved the creation of an integrated multidisciplinary research team of clinicians who evaluated and treated the patients, interventional radiologists who performed the image-guided core-needle biopsy, pathologists and basic scientists who performed the histology reading and biomarker analyses, statisticians who provided the trial design and implemented the algorithm for adaptive randomization, pharmacists who dispensed study medicines, radiologists who evaluated the tumor response, research nurses and research coordinators who worked with patients step-by-step during the entire trial period, and computer programmers who built and maintained the

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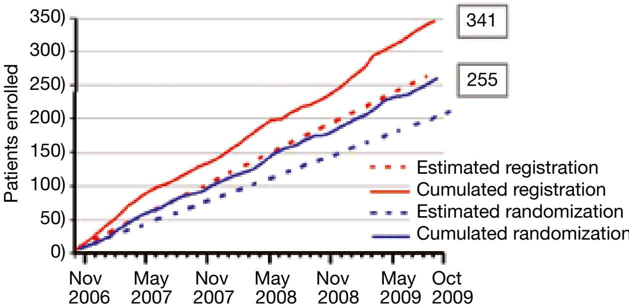

Figure 3 Study accrual and randomization in the BATTLE-1 trial. BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination.

web-interfaced database applications. Everyone in the team worked together to ensure the smooth conduct of the study. Although much effort was required to build and operate a multidisciplinary team for conducting an adaptive trial, as a result, the BATTLE-1 trial was implemented exquisitely and high quality data were collected throughout the trial.

Results of the BATTLE-1 trial

The BATTLE-1 trial was activated in November 2006, and patient accrual was completed in October 2009. In those 3 years, a total of 341 patients were enrolled in the study. Among that total enrollment, 255 patients were randomized to the treatment arms and 86 patients were not randomized because of either concurrent illness, worsening overall condition, a condition preventing a biopsy, or the choice of the patient or the treating physician to seek alternative treatments. *Figure 3* shows the accumulating number of patients enrolled and randomized in the trial over time. The patient accrual rate was about 9.5 patients per month, which was better than the expectation of eight patients per month. On average, 7.1 patients were randomized each month. Both clinicians and patients were enthusiastic to participate in the study. The concepts of personalized medicine and adaptive trial designs were well accepted by the clinicians and patients.

Among the patients who were randomized to treatments within the trial, 244 had an evaluable 8-week disease control status. The overall 8-week DCR was 46%. The marginal DCRs were 34%, 33%, 50%, and 58% for the treatments of erlotinib, vandetanib, erlotinib plus bexarotene, and sorafenib, respectively. The adaptive randomization scheme assigned the most patients (n=105) to receive sorafenib because it had a better marginal DCR compared to the other three treatments (erlotinib: n=58; vandetanib: n=52; erlotinib plus bexarotene: n=36). *Figure 4* shows the distributions of the final randomization probability into the four treatments for marker groups 1, 2, 3, and 5 (marker group 4 is not shown because only six patients belonged to it). Confirming the initial hypothesis, the trial showed that patients in the *KRAS/BRAF* marker group had a much higher DCR (79%) when treated with sorafenib, compared to the DCRs of 14% for erlotinib, 0% for vandetanib, and 33% for the combination of erlotinib and bexarotene. In addition, erlotinib plus bexarotene worked well in the *RXR/* cyclin D1 marker group. We also performed exploratory analyses to identify potential predictive biomarkers. The DCR for patients in the *KRAS* mutation group was higher when treated with sorafenib compared to erlotinib (61% *vs.* 22%). Though erlotinib did not show significantly high DCRs among patients in the *EGFR* marker group, it did have a higher DCR for patients with the single marker of *EGFR* mutation compared to those with the wild-type *EGFR* (71% *vs.* 29%). Of interest, patients with wild-type *EGFR* had a better DCR when treated with sorafenib than with erlotinib (64% *vs.* 29%). More complete results can be found in the original publication of the BATTLE-1 trial (12).

Lessons learned from the BATTLE-1 trial

The BATTLE-1 trial was the first completed, prospective biopsy-mandated, biomarker-based, adaptively randomized clinical trial for patients with relapsed NSCLC. Compared to using tissue samples and biomarker status assessed at the time of diagnosis, the re-biopsy in patients with disease relapse and the real-time biomarker analysis provided an accurate biomarker status for the current treatment assignment in the trial and a wealth of information for future studies. By using a fresh core-needle biopsy, not only did we obtain the tissue samples needed to define the patient's biomarker profile for treatment assignment in the BATTLE-1 trial, but we procured tissue samples that will be available for future studies. In addition, from patients who consented, we collected blood and serum samples at baseline and after treatment. All this information will enable us to discover and validate novel biomarkers in future studies.

The BATTLE-1 study confirmed our pre-specified hypotheses that patients with *EGFR* mutations had better disease control when treated with erlotinib and patients with *KRAS/BRAF* mutations had better disease control

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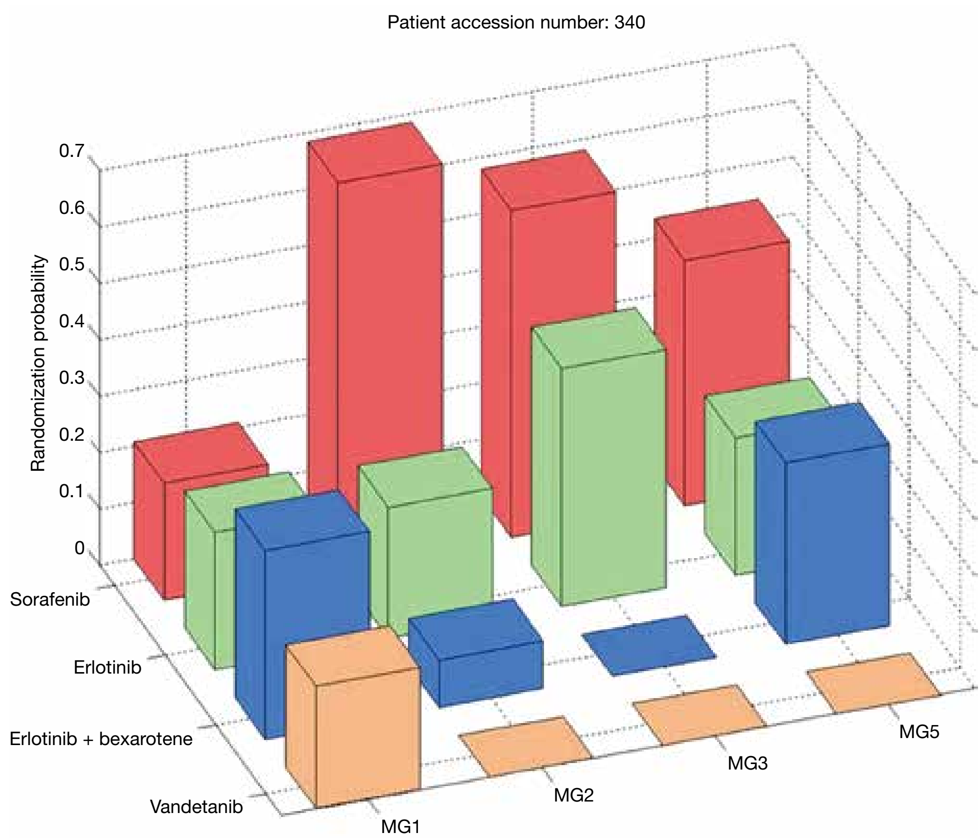

Figure 4 BATTLE-1 trial: probability of adaptive randomization by treatment and marker group. BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination.

when treated with sorafenib. The study also identified some interesting findings, for example, the predictive effects of better DCR for patients with *KRAS* mutations or wild-type *EGFR* who were treated with sorafenib, for patients with high *VEGFR-2* expression who were treated with vandetanib, and for patients with high cyclin D1 expression who were treated with erlotinib plus bexarotene. Of course, all these findings are based on small sample sizes and therefore must be validated in future BATTLE trials and in other studies.

The BATTLE-1 trial used a Bayesian adaptive design. Compared to traditional equal randomization or fixed rate randomization schemes, the outcome-adaptive randomization scheme allows us to adjust the randomization probability as the data accumulate during the trial. By using the accumulating data, our knowledge of the treatment effect can be continuously updated during the trial. Consequently, future patients can be assigned to better treatments with higher probability according to their biomarker profiles. Thus, the design enhances individual ethics. This adaptive feature not only refines our initial assumption of the treatment effect, but, should our initial assumption be wrong, this feature can correct the assumption as the data accumulate such that the amount of information from the trial overwhelms the prior information. Better estimation of the treatment effect can be achieved when a larger sample size is achieved by assigning more patients to more effective treatments in patients with the corresponding predictive markers. As shown, some findings from the BATTLE-1 trial have validated our pre-specified scientific hypotheses regarding biomarkers that are predictive of disease response to targeted agents and, more importantly, the trial has also identified potential new predictive markers to be studied in the future.

The successfully conducted BATTLE-1 trial has made important clinical discoveries and demonstrated the feasibility of its novel design for advancing personalized treatment in NSCLC. It also leaves room for improvement

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in the future. Notably, in the BATTLE-1 trial, the biomarkers were pre-specified in the study based on our experience and the research literature available at that time. However, some of the selected biomarkers did not have any observable prognostic or predictive effects, e.g., RXRs. Furthermore, although the biomarker grouping reduced the number of parameters in the model and simplified the trial design, combining markers weakened the association between the real predictive biomarkers and the treatments. For example, we formed the *EGFR* marker group from three subgroups: *EGFR* mutation, *EGFR* overexpression/amplification, and *EGFR* increased copy number. The predictive effect of the *EGFR* mutation with the erlotinib treatment was very strong but was diluted after grouping it with *EGFR* overexpression/amplification and *EGFR* increased copy number. We have learned that it is not a good idea to pre-select the study markers, particularly in the setting when little is known about the new treatments and their corresponding markers. We also learned that grouping different genetic mutations or characteristics to form fewer marker groups is not desirable because the true marker effect can be weakened by incorporating unimportant markers.

Equal randomization was applied in the first stage of the trial to gather the information required to form the prior distribution that would be used for adaptive randomization in the second stage. We stipulated that the adaptive randomization scheme would start after we enrolled at least one patient in each of the marker-by-treatment subgroups. It turned out that few patients belonged to the *RXR*/cyclin D1 marker group, which unfortunately delayed the start of the adaptive randomization scheme until about 40% of the patients had been equally randomized to the various treatments. Looking back, we determined that we should have allowed the adaptive randomization scheme to start earlier, say, after about 20% to 25% of the patients had been equally randomized, so that more patients could have benefited from adaptive randomization.

Another hurdle that inadvertently impacted the adaptive randomization scheme in the BATTLE-1 trial involved the eligibility criteria specific to each treatment. The unique properties of each treatment required the use of treatment-specific eligibility criteria in addition to the eligibility criteria common to all trial participants. Patients enrolled in the BATTLE-1 trial had advanced stage NSCLC and therefore had already received cancer treatments, typically two to six lines of treatment. Their resulting medical conditions disqualified many of the patients from eligibility for all of the BATTLE-1 treatments. In fact, only 14% of the patients were eligible for all four treatments in the trial. Patients can only be randomized among the treatments for which they are eligible; thus, for 86% of the patients enrolled in the trial, we had to adjust the adaptive randomization according to the patient's eligibility.

It is well known that adaptive trial designs are prone to experience a study population drift (16). The study population in the BATTLE-1 trial was quite stable in general; however, over the course of the study, we found that more smokers and patients who had previously received erlotinib enrolled in the latter part of the study compared to the beginning of the study. Statistical methods such as covariate adjusted regression analysis are available to alleviate the impact of an unbalanced covariate distribution during a trial. Adaptive randomization works best when used with effective treatments and markers that show good predictive performance. The final lessons learned were that some of the pre-specified markers were not predictive of the treatment response and some of the treatments were not as successful as we anticipated, and these factors limited the success that could be achieved in the trial.

Additional publications from the BATTLE-1 trial

The BATTLE program and the first completed trial compose a rich learning environment through which we have explored many topics in NSCLC research, medical practice, clinical trial design and conduct, and the continuing development of novel statistical methods in medical research. Here, we report selected publications from the BATTLE-1 trial. Ihle *et al.* conducted microarray analysis of mRNA expression on frozen core biopsy tumor samples from the patients who participated in the BATTLE-1 trial (17). They found that patients who had either mutant KRAS-Gly12Cys or mutant KRAS-Gly12Val had worse progression-free survival compared with patients who had other mutant *KRAS* proteins or wild-type *KRAS*. Tsao *et al.* performed an analysis that focused on elderly patients (18). Of interest, they found that elderly men showed better clinical benefit from certain targeted agents. For example, men aged 65 to 70 years had better progression-free survival when treated with vandetanib, and men over 70 years of age had better progression-free survival when treated with sorafenib. Tam *et al.* assessed the acquisition of tissue for biomarker analysis using the image-guided percutaneous transthoracic core-needle biopsy and determined that the success rate for obtaining tissue was

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82.9% in patients in the BATTLE-1 trial (14). Byers *et al.* developed and validated a 76-gene epithelial-mesenchymal transition (EMT) signature using gene expression profiles from four microarray platforms of NSCLC cell lines and patients treated in the BATTLE-1 trial (19). The EMT signature predicted resistance to EGFR and PI3K inhibitors and identified Axl as a potential therapeutic target for overcoming resistance to EGFR inhibitors.

Gene expression and the biomarker effect in each of the four targeted agents in the BATTLE-1 trial have been further studied. For example, Tsao *et al.* reported that vandetanib improved progression-free survival in patients with *EGFR* mutation compared to patients with wild-type *EGFR*, if the patients' tumors were resistant to EGFR TKIs (20). For patients treated with sorafenib, three important findings were documented: (I) significant clinical benefit for those with mutated *KRAS* versus wild-type *KRAS*; (II) significant clinical benefit for those with wild-type *EGFR* versus mutated *EGFR*; and (III) the gene expression profiles from NSCLC cell lines and patient tumor biopsies with wild-type *EGFR* were used to develop a sorafenib sensitivity signature that showed improved progression-free survival among patients with wild-type *EGFR* (21).

Related research conducted outside of the BATTLE team by Dragnev *et al.* showed that bexarotene plus erlotinib suppressed lung carcinogenesis independent of *KRAS* mutations in clinical trials and transgenic mouse models (22). Cotargeting cyclin D1 via the retinoid X receptor and EGFR by combining erlotinib and bexarotene is a potentially promising venue for the prevention and treatment of lung cancer (23). In addition, knowledge gained from the mechanistic approach to treating lung cancer in the BATTLE program led to the proposal of the concept of reverse migration as a new strategy for personalized lung cancer prevention (24).

From the statistical methodology point of view, the BATTLE program has inspired the development of Bayesian adaptive trial designs and the evaluation of various trial designs for studying targeted agents (25). Outcome-adaptive randomization has been shown to be very useful when a large difference in efficacy is found among treatments or when the goal is to maximize the overall treatment benefit for patients enrolled in the trial, particularly when the applicable patient population beyond the trial is small (26). Furthermore, in order to select relevant prognostic and predictive markers, a Bayesian 2-step Lasso strategy with a group Lasso approach followed by an adaptive Lasso approach was developed for time-to-event endpoints (27).

Extension of the BATTLE-1 trial: the BATTLE-2 trial

The BATTLE-1 trial demonstrated a new platform for novel adaptive clinical trial design and has allowed the investigators to derive interesting findings for validation in future studies. Major limitations of the BATTLE-1 trial were the pre-selection of biomarkers and bundling the biomarkers into marker groups. To rectify this problem, we have designed a Bayesian 2-stage biomarker-based adaptive randomization trial called BATTLE-2 (13). The BATTLE-2 trial has been designed for the same patient population that was eligible for the BATTLE-1 trial, and uses the same primary endpoint, i.e., the 8-week DCR, which has been shown to be a good surrogate of the overall survival time in BATTLE-1 and other studies (7,8). In BATTLE-2, four treatments were selected: erlotinib (serving as the control group), sorafenib, MK-2206 (an AKT inhibitor) plus erlotinib, and MK-2206 plus AZD6244 (a MET inhibitor). The study schema is shown in *Figure 5*.

The potential prognostic/predictive biomarkers are identified during the training phase (pre-BATTLE-2) on the basis of prior studies and the literature. These putative markers are tested in the first stage and validated in the second stage of the BATTLE-2 trial. The trial is designed to achieve three goals: (I) test the treatment efficacy of the targeted agents and their combinations; (II) identify the corresponding prognostic and predictive markers; and (III) treat patients with the most effective treatment in the study based on the available data. Adaptive randomization is applied in both stages to assign more patients to better treatments based on the individual patient's biomarker profile. In contrast to the BATTLE-1 trial, adaptive randomization in BATTLE-2 starts at the beginning of the study stratified by the *KRAS* mutation status. Note that the randomization probability is set to be bounded between 0.2 to 0.8 to ensure that the AR allows patients to be assigned to all treatment arms with reasonable probabilities in stage 1. A "Go or No-Go" decision is made at the end of the first stage by testing the treatment effect of each individual treatment. If none of the experimental treatments shows any promising effect compared to erlotinib (the control group) in all patients and in any marker subgroup (wild-type or mutated *KRAS*), a "No-Go" decision is rendered and the trial can be stopped early. On the other hand, if a "Go" decision is made at the end of the first stage, the process of

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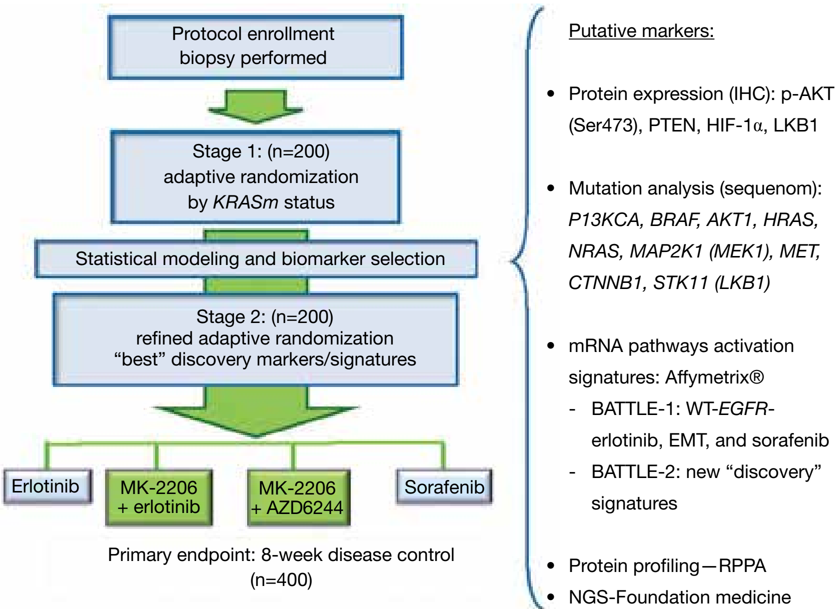

Figure 5 BATTLE-2 study schema. Four treatment arms: EGFR inhibitor erlotinib (control), erlotinib + AKT-inhibitor (MK-2206), MEK-inhibitor (AZD6244) + MK-2206, and sorafenib. EGFR, epidermal growth factor receptor; BATTLE, Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination; EMT, epithelial-mesenchymal transition.

biomarker analysis and selection is implemented to screen for and select additional prognostic or predictive biomarkers based on the data from the first stage and other available information. A refined predictive model is used for the adaptive randomization scheme in the second stage. Note that the randomization probability is set to be bounded between 0.1 to 0.9 to guard against extreme allocation in stage 2.

The plan is to enroll a total of 400 evaluable patients over a 4-year period. With a conservative estimate that 10% of the patients may have incomplete marker profiles due to limited numbers of tumor cells in the biopsy samples or an unevaluable endpoint, a total of 450 patients will be enrolled. Simulations were applied to thoroughly study the operating characteristics of the design, with the goal of achieving at least 80% power at a 10% type I error rate for testing the efficacy of each treatment, as well as yielding at least 80% power for identifying important prognostic and predictive markers.

Patients with a prior history of having received erlotinib treatment are not randomized into the erlotinib-only treatment arm. Treatment effects are tested separately in the patient subgroups stratified by whether or not patients had prior erlotinib exposure as well as in the overall patient groups. As mentioned previously, BATTLE-2 uses a 2-stage design. In the first stage, patients are adaptively randomized based on their *KRAS* mutation status and whether they were previously treated with erlotinib. An early futility stopping rule is activated from the 71st patient. If all the experimental treatment arms do not show evidence of improved efficacy over the control arm (erlotinib) for all patients and any of the biomarker groups (*KRAS* mutation negative or positive) by prior erlotinib treatment status, the trial is stopped early. By the end of stage 1, if the trial has not been stopped, then the biomarker analysis is performed through a training, testing, and validation procedure described as follows.

Before BATTLE-2, over 100 discovery biomarkers were screened to identify putative prognostic and predictive markers. Combining with the finding in the BATTLE-1 trial, promising prognostic and predictive markers are identified in the training step. During stage 1 of the trial, the identified markers are assessed in the patients' tissues and blood samples. Those data, as well as the patients' medical demographic variables and treatment outcomes, supplemented by other up-to-date *in vitro* or *in vivo* data and information from the literature, are combined by the biostatistics and bioinformatics team to propose a refined predictive model to be tested. We test the "best-performing" markers from the data in the first stage of BATTLE-2. For markers passing the training and testing steps, they will be

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further validated in the second stage of BATTLE-2. The predictive markers identified in stage 1 are used for adaptive randomization in stage 2. We apply the Bayesian 2-step Lasso method for variable selection at the end of stage 1. Specifically, the first step of variable selection is a group selection procedure aimed at identifying markers with either prognostic effects or predictive effects. The second step is an individual selection for a marker and its interactions with the treatments. The final decision of biomarker selection is based on statistical strength, biological plausibility, and practical considerations. Upon final selection of markers and the refined predictive model, we amend the protocol for IRB approval and continuously adaptively randomize patients in stage 2 if the trial is not stopped early. By the end of the study, all markers will be evaluated for potential predictive or prognostic effect, and the effective treatment in patients with predictive markers will be declared.

Using the experiences and knowledge gained from the conduct of the BATTLE-1 trial, the BATTLE-2 trial has been designed with more flexibility: no restriction of pre-specified biomarkers, no biomarker grouping, adaptive randomization starting from the beginning of the trial, prognostic/predictive biomarkers being screened and selected in a 3-step process: training, testing, and validation, and the predictive model for adaptive randomization being refined with real-time data observed for the study. Due to its exploratory nature, BATTLE-2 has received the investigational device exemption (IDE) waiver after a meeting with the FDA in January 2013. Like BATTLE-1, BATTLE-2 is being conducted through a web-based application. The first stage of patient accrual was opened at the UT MD Anderson Cancer Center and the Yale Cancer Center in June 2011. Patient accrual and biomarker analyses have continued in the subsequent years.

Impact of BATTLE trials and future directions

The BATTLE program has demonstrated the feasibility and impact of the first biomarker-based, adaptively randomized novel clinical trial platform in NSCLC, and has set an example for the development of targeted agents in cancer. The successful conduct of this program has demonstrated that it is feasible in modern medical practice to undertake real-time biomarker analysis following a tissue biopsy in patients with relapsed disease. The primary paper describing the BATTLE-1 trial (12) has been cited in more than 200 articles and book chapters to date. The successful completion of that study has been called "an important milestone" in the war against cancer (28). In NSCLC diagnosis and treatment, BATTLE-1 is a landmark trial for successfully pairing biomarker-defined cohorts of patients with targeted therapeutics (29). The completion of BATTLE-1 has proven that we can expand the horizon of oncology clinical trial research to incorporate a prospective biopsy and real-time biomarker analysis. This alleviates many problems such as selection bias and the inflation of the type I error rate in retrospective studies based on post-treatment subgroup analysis. It also addresses the problems of biomarker assays obtained from the original diagnostic tissue, which is far from satisfactory because of the changes that may occur in a patient's biomarker profile after the patient receives many lines of treatment. It can help us to achieve a more accurate understanding of the cancer-causing mechanism, to efficiently identify the predictive biomarkers and corresponding targeted therapies, and to use this information to provide better treatment for patients enrolled in the trial.

Applying Bayesian adaptive designs in the BATTLE trials provides excellent examples of how to fill the gap between statistical methodology research and its application in medical practice. Though more researchers are realizing the advantage of using Bayesian adaptive clinical trial designs, real applications of such designs in clinical trials are still limited. It is a common occurrence for there to be a long time lag between the publication of a statistical method and its application in a clinical study. For example, the seminal continuous reassessment method for phase I trials was published in 1990 (30), but was not widely used for some time. Reviewing the Science Citation Index database between 1991 and 2006, it was found that only 1.6% of the 1,235 phase I trials reported used Bayesian adaptive designs (31). A recent review of published Bayesian adaptive clinical trials indicated that the challenges when using Bayesian adaptive trial designs were the difficulties of the Bayesian computations and the lack of user-friendly software for the study design and trial conduct (32). However, more and more tools have been developed in recent years to conquer these computational barriers when using Bayesian methods (33). One notable example is the collection of useful software that is available at the UT MD Anderson Cancer Center software download site (https://biostatistics.mdanderson.org/SoftwareDownload/). The successful conduct of the BATTLE trials and studies that have similarly applied Bayesian adaptive designs, such as the I-SPY2 trial (34), has promoted methodological research in novel clinical trial design and encouraged statisticians and

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clinical trialists to implement more new design methods in their medical research (35). Concurrently, there have been several major attempts to apply similar concepts in the quest to identify effective cancer therapies and associated predictive markers. These include the National Cancer Institute's Molecular Analysis for Therapy Choice Program (MATCH; http://www.cancer.gov/clinicaltrials/noteworthy-trials/match#match), the Lung Cancer Master Protocol (Lung-MAP; http://www.cancer.gov/newscenter/newsfromnci/2014/LungMAPlaunch), and the Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trials (ALCHEMIST; http://www.cancer.gov/clinicaltrials/noteworthy-trials/alchemist).

The BATTLE program has created a new paradigm of prospective biopsy-based, real-time biomarker analysis and adaptive designs in clinical studies. With advancements in biomedical research, we look forward to more such studies increasing trial efficiency and enhancing the benefit to patients while developing more effective treatments. The BATTLE program has opened a new page for clinical trial design and conduct, and has brought us one step closer to personalized medicine.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Lung-MAP—framework, overview, and design principles

Renata Ferrarotto[1], Mary W. Redman[2], David R. Gandara[3], Roy S. Herbst[4], Vassiliki A. Papadimitrakopoulou[1]

[1]Department of Thoracic and Head and Neck Medical Oncology, University of Texas, MD Anderson Cancer Center, USA; [2]Clinical Statistics, Clinical Research Division, Fred Hutchinson Cancer Center, Seattle, WA, USA; [3]University of California Davis Cancer Center, Sacramento, CA, USA; [4]Yale Cancer Center, Yale School of Medicine, New Haven, CT, USA

Correspondence to: Vassiliki A. Papadimitrakopoulou, MD. 1515 Holcombe Blvd., 77030 Houston, Texas, USA. Email: vpapadim@mdanderson.org.

Abstract: Metastatic lung squamous cell carcinoma (SCC) is a common disease with limited therapeutic options and poor patient outcomes. Standard "all comers" clinical trial designs usually benefit only a small population sub-group. Targeted-therapy matched clinical trials have a higher potential to achieve better results, however, given the low frequency of driver genetic alterations, they are associated with a large number of screen-failures, are not cost-effective, and frequently not feasible. Lung-MAP is an umbrella master protocol for recurrent or metastatic lung SCC patients that uses a central genomic profiling screening platform to allocate patients to phase II/III biomarker-matched target therapy clinical trials or to a "non-match" treatment arm; therefore, all eligible patients screened can be treated under the protocol. If evidence of efficacy is seen in the phase II trial portion for a particular treatment/marker combination, that sub-study moves directly to phase III and incorporates the patients treated in phase II. Lung-MAP has an efficient and adaptable structure that allows for sub-studies to open and close based on changes in an evolving cancer research field. It also provides a path for FDA-approval in order to bring promising agents to clinic in a time efficient manner, with the ultimate goal of significantly improving lung SCC patient's quality and length of life.

Keywords: Clinical trial; lung cancer; master protocol; squamous cell carcinoma (SCC)

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Introduction

The expanding application of genomic sequencing in oncology is revealing targetable genetic aberrations including gene mutations, rearrangements, amplifications, and deletions, and creating an immense opportunity to implement personalized therapy with a high potential to improve patients outcomes.

In lung adenocarcinoma, potentially actionable genomic alterations are present in approximately 64% of patients (1). The activity of EGFR and ALK inhibitors in patients harboring respectively EGFR activating mutations (~17%) or ALK translocations (4-8%) has changed the treatment paradigm and significantly improved survival of lung adenocarcinoma patients (2,3). New gene aberrations such as *ROS1* rearrangements, *BRAF* mutation, *ERBB2* mutation, and *RET* fusions, are being targeted in clinical trials, with promising preliminary results (1,4-7). Unfortunately, in spite of squamous cell carcinoma (SCC) being the second most common lung cancer histology, corresponding to approximately 25% of cases, no molecular targeted agents have been developed for patients with this histology thus far.

The Cancer Genome Atlas and other sequencing initiatives shed some light into the genomics of lung SCC and revealed potentially targetable alterations such as FGFR mutations, fusions, and amplifications; PI3KCA mutations; CCND1 amplification; and c-MET amplification and/or protein overexpression (8,9). Successful targeting of driver oncogenes in SCC, mirroring lung adenocarcinoma, could lead to a significant improvement in patient's clinical outcomes and its investigation should be intensively pursued.

The traditional development of oncology therapeutic

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agents is overall a lengthy, expensive, and inefficient process. The time from initial drug discovery to clinical testing and regulatory review can take up to 15 years. The many challenges of this process include difficulties in the recruitment of patients; high number of screen failures, particularly for trials studying a rare biomarker defined subgroup; the bureaucratic process; cost; and lengthy regulatory review (10). Only 3-5% of adult cancer patients enroll in clinical trials in the United States, leading to underpowered studies, early trial discontinuation, lack of feasibility, and inapplicability of trial results in an evolving medical field (11).

In order to overcome the above-mentioned barriers of cancer drug development and ask more questions in a single study, new approaches to clinical trial designs are being explored. For example, BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung cancer Elimination) and I-SPY2 TRIAL (Investigation of Serial studies to Predict Your Therapeutic Response with Imaging And moLecular analysis 2) were pilot studies using adaptive designs combined with biomarker testing. BATTLE accrued refractory non-small cell lung cancer, and based on the results of 11 biomarkers tested in a mandatory fresh biopsy, it adaptively randomized patients to 4 different arms, according to the data obtained during the trial course (12). I-SPY2 is investigating the addition of targeted therapy to chemotherapy in the breast cancer neoadjuvant setting, based on tissue and image biomarkers. It has a master structure that allows up to 5 drugs to be tested simultaneously in independent phase II trials (13).

In order to fulfill the unmet need of lung SCC, the LUNG-MAP (SWOG S1400) protocol was conceptualized by SWOG with the collaboration of public and private groups including the National Cancer Institute, the Food and Drug Administration (FDA), the National Clinical Trials Network, pharmaceutical industry partners, and advocacy organizations. LUNG-MAP is an "umbrella" trial design that facilitates the evaluation of multiple investigational therapies in independently conducted therapeutic studies under a single trial infrastructure in lung SCC population subsets. The goal of this approach is to improve genomic screening and time lines for drug-biomarker testing with the "one-stop-shop" approach allowing for inclusion of the maximum numbers of otherwise eligible patients in comparison with the usually employed "single screen-single trial". This manuscript will discuss the overall protocol and trial design principles.

Lung-MAP framework and overview

Protocol design

Lung-MAP (S1400) is aiming to identify biomarker-drug pairs that will lead to successful therapeutic outcomes and registration of new agents. It is a registration-intent master protocol that includes a screening and a clinical trial component. The clinical trial component includes multiple sub-studies that independently evaluate investigational therapies. It is designed to be modular such that new sub-studies can be added either as other sub-studies close or as new biomarker-drug pairs are identified for testing in the SCC patient population.

Original eligible population

The eligible population consists of adult patients with recurrent or metastatic lung SCC who progressed after first line platinum based chemotherapy and have a performance status ECOG ≤2. Measurable disease and adequate organ function is required. The presence of an EGFR activating mutation or EML4-ALK translocation as defined by the central screening next generation sequencing performed by Foundation Medicine, Inc. (FMI), uncommon in SCC, is an exclusion criterion.

Screening overview

Patient tumor specimens are to be submitted for central testing within 1 day after registration to the screening portion of the trial. Formalin-fixed and paraffin-embedded tissue from archival and/or fresh tumor biopsy must be available for biomarker testing. Submission of either a tumor block or minimum of 12 unstained slides is required for study entry. FMI is performing the biomarker analysis using massive parallel DNA sequencing to detect potentially targetable genomic alterations in cancer related genes. Immunohistochemical assays can also be performed according to the biomarker being investigated. The tests are executed in CLIA (Clinical Laboratory Improvement Amendments) certified laboratories. The turn-around time from tissue submission to reporting of the results is less than or equal to 16 days. Upon completion of biomarker screening, the results are reported to the SWOG statistical center.

Clinical trial component

Within one day of receipt of the tumor profiling results,

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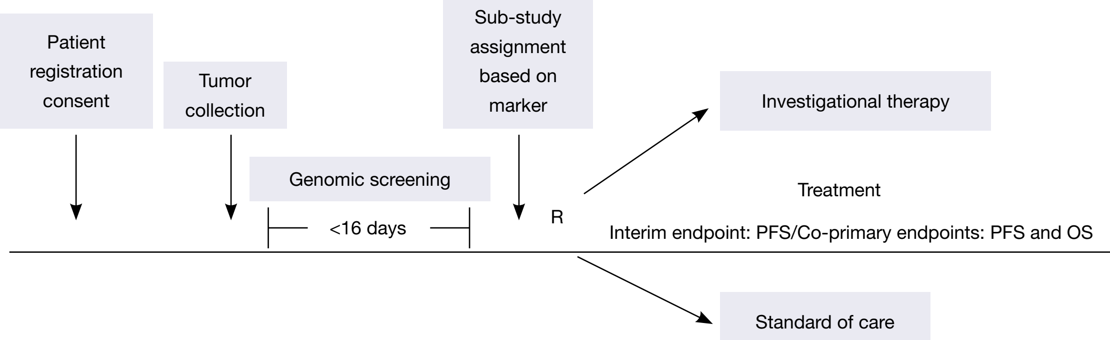

Figure 1 Lung-MAP master protocol schema. The schema demonstrates overall procedures and timeframes from patient's informed consent signature to treatment. The revised protocol allows for genotype pre-screening, which should expedite treatment initiation. R, randomization; PFS, progression-free survival; OS, overall survival.

Table 1 Sub-studies included in the original Lung-MAP protocol

Sub-study	Experimental drug	Target inhibition	Genomic aberrations	Frequency (14,15)
S1400A	MEDI4736	PD-L1	Non-match	NA
S1400B	GDC-0032	PI3K	PIK3CA mutation	~15%
S1400C	Palbociclib	CDK4/6	CDK4, CCND1, CCND2, or CCND3 amplification	~20%
S1400D	AZD4547	FGFR	FGFR1, 2, or 3 mutation, fusion, or amplification	~26%
S1400E	Rilotumumab + Erlotinib	HGF	c-MET IHC positive	~8%

When it initially launched in June 2014, the Lung-MAP had 5 sub-studies. Given lack of efficacy and increased toxicity of rilotumumab in gastric cancer, Amgen suspended all clinical trials with this drug and sub-study E was discontinued.

patients are assigned to a sub-study within the Lung-MAP umbrella. Patients with exactly one of the targeted biomarkers are assigned to the associated sub-study evaluating an investigational therapy targeted against that aberration. For patients with more than one of the targeted biomarkers, assignment is randomized between the sub-studies they are eligible for using an algorithm that gives more weight to studies with lower prevalence biomarkers. Patients whose tumors alterations don't fall into any of the available matched drug-biomarker sub-studies are assigned to a "non-match" sub-study. Therefore all screened patients who satisfy the clinical eligibility criteria have a study in which to enroll. *Figure 1* shows an overview of the study schema. The specific drugs that were included in the original protocol launched in June 2014 are listed in *Table 1*. Docetaxel is considered the standard of care comparison arm for all sub-studies, except for S1400E, where the comparison drug was Erlotinib.

Statistical design

Each of the sub-studies are independently conducted, analyzed and reported. The initial statistical design and the primary design of each of the sub-studies is a seamless phase II/III design (16). The sub-studies employ co-primary objectives: (I) to compare overall survival between the investigational therapy arm and the standard of care arm; and (II) to determine if there is both a statistical and clinically-meaningful difference in progression-free survival (PFS) between the treatment arms on the study. "Clinically meaningful" is defined as at least a 2.25 month difference in median PFS between the two arms for sub-studies evaluating single agent targeted therapy/non-match therapy; and at least a 2.5 months difference for sub-studies evaluating targeted therapy or non-match therapy combinations. The expected median PFS and OS for patients receiving standard of care treatment is 3 and 8 months respectively.

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The phase II is an interim analysis, which evaluate early stopping due to futility, based on PFS. As such, all patients included in the phase II component of the study are included in the phase III analyses. The 'bar' for continuing past the phase II interim analysis is based on phase II design properties and has a much higher chance of stopping the trial than standard phase III interim analyses. This design facilitates both speedy screening of less effective investigational therapies (and thereby closing sub-studies at the phase II interim analysis point which is approximately 100 patient accruals), and accelerates completion of accrual to a phase III and time to a definitive answer for effective investigational therapies. Secondary objectives include a comparison of response rate and toxicity between the arms within a sub-study.

The sample size for each sub-study is determined based on the biomarker prevalence, maintaining all other design parameters the same across sub-studies. It ranges from 68 to 124 patients for a biomarker prevalence of 2.5% to 20%. For the phase III analysis with similar prevalence assumptions, the sample size ranges from 272 to 336. The expected accrual rate in the Master Protocol is 500 to 1,000 patients per year in approximately 400 sites.

Lung-MAP design principles

One of the major principles of Lung-MAP is the rapid implementation of new research findings within the protocol framework and study design. In other words, new sub-studies can enter the trial at any time when relevant drug-biomarker pairs with sufficient proof-of concept become available.

The agent selection for the protocol is the task of a Drug/Biomarker Selection Committee comprised of independent members from the pharmaceutical industry, academia, the Investigational Drug Branch of the NCI, and the lead principal investigators. A formal presentation and scoring procedure is adopted for selection of the best potential candidate in class. The criteria include target appropriateness to lung SCC, drug/biomarker understanding, preclinical data, pharmacodynamics and pharmacokinetics, toxicity, and clinical data with proof of principle in the biomarker-selected population.

According to the results of the futility analysis in the phase II portion, the sub-studies can be quickly closed or move to a phase III registration trial. This strategy significantly reduces time, number of patients, and cost needed to bring promising agents to the clinical setting. The sub-studies are based on the same protocol design and statistical assumptions; therefore, all investigational agents are tested in a comparable manner. The addition of new sub-study populations affects the estimated biomarker overlap prevalence but does not alter the overall design or statistical assumptions.

The use of a common and detailed genotype platform facilitates broad screening and efficient allocation of patients to biomarker-specific sub-studies. The presence of a "non-match" arm, allow for all eligible patients to be accrued. This platform also offers opportunities for exploratory studies incorporating NGS results and clinical outcomes, in order to identify additional predictive biomarkers as well as potential resistance mechanisms. The tissue and blood banking from patients screened for LUNG-MAP will represent one of the largest repositories of squamous cell lung cancer. The master protocol is flexible and adaptable, allowing for incorporation of "new standards" in an evolving field.

Adaptability of the framework

It was with great excitement that the Lung-MAP group of investigators saw the approval of nivolumab (Opdivo) as second line therapy of lung SCC, in the same research space occupied by Lung MAP (17). The Lung-MAP team had recognized the potential of immunotherapies as treatments for lung cancer in its early design by choosing an investigational checkpoint inhibitor from AstraZeneca/MedImmune to be part of the inaugural launch of the trial. The flexibility of the Lung-MAP study design allowed the study team to modify the trial to allow patients to participate when they have received not just one prior, but now two or more lines of systemic therapy, thus allowing patients to receive nivolumab prior to entry on the trial. It also prompted a change in the design of the non-match arm as described below.

The study is also allowing patients to be screened while receiving first-line therapy (pre-screening), which will facilitate and expedite enrollment upon progression. Another important change is the modification of the non-match sub-study to single arm treatment with MEDI4736 an anti-PDL1 monoclonal antibody. An additional change in the trial was the closure of one of the initial sub-studies (S1400E), rilotumumab *vs.* erlotinib because the manufacturer, Amgen, withdrew the drug from its phase III study in gastric cancer on observation of toxicity that was not outweighed by efficacy. The revised study schema is

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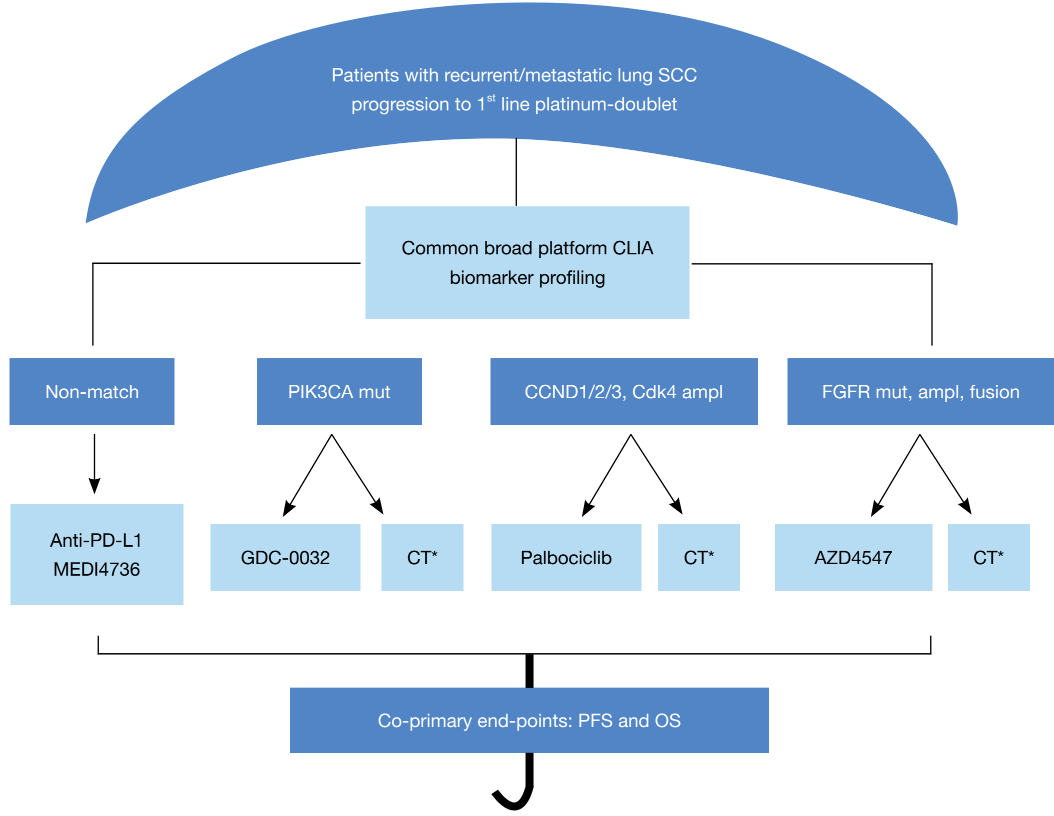

Figure 2 Sub-studies currently open in the Lung-MAP protocol (NCT02154490). The schema demonstrates the sub-studies currently active under the master protocol umbrella. The non-match single arm study tests MEDI4736, an anti-PD-L1 inhibitor. There are three randomized targeted-therapy biomarker matched sub-studies. The drugs being tested are GDC-0032, a PI3K inhibitor; Palbociclib, a CDK4/6 inhibitor; and AZD4547, a FGFR inhibitor. Docetaxel is the control arm for the genotype-matched sub-studies. mut, mutation; ampl, amplification; CT, chemotherapy (docetaxel); PFS, progression-free survival; OS, overall survival.

shown in *Figure 2*. Clearly, as nivolumab becomes second line standard of care therapy for lung SCC, changes are being made to the current non-match sub-study and consideration will also be given to changes in the control arm for the biomarker matched targeted therapy sub-studies.

As of July 2015, a year after activation of the study, 330 patients have been enrolled in the master protocol and 261 have been assigned to a sub-study. Sub-study A (non-match arm) is leading in terms of accrual with 90 patients enrolled and an estimated complete accrual in the fall of 2015. The estimated time to complete accrual for the biomarker-matched sub-studies will range between 14 and 36 months.

Conclusions

LUNG-MAP is an "umbrella," tumor specific protocol, inspired on BATTLE and I-SPY2, but with unique characteristics. It does not use adaptive randomization. The target therapy being tested may not have been validated for a specific biomarker, but have a high potential to be active in the molecular defined population based on promising phase I trials results and strong rationale. The turnaround time of the genetic screening is short and pre-screening is allowed, which is essential in the setting of refractory lung SCC. It also combines the learning (phase II) and confirmatory (phase III) phases into a single seamless phase II-III trial. Counting the patients from the phase II stage towards the phase III portion decreases the total sample size needed. This strategy diminishes administrative burden, cost, and time to get effective therapies to patients, as each sub-studies has the potential for drug registration. It is also appealing for investors and pharmaceutical companies as the phase II study results can be published while the phase III portion is ongoing. Indeed the major challenges to the

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development of the protocol were logistic in nature rather than lack of engagement and enthusiasm by academia and pharmaceutical industry and the protocol has actually been embraced by community practices that have been leading the accrual. The Master Protocol is flexible, allowing sub-studies to enter on a rolling basis as other closes. It also will create a biorepository of refractory lung SCC tissue, blood, and imaging, which will allow future translational research studies. It is frequently revised to accommodate advances made in the evolving field. Finally, it has a great potential to implement personalized cancer care in an organized, cost-effective and timely manner that will ultimately impact the lives and bring hope for innumerous metastatic lung SCC patients.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Designing a definitive trial for adjuvant targeted therapy in genotype defined lung cancer: the ALCHEMIST trials

Ryan S. Alden[1], Sumithra J. Mandrekar[2], Geoffrey R. Oxnard[1]

[1]Dana-Farber Cancer Institute, Boston, MA, USA; [2]Alliance Statistics and Data Center, Mayo Clinic, Rochester, MN, USA

Correspondence to: Geoffrey R. Oxnard. Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. Email: Geoffrey_Oxnard@dfci.harvard.edu.

Abstract: Genotype-directed targeted therapies have revolutionized the treatment of metastatic non-small cell lung cancer (NSCLC) but they have not yet been comprehensively studied in the adjuvant setting. Previous trials of adjuvant targeted therapy in unselected early stage NSCLC patients showed no benefit versus placebo, however retrospective data suggests improved disease free survival (DFS) with epidermal growth factor receptor (EGFR) inhibitors in patients with appropriate molecular alterations. A definitive prospective, randomized, placebo-controlled trial of targeted therapies for NSCLC is needed to determine the efficacy of targeted therapy following surgical resection and standard adjuvant therapy. The principal challenges facing such a trial are (I) identification of actionable alterations in early stage patients; and (II) realization of sufficient enrollment to power definitive analyses. The ALCHEMIST trial (Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trial) was designed to overcome these challenges. Using the national clinical trials network (NCTN) of the National Cancer Institute (NCI), several thousand patients with operable NSCLC will undergo tumor genotyping for *EGFR* mutations or rearrangement of anaplastic lymphoma kinase (*ALK*). Following resection and completion of standard adjuvant therapy, patients with *EGFR*-mutant NSCLC will be randomized to erlotinib versus placebo (1:1), those with *ALK*-rearranged NSCLC will be randomized to crizotinib versus placebo (1:1), while those not enrolled onto the adjuvant trials will continue to be followed on the screening trial. ALCHEMIST also provides for the collection of tissue at baseline and at recurrence (if available) to characterize mechanisms of recurrence and of resistance to targeted therapy. Thus, ALCHEMIST is a platform for validation of targeted therapy as part of curative care in NSCLC and creates an opportunity to advance our understanding of disease biology.

Keywords: Clinical trial design; targeted therapy; adjuvant therapy; non-small cell lung cancer (NSCLC)

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Introduction

Targeted therapies, specifically tyrosine kinase inhibitors (TKIs), have redefined the standard of care for metastatic epidermal growth factor receptor (*EGFR*)-mutant or anaplastic lymphoma kinase *(ALK)*-rearranged non-small cell lung cancer (NSCLC) (1-8). The success of these agents exemplifies the current trend toward rationally designed compounds which target specific molecular alterations in cancer. The benefit associated with these agents is similar to the transformative benefit seen with imatinib in gastrointestinal stromal tumor (GIST) or with tamoxifen or trastuzumab for metastatic breast cancers (9-16). But while imatinib, tamoxifen and trastuzumab have long been employed in the adjuvant setting, targeted therapies have not been comprehensively studied in early stage NSCLC (*Tables 1,2*) (17-20,25-28). About half of all patients who present with potentially curable NSCLC will die from recurrent disease within 5 years (29). By comparison, 5-year survival among 200 patients with high-risk early stage KIT positive GIST treated with imatinib following surgery was 92% (18) and 5-year survival in operable ER positive or

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Table 1 Studies of adjuvant targeted therapies in diseases other than lung cancer

Trial	Disease	Treatment	Exposure	Primary endpoint	DFS	OS
Dematteo *et al.* (17)	KIT+ GIST, >3 cm	Imatinib *vs.* placebo (1:1)	1 year	DFS	HR 0.35 (95% CI: 0.22-0.53) P<0.0001; at 1 year, 98% *vs.* 83%	HR 0.66 (0.22-2.03) P=0.47; at 4 years, 99% *vs.* 98%
Joensuu *et al.* (18)	KIT+ GIST, high-risk	1 year *vs.* 3 years imatinib (1:1)	1 year *vs.* 3 years	DFS	HR 0.46 (0.32-0.65) P<0.001; at 5 years, 66% *vs.* 48%	HR 0.45 (0.22-0.89) P=0.02; at 5 years, 92% *vs.* 81.7%
IBCSG trial 13-93 (19)	ER+ breast cancer, node+	Tamoxifen *vs.* observation (1:1)	5 years	DFS	HR 0.59 (0.46-0.75) P<0.0001; at 5 years, 75% *vs.* 62%	HR 0.86 (0.62-1.19) P=0.36; at 5 years, 87% *vs.* 87%
HERA trial (20)	HER2+ breast cancer	Trastuzumab *vs.* observation (1:1)	1 year	DFS	HR 0.54 (0.43-0.67) P<0.0001; at 2 years, 86% *vs.* 77%	HR 0.76 (0.47-1.23) P=0.26; at 2 years, 96% *vs.* 95%

DFS, disease free survival; OS, overall survival; GIST, gastrointestinal stromal tumor; HR, hazard ratio; CI, confidence interval.

Table 2 Prior studies of adjuvant targeted therapies in NSCLC

Trial	Disease	Treatment	Exposure	Primary endpoint	DFS	OS
NCIC CTG BR.19 trial (21)	NSCLC, no molecular selection	Gefitinib *vs.* placebo (1:1)	2 years	OS	HR 1.22 (0.93-1.61) P=0.15; median 4.2 years *vs.* NR; at 6.3 years, 52% *vs.* 59%	HR 1.24 (0.94-1.64) P=0.14; median 5.1 years *vs.* NR; at 6.3 years, 54% *vs.* 59%
RADIANT trial (22)	NSCLC, EGFR+ by IHC or FISH	Erlotinib *vs.* placebo (2:1)	2 years	DFS	HR 0.90 (0.74-1.10) P=0.3235; median 50.5 months *vs.* 48.2 months	HR 1.13 (0.88-1.45) P=0.3350
D'Angelo *et al.* (23)	NSCLC, EGFR mutant (retrospective analysis)	Erlotinib or gefitinib [84] *vs.* no adjuvant TKI [202]	Median 18.6 months (0.1-51.4)	Retrospective	HR 0.43 (0.26-0.72) P=0.001	HR 0.50 (0.23-1.08) P=0.076
SELECT trial (24)	NSCLC, EGFR mutant	Erlotinib	2 years	DFS	90% at 2 years	92% at 2 years

NSCLC, non-small cell lung cancer; DFS, disease free survival; OS, overall survival; HR, hazard ratio; NR, not reached; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

HER2 positive breast cancers treated with tamoxifen or trastuzumab is 92% (25) and 91% (26,27) respectively. It is fitting to now aim to transform adjuvant therapy of NSCLC through incorporation of targeted agents just as it has been transformed for these other diseases, but to achieve this a prospective controlled trial in a carefully selected population is needed.

Definitive study of adjuvant targeted therapy in NSCLC faces challenges. Such a trial must establish a system to prospectively identify patients with actionable genetic alterations and allocate them appropriately, as targeted therapy does not improve outcomes in unselected populations of early stage NSCLC (*Table 2*) (21,22). Furthermore, a successful trial must overcome the consistently low participation rate (<5%) in clinical trials among adults with cancer in the United States (30). The Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trial (ALCHEMIST) combines the

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Table 3 ALCHEMIST trials

Trial	Disease	Treatment	Exposure	Primary endpoint	DFS	OS
ALCHEMIST EGFR	NSCLC, EGFR mutant	Erlotinib *vs.* placebo (1:1)	2 years	OS	NR	Goal HR 0.67; median 7.5 *vs.* 5.0 years
ALCHEMIST ALK	NSCLC, ALK rearranged	Crizotinib *vs.* placebo (1:1)	2 years	OS	NR	Goal HR 0.67; median 7.5 *vs.* 5.0 years

DFS, disease free survival; OS, overall survival; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NR, not reached; HR, hazard ratio; ALK, anaplastic lymphoma kinase.

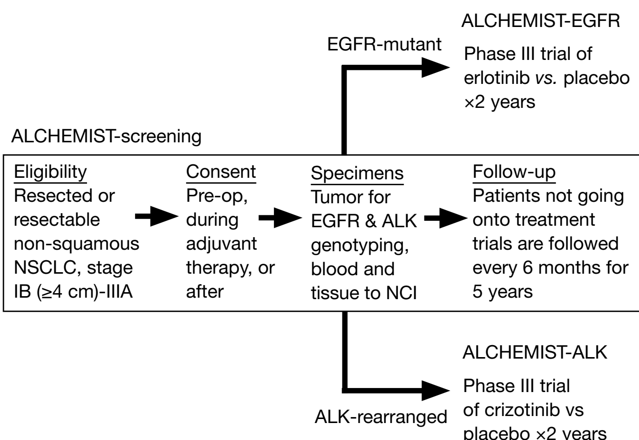

Figure 1 Schema for the three current ALCHEMIST trials.

resources of the National Cancer Institute (NCI) with the broad reach of the NCTN to overcome these challenges. The NCTN, built from the previous cooperative group system, will use its academic and community centers to identify eligible patients who will agree to have tumor genotyping performed. The NCI's Center for Cancer Genetics (CCG) will perform further genomic analysis of all samples. The ALCHEMIST design will allow comprehensive investigation of targeted therapies in resected NSCLC, and will be a platform that can grow with future therapeutic advances (*Table 3*).

Design overview

ALCHEMIST is designed to facilitate prospective screening, identification and enrollment of high-risk early stage (IB-IIIA), genotype-selected NSCLC patients in randomized trials of targeted therapy. ALCHEMIST currently consists of three integrated protocols: a screening study (A151216, coordinated by the Alliance for Clinical Trials in Oncology, PI: Geoffrey Oxnard) and two treatment trials (A081105, coordinated by the Alliance for Clinical Trials in Oncology, PI: Ramaswamy Govindan; E4512, coordinated by the ECOG-ACRIN Cancer Research Group, PI: David Gerber). Additional protocols may be included as other compelling therapies, such as immunotherapy agents, emerge.

All patients must first consent to ALCHEMIST Screening (A151216) to be considered for ALCHEMIST trials of targeted therapy (*Figure 1*). Patients can consent prior to surgery or after resection. Eligible patients must have completely resected non-squamous NSCLC. Tumor specimens will be tested centrally for *EGFR* kinase domain mutations using sequencing, and for *ALK* rearrangements using FISH; patients must undergo confirmatory central genotyping on study regardless of whether any local genotyping was positive or negative for *EGFR* or *ALK*. Remaining tumor tissue will be paired with blood specimens for exploratory genomic analysis by the NCI. Patients whose tumors are found to harbor *EGFR* mutation or *ALK* rearrangement will be offered enrollment onto trials of adjuvant targeted therapy. Patients not enrolled onto the adjuvant trials will be followed on ALCHEMIST screening every 6 months for 5 years. In order to advance the understanding of genomic and biological mechanisms of recurrent or resistant disease, tumor samples from any biopsies performed at the time of recurrence will be collected and studied. While it is understood that not all patients will have tissue available at recurrence for genomic analysis, it is expected that a subset of patients will undergo resection or metastectomy as part of their clinical care and therefore will have a larger specimen available for study.

The flow of biospecimens and clinical data on ALCHEMIST are described in *Figure 2*. All biospecimens are logged and tracked through BioMS, and all genotype and clinical data are collected in Medidata Rave. While

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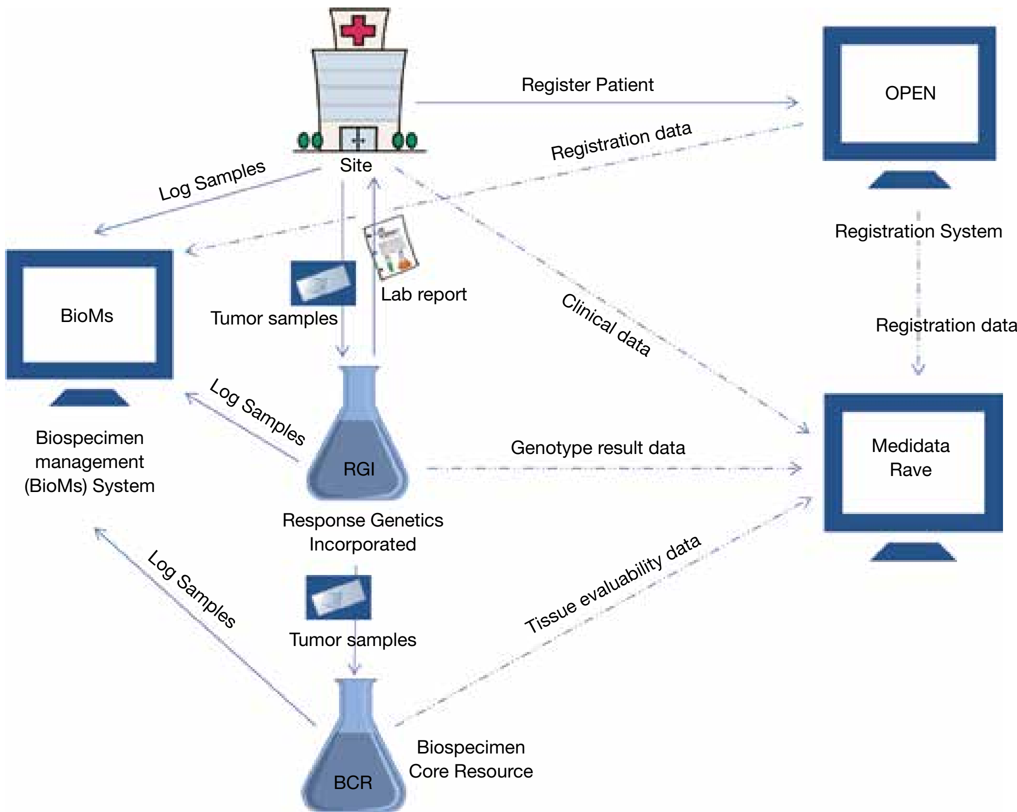

Figure 2 Systems for biospecimen and data flow for ALCHEMIST-Screening trial.

there were some existing integrations between the patient registration system (OPEN) and Medidata Rave, infrastructure to interface with laboratory partners (RGI, BCR) and Medidata Rave were built specifically for ALCHEMIST. As noted above and in *Figure 2*, beyond the predefined molecular alterations to identify potential patients for the EGFR and ALK sub-studies, full genomic characterization of submitted tissue will occur at the NCI-CCG. Regular conference calls and data exchanges between the different entities involved in ALCHEMIST ensure smooth functioning of this complex infrastructure.

Following standard adjuvant therapy, patients with fully resected *EGFR*-mutant NSCLC will be randomized to erlotinib versus placebo (1:1) for two years under ALCHEMIST-EGFR (A081105). Similarly, patients with fully resected *ALK*-rearranged NSCLC will be randomized to crizotinib *vs.* placebo (1:1) for two years under ALCHEMIST-ALK (E4512) following standard adjuvant therapy. As erlotinib and crizotinib are well established standard therapies for advanced NSCLC, it is anticipated that toxicity and compliance will be manageable throughout the NCTN.

The primary endpoint for ALCHEMIST adjuvant therapeutic trials is overall survival (OS). For the *EGFR* trial (A081105), a total accrual of 450 patients will allow for a 5% rate of study withdrawal and 5% non-confirmation rate of *EGFR* mutation via central testing, leaving the target accrual of 410 patients to power definitive analysis of the primary endpoint. This sample size will provide at least 85% power to show a hazard ratio (HR) of 0.67 or better in favor of erlotinib over placebo after 183 events, using a one-sided type I error of 5%. There is one planned interim analysis for futility when 50% of the events have been observed, using the O'Brien-Fleming stopping boundary. If the observed HR is greater than or equal to 0.96 (P value is 0.43 or greater), the recommendation will be to stop further accrual (if the trial is still accruing). It is expected that this trial will complete its targeted accrual. For the *ALK* trial (E4512), a total accrual of 378 patients will allow for a 5% non-confirmation rate of *ALK* rearrangement via central testing, leaving the target accrual of 360 patients to power definitive analysis of OS. This sample size will provide at

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least 80% power to detect a HR of 0.67 or better in favor of crizotinib over placebo after 164 events, using a one-sided type I error of 5%. A continuous efficacy-monitoring plan based on the O'Brien-Fleming group sequential boundary for the sequential testing incorporating the Lan and DeMets methodology for the error spending rate function is proposed: 10 planned interim analyses for OS starting at roughly 25% information (42 events under the alternative hypothesis) and one final analysis. At each interim analysis for efficacy, the study will also be monitored for early stopping in favor of the null hypothesis (i.e., futility) using repeated confidence interval methodology similar to that described by Jennison and Turnbull. At each interim analysis, if the nominal (1-2× alpha) confidence interval on the OS HR does not contain the target alternative of 0.67, then the data safety monitoring committee may consider terminating the study early for overall lack of treatment differences.

Rationale

EGFR TKIs are effective therapies, but must be employed in appropriately selected populations. OPTIMAL, a phase III randomized trial of erlotinib versus carboplatin and gemcitabine (1:1) for metastatic, previously untreated *EGFR*-mutant NSCLC demonstrated a significantly longer progression-free survival (PFS) for patients treated with erlotinib {13.1 [95% confidence interval (CI): 10.58-16.53] *vs.* 4.6 (4.21-5.42) months; HR: 0.16 (95% CI: 0.10-0.26); P<0.0001} (4). The IPASS trial further showed that clinical criteria alone are not sufficient to predict benefit from EGFR TKI—molecular analysis is needed (6,31). A total of 1,217 East Asian former light or non-smokers with treatment naïve advanced adenocarcinoma were randomized to gefitinib or carboplatin-paclitaxel (6). No advantage in OS was observed between the two arms [18.6 months, HR: 0.91 (95% CI: 0.76-1.10)] (31). While PFS (primary endpoint) was improved in the gefitinib arm [5.7 months, HR: 0.74 (95% CI: 0.65-0.85; P<0.001)], PFS benefit was limited to patients with *EGFR* mutations, as gefitinib was associated with shorter PFS for patients with wild-type *EGFR* [HR: 2.85 (95% CI: 2.05-3.98); P<0.001] (31).

No prospective, placebo controlled trial has examined adjuvant treatment with EGFR TKI in an appropriately selected population. The BR19 trial randomized patients with completely resected NSCLC to receive gefitinib or placebo for 2 years (21). The trial closed early due to lack of benefit, enrolling 503 of 1,242 planned patients (21). There was no significant difference in OS (HR: 1.24; 95% CI: 0.94-1.64; P=0.14) or DFS (HR: 1.22; 95% CI: 0.93-1.61; P=0.15) between the two arms (*Table 2*) (21). However, patients were not selected based on *EGFR* mutation status, and only 15 patients were found to have *EGFR* activating mutations (21). The RADIANT trial selected for EGFR expression or amplification, not *EGFR* mutation, in patients with resected NSCLC and showed no improvement in OS for erlotinib *vs.* placebo (*Table 2*) (22,32). Median DFS was improved with erlotinib *vs.* placebo in 161 patients with *EGFR* mutations (46.4 *vs.* 28.5 months; HR: 0.61, 95% CI: 0.38-0.98, P=0.039), however, this result was not statistically significant due to the hierarchical testing procedure employed by the trial (22,32). Retrospective data from MSKCC suggests improved DFS (HR: 0.43, 95% CI: 0.26-0.72, P=0.001) and OS with adjuvant EGFR TKI in resected *EGFR*-mutant NSCLC, but OS figures are not significant (HR: 0.50, 95% CI: 0.23-1.08, P=0.076) (*Table 2*) (23).

Motivated by this retrospective data, the SELECT trial enrolled 100 *EGFR*-mutant NSCLC patients to prospectively test the efficacy and feasibility of adjuvant erlotinib in molecularly-selected patients (24,33). Median DFS and OS have not yet been reached, but the 2-year DFS observed by the trial was 90% (n=89) (*Table 2*) (24). Importantly, many patients underwent dose reduction, which allowed improved compliance for the duration of adjuvant therapy. Building off the feasibility demonstrated in the SELECT trial, ALCHEMIST-Screening (A151216) in conjunction with ALCHEMIST-EGFR (A081105) will identify patients with resected *EGFR*-mutant lung cancer for enrollment onto a randomized, placebo-controlled trial to power definitive analyses regarding efficacy of EGFR TKI in resected *EGFR*-mutant NSCLC.

Phase III trials have also confirmed the efficacy of targeted TKI therapy in advanced *ALK*-rearranged NSCLC, but no prospective studies have examined adjuvant TKI in early stage *ALK*+ patients (7,8). A randomized study of second-line crizotinib *vs.* pemetrexed or docetaxel for *ALK*+ locally advanced or metastatic NSCLC demonstrated a median PFS of 7.7 months for crizotinib *vs.* 3.0 months for chemotherapy (HR: 0.49, 95% CI: 0.37-0.64, P<0.001) (7). These findings, combined with recent data that point to poorer prognosis for early stage *ALK*+ patients (34), make crizotinib an attractive option for incorporation into

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curative therapy for these patients. ALCHEMIST-ALK (E4512) represents a definitive trial to evaluate adjuvant ALK TKI in this subpopulation of NSCLC.

Endpoints and design rationale

The primary objective of ALCHEMIST-Screening is to facilitate accrual to ALCHEMIST trials of targeted therapy. ALCHEMIST-Screening aims to genotype up to 8,000 high-risk early stage NSCLC patients in order to fully accrue ALCHEMIST-EGFR and ALCHEMIST-ALK, based on estimated 15% prevalence of *EGFR* kinase domain mutations and 5% prevalence of *ALK* rearrangements (7,35,36). The desired rate of accrual to the therapeutic randomized trials is 16 patients per month: ~10 patients for the ALCHEMIST-EGFR trial and ~6 per month for ALCHEMIST-ALK trial, in order to reach completion within four years.

OS was chosen as the primary endpoint for ALCHEMIST trials of targeted therapy because it represents the most significant endpoint for patients with curable disease. In this setting, OS is the best measure for absolute benefit of a new treatment relative to standard of care. OS as an endpoint can be diluted by the effect of subsequent therapies, but if the benefit afforded by targeted therapy in the adjuvant setting does not surpass the benefit of targeted therapy at recurrence for control arm patients, then the value of adjuvant targeted therapy is less clear compared to treatment at time of recurrence.

The type I error rate (1-sided 0.05) for the two adjuvant trials is higher than the standard rate (2-sided 0.05) to accommodate the low prevalence of *EGFR* kinase mutations and *ALK* rearrangements in NSCLC and make the studies feasible. OS estimates for placebo arms for the purpose of sample size calculations were based on historical data of standard of care outcomes from unselected early stage NSCLC. It is important to note that, while there is less historical data for the standard of care outcome in early stage *EGFR*-mutant or *ALK*-rearranged patients, there are indications that *EGFR*-mutant NSCLC may predict a better prognosis, while *ALK*-rearranged NSCLC may be associated with a poorer prognosis (23,34,36,37).

The rate of agreement between the local and central testing results for EGFR and ALK will be monitored within the ALCHEMIST screening trial. Specifically, each locally deemed *EGFR*-mutant or wild-type patient will also be classified by central assessment. Similarly, each patient deemed locally as *ALK*-rearranged or not by FISH will be classified by the central assessment. For each locally used assay, agreement will be defined as the proportion of patients deemed mutant (or wild-type) by local and central assessment divided by the number of evaluable patients, where an evaluable patient is one who has a local assessment result and has submitted tissue for central assessment. An agreement rate of 90% or higher between the local assay and the central assessment will be deemed acceptable.

Conclusions

If the ALCHEMIST trials of targeted therapy reach their primary objectives, genotype-directed TKIs will become an important addition to standard curative therapy for early stage NSCLC patients with appropriate molecular alterations. The results of these trials may encourage further study of highly active targeted agents as part of curative therapy for NSCLC. For example, a trial studying adjuvant PD-1 inhibition is in development as part of the ALCHEMIST effort. Tissue collection and analysis through ALCHEMIST-Screening may also reveal new molecular targets, and may in turn pave the way for novel therapies to be studied in the adjuvant setting for appropriate populations.

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Footnote

Conflicts of Interest: GR Oxnard has received consulting fees from Astra Zeneca and Boehringer Ingelheim and honoraria from Chugai.

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Statistical aspect of translational and correlative studies in clinical trials

Herbert Pang[1,2], **Xiaofei Wang**[2]

[1]School of Public Health, Li Ka Shing Faculty of Medicine, Pok Fu Lam, Hong Kong SAR, China; [2]Department of Biostatistics and Bioinformatics, Duke University School of Medicine, Durham, NC, USA

Correspondence to: Herbert Pang. School of Public Health, Li Ka Shing Faculty of Medicine, Pok Fu Lam, Hong Kong SAR, China. Email: pathwayrf@gmail.com; Xiaofei Wang. Department of Biostatistics and Bioinformatics, Duke University School of Medicine, Durham, NC, USA. Email: xiaofei.wang@duke.edu.

Abstract: In this article, we describe statistical issues related to the conduct of translational and correlative studies in cancer clinical trials. In the era of personalized medicine, proper biomarker discovery and validation is crucial for producing groundbreaking research. In order to carry out the framework outlined in this article, a team effort between oncologists and statisticians is the key for success.

Keywords: Big data; bioinformatics; biomarkers; oncology; personalized medicine; translational science

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Introduction

Biomarkers play a prominent role in cancer research and development. Gene expression microarrays and single nucleotide polymorphism arrays were commonly used technologies in earlier research. Today, a platform such as next generation sequencing is often used. This tool can be used to measure gene expression, RNA-Seq, methylation, TF binding Chip-Seq, and genetic variant discovery and quantification. Most of these data are generated from Illumina (Solexa), 454 Roche, and SOLiD sequencing machines. In patients care, biomarkers can potentially be used for risk stratification in terms of clinical outcome and may assist physicians in making treatment decisions. Apart from genetic biomarkers, imaging biomarkers can also serve as a potential surrogate for clinical trial endpoints, or guide the treatment routine. These biomarkers are being integrated into many modern clinical trials (1).

In the discovery and identification of biomarkers from big '-omics' data for clinical outcomes, application of sound statistical approaches is essential. We will discuss several statistical issues and introduce statistical methods and strategies for consideration. Without proper implementation of these steps, the resources spent on designing and running an independent clinical validation may turn out to be unfruitful. In this article, we will define some of the terminologies commonly used, discuss how to build and evaluate classifiers, and describe strategies to validate them retrospectively and prospectively.

Definitions

Biomarkers can mainly be classified into three different groups, depending on their intended use in treatment. The evaluation requirements and validation criteria vary according to the purpose of the usage of the biomarkers.

(I) Prognostic biomarkers, which are associated with patients' overall outcome. A validated prognostic biomarker provides the opportunity to identify patients at high risk and thus a population that may benefit from early or aggressive intervention. For example, KRAS mutation is associated with poor prognosis in non-small cell lung cancer (NSCLC) patients (2).

(II) Predictive biomarkers, which predict the effect of a specific treatment on a clinical endpoint for patients. As an example, advanced pancreatic cancer patients with lower levels of vascular endothelial growth

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factor-D (VEGF-D) benefited from the addition of bevacizumab to standard gemcitabine, while patients with high VEGF-D levels did not (3). Another example is that patients with overexpressed Cyclo-oxygenase-2 (COX-2) who appeared to benefit from the addition of celecoxib (a COX-2 inhibitor) to standard chemotherapy relative to those receiving chemotherapy only (4).

(III) Biomarkers which can potentially serve as a surrogate for the primary endpoint in clinical trials. Analogous to surrogate clinical endpoints (5), surrogate biomarkers can be used as intermediate indicators of treatment efficacy in cancer treatment studies. For example, maximal pain intensity, an individual measure, on the Brief Pain Inventory quality-of-life instruments in the previous 24 hours, has been used as a surrogate endpoint for clinical benefit (6).

Development of cancer biomarkers: planning and design

Clustering or cluster analysis is an algorithm that can be applied to identify groupings of genes or patients. While it is an excellent discovery tool for unsupervised learning, heatmap and clustering methods applied to a genomic feature set do not rigorously define a classifier, defined as a tool that utilizes a patient's genetic characteristics to determine which class or group he/she belongs to. Many traditional statistical methods are not capable of handling the large number of genes and small sample size problems that biomarker discovery often encounters. Therefore, modified and new methods are needed for tackling big '-omics' data problems.

Building a classifier

To build a classifier of a clinical outcome based on the pattern of thousands of biomarkers, such as genes or genetic variants, one often uses supervised learning methods to train the classifier with a data set in which true phenotypes of the outcome is known. Classifiers using different supervised learning algorithms have been proposed, including discriminant analysis, decision trees, random forests, nearest neighbor classifiers, neural networks, and support vector machine classifiers. However, there is no consensus in the statistical and machine learning communities about which particular classifier is superior to others across different data sets. Key considerations for deciding which approach might be more appropriate include the ability to handle missing and/or noisy data, interpretability, and predictive power.

Feature selection

Feature selection is a critical component of building a classifier. In our context, genes are the features that require selection. A good classifier depends on the selection of important features, i.e., features that can help distinguish between the categorical outcomes of interest. As in model building, good classifiers that are parsimonious are easier to interpret. Complicated classifiers with too many features can degrade the performance of the classifier and make external validation more difficult. One can utilize univariate test statistics like the two-sample *t*-test or Wilcoxon rank sum test for all features based on the training set, and then identify the top features by ranking the P values. A classifier is then built on these top features based on the training set. In the survival setting, one may use Cox regression or non-parametric methods to identify top features. Considering the complex relationship of biomarkers with the associated phenotype, one often believes a decision based on multiple biomarkers may potentially be more useful than individual biomarkers. There have also been methods developed to identify multiple genes such as the approach developed by Pang *et al*. [2012] for survival outcomes (7).

Strategies for internal validation

Overfitting happens when the model corresponds too closely to a particular data set. As a result, the model may not predict future observations well. To prevent overfitting the data, validation methods such as cross-validation can be employed. Internal validation uses the data set from the same set of patients as was used to develop the classifier to assess the performance of the classifier. To ensure an unbiased evaluation, one must ensure that the data used for evaluating the predictive accuracy of the classifier be distinct from the data used for selecting the biomarkers and building the supervised classifier. This can be achieved by resampling techniques including hold-out or split sample, k-fold cross validation and leave-one-out cross validation. The hold-out method is usually applied to larger data sets, while the leave-one-out cross validation may provide the best option for smaller data sets. K-fold cross validation with k=5 or 10 are commonly used for various sizes of data. Some investigators will also incorporate permutation and nested cross validation strategies. Other strategies to help reduce overfitting include dimension reduction, penalization, and the use of Bayesian methodology.

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Table 1 Confusion matrix

True class	Predicted class	
	Positive	Negative
Positive	A	B
Negative	C	D

Retrospective validation

After the classifier is built, the next step is to perform retrospective validation, i.e., validation based on existing clinical data and samples. These samples are independent from the original training data in the previous step. A locked down model should be pre-specified. This model is then used to predict the outcome of interest in the independent validation data. The predicted outcome is then compared against true clinical outcomes for concordance and/or accuracy. However, this may not always be possible. Large databases of '-omics' data may turn out to be too heterogeneous for validation, or the patient population may turn out to be different from that used in model-building. Moreover, investigators may face issues such as assay platform changes or differences in sample collection protocol. Despite these potential drawbacks, some researchers turn to biospecimen banks, where samples have been collected from large clinical trials (8), such as the NCI National Clinical Trials Network. One such example is the CALGB 140202 lung cancer tissue bank (9) that has contributed samples to multiple studies, including microRNA signature validation, gene-expression signature validation, The Cancer Genome Atlas (http://cancergenome.nih.gov/), exome-sequencing, blood biomarkers, and protein assay validation.

Sample size calculation

Researchers have taken different strategies in sample size calculations for designing studies assessing '-omics' data. Jung [2005] described an approach for sample size calculation based on false discovery rate control in microarray data analysis (10). Dobbin and Simon [2007] provided a sample size calculation algorithm based on the specification of some level of tolerance within its true accuracy (11). Pang and Jung [2013] developed a sample size calculation method that may be used to design a validation study from pilot data (12). These sample size calculation methods require the knowledge of the expected effect sizes, number of genes on the platform, sample proportions, the desired level of statistical power, and the acceptable type I error or false discovery rate.

Pathway analysis

Biology is generally not dictated by a single gene, but rather a set of genes. Pathways are set of genes that serve different cellular or physiologic functions. Pathways are becoming more important in identifying biomarkers and molecular targets for diagnosis and treatment. These pathways can come from pathway databases such as KEGG or Gene Ontology. In recent years, researchers have developed methods to associate gene expression or single nucleotide polymorphisms with prognosis and identify gene signatures (13,14). Statistical methods for pathway analysis based on machine learning, Bayesian approaches and enrichment tests have been developed in the past few years. These pathway-based approaches allow scientists to focus on limited sets of genes, select targets from multiple biomarkers, and gain insights into the biological mechanisms of the tumor. Using random forests importance measure, one can select features in a pathway-based setting (13,15). Compared to single -gene based analysis, pathway-based methods can identify more subtle changes in expression (16).

Evaluation strategies

To evaluate the accuracy of predicting a binary outcome based on a classifier with two statuses, we often consider the use of a 2 by 2 table. This table is often called the confusion table. The sum of the diagonal values divided by the total number of participants indicates the prediction or classification accuracy. Several other measures based on true positive (TP), true negative (TN), false positive (FP), and false negative (FN), are also important for consideration. Using the values in cells labelled as A, B, C, D in *Table 1*, these measures can be defined as: (I) positive predictive value (PPV) = A/(A + C); (II) negative predictive value (NPV) = D/(B + D); (III) sensitivity = A/(A + B); and (IV) specificity = D/(C + D). The Area Under the receiver operating characteristics (ROC) Curve (AUC) is also commonly used. A value of 0.5 represents a random guess while a 1 represents a perfect prediction.

One approach to assess survival prediction performance is to compare the predicted survival of various risk groups using a log-rank test. This can be coupled with permutation testing when appropriate. To evaluate the accuracy of

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survival prediction without dichotomizing, we can employ the area under the ROC curve (AUC) approach for survival data of Heagerty *et al*. [2000] (17). In this instance, sensitivity and specificity are defined as a function of time, and the time-dependent ROC curve is a plot of sensitivity (t) versus 1—specificity (t). Higher prediction accuracy is supported by a larger AUC value. An alternative would be to use the concordance index (C-index) (18), a measure of how well the prediction algorithm ranks the survival of any pair of individuals. C-index takes values between 0 and 1. A C-index of 0.5 corresponds to a random guess and 1 means perfect concordance.

Prospective trial designs

We briefly discuss three main types of designs for prospective validation: targeted design, biomarker-stratified randomized (BSR) design, and hybrid design. Additional details can be found in Simon 2014 (CCO) (19).

Targeted design

For a targeted design, a biomarker is used to restrict eligibility for a randomized clinical trial comparing an experimental regimen to standard of care or control. Often, the experimental regimen is a targeted agent developed for those patients with a particular mutational status of a biomarker. When evaluating the treatment efficacy of a target agent using a randomized phase III trial, the targeted design can be much more efficient than untargeted design. However, a targeted design prevents the chance to test for interaction between treatment and the biomarker. It also prevents the researcher from validating the performance of the predictive biomarker by restricting enrollment to marker-positive only patients. CALGB 30801 is a good example of such design to validate the findings from CALGB 30203 in which patients whose tumors over-expressed COX-2 were randomized to either celecoxib or placebo (20).

(BSR) design

In BSR designs, biomarker status is a stratification factor. For example, both marker positive and negative patients are randomized to a targeted agent versus standard of care or placebo, with randomization stratified by biomarker status. The BSR design allows testing of whether the marker positive patients benefit from an agent compared to standard of care or placebo, with randomization stratified by biomarker status. testing of an overall treatment benefit, and an evaluation of the predictive classifier's performance in identifying the targeted subgroup of patients. However, the drawback of BSR design is the resources and time needed for the conduct of the trial. The ability to answer several questions comes at a cost of the need of more treated patients, and potentially longer follow-up. If the overall treatment benefit is small and the patient population is predominately marker negative, such a design can be ineffective and unethical for the marker negative patients. However, the BSD does avoid a limitation of the following design (hybrid design) that one must be highly confident that the biomarker can identify the subgroup of patients who may benefit.

Hybrid design

A hybrid design lies between targeted and BSR designs. Like the BSR design, the hybrid design randomizes both marker-positive and marker-negative patients. But to reduce cost and improve study efficiency, for example, only a subset of all marker-negative patients is randomized. The process of selecting which patients to randomize may depend on biomarker prediction, clinical outcome, or other baseline patients' characteristics. The efficiency gain due to a hybrid design could be significant when marker negative patients are predominant in the unselected patient population and auxiliary variables exist to identify those informative patients. If the targeted therapy benefits a subgroup of the patient population, but the biomarker used does poorly in the identification of the group, then a useful therapy could be halted for further investigation. An example of the hybrid design is EORTC 10041 (21), which restricted eligibility to only node-negative breast cancer patients to assess a 70-gene expression profile developed by the Netherlands Cancer Institute.

Reporting guidelines

The Strategy Group of the Program for the Assessment of Clinical Cancer Tests and a working group of a National Cancer Institute-European Organization Research Treatment Collaboration developed the Reporting recommendations for tumor Marker prognostic studies (REMARK) (22,23). Many high profile journals require that submissions be vetted through this guideline. This guideline provides a thorough 20-item checklist on essential

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pieces in the publication of marker-based studies, such as assay methods, study design, and statistical methods. It also focuses on presentation of the study results, with guidelines for data, analysis and presentation.

Discussion

The availability of big '-omics' data presents an exciting opportunity for researchers to translate their findings and discovery into clinical trials and ultimately clinical practice. Presently, biomarker discovery is an integral part of the main clinical study. Special attention in planning the study at the protocol development stage can help facilitate testing of secondary hypotheses, collection of specimens, and statistical analysis. While we have covered multiple aspects of statistical considerations for correlative studies in clinical trials, some important topics not covered include differentially expressed genes (DEGs), prospectively validation study designs for prognostic markers, and multiple hypothesis testing issues. Additionally, specific cancers may have their unique topics (24). As sequencing becomes more affordable, we expect that biomarkers will become a routine component of clinical trials. The big '-omics' data generated from these technologies will prove invaluable in this personalized medicine era.

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Footnote

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Biomarker based clinical trial design

Richard Simon

Biometric Research Branch, National Cancer Institute, Bethesda, MD, USA

Corresponding to: Richard Simon, Chief. Biometric Research Branch, National Cancer Institute, 9609 Medical Center Dr, Rockville, MD 20892-9735, USA. Email: rsimon@nih.gov.

Abstract: The established molecular heterogeneity of human cancers and the subsequent stratification of conventional diagnostic categories require the development of new paradigms for the development of a reliable basis for predictive medicine. We review clinical trial designs for the development of new therapeutics and predictive biomarkers to inform their use. We cover designs for a wide range of settings. At one extreme is the development of a new drug with a single biomarker and strong biological evidence that marker negative patients are unlikely to benefit from the new drug. At the other extreme are phase III clinical trials involving both genome-wide discovery and internal validation of a predictive classifier that identifies the patients most likely and unlikely to benefit from the new drug.

Keywords: Predictive biomarker; clinical trial design; adaptive design; companion diagnostic; enrichment trial

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Introduction

Randomized clinical trials have been key for the development of a reliable evidence based medicine. Randomized trials generally evaluate a treatment relative to a control regimen for a broadly defined population of patients traditionally defined based on primary site, histologic diagnosis, stage and number of prior treatments. One limitation of randomized clinical trials is that they have also led to the over-treatment of broad populations of patients, most of whom don't benefit from the drugs and procedures shown to have statistically significant average treatment effects.

Tumors of a primary site in many cases represent a heterogeneous collection of diseases that differ with regard to the mutations that cause them and drive their invasion. The heterogeneous nature of tumors of the same primary site offers new challenges for drug development and clinical trial design. Physicians have always known that cancers of the same primary site were heterogeneous with regard to natural history and response to treatment. Today we have better tools for characterizing the tumors biologically and using this characterization in the design and analysis of clinical trials that utilize this information prospectively.

Presently, most oncology drugs are being developed for defined molecular targets. In some cases the targets are well understood and there is a compelling biological basis for restricting development to the subset of patients whose tumors are characterized by deregulation of the drug target. For other drugs there are multiple targets and more uncertainty about how to measure whether a drug target is driving tumor invasion in an individual patient (1). It is clear that the primary analysis of the new generation of oncology clinical trials must consist of more than just treating broad patient populations and testing the null hypothesis of no average effect. But it is also clear that the tradition of post-hoc data dredging subset analysis is not an adequate basis for predictive oncology. For establishing practice standards and for drug approvals we need prospective analysis plans that provide for both preservation of the type I experiment-wise error rate and for focused predictive analyses that can be used to reliably select patients in clinical practice for use of the new regimen (2-4). The type I experiment-wise error rate is the probability of making any false positive claim (for the overall population or any subset) based on the analysis of the clinical trial. These two primary objectives involve co-development of a drug and a companion diagnostic.

The ideal approach to co-development of a drug

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and companion diagnostic involves (I) identification of a predictive biomarker based on understanding the mechanism of action of the drug and the role of the drug target in the pathophysiology of the disease. A predictive biomarker is a biological measurement that indicates whether the patient is likely to respond to the particular drug. It is distinguished from a prognostic biomarker which may indicate the pace of progression of the underlying disease. This biological understanding should be validated and refined by pre-clinical studies and early phase clinical trials. The predictive biomarkers for successful cancer drugs have generally involved a single gene or protein rather than a multivariate classifier. Multivariate classifiers have found use as prognostic indicators that reflect a combination of the pace of the disease and the effect of standard therapy (5). They can identify which patients have such good prognosis with conservative management that they do not require more aggressive treatment. Multivariate classifiers have rarely been used as predictive biomarkers for response to specific drugs, however, because their use often reflects an incomplete understanding of the mechanism of action of the drug or the role of its molecular target; (II) development of an analytically validated test for measurement of the relevant biomarker. Analytically validated implies that the test accurately measures what it is supposed to measure, or if there is no gold-standard measurement, that the test is reproducible and robust; (III) use of the defined test to design and analyze a new clinical trial to evaluate the effectiveness of the investigative drug and how the effectiveness relates to the biomarker value.

Phase II trials

Candidate predictive biomarkers are often evaluated in traditional phase II trials for patients with tumors of a single primary site. Pusztai and Hess (6) and Jones and Holmgren (7) have described extensions of Simon's two-stage single arm phase II design to accommodate a single binary candidate marker. These designs are focused primarily on ensuring that promising activity of the drug is not missed in cases where its activity is restricted to test-positive patients, and yet excessive numbers of patients are not required in cases where its activity is sufficiently broad that the marker is not needed. Freidlin *et al*. (8) have described a design for use with a single binary biomarker in a randomized phase II design that enables one to determine whether the drug should be developed in a phase III enrichment trial, an all-comers trial, or dropped from further development.

There are many more complicated phase II settings, where no natural cut-point of the biomarker is known in advance, or where there are multiple candidate biomarkers. The BATTLE I trial in NSCLC is an example of a phase II clinical trial in which four different tests were evaluated in the context of four different drug regimens (9). Treatment assignment among the four regimens was randomized, but the randomization weights varied as the trial went along according to which treatment had the best performance within each of the four biomarker strata using freedom from progressive disease at week 8 as the endpoint. There were two main objectives of the adaptive randomization. One was to efficiently screen four treatments in four pre-determined strata of NSCLC patients. The second objective was to provide patients with a trial in which they could feel that the design was adapting to assign them the drug regimen that was best for their form of the disease. Korn and Freidlin (10) have raised questions about the effectiveness of such response adaptive randomization designs for reducing the number of patients receiving what turns out to be a less active regimen and Simon (2) has raised questions about how efficient this design is relative to use of optimal two-stage designs for each drug-stratum combination. The I-SPY 2 phase II design being conducted in breast cancer also uses an adaptive design with pre-specified biomarker strata and multiple treatments (11).

Phase IIa basket discovery trials

Large tumor sequencing studies (12) like the Cancer Genome Project in the UK and The Cancer Genome Atlas (TCGA) in the US have identified recurrent genomic changes in a variety of primary tumor sites. These data provide a scientific basis for treatment of individual patients based on the biological characterization of their tumors. There are, however, many challenges in moving tumor genomics to clinical oncology. These include challenges of logistics, ethics, bioinformatics, study design, regulatory, analytical assay validation and interdisciplinary collaboration. Moving genomics to therapeutics involves using drugs for new indications and dealing with uncertainties about which mutations in a given gene effect the function of the protein product, which are important for the invasive properties of the tumor and which should be considered "actionable" for administration of a drug that was developed for somewhat different mutations in a different primary site. There is much yet to learn about

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effective matching of drugs to genomically characterized tumors (13). Treating patients with drugs selected based on current knowledge to block the de-regulation caused by genomic alterations can, however, provide a database for improving our knowledge of how to combine tumor genomics with therapeutics. It may be much less informative to treat patients without prospective biological characterization and hope to correlate responses to post-hoc assessed genomic tumor alterations although the latter approach may be useful for trying to understand unusually good responses to standard treatments.

"Umbrella" discovery trials include patients with advanced cancer of multiple primary disease sites which are resistant to standard treatment (14). The patients have their tumor DNA sequenced and it is determined (based on a pre-specified algorithm) whether an actionable mutation is present. Actionable means that a drug is available whose range of molecular targets 'mesh' with the genomic alterations of the tumor in a way that suggest treatment may result in benefit for that patient. The evidence that a drug is actionable for a given mutation varies and is often based on biological or pre-clinical data or on data in a different tumor type. The rules of actionability should be prospectively defined. Basket trials have only a single drug available and attempt to discover the types of patients for whom the drug should be developed in later phase studies. In other cases, multiple drugs are available. In some cases the trial is randomized in which outcome on drugs matched based on actionability rules are compared to outcome on drugs selected based on physicians choice without genomic characterization data. Other trials do not use a control arm.

The randomized discovery designs address two distinct questions (14). One is the testing of the null hypothesis that the policy of trying to match the drug to the genomics of the tumor is no more effective than a physicians' choice strategy without using any tumor characterization beyond that used for standard of care. Whereas most clinical trials evaluate a single drug or regimen, the null hypothesis for multi-drug umbrella trials relates to a matching policy for a given set of drugs and biomarkers available for the study. This makes it particularly important to obtain a broad enough menu of potent inhibitors of their targets. The policy is also determined by the type of genomic characterization performed and by the "rules" for matching drug to tumor. If the matching is done by a tumor board and is not rule-based or if the rules change frequently, the pragmatic value of the clinical trial will be limited. It may also be difficult for regulatory bodies to approve use of investigational drugs for use as decided by a tumor board rather than in a more rule-based manner. Consequently, it is important that the policy of treatment-assignment by genomic characterization be transparent and that the duration of the trial be short so that the rules do not change frequently. The use of a randomized control group ensures that comparisons of progression free survival (PFS) between the matched group and the control group are not biased by differences in patient characteristics or biases in assessment of progression. The proof-of-principle embodied by the null hypothesis may be more meaningful, however, in a multi-drug trial of a single histologic category than in cases where a wide range of primary sites of disease are included.

A second objective of the randomized studies is the screening of individual drugs used in specific tumor contexts. For some primary sites a gene may be mutated sufficiently frequently for the study to provide an adequate phase II evaluation of the drug for that new indication (13). In many cases, however, the available patient numbers will not be adequate for a proper phase II evaluation. Nevertheless, the trial may serve to screen for drug-mutation matches for which there is a substantial degrees of activity. These leads must be confirmed in an expanded cohort of a follow-up trial (13). In this discovery mode, assessment of activity of a drug against tumors with a given gene mutated must take into account the possibility that the primary site may indicate a genomic context which may modulate activity of the drug against the alteration.

The non-randomized trials are sometimes called "N of 1" trials in the sense that each patient is different and the outcome of treatment must be evaluated individually in terms of the individual characterization of his or her tumor. This nomenclature can be misleading, however. The "N of 1" approach traditionally referred to a design in which individual patients were treated sequentially for multiple courses with either a test drug or control, with the sequence of treatment or control determined by randomization. This is clearly not possible for cancer studies however. The only endpoint clearly interpretable for non-randomized studies is objective tumor response. Tumors generally do not shrink spontaneously, and so an objective tumor response can usually be attributed to the effect of the drug. Durable objective responses for patients with far advanced metastatic disease are generally rare and can be used for discovering promising ways to target molecularly characterized tumors. PFS is much less interpretable in non-randomized studies. The pace of disease can vary substantially even in advanced cases and so comparing PFS between different subsets of

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patients is hazardous. PFS is subject to measurement error and ascertainment bias depending on the frequency of surveillance. For a patient who has a PFS prior to entry on study of eight weeks, a PFS ratio (relative to the PFS on the previous treatment) in excess of 1.3 may only mean that progression was not declared at the first eight week follow-up of the genomic based study. This is not strong evidence of an effective treatment effect.

Phase III targeted (enrichment) designs

Designs in which eligibility is restricted to those patients considered most likely to benefit from the experimental drug are called "targeted designs" or "enrichment designs." With an enrichment design, an analytically validated diagnostic test is used to restrict eligibility for a randomized clinical trial comparing a regimen containing a new drug to a control regimen. This approach has now been used for pivotal trials of many drugs whose molecular targets were well understood in the context of the disease. Prominent examples include trastuzumab (15), vemerafinib (16), and crezotinib (17).

Several authors have studied the efficiency of the 'targeted' approach relative to the standard approach of randomizing all patients without using the biomarker test at all (18-22). The efficiency of the enrichment design depends on the prevalence of test positive patients and on the effectiveness of the new treatment in test negative patients. When fewer than half of the patients are test positive and the new treatment is relatively ineffective in test negative patients, the number of randomized patients required for an enrichment design is dramatically smaller than the number of randomized patients required for a standard design. For example, if the treatment is completely ineffective in test negative patients, then the ratio of number of patients required for randomization in the enrichment design relative to the number required for the standard design is approximately $1/\gamma^2$ where γ denotes the proportion of patients who are test positive (2). The treatment may have some effectiveness for test negative patients either because the assay is imperfect for measuring deregulation of the putative molecular target or because the drug has off-target anti-tumor effects. Even if the new treatment is half as effective in test negative patients as in test positive patients, however, the randomization ratio is approximately $4/(\gamma+1)^2$. This equals about 2.56 when γ =0.25, i.e., 25% of the patients are test positive, indicating that the enrichment design reduces the number of required patients to randomize by a factor of 2.56.

The enrichment design was very effective for the development of trastuzumab even though the test was imperfect and has subsequently been improved. Simon and Maitournam (18-20) also compared the enrichment design to the standard design with regard to the number of screened patients. The methods of sample size planning for the design of enrichment trials available on line at http://brb.nci.nih.gov; the web-based programs are available for binary and survival/disease-free survival endpoints. The planning takes into account the performance characteristics of the tests and specificity of the treatment effects. The programs provide comparisons to standard non-enrichment designs based on the number of randomized patients required and the number of patients needed for screening to obtain the required number of randomized patients.

The enrichment design is appropriate for contexts where there is a strong biological basis for believing that test negative patients will not benefit from the new drug. In such cases, including test negative patients may raise ethical concerns and may confuse the interpretation of the clinical trial.

Phase III biomarker stratified design

When a predictive classifier has been developed but there is not compelling biological or phase II data that test negative patients do not benefit from the new treatment, it is generally best to include both classifier positive and classifier negative in the phase III clinical trials comparing the new treatment to the control regimen. In this case it is essential that an analysis plan be pre-defined in the protocol for how the predictive classifier will be used in the analysis. The analysis plan will generally define the testing strategy for evaluating the new treatment in the test positive patients, the test negative patients and overall. The testing strategy must preserve the overall type I error of the trial and the trial must be sized to provide adequate statistical power for these tests. It is not sufficient to just stratify, i.e. balance, the randomization with regard to the classifier without specifying a complete analysis plan. The main value of "stratifying" (i.e., balancing) the randomization is that it assures that only patients with adequate test results will enter the trial. Pre-stratification of the randomization is not necessary for the validity of inferences to be made about treatment effects within the test positive or test negative subsets. If an analytically validated test is not available at the start of the trial but will be available by the time of analysis, then it may be preferable not to pre-stratify the

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randomization process. Several primary analysis plans have been described (23-25) and a web based tool for sample size planning for some of these analysis plans is available at http://brb.nci.nih.gov.

If one has moderate strength evidence that the treatment, if effective at all, is likely to be more effective in the test positive cases, one might first compare treatment versus control in test positive patients using a threshold of significance of 5%. Only if the treatment versus control comparison is significant at the 5% level in test positive patients, will the new treatment be compared to the control among test negative patients, again using a threshold of statistical significance of 5%. This sequential approach controls the overall type I error at 5%. To have 90% power in the test positive patients for detecting a 50% reduction in hazard for the new treatment versus control at a two-sided 5% significance level requires about 88 events of test positive patients. If at the time of analysis the event rates in the test positive and test negative strata are about equal, then when there are 88 events in the test positive patients, there will be about $88(1-\gamma)/\gamma$ events in the test negative patients, where γ denotes the proportion of test positive patients. If 25% of the patients are test positive, then there will be approximately 264 events in test negative patients. This will provide approximately 90% power for detecting a 33% reduction in hazard at a two-sided significance level of 5%. In this case, the trial will not be delayed compared to the enrichment design, but a large number of test negative patients will be randomized, treated and followed on the study rather than excluded as for the enrichment design.

In the situation where one has more limited confidence in the predictive marker, the marker can still be effectively used for a "fall-back" analysis. Simon and Wang (25) proposed an analysis plan in which the new treatment group is first compared to the control group overall. If that difference is not significant at a reduced significance level (such as 0.03), then the new treatment is compared to the control group just for test positive patients. The latter comparison uses a threshold of significance of 0.02, or whatever portion of the traditional 0.05 not used by the initial test. Wang *et al.* have shown that the power of this approach can be improved by taking into account the correlation between the overall significance test and the significance test comparing treatment groups in the subset of test positive patients (26). So if, for example a significance threshold of 0.03 has been used for the overall test, the significance threshold for used for the subset can be somewhat greater than 0.02 and still have the overall chance of a false positive claim of any type limited to 5%. Real world experience with stratification and enrichment designs are described by Freidlin *et al.* (27) and by Mandrekar and Sargent (28).

Karuri and Simon (29) introduced a phase III design for the setting of a single binary biomarker stratification design in which futility monitoring of the test negative patients is performed based on a joint prior joint distribution for the treatment effects in test negative and test positive patients. The prior distribution enables the trialist to represent the prior evidence that the treatment effect will be reduced for test negative patients and use that information in monitoring the clinical trial. Although the formulation is Bayesian, the rejection region based on posterior probability is calibrated so that type I errors satisfy the usual frequentist requirements. The Karuri and Simon approach to interim monitoring permits earlier termination of accrual of marker negative patients than with traditional futility analysis methods.

Hong and Simon developed a run-in design which permits a pharmacodynamic, immunologic, or intermediate response endpoint measured after a short run-in period on the new treatment to be used as the predictive biomarker (30). Simon *et al.* (31) described a prospective-retrospective approach to using archived tumor specimens for a focused re-analysis of a randomized phase III trial with regard to a predictive biomarker. The approach requires that archived specimens be available on most patients, and that an analysis plan focused on a single marker be developed prior to performing the blinded assays. This approach was used in establishing that a K-RAS mutation was a negative predictive biomarker for response of colorectal cancer patients to anti-EGFR antibodies.

Phase III adaptive

Jiang *et al.* (32) reported on a "Biomarker Adaptive Threshold Design" for situations where a biomarker is available at the start of the trial, but a cut-point for converting the value to a binary classifier is not established. Tumor specimens are collected from all patients at entry, but the value of the biomarker is not used as an eligibility criteria. The analysis plan does not stipulate that the assay for measuring the index needs to be performed in real time. At the final analysis Jiang *et al.* (32) determine the optimal threshold for the biomarker; that is, the threshold that

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identifies the subset of patients for whom the treatment effect is maximum, using a pre-specified metric. The null distribution of the treatment effect in the optimally selected subset was determined by repeating the analysis after permuting the treatment and control labels a thousand or more times. This permutation analysis automatically adjusted for the fact that a full range of thresholds were evaluated and automatically adjusts for the correlation of the treatment effects among nested subsets. Jiang *et al.* also described a method of obtaining confidence intervals for the optimal threshold using bootstrap re-sampling. Since the treatment is presumed effective only for patients with biomarker above the threshold, the confidence coefficient associated with a given biomarker value x can be interpreted as the probability that a patient with marker value x benefits from the new treatment.

The adaptive threshold design described above enables one to conduct the phase III clinical trial without pre-specifying the cut-point for the biomarker. It provides for a valid statistical significance test that has good statistical power against alternative hypotheses that the treatment effect is limited to patients with biomarker values above some unknown level, and it provides a confidence interval for estimation of the cut-point. These analyses are, however, performed at the end of the trial and accrual during the trial is not restricted by biomarker value. Several authors have studied adaptive enrichment designs in which eligibility criteria change adaptively during the clinical trial based on interim outcome results. Wang *et al.* (33), Rosenblum and Van der Laan (34), and Karuri and Simon (29) consider the case of two strata, e.g., a biomarker positive stratum and a biomarker negative stratum, and adaptively determine when to terminate accrual in the biomarker negative stratum. Follmann (35) considers the case where there are multiple disjoint strata in the population of initially eligible patients and one can adaptively drop each stratum from accrual. Wang *et al.* (33) and Simon and Simon (36), studied more general models for eligibility modification based on multiple candidate biomarkers. The Simon and Simon (36) model was very general and developed statistical significance tests which remain valid even if outcome distributions change during the trial in a manner that depends on the eligibility modifications. Such tests are very robust for use in phase III clinical trials. Simon and Simon (36) illustrated this framework in the setting of adaptive threshold enrichment of a single biomarker.

Designs such as the "adaptive signature design" have been developed for adaptive multivariate classifier development and internal validation based on high dimensional genomic tumor characterization (37). This design employs a "learn and confirm" structure in which a portion of the patients are used to select the biomarker hypothesis, i.e., to develop an "indication classifier" which identifies the target population of patients in which the test treatment is most likely to be effective, and to use the remainder of the patients to test the treatment effect in that subset. The adaptive signature design does not modify eligibility criteria. It is adaptive in the sense that the treatment effect is tested in a single subset determined based on the clinical trial data but in a manner that separates classifier development from testing of treatment effect. Since the adaptive signature design does not use the patients on which the classifier was developed for the testing of the treatment effect, it thus avoids the inflation of type I error described by Wang *et al.* (38) for other approaches. Scher *et al.* described the use of the adaptive signature design for planning a pivotal trial in advanced prostate cancer (39). The key principle of the adaptive signature approach is to replace multiple significance testing based subset analysis with development and internal validation of a single "indication classifier" that informs treatment selection for individual patients based on their entire vector of covariate values.

The adaptive signature design approach is very general with regard to the methodology applied to the training set for identifying the single candidate subset in which treatment effect will be tested in the validation set. Many methods of predictive classifier development can be developed using the training set. It is important to recognize, however, that one is not developing a prognostic classifier. The classifier is used to classify patients as likely to benefit from the new treatment. Matsui *et al.* (40) used their model to predict a continuous score reflecting the expected benefit for the new treatment relative to the control rather than just classifying patients into one of two subsets. Gu *et al.* (41) have developed a two-step strategy for developing a model for predicting outcome as a function of treatment and selected biomarkers. The biomarkers are selected using a group lasso approach in which the main effects of a biomarker are grouped with the interactions of that marker with treatments and can be used with two or more treatments.

Freidlin *et al.* (42) described further extensions of the adaptive signature approach. They use cross-validation to replace simple splitting of the trial into a training set and test set in order to increase the statistical power.

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Conclusions

Recognition of the molecular heterogeneity of human diseases such as cancers of a primary site and the tools for characterizing this heterogeneity presents new opportunities for the development of more effective treatments and challenges for the design and analysis of clinical trials. In oncology, treatment of broad populations with regimens that do not benefit most patients is less economically sustainable with expensive molecularly targeted therapeutics and less likely to be successful. The established molecular heterogeneity of human diseases requires the development of new approaches to use randomized clinical trials to provide a reliable basis predictive medicine. This paper has attempted to review here some prospective designs for the co-development of new therapeutics with companion diagnostics.

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The spectrum of clinical trials aiming at personalizing medicine

Christophe Le Tourneau[1,2,3], Maud Kamal[1], Marie Alt[1], Loic Verlingue[1], Vincent Servois[1], Marie-Paule Sablin[1], Nicolas Servant[1,3], Xavier Paoletti[1,3]

[1]Institut Curie, Paris, France; [2]Institut Curie, Saint-Cloud, France; [3]Unité INSERM/Institut Curie U900, Paris, France

Correspondence to: Christophe Le Tourneau, MD, PhD. Department of Medical Oncology, Clinical Trial Unit, Institut Curie, 26, rue d'Ulm, 75248 Paris Cedex 05, France. Email: Christophe.LeTourneau@curie.fr.

Abstract: All anticancer molecularly targeted agents on the market today have been approved with one or no companion diagnostic based on a specific genomic molecular alteration. These drugs have followed the same clinical development than chemotherapeutic agents and have been developed in selected tumor types and histologies. Now, some molecular alterations have been described across different tumor types, although with variable prevalence and functional impact. The latter raises the question of whether treatment decision should be mainly based on molecular biology, independently of tumor location and histology. This approach refers to what is commonly named personalized medicine and can today be addressed in clinical trials, since major advances in high throughput technologies allow depicting most druggable molecular alterations for an affordable cost in a timeframe that is compatible with clinical practice. Several studies have been initiated that aim at personalizing medicine in oncology. They include molecular screening programs, as well as personalized medicine trials that can be divided in two categories: (I) stratified clinical trials according to either molecular alterations or tumor types; and (II) algorithm-testing trials evaluating a treatment algorithm instead of drugs efficacy. Multiple challenges are associated with personalized medicine trials, but the main one remains our ability to predict drug efficacy based on molecular alterations. It is expected that taking into account several molecular alterations for the prediction of drug efficacy using systems biology approaches will improve patients' outcome. Bioinformatics research will be an important factor of future progression in this emerging field.

Keywords: Algorithms; biomarkers; clinical trials; high throughput technologies; personalized medicine

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Introduction

Personalized medicine, also called precision medicine, is defined by the National Cancer Institute as "A form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease". This term emerged with molecularly targeted agents 15 years ago. While cytotoxic agents destroy rapidly dividing cells by triggering DNA and cell division machinery, molecularly targeted agents block a peculiar molecular alteration involved in cell proliferation, angiogenesis, metastasis, invasion, etc. Medical indication of around half of the drugs approved for clinical use integrates the presence of a molecular alteration (1). The development of some of these agents in molecularly-defined subgroups of patients has yielded unprecedented efficacy in some tumor types (2-7). The remaining drugs lack a validated predictive biomarker of efficacy, and include for example anti-angiogenic agents or mammalian target of rapamycin (mTOR) inhibitors.

Molecularly targeted agents have consistently followed the same clinical development as cytotoxic agents based on tumor location and histology, although some molecular alterations have been reported across different tumor types (8). The emergence of molecularly targeted agents has not immediately led to a paradigm shift in drug development for the following reasons: (I) molecular alterations were initially thought to be specific of certain tumor types, such as the BCR/ABL fusion gene in chronic myeloid leukemia; (II) the

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functional significance of some molecular alterations varies across tumor types, as illustrated by the limited efficacy of BRAF inhibitors in BRAF V600E-mutated colorectal cancer (9) and the substantial efficacy of these drugs in BRAF V600E-mutated melanoma (3); (III) histology-independent drug development would be challenged by the lack of valid benchmarks represented by data on drug efficacy in patients with any type of cancer harboring a common molecular alteration.

Since recently, advances in high-throughput technologies have allowed depicting most druggable molecular alterations for an affordable cost in a timeframe compatible with clinical practice. Despite the caveats associated with histology-independent drug development mentioned above, the question whether personalized medicine based on the molecular profiling of the tumor of cancer patients would still improve their outcome has arisen and led to set up studies addressing this question. We identified three distinct types of studies aiming at personalizing medicine, including molecular screening programs using molecular profiling of the tumor, as well as two distinct types of personalized medicine trials: (I) stratified clinical trials that can be stratified according to either molecular alterations or tumor types; and (II) algorithm-testing trials that evaluate a treatment algorithm instead of drugs' efficacy. These studies are associated with numerous challenges that are then discussed.

Molecular screening programs

Several molecular screening programs have been set up around the world (*Table 1*). These screening programs seek genomic molecular alterations including DNA mutations and/or gene copy number alterations. The primary objective behind these screening programs is to guide patients to clinical trials evaluating drugs matching identified molecular alterations. Most of these programs have therefore been offered to patients with recurrent and/or metastatic cancer with the aim of better selecting therapy in the absence of standard of care. However, some of the programs are proposed to patients at any stage of their disease. These latter programs might be very costly and restrictions might be needed by targeting specific patient populations. Some of the programs focus on one specific tumor types such as non-small cell lung cancer (NSCLC) and breast cancer, while the remaining majority are opened to patients with any kind of cancer.

Various technologies are used in these screening programs, most of them relying on high throughput technologies, while a few programs only look at a very limited number of molecular alterations using polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH). The use of high-throughput technologies often addressed additional challenges based on the complexity and the size of the data. In practice, the management and the analysis of such data require adequate bioinformatics environment and resources.

In the majority of the screening programs, molecular analyses are proposed on archival tissue from primary tumor with the underlying assumption that molecular alterations identified on the primary tumor would still be relevant at later stages of the disease. Numerous studies have evaluated the concordance rate between primary and metastatic sites in terms of actionable molecular alterations (23). Results of these studies are inconsistent, with discrepancies rates reported being low in colorectal (24) and lung cancer (25) for instance and much higher ones in breast cancer (26,27). Discrepancies may account for several factors, including tumor type, type of molecular alteration and most importantly selection pressure due to molecularly targeted therapy, but also the percentage of tumor cells, tumor heterogeneity and the emergence of sub-clones potentially related to the targeted therapy. As an example, epidermal growth factor receptor (EGFR)-mutated lung cancer patients might ultimately become resistant to EGFR-targeting therapy through the emergence of the T790M EGFR mutation (28). Screening programs based on the identification of molecular alterations on archival tissue are easier to implement than those mandating a tumor sample from a metastatic site. The question remains whether the molecular data collected from the primary tumor is adequate or rather expose patients to be guided to inadequate therapy.

These screening programs provide useful information on the prevalence of specific molecular alterations in various tumor types. They might also identify predictive biomarkers as well as new molecular alterations although this should become infrequent because of the data accumulated by the genome sequencing of hundreds of tumors that are now available. Besides the cognitive aspect, these screening programs might have a direct implication for the patients by allowing them to potentially use the information from their tumor for further therapy. Most of the programs include a retrospective assessment of the efficacy in their objectives. Retrospective analysis of the outcome of cancer patients included in a phase I trial at the M.D. Anderson

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Table 1 Screening programs using molecular profiling of the tumor

Programs	Tumor types	Technology	Patient population
Archival tissue			
Cancer Research UK (Stratified Medicine Programme) (10)	Melanoma NSCLC CRC Breast cancer Prostate cancer Ovarian cancer	PCR FISH	All
Dana-Farber Cancer Institute (PROFILE) (11)	All	OncoMap	All
Massachusetts General Hospital (12)	NSCLC	SNaPshot FISH	All
Val d'Hebron Institute of Oncology (13)	All	Sequenom	Phase I
Memorial Sloan-Kettering Cancer Center (IMPACT) (14)	All	Illumina Sequenom	R/M eligible for a clinical trial
Vanderbilt-Ingram Cancer Center (PCMI) (15)	Melanoma NSCLC CRC Breast cancer	SNaPshot	NS
Princess Margaret Hospital (IMPACT) (16)	Selected	MiSeq Sequenom	R/M
Centre Leon Berard (PROFILER) (17)	All	Ion Torrent CGH	All
Fresh biopsy			
MD Anderson Cancer Center (IMPACT) (18)	All	FISH PCR	Phase I
National Cancer Institute (MATCH) (19)	All	NS	R/M eligible for a NCI clinical trial
Netherlands (20)	All	Ion Torrent	All
Institut Gustave Roussy (MOSCATO) (21)	All	CGH Ion Torrent	All
UNICANCER (SAFIR 01) (22)	Breast cancer	CGH/FISH PCR	All

NSCLC, non-small cell lung cancer; CRC, colorectal cancer; PCR, polymerase chain reaction; FISH, fluorescent *in situ* hybridization; CGH, comparative genomic hybridization; NS, not specified; R/M, recurrent and/or metastatic; NCI, National Cancer Institute.

Cancer Center based on a molecular alteration identified on metastatic tumor samples showed that it was better than the outcome of patients who entered a phase I trial without matching in terms of overall response rate, failure-free and overall survival (18). The ratio of progression-free survival (PFS) on phase I therapy to PFS on last therapy was also substantially longer in the former group of patients. Results in terms of PFS ratio were reproduced in the similar MOSCATO study (21). In contrast, disappointing results were reported in a subgroup of patients with metastatic colorectal cancer referred to phase I trials at the Val d'Hebron Hospital (29).

Overall, these retrospective analyses of the utility of screening programs suggest that guiding patients to molecularly targeted therapy would improve their outcome. This has constituted the rationale for designing prospective personalized medicine clinical trials, including stratified and algorithm-testing personalized medicine trials (*Figure 1*). The

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	Personalized medicine trials			
	Stratified trials		Algorithm-testing trials	
Test	Test drugs efficacy		Test algorithm efficiency	
	Molecularly-stratified	Histology-stratified	Non-randomized	Randomized
Tumor types	1	N	1 or N	
Molecular alterations	N	1 or N	N	
Treatments	N	1	N	
Design	- Often use an adaptive design in order to prematuraly close treatments with low efficacy and expand promising treatments - Possibility of randomization		- Patients often used as their own controls to assess efficacy - Stratification may be needed to control for heterogeneity	

Figure 1 Personalized medicine trials.

former ones evaluate the efficacy of drugs while the latter ones evaluate the relevance of a treatment algorithm.

Stratified personalized medicine trials

Stratified personalized medicine trials include two distinct types of trials: molecularly- and histology-stratified trials (*Table 2*). Molecularly-stratified trials usually evaluate several drugs but focus on one specific tumor type, while histology-stratified trials, also called basket trials, focus on one specific drug across diverse tumor types.

Molecularly-stratified trials

Molecularly-stratified personalized medicine trials allocate drugs or drug combinations to patients based on the presence or the absence of specific molecular alterations. These trials sometimes use adaptive designs so that arms with little efficacy can be closed early, whereas arms that show hints of efficacy can be expanded.

The BATTLE trial is the first molecularly-stratified adaptive randomized clinical trial (30). The objective of this trial was to test several biomarkers together with several drugs and to find out what were the most successful associations in patients with advanced lung adenocarcinoma. Patients were randomized between four treatments arms: erlotinib, vandetanib, erlotinib plus bexarotene, and sorafenib. All patients included in the trial were tested for the following molecular alterations on a tumor sample from a mandatory biopsy: EGFR mutation/amplification, KRAS/BRAF mutation, VEGF/VGFR2 expression, RXR/Cyclin D1 expression and CCND1 copy number. Real-time analysis of efficacy using a Bayesian model led to further evaluation of the efficacy of sorafenib in patients whose tumor harbored a KRAS mutation. The BATTLE-2 trial (NCT01248247) allocates advanced lung cancer patients who progress on first line chemotherapy to one of the four following drugs or drug combinations based on the analysis of 11 biomarkers: erlotinib, erlotinib in combination with MK2206 (AKT inhibitor), MK2206 in combination with

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Table 2 Stratified personalized medicine trials

Trial's name	Tumor type	Setting	Design	Molecular alterations	Treatment arms
Molecularly-stratified trials					
BATTLE (30)	NSCLC	>1 line R/M	Adaptive randomization	EGFR mutation/amplification	Erlotinib
				KRAS/BRAF mutation	Sorafenib
				VEGF/VEGFR2 expression	Vandetanib
				RXR/Cyclin D1 expression	Erlotinib + bexarotene
				CCND1 copy number	
BATTLE-2 (31)	NSCLC	>1 line R/M	Adaptive randomization	11 biomarkers	Erlotinib
					Erlotinib + MK2206
					AZD6224 + MK2206
					Sorafenib
BATTLE-FL (32)	EGFR wt NSCLC	1st line R/M	Adaptive phase 2	NS	CP + bevacizumab
					CP + cetuximab
					CP + cituxumumab (IGF1R inh)
FOCUS 4 (33)	CRC	16 wks non PD 1st line	Adaptive randomization	BRAF mutation	BRAF + EGFR ± MEK inh
				PI3KCA mutation/PTEN loss	PI3KCA ± MEK inh
				KRAS/NRAS mutation	AKT + MEK inh
				All wild type	HER1-3 inh
				Unclassified	Capecitabine
I-SPY 2 (34)	Breast cancer	Neoadjuvant	Adaptive phase 2	NS	NS
Histology-stratified trials					
V-BASKET (35)	All	R/M	Stratified phase 2	V600E BRAF mutation	Vemurafenib
CREATE (36)	All	R/M	Stratified phase 2	ALK/MET activation	Crizotinib

NSCLC, non-small cell lung cancer; CRC, colorectal cancer; wt, wild type; R/M, recurrent and/or metastatic; PD, progressive disease; CP, carboplatin + pemetrexed; inh, inhibitor; NS, not specified.

AZD6224 (MEK inhibitor), and sorafenib (31). The trial plans to accrue 450 patients. BATTLE-FL (front-line) (NCT01263782) allocates treatment-naïve metastatic and/or recurrent EGFR wild-type lung cancer patients to one of the three following drugs in combination with the doublet chemotherapy carboplatin and pemetrexed based on the molecular profile established on a mandatory biopsy of a metastatic site: bevacizumab, cetuximab and cituxumumab (anti-IGF1R inhibitor) (32). The BATTLE-2 and BATTLE-FL trials are ongoing.

The FOCUS 4 is a phase II/III trial that involves metastatic colorectal patients without progression after first-line therapy at 16 weeks (33). Following molecular alterations identified on a tumor sample from a metastatic site, patients are allocated to one of the five maintenance treatment arms depending on the molecular alterations identified: (I) a combination of a BRAF inhibitor, an EGFR inhibitor with or without a MEK inhibitor in case of KRAS, BRAF, or NRAS mutation; (II) a PI3KCA inhibitor with or without a MEK inhibitor in case of PI3KCA mutation; (III) a combination of an AKT inhibitor and a MEK inhibitor in case of PTEN loss; (IV) a pan-HER inhibitor if wild type for all previous molecular alterations; and (V) capecitabine if unclassified. Twenty four hundred patients will be included with the aim of randomizing 1,536 patients. In each treatment arm, patients will be randomized against placebo with a 2:1 ratio. An adaptive design is used so that promising treatment arms can switch from a phase II to a phase III. A substantial advantage of this trial is its ability to include any colorectal cancer patient as molecular stratification covers all molecular subgroups.

The I-SPY 1 study had identified clinical, imaging and genomic predictive markers of pathological complete response based on the analysis of 221 early breast cancer

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patients treated with neoadjuvant chemotherapy (37). Based on the hypotheses generated by this study, the I-SPY 2 trial (NCT01042379) has been then set up in the same patient population (34). Patients with stage 3 breast cancer are randomized between standard neoadjuvant chemotherapy and the same treatment combined with a molecularly targeted agent based on molecular alterations identified on tumor biopsy. The primary endpoint is the pathological complete response rate. The protocol allows opening new treatment arms during the trial, to early close presumably non-efficient treatment arms and to expand promising arms. A Bayesian framework is used meaning that the error rate is not controlled according to usual standards. Randomization allows comparing the efficacy of the different experimental arms to standard of care.

All these molecularly-stratified trials evaluate different treatment strategies in different tumor types and settings. However, they do not provide the same level of evidence as 2-arms randomized trials. To date, the operating characteristics of most of these designs have been evaluated in a limited number of settings. As opposed to molecularly-stratified trials, histology-stratified or basket trials evaluate the efficacy of one drug in multiple tumor types based on the presence or the absence of specific molecular alterations, usually matching the targets of the drug under evaluation.

Histology-stratified trials

The V-BASKET trial (NCT01524978) seeks for signals of efficacy in recurrent and/or metastatic cancer patients whose tumors harbor a BRAF mutation, except in patients with V600E BRAF-mutated melanoma (35). The CREATE trial (NCT01524926) is a similar trial with crizotinib intended for the same patient population whose tumors harbor a ALK or MET molecular alteration except for ALK-translocation in lung adenocarcinoma (36). Both trials allow the molecular analyses to be performed on archival tissue, therefore offering the possibility to perform the molecular analyses while the patient is on another treatment. The caveat of this strategy is prescribing a drug in the recurrent and/or metastatic setting based on a molecular alteration detected on the primary tumor.

The main drawbacks of these histology-stratified trials are that (I) they may require to screen many patients who will not be treated with the matching targeted therapy if the incidence of the molecular alterations is low, except if they are concomitantly included in a screening program; and (II) they are not randomized that means, the activity is compared to some theoretical value that is very difficult to interpret due to the variety of tumor types that are included. In all these trials, sample size is calculated so that the efficacy of a drug or drug combination can be adequately assessed with a pre-specified power in any type of cancer.

Algorithm-testing personalized medicine trials

Clinical trials that include patients with multiple tumor types and in which multiple molecular alterations are tested for further treatment allocation can formally evaluate the efficacy of a specific drug (or drug combination) in a specific molecularly-characterized subgroup of patients with a same tumor type only if (I) the trial is stratified on all subgroups of patients; and (II) a formal sample size has been calculated in each subgroup for each drug (or drug combination) to get enough power to conclude based on pre-specified types I and II errors. In other words, except if the sample size is huge, results from such trials will not allow drawing any robust conclusion regarding the potential efficacy of a specific drug or drug combination in a given molecularly-defined subgroup of patients. These trials in fact can only evaluate the treatment algorithm that has been set up to allocate treatments to patients, regardless of the treatment administered. One fundamental requirement in these trials is that the treatment algorithm is not modified during the study. Algorithm-testing trials include non-randomized trials that usually use patients as their own control to assess efficacy, and randomized trials that address various questions (*Table 3*).

Non-randomized clinical trials

Von Hoff's study is the first published histology-independent clinical trial using tumor molecular alterations to select treatment (38). Patients with any type of recurrent and/or metastatic cancer that was refractory to standard of care had selected molecular alterations analyzed using immunohistochemistry (IHC), FISH and oligonucleotide microarray gene expression assays. Based on the detected molecular alterations, a drug or drug combination was prescribed. Eighteen of the 66 treated patients (27%) had a ratio of the time to progression (TTP) on matching targeted treatment to the TTP under the last previous treatment >1.3, which was statistically different from the hypothesis that this ratio is one in the absence of treatment effect.

More recently, the WIN consortium launched the WINTHER trial (NCT01856296) that is currently open

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Table 3 Algorithms-based personalized medicine trials

Trial's name	Tumor type	Setting	End point	Technology	Treatment arms	Control arm
Non-randomized trials						
Von Hoff study (38)	All	>1 line R/M	PFS ratio	Gene expression FISH IHC	Chemotherapy MTA	NA
WINTHER (39)	All	>1 line R/M	PFS ratio	NGS CGH Gene expression	Phase I trial Off-label Chemotherapy	NA
Randomized trials						
SHIVA (40)	All	R/M	PFS	Targeted sequencing Cytoscan HD IHC	Eroltinib Lapatinib + trastuzumab Sorafenib Dasatinib Everolimus Imatinib Vemurafenib Abiraterone Tamoxifen/letrozole	Conventional chemotherapy
MPACT (41)	All	R/M	PFS	Targeted sequencing CGH IHC	Temozolomide + ABT888 Everolimus Trametinib Carboplatin + MK1775	One of the 3 non-matching therapy arms
SAFIR 02 Lung (22)	Non-EGFR mutated/ ALK-translocated NSCLC	1st line R/M	PFS	Targeted sequencing CGH	AZD2014 AZD4547 AZD5363 AZD8931 Pemetrexed Erlotinib Selumetinib Vandetanib	Pemetrexed (squamous) Erlotinib (non-squamous)
SAFIR 02 Breast (22)	ER+/HER2– Breast	1st-3rd line R/M	PFS	Targeted sequencing CGH	AZD2014 AZD4547 AZD5363 AZD8931 Selumetinib Vandetanib Olaparib Casodex	Maintenance chemotherapy
MOST (42)*	All	>1st line R/M	PFS	Targeted sequencing Cytoscan HD	Nilotinib Everolimus Sorafenib Lapatinib Pazopanib Vemurafenib Crizotinib	No treatment

*, discontinuation randomized trial. R/M, recurrent and/or metastatic; PD, progressive disease; PFS, progression-free survival; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; CGH, comparative genomic hybridization; NGS, next generation sequencing; NA, not applicable; NCSLC, non-small cell lung cancer; MTA, molecularly targeted agent.

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in several countries (39). This trial is open to patients with any kind of refractory advanced cancer. Two samples are taken from the patient, one from a metastatic site and the second from adjacent normal tissue. Druggable molecular alterations are first investigated from the tumor sample using next generation sequencing for mutations screening and CGH array for gene copy number alterations profiling. If a druggable molecular alteration is identified, patients are either guided to a phase I clinical trial with an agent presumably matching the molecular alteration or are being prescribed an already approved molecularly targeted agent off-label. If no druggable molecular alteration is detected, data from tumoral RNA and RNA from normal adjacent tissue are analyzed in order to identify gene expression profiles that can orient the patient to the best therapy. Both treatment arms will be analyzed separately using PFS ratio as a primary endpoint. It is planned to treat 200 patients, 80 in the former arm and 120 the latter one, based on the assumption that a druggable molecular alteration will be present in 40% of patients. The main advantage of this trial is that all included patients will be treated.

Given the multiple tumor types included in these non-randomized trials, one way to evaluate treatment efficacy has been to use patients as their own controls assessing the PFS ratio. The main criticism to the use of the PFS/TTP ratio as a primary endpoint in these studies is the assessment of PFS/TTP on the last therapy outside of the clinical trial. In addition, the underlying assumption behind this endpoint is that the natural history of disease is linear over time, in other words that the two PFS/TTP are correlated, which might not be true. For these reasons, the use of randomization has been suggested (43).

Randomized clinical trials

Algorithm-testing randomized clinical trials have been set up either for all types of cancers or in specific tumor types.

The SHIVA trial (NCT01771458) is a proof-of-concept randomized phase II trial comparing molecularly targeted therapy based on tumor molecular profiling versus conventional chemotherapy in patients with any type of cancer that is refractory to standard of care (40). The primary endpoint is PFS. The trial is stratified on (I) the patient's prognosis using the Royal Marsden Hospital prognostic score for phase I cancer patients (44); and (II) the signaling pathway the selected molecular alteration belongs to. Molecular alterations are evaluated on a tumor sample from a metastatic site using the AmpliSeq Cancer panel on Ion Torrent sequencing (Life Technologies) for mutations screening, the Cytoscan HD technology (Affymetrix®) for gene copy number alterations profiling, and IHC for estrogen, progesterone and androgen receptors expression analyses. Only marketed molecularly targeted agents are used in this trial according to a pre-specified treatment algorithm. Eleven molecularly targeted agents are available within the clinical trial, whereas conventional chemotherapy is prescribed at the physician's discretion in the control arm. Cross-over is proposed in both arms at disease progression, allowing the evaluation of tumor growth kinetics on both treatments for each patient (45). Physicians are being told the molecular alteration of interest for their patient only at the time they are about to be treated in the experimental arm. The randomization of 200 patients is planned to detect a significant difference in 6-month PFS with a bilateral type I error of 5% and an 80% power. Feasibility results on the first 100 included patients have shown that biopsies are safe and that 40% of patients were detected a molecular alteration that allowed them to be randomized (46). Ancillary studies include the evaluation of the ability of circulating DNA to predict treatment efficacy or resistance, as well as a medico-economic evaluation of the experimental strategy. Efficacy results should be available in 2016.

The MPACT trial is a randomized phase II trial that will include the same patient population asin the SHIVA trial (41). A tumor sample of a metastatic site will also be mandatory. Molecular alterations will be detected using the Ion Torrent sequencing (Life Technologies) for mutations screening, CGH array (Agilent) for gene copy number alterations profiling, and IHC for protein expression evaluation. Patients will be randomized between therapy matching the detected molecular alteration and therapy not matching the detected molecular alteration. Cross-over will be proposed at disease progression for patients randomized in the non-matching treatment arm. Sample size calculation based on the results of Tsimberidou *et al.*, with an expected overall response rate of 25% in the matching treatment arm versus 5% in the non-matching treatment arm (18). The randomization of 200 patients is planned. Although it might be difficult for patients to accept the randomization in the non-matching treatment arm, this design is the only one that evaluates solely the treatment algorithm. Accrual should start in 2014.

The MOST trial (EudraCT: 2012-004510-34) is a randomized discontinuation trial for patients who have progressed on first-line treatment for a recurrent and/ or metastatic cancer (42). Molecular alterations will

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be identified on a sample from either a metastatic site or the primary using the Ion Torrent technology (Life Technologies) for mutations screening and CGH array (Agilent) for gene copy number alterations profiling. Patients will be treated during three months according to a pre-specified algorithm with one of the seven available already marketed molecularly targeted agents. Responding patients will continue on therapy, while progressive patients will be taken off study. Patients with stable disease will be randomized between treatment continuation and discontinuation for three months. The MOST trial will provide a more accurate evaluation of efficacy than a single-arm study by deciphering between disease stabilization related to the natural history of the disease and disease stabilization related to a cytostatic effect of molecularly targeted therapy. The trial has recently opened.

The SAFIR 02 trials are tumor-specific randomized trials evaluating maintenance therapy (22). SAFIR 02 Breast will include patients with HER-2 negative and estrogen receptor positive recurrent and/or metastatic breast cancer who have not progressed after four to eight cycles of first- to third-line chemotherapy, while SAFIR 02 Lung will include patients with EGFR and ALK wild types recurrent and/or metastatic lung cancer who have not progressed after four cycles of first-line platinum-based chemotherapy. All patients will have a tumor sample taken from a metastatic site in order to seek molecular alterations using targeted sequencing for mutations analysis and CGH array for gene copy number alterations analysis. Patients will be randomized between a molecularly targeted agent from AstraZeneca matching the detected molecular alteration and maintenance chemotherapy (pemetrexed for squamous lung cancer and erlotinib for non-squamous lung cancer). The primary endpoint will be PFS. These trials have opened in 2014 as well. These trials evaluate the utility of a treatment algorithm for selecting maintenance therapy following chemotherapy in recurrent and/or metastatic luminal breast cancer and lung cancer not eligible for molecularly targeted therapy.

All these algorithm-testing clinical trials base their algorithm on DNA analysis, except the WINTHER trial that analyses also gene expression in case no molecular alteration has been detected on DNA. These trials include only so far patients with recurrent and/or metastatic cancer. All these trials ultimately address with different angles the question of whether the use of tumor molecular profiling would improve the outcome of these patients. Results of these trials are highly expected as they will provide meaningful information as of high-throughput technologies should or should not be used in routine in the future. None of these trials is powered to adequately assess the efficacy of any treatment in any molecularly-defined subgroup of patients with a same tumor type and histology. Except for the MPACT trial (41), the treatment effect is confounded with the treatment algorithm for treatment allocation. If a targeted agent had a tremendous effect regardless of the molecular alteration, the whole arm (or period in trials using the PFS/TTP ratio) would appear to be superior and one might erroneously conclude that selecting the treatment based on the molecular profile is superior to not using the treatment algorithm.

Challenges

Given their complexity, personalized medicine trials are associated with numerous challenges. These trials indeed involve several different crucial stakeholders, including physicians, radiologists, pathologists, biostatisticians, sequencing platforms managers, bioinformaticians and biologists. While the four former ones had been used to work together in a clinical setting, the latter ones usually work with researchers and are not used time constraints related to patients care. The novelty with personalized medicine trials is that all these people have to coordinate their actions so that treatment decisions for cancer patients are timely taken.

Tumor tissue

Many clinical trials require having a tumor sample taken from a metastatic site. While biopsies of metastatic sites have been long shown to be feasible without excessive complications (46), a metastatic site might not always be easily accessible for sampling. Moreover, some metastatic sites might still be accessible but not appropriate for high-throughput technologies such as bone where the sequencing failure rate is high (22). Developments of less invasive and more convenient procedures are highly expected. In addition, these samples cannot appreciate tumor heterogeneity.

Most of developments have focused so far on liquid biopsies, including circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). The prerequisite to use this material is obviously to be able to detect it in patients' blood, which might not always be the case. While it has been reported that specific molecular alterations can be detected in CTCs or ctDNA, it remains to be demonstrated

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whether high-throughput technologies can be successfully applied on such material. These liquid biopsies also present a priceless advantage of allowing sequential sampling with the premise of identifying pharmacodynamic biomarkers of efficacy as well as resistance biomarkers. In addition, they might theoretically be able to appreciate tumor heterogeneity if circulating tumor material reflects the tumor burden.

Technology

The use of high-throughput technologies is a multistep process necessitating in the clinics the interaction between multiple actors in order to get real time results for treatment decision making. These steps include tumor sampling by surgeons or interventional radiologists, histological diagnostic confirmation by pathologists, DNA extraction, sequencing, bioinformatics analyses and biological validation by biologists. All these steps are associated with error margins (47), and the whole multistep process might as a consequence be associated with an even increased error margin. Quality controls must therefore be performed at each step of the process and traceability is required.

While the implementation of high-throughput technologies in clinical trials is challenging, the techniques per se represent another challenge. First, the choice of the techniques, including DNA extraction kits, sequencers, pipelines, bioinformatics pipelines must be carefully decided, so that the rates of false negatives and positives reach a reasonable threshold in regards to the project. Different sequencers might also produce different results, although discrepancies are infrequent (48). Second, the techniques have to be validated before being implemented in any research program. No recommendations exist to date regarding these validations. Do we need to validate the technology and some case control somatic mutations by Sanger sequencing in a pilot study before starting any program or should we validate any detected mutation during the program? Finally, techniques evolve so quickly that one might not have any other choice than to implement changes during a trial, simply because the technique used initially is not available any more. In any case, techniques changes must be precisely described when results of clinical trials are reported. The National Cancer Institute, in collaboration with scientists representing multiple areas of expertise relevant to ‘omics’-based test development, has developed a checklist of criteria that can be used to determine the readiness of omics-based tests for guiding patient care in clinical trials (49).

Biology

Behind all these new technologies that are implemented in clinical trials, the real challenge relies on the biological assumptions made in the treatment algorithms used. The elaboration of treatment algorithms have to be supported by strong biological hypotheses. The biological assumptions can either be based on clinical data or on preclinical data only. The key question is the choice of the level of evidence required to set up a treatment algorithm. Is clinical efficacy demonstrated with trastuzumab in HER-2-amplified breast and gastric cancer patients enough to justify the use of this drug in any HER-2-amplified cancer patients? Is the preclinical description of an activating molecular alteration in one single paper enough to incorporate it in the algorithm?

It is now clear that the biological significance of some molecular alterations varies depending on the tumor types, such as the BRAF V600E mutation. Most of melanoma patients respond to BRAF inhibition whereas only a low proportion of colorectal cancer patients do. Preclinical data have shown that colorectal cancer cells may escape via a feedback loop involving EGFR, which is often expressed in colorectal cancer as opposed to melanoma (9). The differential biological significance observed for a specific alteration might indeed be explained by other molecular alterations. Taking into account multiple molecular alterations (or modulations) to predict treatment efficacy opens the field of “systems biology”. The “systems biology” approach will need considerable bioinformatics research to produce valid tools to be used in the clinic. It remains to be demonstrated that a systems biology approach can improve the patients’ outcome. It will also surely imply that several pathways are implicated which raises the critical question of drug combinations that are not easy to manipulate. To date, only single molecular alterations are used to drive treatment selection. No multidimensional algorithm has been proved to be superior.

Another issue pertains when several druggable molecular alterations are detected. The ideal solution would be to target all of them with matching drugs. However, drugs are not easy to combine because of their often overlapping toxicities (50). Treatment priorities have to be established based on strong preclinical data whenever possible. Sequential use of drugs would be worth evaluating especially if pharmacodynamic markers are easily assessable.

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However, from a clinical research perspective, it is essential that the treatment algorithm does not change all along the trial for obvious reproducibility purposes. It is reasonable however considering an enrichment of the algorithm if it does not question previous treatment decisions.

Statistics

Personalized medicine trials that might involve several tumor types, several molecular alterations and matching treatments represent a new challenge for biostatisticians. The ideal clinical trial design would determine a sample size so that a treatment effect can be evaluated with enough statistical power in any subgroup of patients with a specific tumor type harboring a specific molecular alteration and treated with a specific treatment. This type of clinical trial would obviously require thousands of patients and would not be feasible in practice. Compromises have therefore to be made, including stratification in randomized trials that allows controlling for heterogeneity or taking patients as their own controls in non-randomized trials.

Costs

Although the costs of high-throughput technologies are decreasing exponentially, the overall cost of personalized medicine trials remains high. These costs include the sequencing per se, but also the associated bioinformatics analyses and data storage. Trials using drugs that are already on the market should preferentially have the drugs funded by the companies. This might be tricky to get agreements with pharmaceutical companies if several drugs are used, especially if the trial is not powered to evaluate the efficacy of the drug which is less appealing from a pharmaceutical company's point of view. In the case companies refuse to provide their drugs, drugs have then to be funded by the sponsor, which might substantially increase the budget of the trial, especially when patients fortunately display prolonged tumor responses. If studies eventually demonstrate that the use of high-throughput technologies improves patients' outcome, the cost of the implementation of these technologies in routine will have to be precisely determined. The implementation of high-throughput technologies may need a complete restructuration of hospitals unless these analyses are outsourced. In any case, discussions with health authorities but also with pharmaceutical companies will have to be engaged to discuss cost-sharing. Pharmaceutical companies may indeed derive benefits from such implementations that allow patients to be guided in specific molecular-based clinical trials. Medico-economic analyses associated to personalized clinical trials are in that sense crucial.

Ethics

Personalized medicine trials rise ethical considerations if constitutional DNA is needed for genomic analyses (e.g., for exome sequencing). Once constitutional DNA of a given patient is available, following questions have to be answered: Are there genomic data that have to be investigated? What information should be brought back to the patients? What if specific information that concerns the descendants becomes available after the patient's death? It is therefore essential that consent forms used in personalized medicine trials precisely anticipate these questions. They should preferably be discussed with patients' advocates.

Drugs

Access to drugs represents an important issue in personalized medicine trials, especially when several drugs are used. As mentioned earlier, pharmaceutical companies may not be very keen to provide their drugs in multi-drugs trials, all the more if the drugs are in clinical development. Drug combinations with drugs from different companies are almost impossible to obtain, while obtaining drugs from a same company for a drug combination still remains challenging. In addition, algorithm-testing personalized medicine clinical trials do not directly benefit pharmaceutical companies, since these trials evaluate treatment algorithms and not drugs' efficacy. However, they might still derive indirect benefits by the use of systematic molecular profiling of patients that might improve the inclusion rate in clinical trials based on specific molecular alterations. It is therefore urgent that pharmaceutical companies adhere to such trials, as AstraZeneca in the SAFIR 02 trial that provided part of its pipeline.

Discussion

The emergence of cytotoxic chemotherapy after the Second World War has led to a significant improvement in cancer cure. While molecularly targeted therapy has clearly modified the prognosis of some cancers such as chronic myeloid leukemia or subgroups of cancers such as HER-2-overexpressing breast cancer, the cure rates of

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cancer patients has not increased substantially. Two reasons may explain this. First, a minority of cancer patients is today eligible for molecularly targeted therapy. Second, molecularly targeted therapy is mostly approved in the recurrent and/or metastatic setting where they prolong survival but do not cure. Only trastuzumab in HER-2-overexpressing breast cancer and imatinib in c-KIT-overexpressing gastrointestinal stromal tumors are approved in the adjuvant setting (51,52). The substantial decrease of recurrences in these two settings likely provides an indirect demonstration that these two agents are able to cure cancer. The fundamental question we have today is whether the use of high-throughput technologies will increase the rate of cancer cure. The personalized medicine trials described above are almost all performed in patients with recurrent and/or metastatic cancer and will certainly not lead to an increased cure rate of cancer even if they are positive. Only the evaluation of such strategies at earlier stages of the disease could potentially lead to substantially improve the rate of cancer cure. While positive results of these trials would undoubtedly accelerate the implementation of high-throughput technologies in routine, negative results should not be interpreted as a failure of the overall strategy. Subgroup analyses might also pinpoint potential biomarkers that might after clinical validation improve treatment efficacy when taken into account. Patients are indeed usually heavily pretreated in these trials. In addition, patients are usually proposed single agent molecularly targeted therapy, which we know is often insufficient to achieve prolonged efficacy. Last, the treatment algorithms used in these trials have not been validated. Bioinformatics and research in biology will be critical to improve them, using systems biology approaches, along with functional validation in preclinical studies.

The personalized medicine trials described above focus on the use of genomic alterations to decide molecularly targeted therapy. Other approaches to treat cancer have recently emerged and appear to be very promising, including immunotherapy and therapies targeting the microenvironment. Restoring an efficient immune response by targeting CTLA4 in melanoma patients has been demonstrated to improve their outcome in the recurrent and/or metastatic setting (53). Ten to fifteen percent of patients are long responders to this treatment, which is unprecedented. The future will tell whether they are cured, which might be plausible given the mechanism of action of these drugs. Outstanding results have also been reported in several tumor types with drugs targeting the PD-L1/PD1 axis (54-56). Other drugs that target the microenvironment such anti-CSF-1R antibodies that target activated macrophages on the surface of which CSF-1R is present are in clinical development.

Ultimately, it is very likely that cure of cancer will substantially increase thanks to a combination of molecularly targeted therapy that will be more adequately implemented using high-throughput technologies and novel therapies such as immunotherapy and therapies targeting the microenvironment. The integration of these latter therapies to molecularly targeted therapy opens an important field in cancer research. The development of ctDNA will hopefully also help circumvent the issue of intra-tumor heterogeneity (57).

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Adaptive randomized phase II design for biomarker threshold selection and independent evaluation

Lindsay A. Renfro[1], Christina M. Coughlin[2], Axel M. Grothey[3], Daniel J. Sargent[1]

[1]Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN, USA; [2]Novartis Oncology, East Hanover, NJ 07936, USA; [3]Department of Oncology, Mayo Clinic, Rochester, MN, USA

Correspondence to: Lindsay A. Renfro, Ph.D. Division of Biomedical Statistics and Informatics, Mayo Clinic, 200 First St SW, Rochester, MN 55905, USA. Email: Renfro.lindsay@mayo.edu.

Background: Frequently a biomarker capable of defining a patient population with enhanced response to an experimental agent is not fully validated with a known threshold at the start of a phase II trial. When such candidate predictive markers are evaluated and/or validated retrospectively, over-accrual of patients less likely to benefit from the regimen may result, leading to underpowered analyses or sub-optimal patient care.

Purpose: We propose an adaptive randomized phase II study design incorporating prospective biomarker threshold identification (or non-identification), possible early futility stopping, potential mid-trial accrual restriction to marker-positive subjects, and final marker and treatment evaluation in the patient population identified as most likely to benefit.

Methods: An interim analysis is used to determine whether an initially unselected trial should stop early for futility, continue without a promising marker, or adapt accrual and resize (up to a pre-determined maximum) according to a promising biomarker. Final efficacy analyses are performed in the target population identified at the interim as most likely to benefit from the experimental regimen. Simulation studies demonstrate control of false-positive error rates, power, reduced average sample size, and other favorable aspects.

Results: The design performs well at identifying a truly predictive biomarker at interim analysis, and subsequently restricting accrual to patients most likely to benefit from the experimental treatment. Type I and type II error rates are adequately controlled by restricting the range of marker prevalence via the candidate thresholds, and by careful consideration of the timing of interim analysis.

Conclusions: In situations where identification and validation of a naturally continuous biomarker are desired within a randomized phase II trial, the design presented herein offers a potential solution.

Keywords: Adaptive design; randomized clinical trial; threshold identification; predictive biomarker; phase II trial; interim futility analysis

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Introduction

Development of targeted therapies is accelerating in response to widespread identification of hypothesized biomarkers. Of particular interest are candidate predictive markers believed to be related to the efficacy of an experimental treatment under study, where co-primary aims of a phase II trial may be determination of the marker's predictive value and identification of the marker-related subpopulation most likely to benefit. Before a new biomarker can be used to guide treatment decisions and patient care, however, a lengthy process from marker identification to validation must occur. For quantitative biomarkers (e.g., circulating levels of a target), a threshold to distinguish marker-low from marker-high patients may additionally be required. This process becomes inefficient when individual steps are accomplished in a post-hoc manner using data from multiple and potentially

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disparate sources, and results may be biased or confounded if marker identification and marker evaluation studies are performed separately or without a prospective framework.

Existing biomarker-based adaptive designs are either not truly adaptive (in the sense that adaptations are applied retrospectively), or rely on a dichotomous marker or previously defined marker threshold. Freidlin and Simon (2005) proposed a two-stage "adaptive signature design", where a set of genes sufficiently predictive of treatment efficacy among patients enrolled during the first stage of a phase III trial are subsequently used to classify the remaining patients as "sensitive" or "not sensitive" in the second stage (1). This design, which was subsequently expanded to incorporate cross-validation (2), does not restrict accrual based on interim results, and thus is not truly adaptive in the sense that adaptations are not applied during the course of the trial. Other proposed "retrospective-adaptive" designs (3-5) similarly do not affect treatment of patients on-study, though one such design by Jiang, Freidlin, and Simon (2009) does include retrospective identification of a continuous marker threshold (4). Of those existing truly adaptive designs (i.e., allowing for interim changes or restrictions to accrual to marker-defined subpopulations), most assume that dichotomizing thresholds for marker(s) of interest have already been established (6-11).

Here, we propose a novel phase II biomarker-based design that prospectively integrates four key desired features: (I) an interim analysis for continuous biomarker threshold selection (or non-selection); (II) possible futility stopping in either the overall or marker-defined populations; (III) potential restriction of accrual to the marker-based population of patients who, based on preliminary data, are most likely to benefit from the experimental treatment; and (IV) fully-powered final analyses in the population identified as benefitting at interim, where these analyses are based on an independent set of marker-positive patients in the event a promising marker exists. At the interim analysis, a pre-specified candidate biomarker is evaluated for its ability to predict the treatment effect, and if sufficiently promising, a threshold is chosen to distinguish marker-negative from marker-positive subjects. Depending both on the presence/absence of a predictive biomarker and marker subgroup-specific/overall performance, the trial may stop for futility, continue accrual to both marker groups, or restrict accrual to the marker-positive group. In the event a promising biomarker is identified at the interim analysis, the design includes subsequent final evaluation of the marker in an independent set of patients from the target subpopulation of interest.

Methods

Application context

Throughout, we describe our design in the context of our experience developing an actual randomized phase II trial to include biomarker identification and subsequent independent evaluation. This oncology trial—now ongoing—was originally planned as a simple randomized phase II design with retrospective evaluation of candidate biomarkers. In this framework, the design called for a maximum accrual of 160 patients randomized to an experimental arm versus placebo in a 2:1 ratio, which provided 80% power to detect a hazard ratio (HR) of 0.60 based on 107 progression-free survival (PFS) events with a one-sided type I error rate of α =0.05. During study development, investigators identified a candidate predictive biomarker; that is, a marker with the potential to identify a subgroup of patients who would achieve substantial benefit from therapy. A modified design was desired, to include prospective assessment of the continuous, serum-based baseline marker for prediction of treatment benefit, and further, to identify a threshold for classification of patients into positive (treatment responsive) versus negative (treatment resistant) marker status. Also desired were interim futility stopping rules in both the overall and biomarker-defined populations, and possible interim accrual restriction and final treatment evaluation in the preliminarily identified "treatment-responsive" or biomarker-positive population.

Below, we describe details of the final design solution in terms of the algorithm we used for its implementation. Here, primary interest lies in a time-related endpoint (PFS), where a single interim check incorporates a series of analyses for predictive marker evaluation, cut-point selection, futility, and possible restriction of accrual. A design overview and schema are presented in *Figure 1*. In specific application to this study, we performed interim and final analyses with the numerical settings and thresholds as described in the algorithm below, but note that particular study characteristics (e.g., primary endpoint, randomization ratio, and timing of interim analyses) may be easily generalized to extend the design to other settings. A discussion intended to guide selection of these trial-specific design quantities follows presentation of the algorithm, to facilitate the reader's implementation of the design in future contexts.

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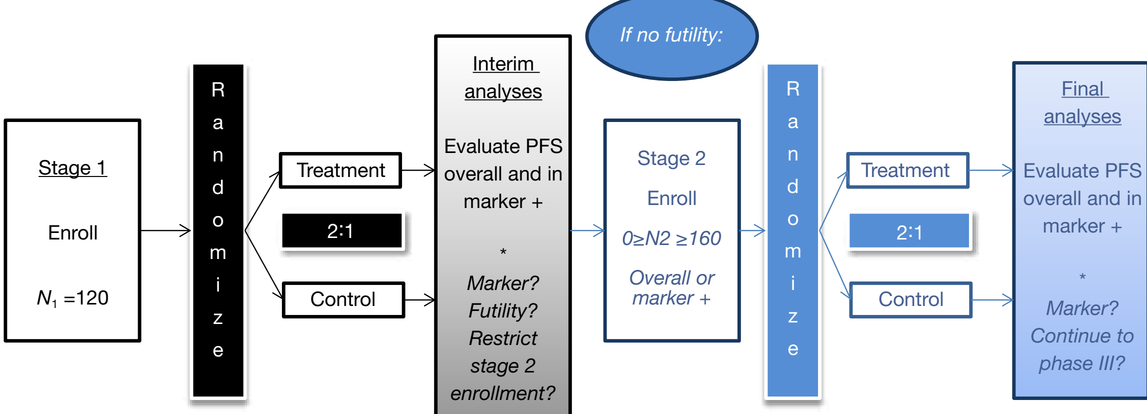

Figure 1 Adaptive design schema.

Study algorithm and analyses

We assume existence of a single continuous marker, possibly predictive of treatment effect, but with unknown distribution in the study population. Based on the sponsor's prior experience with the marker and preliminary data, possible dichotomizations of the marker are considered that result in marker(-positive) prevalence in the range of 25% to 75%. In the event the marker is unrelated to treatment effect in the interim analyses, the sponsor wishes to limit enrollment to the originally planned 160 patients. However, if the marker demonstrates sufficient association with the treatment effect in the interim analyses, the sponsor is willing to enroll up to an additional 160 patients to confirm efficacy in the tentatively identified benefit population (overall or marker-positive). We note that the timing of the interim analyses was chosen in simulations to provide the minimum acceptable power for the treatment-by-biomarker interaction and efficacy tests described in the algorithm below. Additional practical details are provided in the subsequent discussion.

Step 1: interim analyses for marker identification

After N_1 =120 stage I patients (80 on the treatment arm, 40 on placebo) are enrolled and followed for at least eight weeks, interim analyses will be performed. At that time, possible cut-points of the candidate biomarker are explored, restricted to those cut-points that result in a 25% to 75% marker prevalence. A series of Cox proportional hazards (PH) regression models are fit across a reasonably fine grid of possible cut-points for the biomarker. Each Cox model treats (possibly right-censored) PFS as the outcome, and treatment assignment, dichotomous biomarker status, and treatment-by-biomarker interaction effect as covariates. The cut-point associated with the strongest interaction effect (potentially after smoothing of these effects over neighboring cut-points) is used in subsequent interim analyses, and potentially in the test for subpopulation benefit in final analyses, assuming the interaction effect is associated with significance $p \leq P_{int}$. Thus, at the conclusion of the stage I enrollment and interim analysis, we establish two scenarios:

Scenario 1: promising biomarker. A promising biomarker is considered to have been identified when, according to the best-identified cutpoint, the interaction P-value is less than or equal to P_{int} and the treatment demonstrates greater benefit in the biomarker-high group relative to the marker-low group.

Scenario 2: no promising biomarker. No promising biomarker is considered to have been identified when, according to the best-identified cutpoint, the interaction P-value is greater than P_{int} or the treatment demonstrates greater benefit in the biomarker-low group relative to the marker-high group.

In practice, P_{int} is chosen via simulation to optimize the design's operating characteristics (e.g., desired power or type I error), given practical constraints such as stage I sample size, distribution of the primary study endpoint, anticipated or targeted clinical benefit, and level of censoring for a time-to-event endpoint. According to Scenarios 1 and 2 defined above, the following additional analyses will be performed.

Scenario 1: test for the treatment effect in subgroups. If the biomarker is promising for prediction of treatment

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effect (Scenario 1), then log-rank tests for the superiority of treatment versus placebo are performed within each marker subgroup defined by the newly-selected cut-point. Cox PH models are also used to compute the HR in the marker-high and marker-low patients, HR_L and HR_H respectively, for treatment versus placebo.

Scenario 2: test for overall treatment effect. If no promising biomarker exists at stage I (Scenario 2), a log-rank test for the superiority of the treatment arm versus placebo is performed using data from all (biomarker low and high) stage I patients. A Cox PH model is used to compute the interim HR of treatment versus placebo in terms of overall PFS.

Step 2: stage I futility stopping rules

Immediately following the interim analyses for stage I patients, futility stopping may be invoked according to Scenarios 1 and 2 defined in Step 1.

Scenario 1: promising biomarker. If the biomarker is promising for prediction of differential treatment effect, futility is separately evaluated within marker-low and marker-high subgroups as follows: if the one-sided P-values from both subgroups' log-rank tests for superiority are greater than P_{fut} (approximately corresponding to a HR greater than HR_{fut}), the trial terminates for futility. If the one-sided P-value is greater than P_{fut} for marker low but not marker high patients, accrual to stage II will continue only in marker high patients, as described in Step 3.

Scenario 2: no promising biomarker. If the biomarker is not promising for prediction of differential treatment effect, futility is evaluated as follows: if the P-value associated with the overall log-rank test for superiority is greater than P_{fut} (approximately corresponding to a HR greater than HR_{fut}), the trial terminates for futility. Similar to P_{int}, the futility stopping boundary P_{fut} is chosen via simulation to optimize the operating characteristics of the design for a given application.

Step 3: stage II accrual restrictions and trial resizing

If the study is not stopped for futility based on the interim analyses and decision rules described in Step 2, an additional N_2 patients are accrued in stage II, taking into account sponsor-defined enrollment caps on total enrollment and marker-low enrollment, given by N_{cap} and N_{cap}^L, respectively. In practice, N_{cap} and N_{cap}^L are chosen jointly by the sponsor and statistical team, such that the design operating characteristics (i.e., power and type I error) may be optimized for the specific study objectives and resource constraints. For our trial, we set N_{cap} =280 and N_{cap}^L =90 and proceed with accrual and corresponding primary endpoint analyses according to Scenarios 1 and 2, where special subcases of Scenario 1 (A and B) are defined below.

Scenario 1A: promising biomarker, restricted accrual. If the biomarker is identified as promising at interim Step 1 (Scenario 1), but subgroup analysis of marker-low patients (Step 2) shows that treatment provides no meaningful benefit relative to placebo in terms of PFS by the futility threshold P_{fut}, accrual to stage II proceeds only in the marker-high group. Henceforth, we refer to this scenario as Scenario 1A or "restricted accrual." In this case, stage II sample size is N_2 =160 *marker-high* patients, to achieve 80% power to detect HR_{H} =0.60 for treatment versus placebo with 1-sided α =0.05 based on 107 PFS events. Under this scenario, the total trial size is $N = N_1+N_2$ =280 combined (120 stage I +160 stage II) patients, such that $N = N_{cap}$.

Scenario 1B: promising biomarker, unrestricted accrual. If the biomarker is identified as promising at interim Step 1 (Scenario 1), and corresponding subgroup analysis of marker-low patients (Step 2) shows that treatment may still hold promise versus placebo in terms of PFS by the futility threshold P_{fut}, accrual to stage II continues to both biomarker groups, but in accordance with N_{cap}^L =90 total marker-low patients in the trial. If N_{cap}^L has already been reached at the time of interim analysis, stage II accrual continues only to the marker-high group. Regardless of whether N_{cap}^L is already reached at interim, we refer to this scenario as Scenario 1B or "unrestricted accrual." In this case, sample size for stage II is based on achieving 160 total (stage I and stage II) *marker-high* patients, to provide 80% power to detect HR_H =0.60 for treatment versus placebo with 1-sided α =0.05 after 107 PFS events have occurred in the marker-high group. Under this scenario, the total trial size $N = N_1+N_2$ falls between 214 and 250 combined (120 stage I +94 to 130 stage II) patients, depending on marker prevalence falling between 25% and 75%, such that $N < N_{cap}$.

Scenario 2: no promising biomarker. If in the Step 1 analysis the biomarker is not promising for prediction of treatment effect, the trial is not resized, and accrual to stage II continues to all patients regardless of biomarker status. In this case, the final analysis of treatment versus placebo ignores the biomarker and follows the original design; i.e., an additional N_2 =40 patients are enrolled regardless of biomarker status in stage II, to yield a total trial size of $N = N_1+N_2$ =160 patients to detect HR =0.60 for PFS with at least 80% power and 5% one-sided type I error after 107 events

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have occurred. After the trial's conclusion, retrospective exploratory analyses of the biomarker (or other potential biomarkers) may be performed.

Step 4: final efficacy testing in the biomarker-based benefit population

Final tests for efficacy are performed in either the marker-high (Scenario 1A or 1B) or overall (Scenario 2) benefit population as follows.

Scenario 1A: promising biomarker, restricted accrual. If a promising marker is identified at the interim analysis and stage II accrual is restricted to the marker-high group (Scenario 1A), the primary difference in PFS between treatment and placebo is tested using a log-rank test with *stage II marker-high patients only*. This is to preserve independence of the (marker unrestricted) stage I patients that were used to identify the marker effect from the (marker-restricted) stage II patients to be used to confirm efficacy in patients defined by the marker. This case, interim testing of stage I patients results in a permanent change to the trial's population of interest for testing efficacy where marker-low patients are no longer considered for enrollment. To address this lack of exchangeability of stage I and II patients, only stage II marker-high patients are used in the final log-rank test for efficacy, while stage I patients are not used in the primary efficacy analysis. The decision rule considers the treatment promising in the marker-high subpopulation if the P-value associated with a one-sided log-rank test is $p \le P_{eff}$ in favor of treatment.

Scenario 1B: promising biomarker, unrestricted accrual. If a promising marker is identified at the interim analysis but stage II accrual is unrestricted (Scenario 1B), a treatment versus placebo difference in PFS within the marker-high subgroup is tested using stage I and II patients, as stage I and stage II patients were enrolled from the same (marker-unrestricted) population. While it is true that the primary treatment effect will be tested in the marker-high population at the trial's conclusion, reuse of stage I patients in the final analyses is justified as stage I and stage II patients are exchangeable; specifically, interim testing of stage I patients has not changed the population of patients (both marker-high and marker-low) enrolled to the trial. In this case, the treatment is considered promising in the marker-high subpopulation if the P-value associated with a one-sided log-rank test is $p \le P_{eff}$ in favor of treatment. As an independent test of the biomarker under scenario 1B, a log-rank test using only stage II marker-high patients may be performed.

Scenario 2: no promising biomarker. If the biomarker was not promising at the interim analysis and accrual was limited to the 160 originally-planned patients, then treatment will be considered promising overall if the p-value associated with a one-sided log-rank test is $p \le P_{eff}$ in favor of treatment. As exploratory analyses, additional biomarker explorations and subgroup analyses may be performed.

Design evaluation approach

A simulation study was performed to investigate the operating characteristics of the design. Throughout, settings and assumptions were chosen to reflect the particular oncology study for which the design was created.

All simulation scenarios were performed with 10,000 iterations (hypothetical trials), with interim analyses performed after eight weeks follow-up on the 120th patient enrolled. Trials for which futility was not reached at the interim analysis were allowed to enroll up to a financially dictated, sponsor-defined cap N_{cap} =280 total patients, with a marker-low enrollment cap of N_{cap}^{L} =90 patients ensuring an adequate number of marker-high patients are enrolled to power the stage II analyses. Throughout, we assume uniform accrual at the rate of four patients per week, exponentially-distributed PFS, and a median PFS of eight weeks for the control arm, regardless of biomarker status. Patients were randomized in a 2:1 ratio to experimental versus control treatments, respectively.

Throughout, we fix P_{int} =0.50, P_{fut} =0.60, and P_{eff} =0.10 to maximize overall power, given the possibilities of low marker prevalence and imperfect interim marker identification. Other values of these thresholds were considered via simulation (results not shown), and for a given new application of this design, possible adjustments should be studied accordingly. We constructed simulation scenarios using three different values of biomarker prevalence, representing relatively extreme levels of prevalence (25% and 75%) as well as moderate prevalence (50%). Within each of these levels, we vary both the HR in the marker-low subgroup (HR_L) and the HR in the marker-high subgroup (HR_H), considering a sequence of cases where $HR_L \ge HR_H$.

Results

Simulation-based operating characteristics for the proposed design are presented by marker prevalence and hypothesized marker subgroup-specific HRs in *Table 1*. In the null case

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Table 1 In each section of the table, mutually exclusive special cases such as "Marker" and "No marker" are indented and presented with plain text, while primary parent outcomes of interest are presented in bold text. Rates in parentheses are conditional on biomarker detection at interim analysis

Average/percent	HR_L =1.2 HR_H =1.2	HR_L =1.2 HR_H =1.0	HR_L =1.0 HR_H =1.0	HR_L =1.0 HR_H =0.8	HR_L =1.0 HR_H =0.6
25% marker prevalence					
Trial size	166	187	176	198	224
Interim marker	26.3%	39.2%	25.8%	42.4%	63.5%
Restricted accrual	21.4%	31.5%	15.8%	24.3%	32.2%
Interim futility	55.7%	41.2%	29.6%	18.2%	7.7%
No marker	53.6%	39.4%	29.3%	17.9%	7.6%
Marker	2.1%	1.8%	0.3%	0.3%	0.1%
Final efficacy	1.7% (2.3%)	6.4% (12.0%)	12.1% (16.3%)	30.9% (51.7%)	67.0% (91.8%)
No marker	1.1%	1.7%	7.9%	9.0%	8.7%
Marker	0.6%	4.7%	4.2%	21.9%	58.3%
Final marker	0.5% (1.9%)	3.8% (9.7%)	2.6% (10.1%)	17.2% (40.6%)	54.0% (85.1%)
50% marker prevalence					
Trial size	159	186	175	201	232
Interim marker	25.2%	40.1%	26.2%	43.5%	69.1%
Restricted accrual	17.8%	31.5%	18.4%	27.9%	35.4%
Interim futility	59.7%	37.9%	31.0%	13.8%	2.5%
No marker	53.8%	33.6%	29.0%	12.8%	2.3%
Marker	5.9%	4.3%	2.0%	1.0%	0.2%
Final efficacy	2.1% (2.8%)	7.6% (12.0%)	12.0% (15.6%)	36.1% (52.9%)	78.9% (93.3%)
No marker	1.4%	2.8%	7.9%	13.1%	14.4%
Marker	0.7%	4.8%	4.1%	23.0%	64.5%
Final marker	0.5% (2.0%)	3.6% (9.0%)	2.4% (9.2%)	16.4% (37.7%)	55.0% (80.0%)
75% marker prevalence					
Trial size	151	181	171	200	228
Interim marker	25.5%	38.4%	25.1%	40.7%	63.4%
Restricted accrual	13.8%	28.0%	18.4%	29.4%	37.7%
Interim futility	65.3%	37.5%	34.0%	10.7%	1.0%
No marker	54.2%	29.8%	30.2%	9.2%	0.9%
Marker	11.1%	7.7%	3.8%	1.5%	0.1%
Final efficacy	1.8% (2.0%)	8.1% (10.7%)	11.2% (13.9%)	39.1% (50.4%)	85.2% (92.6%)
No marker	1.3%	4.0%	7.7%	18.6%	26.5%
Marker	0.5%	4.1%	3.5%	20.5%	58.7%
Final marker	0.3% (1.2%)	3.0% (7.8%)	2.2% (8.8%)	14.8% (36.4%)	46.7% (73.7%)

(HR_L = HR_H =1), the type I error rate (concluding the experimental treatment is efficacious when it is not, with or without a biomarker) is controlled at 12.1% or less for our specific choice of design parameters. This rate was deemed acceptable by the sponsor of our motivating study. In practice, design thresholds should be modified to achieve the specific desired type I target. Among the same scenarios, futility stopping after the initial 120 patients

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occurred in 29.6% of cases for low marker prevalence, and at higher rates for higher prevalence. When the control arm was slightly inferior, false positive results were obtained in a maximum of 8.1% of cases across prevalence levels with HR_L =1.2 and HR_H =1, and a maximum of 2.1% of cases with HR_L = HR_H =1.2. Under these same scenarios and regardless of prevalence, futility stopping rates were at least 37.5% and 55.7%, respectively, and reached as high as 65.3%.

Among scenarios with no treatment effect for marker-low patients but a beneficial effect for marker-high patients (HR_L =1 and HR_H <1), overall power increases with marker prevalence, as expected. For example, with HR_L =1 and HR_H =0.60 (the latter being the targeted treatment effect), overall power (defined as a significant result overall or within the marker-high subgroup) is 67.0% for 25% marker prevalence, 78.9% for 50% prevalence, and 85.2% for 75% marker prevalence. We note that power is highly dependent upon successful identification of the true predictive marker at the time of interim analysis. Specifically for the case of HR_L =1 and HR_H =0.60, the probability of reaching a positive trial result given successful interim marker identification ("conditional" power) is 91.8% to 93.3% across possible values of marker prevalence. This critical identification of a truly predictive marker depends not only on marker prevalence and magnitude of the effects, where HR =0.60 was the largest effect reasonably expected by the sponsor, but also on timing of the interim analysis. Where the total sample size is large enough to justify a later interim analysis, or where larger treatment effects than HR =0.60 might reasonably be expected (simulations not shown), the power to detect a truly predictive marker at the time of interim analysis will be increased.

Among the scenarios considered along the continuum from no biomarker (HR_L = HR_H =1) to best-case biomarker (HR_L =1 and HR_H = 0.60), the chance of identifying a biomarker at the interim analysis ranged from 26.2% to 69.1% with 50% marker prevalence. Though greater differentiation of the no marker and promising marker cases would certainly be desired, in simulations, these probabilities of false positive and successful marker detection could not be further separated without modifying one of two design factors: an increase in the targeted differential treatment effect between marker-based subgroups (which was not deemed plausible in our case), or an increase in the maximum trial size (also constrained), which in turn allows a later interim analysis based on a greater number of events. Under our best-case marker scenario (HR_L =1 and HR_H =0.60), given marker identification at interim, the conditional rate of marker validation ranged from 73.7% to 85.1% across levels of marker prevalence. These rates were deemed acceptable by the sponsor; see Section 4 for additional discussion of the power of treatment-by-marker interaction tests.

Discussion

Practical considerations

We note that the final version of our design presented in Section 2 is the result of a number of iterative decisions, beginning with initial modifications made to an original, biomarker-free design with 160 patients. In the original design, an interim analysis was planned after the first 80 patients. In simulation studies, however, this stage I sample size was identified as too small to detect a meaningful predictive biomarker using treatment-by-marker interaction tests; thus, the decision was made to postpone the interim analysis until after 120 patients were enrolled and followed. With this timing, false-positive marker identification was controlled to approximately 25% across prevalence levels for the null case, and an interim marker identification rate of 69% was observed under the best-case marker scenario considered for this specific application (HR_L =1 and HR_H =0.60). These rates were deemed acceptable by the sponsor, given the maximum allowed sample size and interim timing constraints. In particular, the sponsor was motivated by the fact that liberal identification of a marker at interim analysis would be balanced by stringent confirmation of the marker's predictive value at the time of final analysis.

It should be noted that prevalences (cutpoints) outside of the range of 25% to 75%, while plausible in some settings, might result in dramatically reduced power at the time of marker detection at the interim analysis. In this case, there may be less enthusiasm for use of this design. Indeed, the post-interim performance of this design is conditional upon successful identification of truly predictive biomarkers, and to a lesser degree, successfully concluding at the interim analysis when a biomarker indeed does not exist. While performing an earlier interim analysis for futility might yield (on average) a smaller trial, an earlier interim analysis would also yield less power to detect a truly predictive biomarker or early overall efficacy. In application of this design, timing of interim analyses for efficacy and futility should be studied via simulation against the corresponding trade-offs.

A 2:1 randomization ratio was utilized in our motivating study, specifically to motivate accrual given the required possibility of randomization to a placebo control arm. Data and Safety Monitoring Board (DSMB) surveillance was in

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place to ensure patient safety as greater than half of enrolled patients received an active experimental agent with possible associated toxicities. In other applications of this design, straightforward 1:1 randomization may be sufficient. In this case, all other things being unchanged, a smaller sample size will be required for 1:1 randomization, or higher power will be achievable with the same sample size. A smaller sample size or greater power to detect treatment and interaction effects may also result in settings where treatment effects larger than HR =0.60 are considered possible to observe. In our study, larger effects were deemed highly unlikely by the sponsor and investigators and were thus not studied in simulations. Given the early stage of drug development this trial was intended to address and the relatively small sample size, early stopping rules for efficacy were not considered.

Our design also assumes a continuous marker such that the experimental treatment is hypothesized to work better for marker-high patients than marker-low patients. That is, a larger treatment effect among marker-low patients than marker-high patients would not be of interest in our setting, but may be possible in others. From our point of view, if a marker is so new (having not been previously studied, at least retrospectively, in earlier trials) or lacking in scientific rationale such that the sponsor does not have a clear idea of the anticipated (marker high versus marker low) direction of benefit, we would caution against use of a marker-based design altogether—especially adaptive designs such as ours where the trial conduct may change based on preliminary results. For comparison against a general one-stage randomized design with no biomarker, the results presented in Section 3 may be compared against the operating characteristics of the original, biomarker-free design described in the first paragraph of Section 2.1.

Implementation

To facilitate adaptation of our proposed design to a new trial setting where a candidate predictive biomarker exists, we suggest the following algorithm. First, the sponsor and study statistician(s) should jointly determine a value of N_{cap} beyond which the objectives of the trial would be too costly to pursue, where the statistician's role is to convey feasibility of the design under a range of enrollment limits. Given a plausible N_{cap}, a study-relevant randomization ratio (e.g., 1:1 versus 2:1), a reasonable range of differential treatment effects (e.g., HRs) within each marker-defined subgroup, and desired levels of power and type I error, the statistician then determines via simulation the optimal stage I sample size N_1, such that the interim analysis occurs after an adequate subset of those patients experience the primary event of interest (e.g., PFS). We suggest tentatively setting $N_1 = N^*/2$, where N^* is the total sample size required to power a biomarker-free design with the same operating characteristics, and then increasing N_1 to find the value associated with the optimal interim biomarker detection rates allowed by both N_{cap} under the best-case marker scenario ($HR_H < HR_L$ =1) and reasonable values of marker prevalence. The stage II sample size N_2 is then chosen by adequately powering the final analysis in the marker-positive benefit population under Scenario 1A, subject to constraints given by N_{cap}, the best-case HR_H, and the desired type I error α. As in our motivating study, it may be necessary to impose a limit N^L_{cap} on the total number of marker-low patients enrolled under Scenario 1B. In practice, this value should be chosen such that a sufficient amount of stage II accrual is reserved for the marker-high patients required to power the final analysis. While our application intentionally uses a smaller N_2 under Scenario 1B (unrestricted accrual) than under Scenario 1A (restricted accrual), such that N is guaranteed to be less than N_{cap} for the former scenario, it may be important in certain applications to continue observing the effect of experimental treatment on as many marker-low patients as allowed by N_{cap}; e.g., when these patients show a non-negligible response to treatment (Scenario 1B). Jointly in simulation studies, the statistician should consider the operating characteristics as a function of marker prevalence, such that minimum and maximum values of the threshold distinguishing marker-positive from marker-negative patients may be prospectively defined. The R program used to design our motivating study and conduct simulations is available from the first author upon request.

Limitations

Some limitations not already mentioned and inherent to our proposed design, and biomarker designs in general, should be noted. First, we perform dichotomization of a continuous biomarker, to achieve the simplicity and interpretability similar to the dichotomous marker assumptions present in most existing biomarker design literature. In some cases, maintaining a marker as continuous in the interaction modeling process may be more appropriate. On a related note, we acknowledge an often-cited limitation of interaction testing, namely that the power to detect a treatment-by-marker interaction may be low relative to the power to detect a treatment effect using the same sample size. Nonetheless,

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rather than perform an overall test for treatment benefit followed by a test within a marker-defined subgroup in the event the overall test is negative, as is common in the marker design literature, we utilized a formal treatment-by-marker interaction test within a Cox model at the interim analysis to check for early evidence of a marker-treatment-outcome relationship. We maintained this test specifically to address the question of whether the marker is truly predictive in nature, as opposed to merely prognostic, which may actually be the case if a treatment effect is weak in the overall population but strong in a marker positive subgroup. Another limitation generally common to multi-stage classical designs is that variability inherent to the interim and final analyses are not comprehensively addressed in a formal manner, e.g., via Bayesian posterior distributions with accompanying decision rules, though we note that this will be an area of future exploration. Lastly, we acknowledge that in practice, limited sponsor resources may restrict the total sample size to an extent that may impact the design's performance or make its use impractical relative to other existing designs. Despite these limitations, which elicit important topics for further research, we expect the prospective and adaptive biomarker-based design presented here to prove both efficient and practical for phase II screening of targeted therapies and companion biomarker diagnostics for subsequent study in phase III trials.

Conclusions

The prospective biomarker-based design to evaluate a time-related endpoint (e.g., PFS) presented herein provides a potentially powerful and useful tool in the situation where limited information exists regarding the predictive ability of an exploratory, continuous biomarker. This design could easily be modified to address alternative endpoints, such as tumor response within a pre-specified time period. Making timely use of available patient data, the design yields a candidate marker threshold, identifies the population (overall or marker-based) most likely to benefit from the experimental treatment, and subsequently optimizes enrollment for members of this population. Prospectively including predictive biomarker identification within a phase II study of a novel experimental treatment will not only significantly shorten development timelines by removing the second phase II study required for biomarker validation, but also reduce the population required for a selected phase III trial, compared with a trial in which an unselected population is studied. Through integration of a panel of important phase II objectives—biomarker threshold selection (or non-selection), determination of overall or subgroup-specific futility, possible accrual restriction to the hypothesized treatment benefit population, and timely independent marker evaluation in patients from the same trial—this design allows flexible specification of parameters to suit sponsor interests and objectives, while also encouraging efficient and ethical use of patient resources.

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Drug designs fulfilling the requirements of clinical trials aiming at personalizing medicine

Sumithra J. Mandrekar, Daniel J. Sargent

Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN 55905, USA

Correspondence to: Sumithra J. Mandrekar, Ph.D. Professor of Biostatistics, Division of Biomedical Statistics and Informatics, Mayo Clinic, 200 First St SW, Harwick 8, Rochester, MN 55905, USA. Email: Mandrekar.sumithra@mayo.edu.

Abstract: In the current era of stratified medicine and biomarker-driven therapies, the focus has shifted from predictions based on the traditional anatomic staging systems to guide the choice of treatment for an individual patient to an integrated approach using the genetic makeup of the tumor and the genotype of the patient. The clinical trial designs utilized in the developmental pathway for biomarkers and biomarker-directed therapies from discovery to clinical practice are rapidly evolving. While several issues need careful consideration, two critical issues that surround the validation of biomarkers are the choice of the clinical trial design (which is based on the strength of the preliminary evidence and marker prevalence), and biomarker assay related issues surrounding the marker assessment methods such as the reliability and reproducibility of the assay. In this review, we focus on trial designs aiming at personalized medicine in the context of early phase trials for initial marker validation, as well as in the context of larger definitive trials. Designs for biomarker validation are broadly classified as retrospective (i.e., using data from previously well-conducted randomized controlled trials (RCTs) versus prospective (enrichment, all-comers, hybrid or adaptive). We believe that the systematic evaluation and implementation of these design strategies are essential to accelerate the clinical validation of biomarker guided therapy.

Keywords: All-comers design; adaptive design; biomarker; enrichment design; hybrid design; randomized controlled trial (RCT)

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Introduction

The development of new therapeutics in oncology typically follows the phase I, phase II, and phase III drug development paradigm. In phase I, the primary goal is to understand the safety profile of a new treatment in a small group of patients (typically including multiple tumor types) for further investigation. In the phase II setting, the primary goal is to better understand the efficacy profile in order to make a determination of whether the treatment is worthy of further investigation with a secondary goal of gaining a better understanding of the treatment's safety. Traditionally, this has been accomplished through a single arm or a randomized trial in a homogenous group of patients, with the trial size varying from 30 to 100 patients. If the agent is considered safe with a promising efficacy signal, it is then taken forward into a phase III trial, where the primary goal is to compare the new treatment or regimen with the standard of care to demonstrate a clinical benefit, and/or in some cases, cost-effectiveness. Phase III trials are usually large (few hundreds to few thousands of patients), and are done in a homogenous group of patients in a multi-institution setting.

This traditional drug development paradigm is challenged by the fact that cancer is increasingly becoming a "rare" disease with the use of targeted therapeutics and biomarker assessment for medical treatment. Medical treatment for oncology patients is driven by a combination of the expected outcome for the patient (prognosis), and the ability for treatment to improve the expected outcome (prediction). The standard paradigm of drug development is

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thus called into question in the setting of biomarker based trials. In the context of personalized medicine utilizing biomarkers and targeted therapeutics, a "phase I" study tests the methods of assessment of marker alteration in normal and tumor tissue samples and guides in the determination of cut points, if applicable, for quantitative and meaningful interpretation of results. The feasibility of obtaining the specimens as well as the reliability and reproducibility of the assay is often established at this stage. A "phase II" study is typically a careful retrospective assessment of the marker to establish clinical value, and phase III trials are prospective confirmatory trials that attempt to validate the marker through large randomized controlled trials (RCTs) in a multi-center setting (1).

Biomarkers can be classified into three categories: prognostic biomarkers, predictive biomarkers, and surrogate endpoints, with the recognition that some biomarkers may fall into more than one category (2,3). Prognostic and predictive biomarkers focus on individual patient risk-classification and treatment selection respectively, whereas biomarkers used as surrogate endpoints aid in the evaluation of the efficacy of a new treatment. The ultimate intended usage of a biomarker usually determines its definition and the required validation methods. A prognostic biomarker predicts the natural history of the disease process in a given individual, and thus aids in the decision of whether a patient needs an intensive and possibly toxic treatment as opposed to no treatment or standard therapy. Prognostic marker validation can be established using the marker and outcome data from a cohort of uniformly treated patients with adequate follow-up since a prognostic marker is associated with the disease or the patient, and not a specific therapy. The validation data source may be from a clinical trial (due to availability of follow-up information) with patients treated on the standard of care arm (or placebo if that is the standard of care), but a clinical trial is not necessarily required.

A predictive biomarker predicts whether an individual patient will respond to a particular therapy or not, and hence its clinical utility is in allowing for individualized therapy. Designs for predictive marker validation are more complex and require at a fundamental level data from a randomized study (4). A surrogate endpoint biomarker replaces the ultimate clinical outcome (i.e., usually overall survival) and informs the efficacy of a new treatment with greater cost-effectiveness than the primary clinical outcome at the population level (5,6). We focus our attention on predictive markers in this review article. We start with a discussion of phase I dose-finding trials, followed by designs for initial marker validation in a phase II setting. Trial designs for definitive marker validation have been extensively discussed in the literature (3,4,7-12); here we provide a summary of the key features and requirements of phase III designs for definitive predictive marker validation. Finally, we will provide examples of real clinical trials that have utilized some of the designs discussed in this article, and conclude with our thoughts on the future perspectives of drug designs aimed at personalizing medicine.

Early phase dose finding trials

While assessing the safety profile and establishing the maximum tolerated dose (MTD) remains the primary focus of phase I trials for all agents, establishing a preliminary efficacy signal and/or identification of subsets of patients most likely to benefit from the new treatment are increasingly assessed as part of phase I trials of targeted therapeutics. The choice of endpoints, patient selection, model-based versus rule-based design algorithms and inclusion of expansion cohorts need carefully consideration in this setting. The historical paradigm of the higher the dose, the greater the chance of efficacy is called into question when evaluating molecularly-targeted therapies, vaccines and immunotherapy agents. While a monotonically non-decreasing dose-toxicity curve definitely continues to be appropriate from a biological standpoint for molecularly-targeted therapies, the dose-efficacy curves for these novel therapies may follow a non-monotone pattern such as a quadratic curve or an increasing curve with a plateau, and often times not well understood at the beginning of the trial (13). Dose finding studies for such agents should therefore incorporate a measure of efficacy in addition to assessment of toxicity with the aim to identify the biologically optimal dose (BOD), or the minimum effective dose (MED) instead of just the MTD. While the incorporation of an early efficacy measure seems straightforward, it poses many inherent challenges. Firstly, should the efficacy measure be based on a biomarker response, or a clinical response? If it is a clinical response, can it be assessed easily and quickly, and does early change to the clinical outcome provide evidence of a sustained clinical benefit? Secondly, if a biomarker response is sought, is there sufficient evidence demonstrating that the impact on the biomarker would translate to a meaningful clinical response? What are the characteristics of the assay(s) to assess the biomarker in terms of reproducibility, validity etc.? Thirdly, can the biomarker endpoint be assessed in real

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time to inform the dose escalation decision process?

Assuming that an efficacy endpoint that is a meaningful "surrogate" for the clinical outcome is available, and one that can be measured reliably and in real time, the question of how best to incorporate this endpoint in the dose escalation/recommended phase II dose determination process needs considerable thought. Phase I trial designs can be broadly categorized into model-based versus rule-based (or algorithm-based) designs (13). In the rule-based designs, small numbers of patients are treated starting at the lowest dose level and the decision to escalate, de-escalate or treat additional patients at the same dose level is based on a pre-specified algorithm related to the occurrence of unacceptable dose-limiting toxicity (DLT). The classical cohorts of 3 design and its many variants (accelerated titration design, two-stage design) are rule-based designs that have been and continue to be widely utilized in oncology (14).

Several ad-hoc rule-based designs have been used in practice in oncology for vaccine based and immunotherapy trials such as: (I) randomize a pre-specified number of patients to all dose levels under investigation (this assumes that there are no safety concerns); or (II) use a rule-based algorithm based on toxicity and efficacy to guide dose escalation (de-escalation), for example, if the observed unacceptable toxicity rate is <33%, and the observed efficacy is below (or above threshold) escalate (or de-escalate/stay at the same level) etc. While these are attractive from an implementation/clinical appeal standpoint, the statistical properties (precision, and sample size) of these designs are often not explored and in many cases can be sub-optimal. On the other hand, model-based designs utilize a statistical model for the dose-toxicity (and dose-efficacy in some cases) relationship to guide the dose-finding process (15-25). Designs that utilize both a safety and efficacy outcome during dose escalation or dose escalation based on safety and inclusion of expansion cohorts to understand efficacy are clearly essential in the context of targeted therapeutics. Model-based designs offer the flexibility to model different possible dose-efficacy curves that can realistically represent the true but unknown underlying dose-efficacy profile of a targeted agent, and allow for flexibility in trial conduct, with improved precision in estimating the dose to take forward for further testing.

Expansion cohorts and patient selection are increasingly being utilized as part of the dose-finding process (either during the dose escalation phase or as part of a dose expansion phase) to try and identify subsets of patient who might benefit most from the treatment (26). Lack of validated assays and/or markers of efficacy, inability to have a real-time assessment of the biomarker, and incomplete understanding of the metabolism of the drug or its pathway often make an enrichment strategy at this stage of agent development non-viable. Notable exceptions include the development of vemurafenib for patients with BRAF mutant melanoma, and crizotinib for ALK positive non-small cell lung cancer (NSCLC) patients, both of which adopted the enrichment strategy from the get go (27,28).

Phase II trials for initial marker validation

Phase II trials have at least three main purposes: assess the regimen/drug under investigation for evidence of efficacy; to evaluate the patient population for a phase III study and to more fully assess the toxicity profile in a larger number of patients than that of a phase I study. A single arm two-stage design could be used to determine if a drug is likely to have a certain level of activity in unselected patients, and if activity is below the level of interest, whether a particular patient selection method can enrich the responding population to meet the targeted level of activity in the selected group (29). Such single-arm designs however conduct comparisons against historical controls, which may be inaccurate given changes in patient population based on biologic sub-setting and/or evolution in imaging technologies (30). McShane *et al.* (31) show through simulations how misleading the results of a single-arm phase II trial in a selected patient population can be when the benchmark estimate is from prior trials of "unselected" patients and thus inappropriate for the enriched study population. In contrast, a RCT includes a control arm for comparison, thereby assuring that patients who are treated with the agent for whom the marker is purported to be predictive are comparable to those who are not. RCTs are essential for making the distinction between a prognostic and predictive marker, as well as to isolate any causal effect of the marker on therapeutic efficacy from the multitude of other factors that may influence the decision to treat or not to treat a patient (4). In the setting of phase II trials, RCTs also provide the opportunity to simultaneously assess multiple promising therapies (and multiple possible markers) for a given disease.

Several prospective designs for biomarker-directed therapy have been proposed, differing primarily in the study population, randomization scheme, or both (4). Most of these designs were intended primarily in a phase III

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setting, but have evolved since then to be applicable in an initial marker validation setting, i.e., a phase II setting (32). The design choice is driven by scientific rationale, marker prevalence, strength of preliminary evidence, assay performance, and turn-around times for marker assessment. An all-comers design with retrospective marker evaluation is a reasonable approach when multiple biomarkers are to be assessed and there is insufficient knowledge to use a specific marker to drive design properties. However this is subject to insufficient power determined by the marker prevalence, for example in a study of 100 patients (50/arm), a marker with 10% prevalence would only have approximately five patients per arm on which to base the subgroup evaluation. A slight variation to the all-comers designs is the multiple hypotheses design that specifies prospectively a test for a treatment effect in the overall population and within pre-specified marker subgroups. A biomarker-stratified design specifies accrual target within biomarker-defined subgroups. The fundamental difference between this design and the traditional RCT is that only patients with a valid marker result are eligible and randomized. A separate evaluation of the treatment effect can be tested in the different marker-defined subgroups, or a preliminary test of marker by treatment interaction can be carried out first. Different sequential analysis plans can also be implemented (4,33). Enrichment or targeted designs enroll only patients with a particular marker profile, compared to hybrid designs where only a certain subgroup of patients based on their marker status are randomized between treatments, however patients in the other marker-defined subgroups are assigned the standard of care treatment(s). Finally, recent emphasis has been on outcome adaptive randomization designs that evaluate the success of the drug-biomarker subgroup in an ongoing manner. These designs allow either for the randomization ratio to be altered in order to place more patients on the most promising arm(s), and/or allow for the elimination of the under-performing drugs and/or the biomarker subgroups midway through the trial (34-36). A much simpler alternative to this completely adaptive approach is the direct assignment option design, which allows the option to stop randomization and assign all patients to the experimental arm based on interim analysis results (37). The key considerations for the choice of the phase II design are outlined in *Table 1* [adapted from Mandrekar *et al*. (32)].

Definitive phase III trials for marker validation

Frequently, a complete understanding of the drug metabolism pathway or the underlying biology prior to the testing of a therapy (and even approval of the therapy in some cases) is not possible. Similarly, an established cut point to classify patients into different marker subsets is also often not available prior to the start of a phase III trial. Retrospective validation can aid in such situations by bringing forward effective treatments to marker-defined patient subgroups (4). This is likely the only possible solution in cases of where there are approved therapies for an indication, since once a therapy is approved for common use, designs that randomize patients to not use that therapy become exceedingly difficult. The important components of a retrospective validation include availability of clinical and biomarker data from a well-conducted RCT, well established assay characteristics (analytical and clinical validity), availability of samples from a large majority of patients from the RCT to avoid selection bias, and finally and most importantly, well defined prospectively stated hypothesis, sample size and power calculations, analytical techniques, and patient sub-populations to ensure statistical rigor and clinical confidence in the findings. An example of a successful retrospective predictive biomarker validation is the establishment of mutant KRAS status as a predictor of lack of efficacy from panitumumab and cetuximab therapy in advanced colorectal cancer (38-40).

As with initial marker validation in a phase II setting, prospective trials for definitive marker validation also fall under the same classifications, but require greater rigidity than the phase II setting for the statistical parameter standards of type I and type II errors (typically set at 2-sided level of 5% and 10% respectively), and multiplicity adjustments. Specifically, the designs can be classified into enrichment, all-comers (with marker stratified, sequential testing strategy, multiple hypotheses or hybrid designs), or outcome based adaptive randomization designs (3,4). A number of innovative statistical designs using an adaptive strategy for analysis have also been proposed for definitive marker validation. The first is an adaptive accrual design that modifies accrual to two pre-defined marker-defined subgroups based on an interim futility analysis (41). In this design, if the treatment effect in one of the subgroups fails to satisfy a futility boundary, accrual to that subgroup is terminated, and accrual is continued to the other subgroup until the planned total sample size is reached, including accruing subjects that had planned to be included from the terminated subgroup. This design has demonstrated greater power than a non-adaptive trial in simulation settings; however, this strategy might lead to a substantial increase

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Table 1 Considerations for the choice of phase II design for initial validation of predictive marker [Adapted from Mandrekar *et al*. (32)]

Considerations	Phase II design for initial marker validation			
	Enrichment/ Targeted	All-comers/ unselected	Direct assignment option	Outcome based adaptive randomization
Preliminary evidence				
Strongly suggest benefit in marker defined subgroups	Appropriate	Not recommended	Appropriate (with an early single IA, or two IA with option for direct at both IA)	Appropriate (assess multiple treatments/biomarker subgroups)
Uncertain about benefit in overall population versus marker defined subgroups	Not appropriate	Appropriate	Appropriate (direct assignment option within the biomarker positive and negative cohorts)	Appropriate (learn and adapt as the trial proceeds)
Assay performance				
Excellent (high concordance between local and central testing; commercially available kits; well established marker cutpoint etc.)	Required	Appropriate	Required	Required
Questionable	Not recommended	Appropriate	Not applicable	Not applicable
Turn-around times				
Rapid (2-3 days; without causing delay in the start of therapy)	Optimal	Optimal	Optimal	Optimal
Slow to modest (one week or more)	Not recommended	Appropriate (retrospective marker subgroup assessment)	Appropriate in some cases	Appropriate in some cases
Marker prevalence				
Low (<20%)	Optimal	Not recommended	Appropriate (with an early single IA, or two IA with option for direct at both IA)	Appropriate
Moderate (20-50%)	Appropriate	Appropriate (stratified by marker status)	Appropriate, with two IA with direct assignment option only at the second IA	Appropriate
High (>50%)	Appropriate	Appropriate	Appropriate	Appropriate

IA, interim analysis.

in the accrual duration depending on the prevalence of the marker for the subgroup that continues to full accrual. The second design adaptively modifies accrual, where in the first stage of the trial, only the marker positive group patients are accrued (42). Based on promising interim analysis results, the second stage can continue accrual to the marker positive cohort and also include marker negative patients. If the first stage shows no benefit in the marker positive cohort, then the trial will be closed permanently.

Sequential testing designs are yet another category of phase III designs for validation of markers that control for the type I error rates associated with multiple testing (43-45). These designs are similar to a traditional RCT in that they have a single primary hypothesis, which is either tested in the overall population first and then in a prospectively planned subset if the overall test is not significant, or in the marker-defined subgroup first, and then tested in the entire population if the subgroup analysis

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is significant (also known as closed testing procedure). The first is recommended in cases where the experimental treatment is hypothesized to be broadly effective, and the subset analysis is ancillary; the second is used when there is strong preliminary data to support that the treatment effect is strongest in the marker-defined subgroup, and that the marker has sufficient prevalence that the power for testing the treatment effect in the subgroup is adequate. A sequential testing strategy can induce potential correlation from testing the overall treatment effect and the treatment effect within the marker-defined subgroup from the same trial population; an approach to appropriately control for such correlations has also been proposed in the literature (45). Freidlin *et al*. (46) argue that sequential testing designs of the overall/marker-defined subgroup (say, marker-positive) do not appropriately control for the type I error rate for the complementary marker-negative subgroup (i.e., result in higher than acceptable error rate of falsely recommending the new treatment for the marker-negative cohort), when the treatment benefits only the marker-positive cohort. The authors propose instead a strategy called the Marker Sequential Test (MaST) design that allows for sequential testing of the treatment effect in the marker-positive and the marker-negative groups as well as the entire population while appropriately controlling for the type I error rates (46). A more recent class of sequential testing strategy designs specifically applicable to biomarker validation are the adaptive threshold (AT) and the adaptive signature (AS) designs (47-49). AT is useful in cases where a marker is known at the start of the trial, but a cut-point for defining marker positive and marker negative groups is not known. AS is useful when information regarding the marker and the threshold are both unknown; AS design allows for the "discovery and validation" process of the marker within the realm of the single phase III trial, using either a cross validation approach or the split-alpha approach in cases when the treatment is not broadly effective in the overall unselected population (47-49). At least two issues need further consideration with such designs: (I) the added cost of a somewhat larger sample size and/or redundant power dictated by the strategy of partitioning the overall type I error rate; and (II) use of data from the same trial to both define and validate a marker cut-point (3).

Finally, in the current era of stratified medicine, phase II/III designs are gaining popularity as they enable us to use small patient subsets most effectively (50,51). These designs, known as multi-arm multi-stage (MAMS) designs, enable the simultaneous assessment of multiple experimental agents against the standard of care in the phase II portion using an intermediate (or surrogate) endpoint. This eliminates the need to conduct separate (large-scale) phase II trials to evaluate each experimental regimen. The phase III portion will subsequently continue with the promising experimental arms from the phase II portion, comparing them to the standard of care (50,51).

Examples of biomarker-based trials in the phase II and phase III setting

In this section, we provide examples of ongoing, in development or completed cancer clinical trials that utilized a design strategy aiming at personalized medicine.

Outcome based adaptive randomization design

The biomarker-integrated approaches of targeted therapy of lung cancer elimination (BATTLE) trial used an outcome based adaptive randomization design for randomizing patients to treatment choices based on multiple biomarker profiles in NSCLC (9). This trial is completed and accrued ~200 patients who had their tumors tested for 11 different biomarkers (categorized into five biomarker subgroups), and were randomized to one of four treatment choices. The first 97 patients were assigned using a balanced randomization to one of the four treatments equally. All subsequent patients were adaptively randomized, where the randomization rate was proportional to the eight week disease control rate. As hypothesized, the results from the trial showed that each drug works best for patients with a specific molecular profile (52). Two successor trials, BATTLE 2 and BATTLE 3, also following an adaptive design strategy, will attempt to confirm these initial findings.

Multiple hypothesis design

A subgroup-focused, multiple-hypothesis design was utilized in the phase III SWOG S0819 that incorporates co-primary endpoints to assess cetuximab in both the overall study population (all-comers) as well as within a pre-specified biomarker subgroup (epidermal growth factor receptor (EGFR) fluorescent in-situ hybridization (FISH)-positive, FISH+), with the sample size determined based on evaluation in the EGFR FISH(+) group (53). This trial evaluates both the value of cetuximab in this setting (overall general efficacy objective) and EGFR FISH as a predictive

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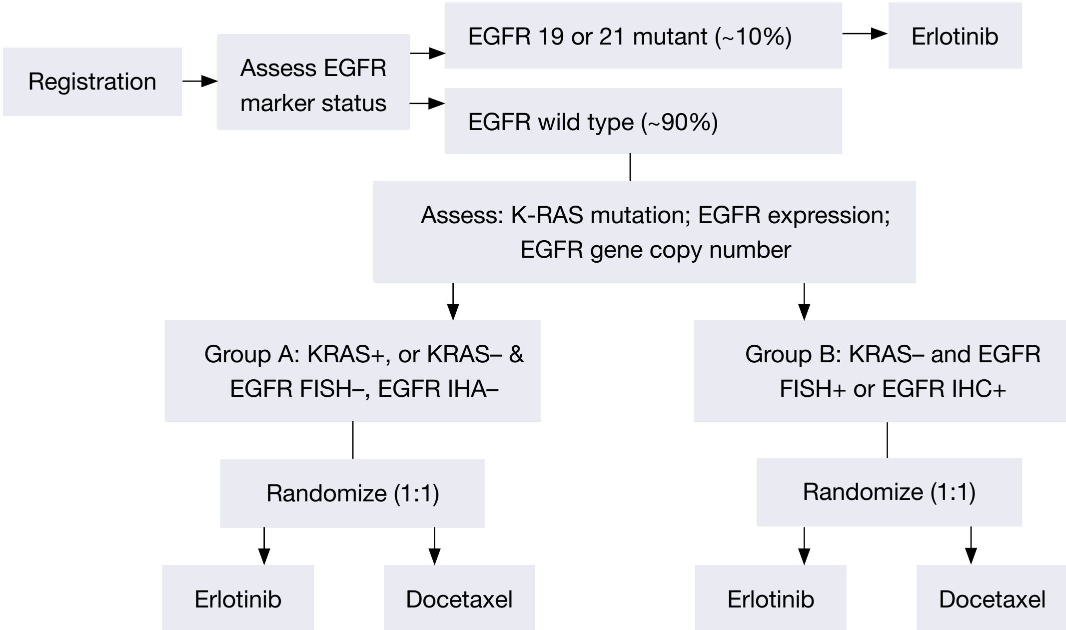

Figure 1 Phase III Marker Validation Combination Design Strategy (Tailor): enrichment followed by a biomarker stratified design. EGFR, epidermal growth factor receptor; FISH, fluorescent *in-situ* hybridization; IHC, immunohistochemistry.

biomarker. With progression-free survival as the primary endpoint (targeting an improvement of 20% in the overall and 33% in the subgroup), and allocating 80% of the type I error rate to the subgroup hypothesis (1-sided study wide type I error of 2.5%), the sample size for this trial is 1,420 patients, with 564 in the EGFR FISH+ group (53).

Combination designs

An example of a phase III trial utilizing an enrichment followed by a marker by treatment interaction design to validate the predictive value of the K-ras mutation, EGFR protein expression, and EGFR gene copy number is the Tailor trial in second line NSCLC (54) (*Figure 1*). The primary hypotheses, based on a 2-sided interaction test with 95% power, is that docetaxel (D) is better than erlotinib (E) in Group A (30% improvement in OS, for a HR of 1.43 in favor of D), and E better than D in Group B (21% improvement in OS, for a HR of 0.79 in favor of E). A limitation of this trial is that the secondary within group comparisons are not adequately powered to detect clinically relevant differences in outcomes. Another example of a phase III trial using a combination design of an enrichment strategy followed by a marker-based strategy design is trial 0601, coordinated by the Spanish Lung Cancer Group, comparing erlotinib with chemotherapy in stage IV NSCLC patients with EGFR mutations (55) (*Figure 2*).

National Cancer Institute (NCI) precision medicine initiative

The NCI's recent focus is to develop trials where patients are screened for certain molecular characteristics that may predict for response to a targeted therapy, the so-called genotype to phenotype initiative. At least three trials are in development to address this paradigm: the adjuvant lung cancer enrichment marker identification and sequencing trial (ALCHEMIST) (*Figure 3*), the molecular profiling based assignment of cancer therapeutics (M-PACT) (*Figure 4*), and the molecular analysis for therapy choice (NCI-MATCH) (*Figure 5*).

Concluding remarks

Cancer is increasingly becoming a "rare" disease with the use of targeted therapeutics and biomarker assessment for medical treatment. Design of phase I, phase II and phase III trials has thus undergone a rapid evolution in the last decade. The focus has shifted from predictions based on the traditional anatomic staging systems to guide the choice of treatment for an individual patient to an integrated approach using the genetic makeup of the tumor and the genotype of

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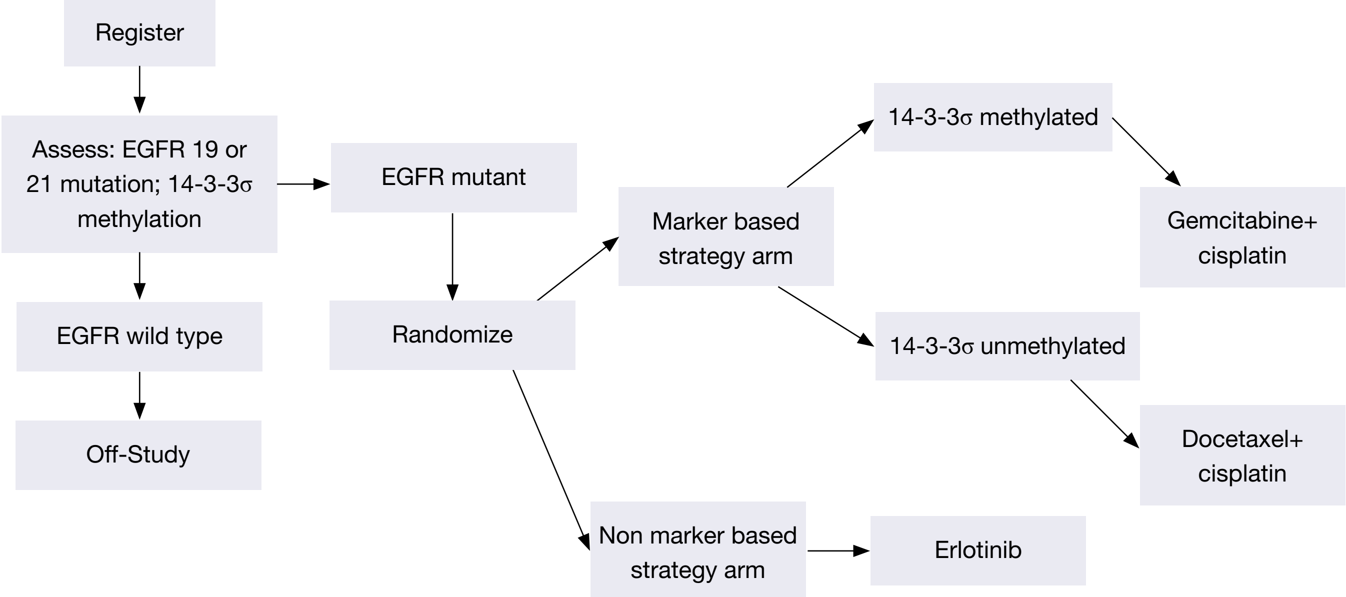

Figure 2 Phase III Marker validation combination design strategy (0601): enrichment followed by a marker-based strategy design. EGFR, epidermal growth factor receptor.

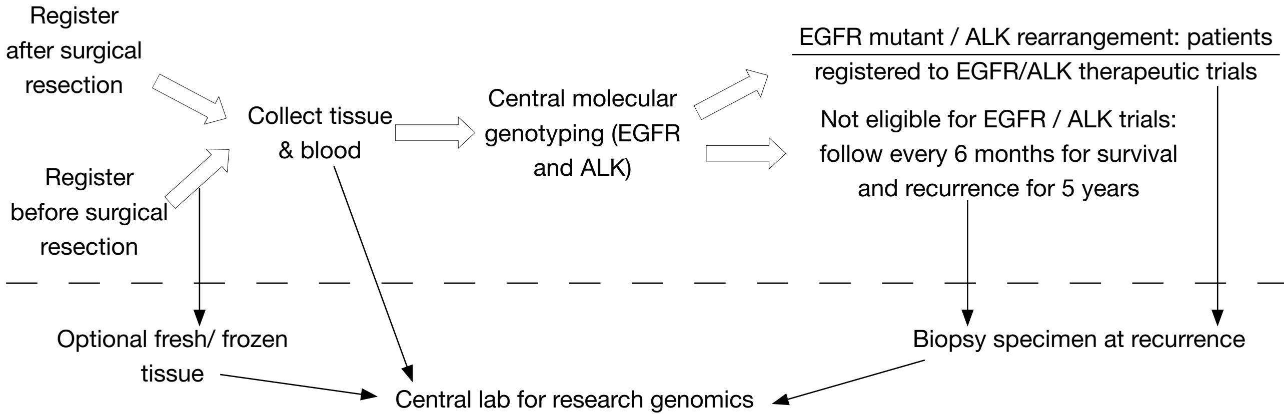

Figure 3 ALCHEMIST trial design for early stage resectable lung disease. ALCHEMIST, adjuvant lung cancer enrichment marker identification and sequencing trial; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase.

the patient. In the setting of early phase dose-finding trials, identification of the MTD and assessment of the safety profile is no longer the only goal; a preliminary assessment of efficacy has become a necessity in order to identify a so-called MED to take forward into phase II trials. A better understanding of the tumor biology (identifying patient subsets, rare tumor subtypes, etc.), advancement in assay techniques, and availability of commercial kits with rapid turn-around times have popularized enrichment designs in phase II and phase III trials, allowing only patients with a particular molecular profile to be eligible for the trial. Tailored treatments with effective biomarker-driven hypotheses are leading to smaller clinical trials targeting larger treatment effects. Phase II/III designs are gaining popularity as small patient subsets will require us to not 'waste' patients. The NCI's initiative to promote and focus on molecularly driven trials has provided impetus to design trials that match the right patient to the right drug. Finally,

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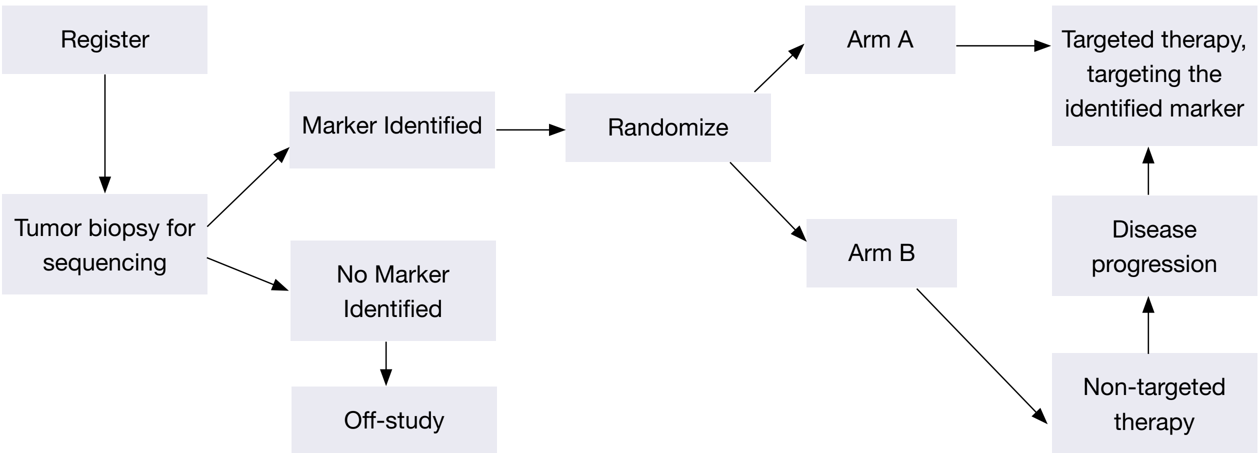

Figure 4 M-PACT trial design (endpoints: response rate and progression-free survival). M-PACT, molecular profiling-based assignment of cancer therapeutics.

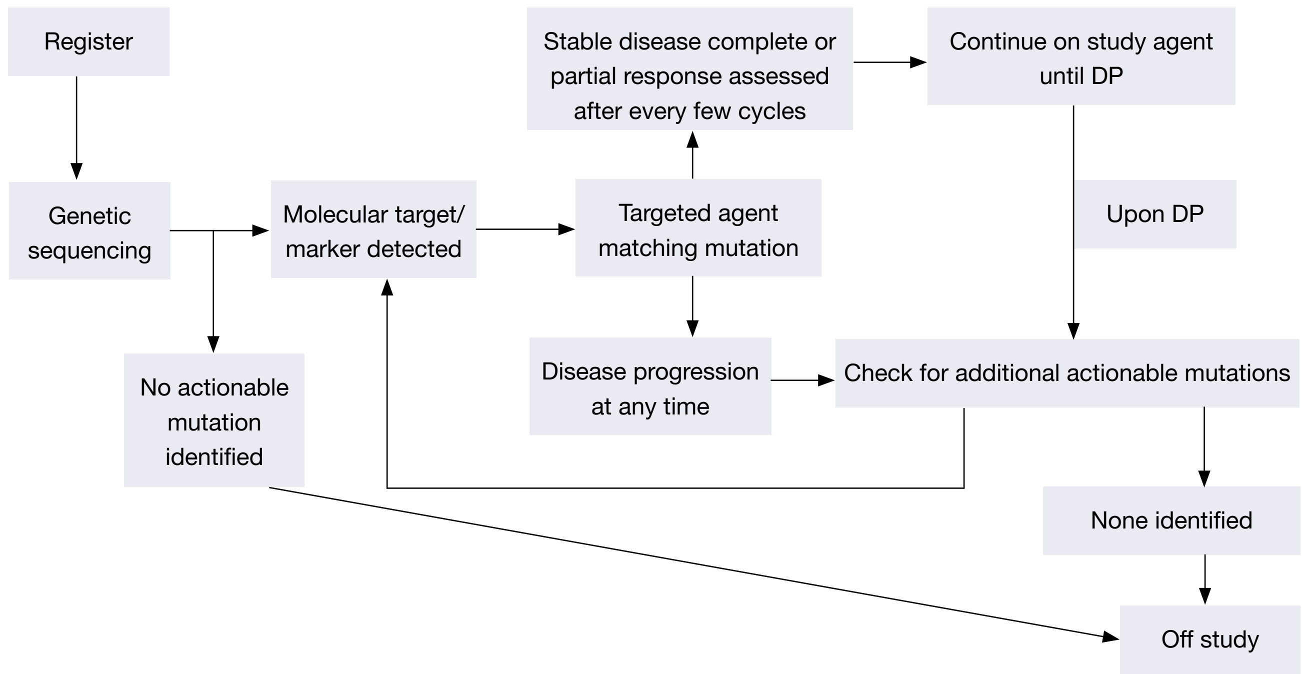

Figure 5 NCI-MATCH trial design (endpoints: response rate and 6-month progression-free survival rate). NCI-MATCH, National Cancer Institute molecular analysis for therapy choice; DP, disease progression.

advancements in technology such as mobile computing, electronic data capture, and integration of research records with electronic medical records has made real time access to clinical trial and biomarker data a reality, allowing adaptive designs to take on a much greater role in clinical trials.

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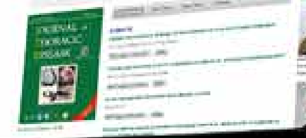

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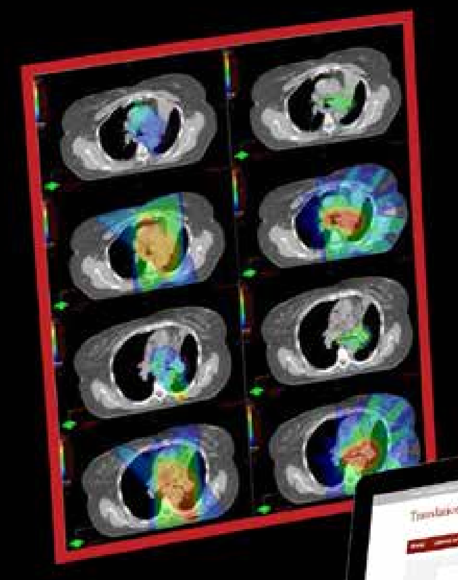

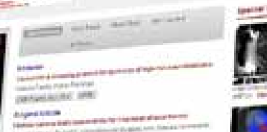

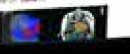

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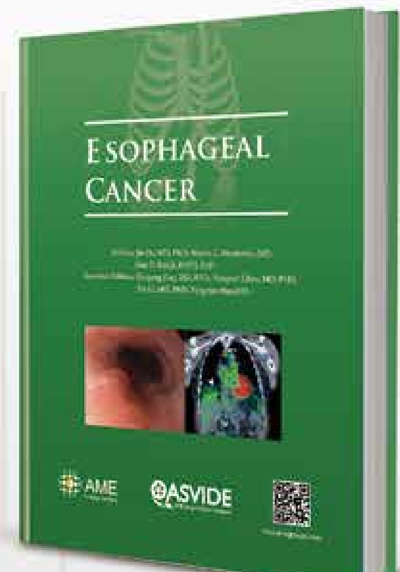

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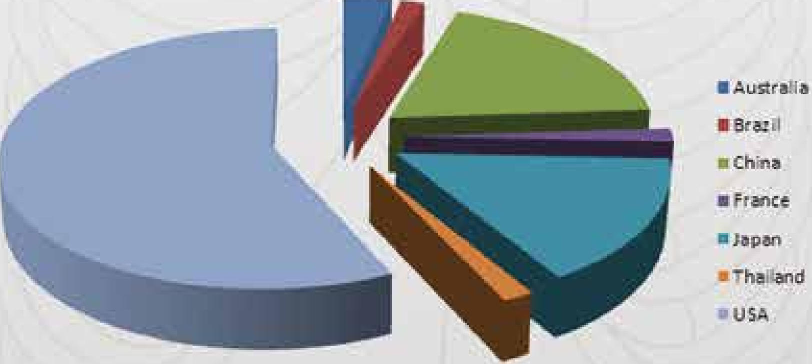

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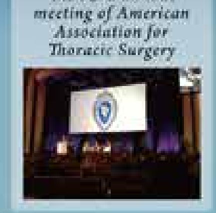

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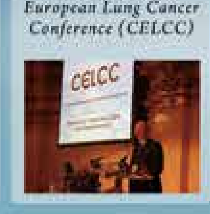

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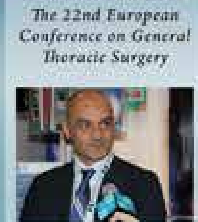

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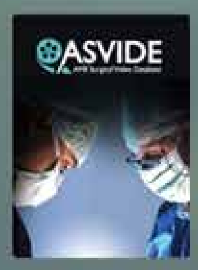

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