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Targeted Therapy for Lung Cancer: Afatinib Focused

Editors: Yilong Wu
Rafael Rosell
Trever G. Bivona

AME Medical Book 1A037

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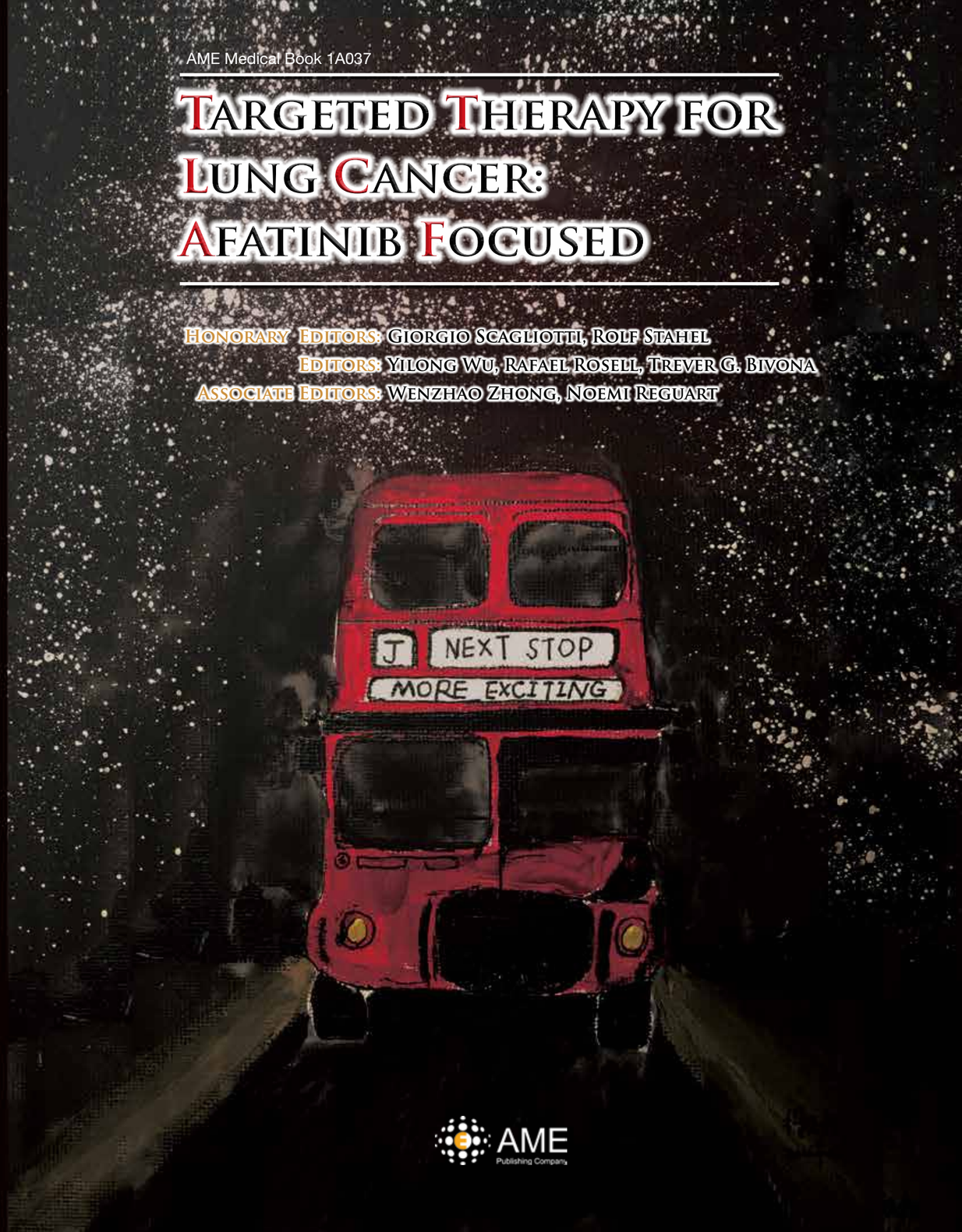

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Targeted Therapy for Lung Cancer: Afatinib Focused (FIRST EDITION)

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We are pleased to announce that the "AME Research Time Medical Book Series" launched by AME Publishing Company have been published as scheduled.

Finishing my medical degree after 4 years and 3 months of study, I decided to quit going on to become a doctor only after 3 months of training. After that, I had been muddling through days and nights until I started engaging in medical academic publishing. Even 10 years after graduation, I had not totally lost the affection for being a doctor. Occasionally, that subconscious feeling would inadvertently arise from the bottom of my heart.

In April 2011, Mr. Tiantian Li, the founder of DXY.cn, and I had a business trip to Philadelphia, where we visited the Mütter Museum. As part of The College of Physicians of Philadelphia, the museum was founded in 1858 and has now become an exhibition hall of various diseases, injuries, deformities, as well as ancient medical instruments and the development of biology. It displays more than 20,000 pieces of items including pictures of wounded bodies at sites of battle, remains of conjoined twins, skeletons of dwarfs, and colons with pathological changes. They even exhibited several exclusive collections such as a soap-like female body and the skull of a two-headed child. This museum is widely known as "BIRTHPLACE OF AMERICAN MEDICINE". Entering an auditorium, we were introduced by the narrator that the inauguration ceremony of the Perelman School of Medicine at the University of Pennsylvania would take place there every year. I asked Mr. Li, "If it was at this auditorium that you had the inauguration ceremony, would you give up being a doctor?" "No," he answered.

In May 2013, we attended a meeting of British Medical Journal (BMJ) and afterwards a gala dinner was held to present awards to a number of outstanding medical teams. The event was hosted annually by the Editor-in-Chief of BMJ and a famous BBC host. Surprisingly, during the award presentation, the speeches made by BMJ never mentioned any high impact papers the teams had published in whichever prestigious journals over the past years. Instead, they laid emphasis on the contributions they had made on improving medical services in certain fields, alleviating the suffering of patients, and reducing the medical expenses.

Many friends of mine wondered what AME means.

AME is an acronym of "Academic Made Easy, Excellent and Enthusiastic". On September 3, 2014, I posted three pictures to social media feeds and asked my friends to select their favourite version of the AME promotional leaflet. Unexpectedly we obtained a perfect translation of "AME" from Dr. Yaxing Shen, Department of Thoracic Surgery, Zhongshan Hospital, Shanghai, who wrote: enjoy a grander sight by devoting to academia (in Chinese, it was adapted from the verse of a famous Chinese poem).

AME is a young company with a pure dream. Whilst having a clear focus on research, we have been adhering to the core value "Patients come first". On April 24, 2014, we developed a public account on WeChat (a popular Chinese social media) and named it "Research Time". With a passion for clinical work, scientific research and the stories of science, "Research Time" disseminates cutting-edge breakthroughs in scientific research, provides moment-to-moment coverage of academic activities and shares rarely known behind-the-scene stories. With global vision, together we keep abreast of the advances in clinical research; together we meet and join our hands at the Research Time. We are committed to continue developing the AME platform to aid in the continual forward development and dissemination of medical science.

It is said that how one tastes wine indicates one's personality. We would say how one reads gives a better insight to it. The "AME Research Time Medical Books Series" brings together clinical work, scientific research and humanism. Like making a fine dinner, we hope to cook the most delicate cuisine with all the great tastes and aromas that everyone will enjoy.

Stephen Wang
Founder & CEO,
AME Publishing Company

In the era of molecular and personalized therapeutics, the discovery of sensitizing in epidermal growth factor receptor (EGFR) in 15%–20% of lung adenocarcinomas and the associated response to EGFR-targeting tyrosine kinase (TK) inhibitors have provided a successful avenue of attack in high-stage adenocarcinomas. In a period of time of approximately 15 years we had the tremendous clinical opportunity to test and implement in our clinical practice three different generation of EGFR-TKI, learning progressively about respective level of activity and toxicity profiles as well as understand every year better the biological basis of acquired resistance to EGFR-TKI. There is no question that in the appropriate subgroup of patients as defined by molecular screening these agents have shown a clear-cut superiority over cytotoxic chemotherapy and significantly prolonged survival.

While most of the clinical development has been focused on common sensitizing mutations more recently investigators started focusing on uncommon mutations and the contribution of HER2 associated genomic changes in lung cancer to better understand if a consensus may be obtained around those rare clinical conditions. In the specific case the rarity of the molecular alterations leads to the uncertainty of clinical evidence and in this setting dedicated trials have to be implemented.

The straightforward clinical improvements have been paralleled by significant achievements on the diagnostic side. While up to few years ago to monitor molecular changes in the context of the EGFR-mutated tumor the only viable option was the repeated tissue biopsy with all associated hurdles such as size and site of progression or relapse, tumor necrosis, side effects related to the diagnostic procedure among others. Nowadays we are entering in a new diagnostic era where several genomic tests are feasible in different biological fluids, from blood to urine, pleural effusion and cerebral-spinal fluid. While some blood-based tests are already approved for clinical use the vast majority of these tests are still restricted to the context of clinical trials but they will definitively represent a step forward to better understand tumor heterogeneity and will contribute to a real-time monitoring of the disease. In a long-term perspective those tests will be potentially useful in early detection strategies, in monitoring tumor dynamics, evaluation of early treatment response and monitoring of minimal residual disease.

This book represents an outstanding piece of work with the contribution of several key opinion leaders in the field that summarizes the state of the art about the current and future knowledge for the appropriate application of targeted therapies in the context of non-small cell lung cancer.

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Lung cancer is the leading cause of cancer-related mortality in both more and less developed countries (1). The past few years have witnessed a great change in the diagnosis and treatment of patients with advanced lung cancer. Surgery, radiofrequency ablation, radiation therapy and chemotherapy are used to be the basic treatments for NSCLC patients, but in recent years, immunotherapy and targeted therapy are increasingly important. Experts get to know the pathobiology of non-small-cell lung cancer (NSCLC) on a deeper level, which then accelerates our better understanding of certain proteins and small molecules (2).

Epidermal growth factor receptor (EGFR) has been proved to be the key molecule associates to lung cancer and it has become a significant therapeutic target for NSCLC (3). EGFR mutations predict responses to EGFR tyrosine kinase inhibitors (TKIs). In the beginning section of this new book *Targeted Therapy for Lung Cancer: Afatinib Focused*, we first introduce some topics about EGFR mutations, such as tumor heterogeneity, circulating DNA, molecular methods for somatic mutation testing, Kinase inhibitor-responsive genotypes and advances on EGFR mutation. It is well known that HER2 mutation is an oncogenic driver in lung cancer and it is responsible for 2% to 6% of lung adenocarcinomas (4). Therefore, in the second section of the book, we briefly review two papers about HER2 driven NSCLC.

It is well established that the progression-free survival (PFS) for patients receiving TKIs varies among different EGFR mutations (5). Gefitinib, erlotinib, afatinib and osimertinib are the options for treatment of patients with EGFR mutations. In the third and fourth section of the book, it gives an overview and future perspectives on the EGFR TKIs and lung cancer metastasis.

In recent years, physicians gradually recognize the role of afatinib in treating patients with metastatic NSCLC whose tumors have EGFR exon 19 deletions or exon 21 L858R substitutions as it was first approved by the Food and Drug Administration (DFA) in 2013. And now in Jan. 2018, the FDA expanded approval of afatinib (Gilotrif) to treat patients with lung cancers with EGFR L861Q, G719X, and S768I (6). Therefore, some hot and controversial topics about afatinib will be presented in the fifth section of the book.

The occurrence of intrinsic or acquired resistance may hinder the efficacy of EGFR TKIs, so the deeper understanding of mechanisms leading to inhibitor resistance will benefit the exploration of new therapeutic strategies. In the second last section of the book, it mainly focuses on the resistance mechanism of EGFR TKIs. Last, in the era of precision medicine, it is indispensable to study patients with lung cancer in a personalized way.

We hope all physicians and other interested readers will enjoy this book and find available and helpful in the daily clinical practice.

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The book, *Targeted Therapy for lung cancer: Afatinib focused* is one of the most positively surprising enterprises realized by AME Publishing. It's on the cutting edge of novel forms of lung cancer, especially treatable subclasses of lung adenocarcinoma, such as, EGFR mutant driven non-small-cell lung cancer (NSCLC). The work also encompasses multiple different subtypes of lung cancer, including salient aspects of squamous cell carcinoma of the lung, as well as the elusive, SCLC.

In cancer medicine, EGFR mutation driven NSCLC has become the paramount of success with targeted therapy. There have been continuous new discoveries and challenges regarding multiple forms of cancer resistance and both clinical investigators and laboratory researchers, alike, have never before seen cancer so up-close. Nevertheless, in spite of all the multi-tasked scientific endeavors, cancer cells are still capable of surviving and, ultimately, fulfill their function of consuming the patient.

The book is particularly useful since it has gathered together an impressive group of dedicated investigators, from bench to bed, who provide their expert opinion in several organized chapters.

The reader will be surprised from the first chapter to the last and will find all the elements of current knowledge easily absorbed with great satisfaction, thus encouraging them to join the knowledgeable investigators and coauthors in this ceaseless research towards the curability of lung cancer. The book has been structured in such a manner that the reader can choose to start reading in the order most suited to them. The book is mandatory for both medical oncologists in training and laboratory investigators, in order to get a closer glance at the lung cancer patient. The book amalgamates extraordinary authors from various geographic regions with different areas of skills and expertise and focuses on providing the most modern and satisfactory therapy in lung cancer, in all subclasses of lung cancer, especially EGFR mutation driven NSCLC.

In the last 60 years considerable progress has been made in cancer treatment and the book covers some astonishing advances accomplished within just the past decade. The fact that the book is guided by several experts in lung cancer permits us to travel through different and intriguing facets of the disease. Any advance is followed by a new failure or mechanism of resistance, prompting us to find a new solution.

In summary, it is without a doubt a great privilege and honor to be illuminated by such high regarded authors contributing with their deep experience and profound knowledge towards a momentous breakthrough in lung cancer therapy.

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Genotype-directed diagnosis and treatment of lung cancer: EGFR and HER2 as molecular paradigms

Lung cancer is the leading cause of cancer mortality worldwide. Great strides have been made against this disease through the identification and therapeutic targeting of oncogenic driver alterations. A paradigm-defining example of the success of this targeted therapy approach is EGFR-mutated lung adenocarcinoma. EGFR-mutated lung adenocarcinoma afflicts approximately 10-30 percent of patients and illustrates both the success and challenges facing the field of precision medicine in oncology.

EGFR inhibitor treatment is widely effective in many EGFR-mutated lung adenocarcinoma patients. However, not all patients respond to treatment and all patients who do respond eventually succumb to disease progression that arises due to acquired resistance to the targeted treatment. Understanding the basis of primary and acquired resistance to EGFR inhibitor treatment is essential in order to devise strategies to prevent or delay this resistance, thereby prolonging patient survival.

In this comprehensive book, we review the current knowledge of the genetic and epigenetic factors that underlie both the response and resistance to EGFR inhibitor treatment in EGFR-mutated lung adenocarcinoma. The underlying biological events contributing to the lack of complete and sustained response to treatment in EGFR-mutated lung cancer are multifactorial. Therefore, the discussion presented in the chapters in this book highlights both tumor-cell intrinsic and extrinsic factors, the role of on-target secondary mutations in EGFR in causing resistance to first- and later-generation EGFR inhibitors, and the emerging understanding of the role of intra- and inter-tumor heterogeneity in modulating response and resistance to first and later-generation EGFR inhibitors.

As the related EGFR family member HER2 is also recurrently mutated as an oncogenic driver in lung cancer, this book also contains the state of the art view on the diagnostic role and therapeutic targeting of mutant HER2 in this disease. Themes arising in EGFR-mutated lung adenocarcinoma are echoed and expanded in the discussion of HER2-driven lung cancer.

In conclusion, the discussions presented herein will serve to summarize the important progress made through genotype-directed therapy in lung cancer through the lens of the EGFR- and HER2-driven molecular subtypes of this disease. Furthermore, factors limiting response and preventing cure are highlighted with the overall goal of charting the future course of basic and translational research that holds promise for improving the depth and duration of therapy response. Ultimately, the goal of these discussions is to stimulate the research community to devise novel strategies that can help transform lung cancer from a lethal disease into a chronic, or even curable condition.

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I would like to welcome readers to this new book of AME Publishing Company. The purpose of this book is to provide you with the most recent and updated insights into the molecular background of lung cancer focusing on epidermal growth factor receptor (*EGFR*) mutations, targeted therapies with tyrosine kinase inhibitors (TKIs) and the molecular mechanisms underlying inherent or acquired resistance to these targeted therapies.

Lung cancer has been always considered a highly aggressive and difficult to treat disease and the majority of patients are diagnosed when the disease is in advanced stage. Chemotherapy has been for many decades the cornerstone of lung cancer treatment and few therapeutic options were available beyond cytotoxic chemotherapy for those patients with advanced disease. Fortunately, for the first time in many decades, we are witnessing dramatic changes in the way lung cancer is treated and conceptualized.

Two major 'sightings' have heralded the paradigm shift in the management of non-small cell lung cancer (NSCLC): the identification of alterations in genetic drivers with potential for target inhibition and the elucidation of the immunogenic properties of lung cancer. The incorporation in the clinical practice of comprehensive mutational analysis technologies has definitely accelerated the identification of several genetic drivers beyond *EGFR*, such as *HER2*, *MET* splice site mutation, *BRAF* mutations and gene rearrangements at *ALK*, *ROS1*, *RET* or *NTRK* and research efforts continue to identify other additional driver candidates (1,2). Today, the use of molecular targeted agents, designed to target driver mutations, and those that target immune checkpoints molecules have overcome a new standard for lung cancer treatment. Inconceivable a few years back and for the first time, both targeted therapies and immune checkpoints have displaced chemotherapy from first-line setting in a subset of molecular-selected lung cancer patients (3-6). Consequently, molecular testing is now crucial in the diagnostic algorithm of this disease.

Scientific community is now pooling all their expertise and knowledges towards a common goal: to convert lung cancer into a chronic disease. This is the real challenge of our time. To do so, we will need to overcome new obstacles in the way by identifying new prognostic and predictive markers of response, learning how to choose among different effective treatments (TKIs *vs.* chemotherapy *vs.* immunotherapy *vs.* combinations), developing novel and more potent inhibitors, understanding the mechanisms that lead resistance and learning how to enhance antitumor inmune responses. It is through the tireless efforts of scientific community that we will be able to progress day by day providing new hope for lung cancer patients.

This new book highlights the most relevant cutting-edge advances in one of the 'hot topics' in the field, *EGFR*-mutant lung cancer. This book has been divided into several sections. The first section namely—*EGFR* mutation and lung cancer—offers a state of the art overview related to this molecular aberration, describing not only the most common types of *EGFR* mutations, indels and point mutations, but other less common genomic events such as duplications and rearrangements involving alternative sites of kinase domains. It also addresses current development of molecular assays for somatic mutation testing not only in tissue but by using novel and less invasive techniques that allow DNA mutation detection and monitoring in blood.

In the second section *HER2*-driven NSCLC is the focus of the topic discussing the genetic alterations that are felt to mediate its oncogenic functions in NSCLC, epidemiology and a detailed overview of new investigational anti-*HER2* therapies that are currently explored in ongoing clinical trials applied to NSCLC.

In the next sections targeted therapies move back into attention addressing areas of huge interest for readers including an up-to-date review of available data from selected pivotal trials with first and second TKIs (focusing on afatinib), as well as an outline of new third generation irreversible and covalent inhibitors with potential to overcome the most frequent cause of acquired resistance related to T790M. This section makes attention to other hot topics in the field such as the controversial role of targeted therapies and immune checkpoint inhibitors with or without radiotherapy in the treatment of brain metastasis.

The last chapter outline the topic of acquired resistance in *EGFR*-mutated NSCLC patients and potential novel strategies to restore the sensitivity with new generation T790M inhibitors. Last but not least a mention to precision medicine, a clear example of implementation and success in lung cancer management.

I would not like to conclude without expressing our most sincere gratitude to all the authors who have contributed to this book. It is their knowledges and insights that have ensured the quality of the content. We extend our thanks to the editors-in chief, Dr./Prof. Yi-Long Wu and Dr./Prof. Rafael Rosell, who worked tirelessly to put this issue together.

We foresee that the content of this new book will be a valuable, helpful and an educational resource for all readers interested in lung cancer disease.

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Tumor heterogeneity: evolution through space and time in EGFR mutant non small cell lung cancer patients

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Abstract: NSCLC patients with mutations in epidermal growth factor receptor (EGFR) gene have dramatic responses with the EGFR tyrosine kinase inhibitors (TKI) in the majority of patients. However, all patients will eventually present progression of disease because of both primary and acquired resistance to EGFR TKI. In the recent years several studies have identified mechanisms involved in primary and secondary resistance to EGFR TKI treatment that can also be potential therapeutic strategies, although up to 30% of cases of acquired resistance to EGFR TKI are still unexplained.

In this review we describe the mechanisms of resistance to EGFR TKIs in NSCLC patients that have been discovered and potential therapeutic strategies to overcome EGFR TKI resistance. Additionally we highlight the importance of performing additional biopsies not only at time of acquired resistance to EGFR TKI but also immediately after initiation of therapy to discover the remaining unknown mechanisms of acquired resistance to EGFR TKI as well as the underlying molecular basis of the heterogeneity in response to EGFR TKI.

Keywords: Primary resistance; acquired resistance; epidermal growth factor receptor; non-small cell lung cancer

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality worldwide, and traditional chemotherapeutic drugs are only modestly effective. Most lung cancer patients usually present with advanced stage disease, where the efficacy of chemotherapy is low, with a 5-year survival rate lower than 15% (1).

The discovery of mutated oncogenes encoding activated signaling molecules that drive cellular proliferation and promote tumor growth has led to the development of more effective and less toxic targeted therapies for NSCLC patients. Particularly, NSCLC patients with mutations in epidermal growth factor receptor (EGFR) gene have dramatic responses and better outcome with the EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib (1-9).

The EGFR is a well characterized mutated oncogene in NSCLC that is associated predominantly with adenocarcinoma histology. EGFR-mutated tumors depend to EGFR signaling for their proliferation and survival. Nearly 90% of lung-cancer-specific EGFR mutations comprise a leucine-to-arginine substitution at position 858 (L858R) and deletion in exon 19 that affect the conserved sequence LREA (delE746-A750) (3,8,10,11).

Unfortunately, despite the dramatic efficacy of EGFR TKI in NSCLC patients with EGFR activating mutations, all patients eventually acquire resistance, with progression of disease occurring in patients around 10-13 months after starting treatment (2,7,12). There are two main mechanisms of resistance to EGFR TKI: the lack of an initial response to therapy, also called de novo or primary resistance to EGFR TKI, and resistance that develops following an initial response to EGFR TKI, also called acquired resistance to EGFR TKI.

To discover those mechanisms involved in EGFR TKI

resistance is a significant challenge in order to develop more effective targeted therapies alone or in combination with EGFR TKI for patients with NSCLC and EGFR mutations. In this article we review the molecular basis of resistance of EGFR mutant NSCLC patients to EGFR TKI and rebiopsy strategies to better understand the underlying molecular basis of resistance.

Primary resistance to EGFR TKIs

Patients with NSCLC and EGFR activating mutation will experience significant tumor regression with EGFR TKI in approximately 70% of cases (5), which means a lack of an initial response in about 30% of patients. Those patients will present primary or de novo resistance to EGFR TKI.

To date, two main mechanisms of primary resistance to EGFR TKI in EGFR mutant NSCLC patients have been described: first, the presence of secondary alterations in EGFR that prevent inhibition of EGFR by an EGFR TKI (also known drug resistant EGFR mutation), and second, the presence of additional genetic alternations that occur together with EGFR mutation.

Secondary alterations in EGFR

EGFR exon 20 insertions

EGFR Exon 20 insertions comprise approximately 4% of all EGFR mutant NSCLC (13) and are associated with lower sensitivity to the reversible EGFR TKIs both in preclinical models and in patients that have experienced a lack of response when treated with gefitinib or erlotinib (14-16). The irreversible EGFR TKIs could be more effective in these mutations (15,17-19).

EGFR T790M (c.2369C>T) mutation in non-small cell lung cancer

The T790M mutation results in an amino acid substitution at position 790 in EGFR, from a threonine (T) to a methionine (M). This gatekeeper mutation also occurs within exon 20, which encodes part of the kinase domain EGFR and alters the binding of EGFR TKI to the ATP-binding pocket, and therefore EGFR TKI are unable to block EGFR signalling (20-22). These pretreatment T790M mutations generally occur together with another EGFR sensitizing mutation and have been found to be associated with decreased sensitivity to EGFR TKIs (16). Additionally, the baseline T790M mutations may be present as an underlying germline mutation at a low frequency (0.5% of never smokers with lung cancer) (23) and may be associated with familial cancer syndromes (24).

Rosell *et al*. assessed the T790M mutation in pretreatment diagnostic specimens from 129 EGFR TKI treated advanced NSCLC patients with EGFR mutations, and found that EGFR T790M mutation was present in 45 of 129 patients (35%). Progression-free survival was 12 months in patients with and 18 months in patients without the T790M mutation (P=0.05). Additionally, it was found that low BRCA-1 levels neutralized the negative effect of the T790M mutation and were associated with longer progression-free survival to erlotinib, whereas high levels of BRCA-1 may lead to de novo resistance through increased DNA damage repair capacity, suggesting that pretreatment assessment of both T790M mutation and BRCA1 expression could be useful to predict outcome (25). Additionally, in the EURTAC trial the T790M mutation was detected in 38% of the pre-treatment specimens analysed (26).

Fujita *et al*. evaluated the incidence of T790M in pretreatment tumor specimens using highly sensitive colony hybridization technique and was detected in 30/38 resected tumor tissues of patients with the EGFR mutation (79%). The median time to treatment failure was 9 months for the patients with pretreatment T790M and 7 months for the patients without the T790M mutation (P=0.44), and suggested that patients with high proportion of T790M allele may have a relatively favorable prognosis (27).

In addition to EGFR T790M, primary EGFR TKI resistance may also be due to other secondary mutations in EGFR (e.g., D761Y) that can occur concurrent with an activating EGFR kinase domain mutation (e.g., L858R) (28).

Genetic alternations with EGFR mutations

Other genetic alterations may occur together with EGFR mutation causing EGFR TKI resistance by preserving cell survival even with EGFR inhibition. These additional genetic alterations that promote EGFR pathway include:

Activation of phosphoinositide-3-kinase (PI3K)/AKT signaling

Phosphatase and tensine homolog (PTEN) acts as a tumor suppressor by negatively regulating the PI3K/AKT signaling pathway. In preclinical studies, loss of PTEN was associated with decreased sensitivity of EGFR mutant lung

tumors to EGFR TKI by increased activity of the PI3K-AKT pathway, and degradation of activated EGFR (29,30).

Somatic mutations in PIK3CA have been found in 1-3% of all NSCLC (31,32). These mutations usually occur within two "hotspot" areas within exon 9 (the helical domain) and exon 20 (the kinase domain). Preclinical data has shown that introduction of activating PIK3CA mutants into EGFR mutant lung cancer cell lines confers resistance to EGFR TKI (33).

Crosstalk with the IGF1R pathway

Resistance to EGFR TKI in cell lines with EGFR activating mutations through crosstalk with the IGF1R pathway has been observed through in preclinical models. For example, some EGFR-mutant cells undergo only G1 cell cycle phase arrest in the presence of erlotinib, but undergo apoptosis when co-treated with an IGF1R-specific antibody (34). In another study, EGFR mutant NSCLC cell lines persisting after EGFR TKI treatment were enriched for a drug-tolerant subpopulation that may have existed prior to treatment that showed a distinct chromatin state that is regulated by IGF1R signalling (35).

Activation of NFκB signaling

NFκB is a protein complex that controls the transcription of DNA. NFκB signaling has been associated with cancer and inflammation (36), and it has also been suggested that activation of NFκB signaling may cause primary resistance to EGFR TKI treatment in EGFR mutant lung cancer patients.

Bivona *et al*. used a cell line (H1650) with EGFR mutation but resistant to EGFR TKI and showed that inhibition of the NFκB pathway enhanced cell death by EGFR TKI whereas activation of NFκB rescued EGFR-mutant lung cancer cells from EGFR TKI treatment. Additionally, genetic or pharmacologic inhibition of NFκB enhanced erlotinib-induced apoptosis in erlotinib-sensitive and erlotinib-resistant EGFR-mutant lung cancer models, and increased expression of the NFκB inhibitor IκB, predicted for improved response and survival in EGFR-mutant lung cancer patients treated with EGFR TKI. Importantly, IκB status was not predictive of outcomes in EGFR mutant lung cancer patients treated with surgery or chemotherapy, indicating NFκB signaling is specific biomarker of EGFR TKI response in this patient population (37). These data identify NFκB as a potential drug target, together with EGFR, in EGFR-mutant lung cancers.

High BIM expression levels

BIM, also known as BCL2-like 11, is a proapoptotic protein that is overexpressed in different malignancies (38,39). Various chemotherapeutic agents use BIM as a mediating executioner of cell death. Hence, BIM suppression supports metastasis and chemoresistance. BIM upregulation is required for apoptosis induction by EGFR-TKIs in EGFR-mutant NSCLC. Low BIM mRNA levels could lead to gefitinib resistance in NSCLC with EGFR mutations and could be a marker of primary resistance. The extracellular regulated kinase (ERK) pathway also negatively regulates BIM expression in NSCLC with EGFR mutations (40-42). Components that cause induction of BIM may have a role to overcome resistance to EGFR TKI in NSCLC with EGFR mutations. Recent studies have showed that HDAC inhibition can epigenetically restore BIM function in vitro and death sensitivity of EGFR-TKI, in cases of EGFR mutant NSCLC where resistance to EGFR-TKI is associated with a common BIM polymorphism (43).

Treatment approaches to overcome primary resistance

For lung cancer patients harboring secondary alterations in EGFR, more effectively EGFR TKI is needed. Second-generation irreversible EGFR TKI have shown to be more active targeting T790M or EGFR exon 20 insertion mutation than gefitinib or erlotinib (44-46). Additionally, the Spanish Lung Cancer Group is conducting a phase Ib/IIb Study to evaluate the role of gefitinib in combination with olaparib in NSCLC patients with EGFR mutation to overcome primary resistance in those patients with high BRCA1 levels (NCT01513174). For lung cancer patients harboring other genetic alterations with EGFR mutation the use of polytherapy could overcome primary resistance. For example, a phase II trial of erlotinib and AT-101 (BCL-2 pan inhibitor) in NSCLC patients with EGFR mutations has been performed, although no results have been presented, yet (NCT00988169).

Additionally, a combination of an EGFR TKI with PI3K-AKT, IGFR, NFκB or BIM inhibitors could also play a role in those alterations co-occur causing EGFR TKI resistance.

Acquired resistance to EGFR TKI

Several mechanisms of acquired resistance to EGFR TKI in EGFR mutant NSCLC patients have been reported, which

could be grouped in four main categories: first, the presence of secondary mutations in EGFR; second, the presence by-pass tracks activation; third a phenotypic transformation; and fourth, additional genetic alternations that occur together with EGFR mutation. Up to 30% of cases are still unexplained.

Second-site mutations in EGFR

Approximately 50-60% of cases with acquired resistance to EGFR TKI therapy have a second-site mutation T790M ("gatekeeper mutation") in the kinase domain of EGFR that coexists with the EGFR activating mutation (21,47). Conversely to primary T790M mutation, acquired resistance by T790M mutation identifies a subset of EGFR-mutant lung cancers with indolent growth in preclinical (48) and clinical set (49).

The subclonal populations of EGFR mutant tumor cells with and without the EGFR T790M can coexist in an EGFR mutant NSCLC with acquired resistance to EGFR TKI. This heterogeneity would explain both the "flare" phenomenon (rapid tumor regrowth upon withdrawal of an EGFR TKI) observed upon discontinuation of an EGFR TKI and also the finding that EGFR mutant NSCLC patients may respond to subsequent EGFR TKI treatment after initial discontinuation of therapy (50-53).

In addition to EGFR T790M mutation, there are other mutations that have been associated with acquired EGFR TKI resistance: T854A in exon 21 (54), L747S (55), and D761Y (28), both in exon 19. However, the frequency of all such mutation appears to be very low in comparison with the T790M mutation.

By-pass tracks activation

Other mechanism of acquired resistance to EGFR TKI is the activation of parallel pathways in which the key downstream targets of EGFR are activated independently of EGFR. These mechanisms include MET amplification and HGF overexpression. Amplification of the receptor tyrosine kinase MET leads EGFR inhibitor resistance by causing phosphorylation of ERBB3, which in turn sustains the activation of the PI3K/Akt signal downstream, providing a bypass signalling even in the presence of EGFR inhibitor. MET amplification was detected in 22% of lung cancer specimens that developed acquired resistance to EGFR TKI and inhibition of both EGFR and MET was required to kill the resistant cells, suggesting a persistent oncogenic addiction to EGFR pathway beyond to acquired resistance to EGFR TKI (56-58). In the clinic, MET amplification was reported in 4% of patients. The prevalence of MET-dependent resistance may depend upon the assay used (59).

Although MET amplification can occur with the EGFR T790M mutation, about 60% of MET amplification is independent of T790M mutation. There is an inverse relationship between the presence of T790M and MET gene copy number, suggesting a complementary role of the two mechanisms in the acquisition of resistance. In preclinical models, MET inhibitors may be able to overcome MET-mediated resistance, even in cells that harbour the T790M mutation (60). Concurrent inhibition therapy might be essential for outcome improvement (61). MET activation by overexpression of its ligand, HGF, also induced drug resistance *in vitro* and *in vivo* through GAB1 signalling, which directly activates PI3K/Akt pathway (62). In patients with paired tumor specimens, HGF expression was higher in drug-resistant specimens than in the pretreatment specimens (P=0.025) (63) and in other study with 23 acquired resistance tumors, high-level HGF expression was detected in higher proportion than T790M mutation (62). Japanese patients with weak HGF expression by immunohistochemistry tend to have lower 5-year OS than those with overexpression (22.2% *vs.* 75%, P=0.259) (64). Of note, MET amplification has also been observed in EGFR mutant NSCLC patients prior to EGFR TKI and was associated with the development of acquired resistance to EGFR TKIs (60), suggesting that EGFR TKI may select for preexisting cells with MET amplification during the acquisition of EGFR TKI resistance.

Phenotypic transformation

This acquired resistance mechanism includes the histological transformation to small cell lung cancer (SCLC) and the epithelial to mesenchymal transition (EMT), with an incidence of 14% and 5%, respectively (58). These new SCLC retain the original EGFR-sensitizing mutation and respond to standard small cell carcinoma chemotherapy, but the exact mechanism for this histological transformation is unknown.

EMT is a phenomenon characterized in which the cancer cell looses its epithelial morphology and develops a more spindle-like mesenchymal morphology with often associated with a shift in expression of specific proteins (for example,

loss of E-cadherin and gain of vimentin) resulting in a more invasiveness phenotype (65). The exact mechanism for the acquisition of the EMT phenotype remains unclear; some studies have found an upregulation of NOTCH-1 expression (66), the aberrant expression of transforming growth factor (TGF)-β (67,68), and phosphorylation of MEK (69). Increased expression of E-cadherin, has been associated with clinical activity of EGFR TKI in NSCLC patients (70,71). EMT has been also associated with acquired resistance to EGFR TKI in preclinical models (65,71) as well as in several studies (58). It is unknown if mesenchymal-like cells in the acquired resistant tumors are exist prior to therapy or are induced upon drug treatment. It has been recently described that activation of the AXL receptor tyrosine kinase by overexpression or upregulation of its ligand GAS6 confers acquired resistance to EGFR TKI in preclinical models, and the inhibition of AXL restored erlotinib sensitivity. Upregulation of AXL was associated with the development of an EMT in EGFR mutant NSCLC with acquired resistance. Approximately 20% of the EGFR TKI resistant tumors showed increased AXL expression (72).

Additional genetic alternations

PIK3CA mutation

Mutation in PIK3CA was identified in 5% of EGFR mutant lung cancers that developed acquired EGFR TKI resistance as well as in preclinical models (58).

PTEN mutation

In preclinical models, loss of PTEN expression contributes to TKI resistance in NSCLC (73). Cells with knockdown of PTEN, with constitutive PI3KCA activation, have a deficient homologous recombinant DNA repair and increased sensitivity to cisplatin and PARP inhibitors (74).

HER2 amplification

HER2 amplification has been recently detected in 12% of tumors with acquired resistance to EGFR TKI, and only in 1% of untreated EGFR mutant NSCLC cells. This new mechanism of acquired resistance was exclusive with T790M mutation (75). Interestingly, in preclinical models the combination of afatinib plus cetuximab significantly inhibited HER2 phosphorylation. These results implicate HER2 as a novel protein involved in the sensitivity or resistance of EGFR mutant NSCLC providing a rationale to assess its status and target HER2 in such tumors.

MAPK1 amplification

MAPK1 amplification was described in approximately 5% of clinical specimens from patients with acquired resistance to EGFR TKI treatment and was mutually exclusive with the T90M mutation or MET amplification (76).

BRAF mutation

RAS pathway mutations are rare, but BRAF mutations (V600E, G469A) can occur in 1% of tumors with acquired resistance to EGFR TKI (77).

JAK2

In a preclinical cell line model, the activation of JAK2 (an upstream STAT signal pathway) caused acquired EGFR TKI resistance. Combined treatments of erlotinib plus a JAK2 inhibitor (JSI-124) restored sensitivity to erlotinib in PC-9/ERB3 cells and reduced tumors in a murine xenograft model (78).

IGFR

In vitro data showed that the increased IGF-1R signalling through the loss of IGF inhibitory proteins may also mediate resistance to EGFR TKI by activating downstream targets that bypass dependency in EGFR (79).

Loss of activating EGFR mutant gene

Loss of activating EGFR mutant gene contributes to acquire resistance to EGFR TKI in lung cancer cells. This loss of addiction to mutant EGFR resulted in gain of addiction to both HER2/HER3 and PI3K/AKT signalling to acquire EGFR TKI resistance (80).

Treatment approaches to overcome acquired resistance

Given this role of persistent EGFR signalling in causing resistance to TKI, a second generation irreversible EGFR TKI bind to a different EGFR tyrosine kinase domain have shown activity against lung cancer cells harboring both EGFR activation mutations and the T790M resistance-mutation (17,45,81,82). A phase III trial of afatinib versus placebo in patients with acquired resistance to EGFR TKI demonstrated a 2-month improvement in progression free survival; although no significant benefit in overall survival was observed (83).

A more recent strategy for intensification of EGFR inhibition has been the addition of monoclonal antibodies targeting EGFR, such as cetuximab. Combined treatment

Table 1 Summary of rebiopsy studies and the molecular and histological alterations

Rebiopsy studies	Mechanisms of resistance to EGFR inhibitors analyzed	Histological alterations in the resistant tumor
Arcilla *et al*.	Pretreatment *EGFR* mutation: 100%	
	T790M:	
	Standard sequencing: 49%	
	Fragment Analysis 53%	Not performed
	Combined standard and LNA-PCR/sequencing: 70%	
	MET amplification: 11%	
Sequist *et al*.	Pretreatment *EGFR* mutation: 100%	
	T790M: 49%	SCLC transformation: 14%
	MET amplification: 5%	
	PIK3CA mutation: 5%	ETM: 8%
	β-catenin mutations: 5% (all with T790M mut)	
Oxnard *et al*.	T790M: 62%	Not performed
Ohashi *et al*.	B-RAF: 1%	Not performed

SCLC, small cell lung cancer; EMT, Epithelial to mesenchymal transition

with afatinib and cetuximab induced regression in T790M transgenic murine and mice models with erlotinib resistant lung tumors (84). This synergistic activity has been confirmed in phase I/II clinical trial, with a response rate of 32% in heavily pre-treated population with T790M-positive and T790M-negative tumors and a median Progression free survival of 4.67 months (85). Erlotinib plus cetuximab has showed to overcome T790M-mediated drug resistance in preclinical data (86). However, this strategy did not show significant activity in a phase I/II trial in patients with acquired resistance to erlotinib (85). The new T790M specific inhibitor WZ-4002 is also under investigation, and has demonstrated to induce greater growth inhibition *in vitro* and *in vivo* against T790M than against WT EGFR (87). Indeed, the FLT3 inhibitor, an indolocarbazole compounds, is under investigation as potent and reversible inhibitor of EGFR T790M that spare wild-type EGFR in the context of T790M-mediated drug resistance in NSCLC (88).

Combined treatments of erlotinib plus therapies targeting compensatory pathways that lead to acquired EGFR TKI resistance may overcome resistance. The addition of a MET inhibitor may benefit those patients with EGFR mutant NSCLC and MET amplification. Antibodies targeting the MET ligand HGF (AMG102), MET itself (MetMAb), and small molecule inhibitors against MET are in clinical development. The combination of AXL inhibitors, such as XL880, MP-470 or SGI-7079, with an EGFR TKI is also a potential approach to overcome resistance associated with EMT (89).

Furthermore, inhibition of NOTCH-1 can be a novel strategy for the reversal of the EMT phenotype thereby potentially increasing therapeutic drug sensitivity to lung cancer cells. BEZ235, a dual inhibitor of PI3K and mTOR, would overcome EGFR-TKI resistance induced by HGF in an EGFR mutant lung cancer cell lines (90).

Finally, combination therapy with EGFR TKI and PI3KCA inhibitor, PARP inhibitors (in PTEN mutant patients), HER2 inhibitors, B-RAF inhibitors or IGFR inhibitors could have a therapeutic effect in tumors with acquired resistant to EGFR TKI by those mechanisms and some of them are being investigated in clinical trials (91).

Strategies to determine molecular basis of resistance to EGFR TKI in NSCLC with EGFR mutations

As commented previously, the biological basis underlying acquired EGFR TKI resistance is unknown in approximately 30% of patients. Some of these previously described mechanisms of resistance to EGFR TKI that have

been identified in preclinical models and have not been validated in patients with acquired resistance. The analysis of clinical specimens is crucial to discover the remaining unknown mechanisms of EGFR TKI resistance. In the last years many authors have published their own experience with rebiopsies on patients with EGFR mutant NSCLC at the time of progression in order to identify how EGFR mutant NSCLC acquire resistance to EGFR TKI (*Table 1*).

Arcila *et al*. undertook a rebiopsy study to determine the feasibility of rebiopsy in patients with EGFR mutant NSCLC with acquired resistance to EGFR TKI and to evaluate the spectrum of EGFR mutations and MET amplification in tumors at progression. One hundred and fifty three samples were obtained from 121 patients including frozen samples, fresh fluids, FFPE tissue and cytologies from fine needle aspirates (FNA); eighty-two per cent were successfully analyzed. Biopsies provided the highest success rate followed by FNA and pleural fluids. Pathologic confirmation was performed in 106 resistant tumors: one hundred and two adenocarcinomas, one squamous cell carcinoma, two small cell carcinomas and 1 with a mixed histology (combined large cell carcinoma/adenocarcinoma in one sample and a high grade neuroendocrine carcinoma in a second). EGFR mutations (exons 19 and 21) were found in 100/104 in resistant samples, seventy-one per cent had EGFR exon 19 deletions, one per cent had an insertion in exon 18 and 28% had an exon 21 point mutation. Of note, patients with multiple tissue sampling had the same mutation in all tumor sites, and all patients maintained the baseline sensitizing mutation. The T790M mutation was detected in 51% of mutant samples by standard analysis, and the retest of 30 negative patients by the LNA-PCR/sequencing method detected 11 additional mutants, raising the T790M mutation rate to 70%. MET amplification was found in 11% (4 patients), three of them also harbored the EGFR T790M mutation (57).

Sequist *et al*. performed rebiopsies on 37 EGFR mutant NSCLC patients with acquired resistance to identify the mechanisms of resistance to EGFR inhibitors. Pre- and post-EGFR TKI tumor samples were analyzed for the presence of genetic alterations with a genotyping platform (SNaPshot assay), and EGFR and MET amplification with fluorescence in situ hybridization (FISH). Eighteen (49%) patients acquired the T790M mutation, and two (5%) patients developed MET amplification, which was not present in the pretreatment specimen. Two (5%) patients showed acquired PIK3CA mutations, two (5%) cases had β-catenin mutations (together with the T790M mutation). Fifteen (41%) rebiopsies didn't reveal any new mutations. The authors also found significant histological alterations in the resistant tumor; five patients (14%) had a diagnosis of SCLC, all maintaining the original EGFR mutation. Additionally, three resistant specimens had phenotypic changes consistent with a mesenchymal, supporting an ETM, none showed another identified resistance mechanism while maintained their original EGFR mutation. Of note, EMT or SCLC were not observed in biopsies from EGFR wild-type tumors resistant to chemotherapy (58).

Interestingly, multiple biopsies over the course of the disease were performed in 3 patients showing gain and loss of the T790M mutation in multiple biopsies from the same anatomical location during the clinical course in two of them at time of progression or when de EGFR TKI was interrupted. The rebiopsy from the third patient showed SCLC transformation with the original EGFR L858R mutation plus an acquired PIK3CA mutation. However, those changes were not observed at progression to treatment for SCLC, where adenocarcinoma histology with EGFR L858R mutation was again demonstrated (58). These results explain why retreatment of NSCLC patients with EGFR TKI who had experienced favorable results from their initial treatment could benefit some patients (53,92).

Oxnard *et al*. performed a rebiopsy protocol in EGFR mutant lung cancer patients with acquired resistance to EGFR TKI comparing for the presence of the T790M. T790M was identified in 62% of patients in the rebiopsy specimens with longer survival after progression than patients without T790M (49,59).

Finally, Ohashi *et al*. systematically screened for recurrent mutations in *RAS/NRAS/BRAF/MEK1* in nearly 200 tumor samples from patients with acquired resistance to EGFR TKI. They found two BRAF mutations: one case with concurrent *EGFR* exon19 deletion and *EGFR* T790M and *BRAF* V600E mutations and another case with *EGFR* exon19 deletion and the *BRAF* G469A mutation (2/195, 1.0%). They studied further the biological and therapeutic consequences of acquired *NRAS* and *BRAF* mutations in *EGFR*-mutant lung tumor cells and showed that these tumor cells were resistant to erlotinib alone but were sensitive to combination treatment with EGFR and MEK inhibition (77).

There is no doubt that identifying the molecular mechanisms underlying variable response and resistance to EGFR TKI in EGFR mutant NSCLC is a major obstacle to optimize EGFR TKI therapy. A more comprehensive

analysis of clinical specimens from EGFR TKI-treated patients should offer a better knowledge about if known mechanisms of resistance occur exclusively and concomitantly to promote clinical resistance. This is a key issue to resolve because we will need to determine whether to target individual or multiple drivers of resistance with targeted therapies in patients according to their molecular alterations present in their tumors.

Additionally, multiple rebiopsy studies also suggest that genetic mechanisms of resistance are potentially reversible, and therefore, a static diagnostic biopsy may be insufficient to guide therapeutic decision making throughout the course of a patient's disease (58). To perform a rebiopsy at time of progression in EGFR mutant NSCLC patients is becoming more and more standard.

However, the underlying molecular basis of the heterogeneity in response to EGFR TKI has never been explored in patients immediately after initiation of therapy. This information would be crucial to study the early changes that can compromise response and progression and would help to uncover the molecular causes of treatment resistance and optimize the EGFR TKI therapy. Characterizing the complete molecular landscape of response to EGFR TKI in EGFR mutant NSCLC specimens from patients before and serially during treatment would reveal not only novel biomarkers of response to therapy but also potential new therapeutic targets to prevent or overcome resistance to EGFR TKI in NSCLC patients.

Summary

Several studies have showed that rebiopsy of EGFR mutant NSCLC patients with acquired resistance to EGFR TKI is feasible and provides sufficient material for mutation analysis in most patients. Interestingly, a wide heterogeneity in resistance mechanisms has been observed, each of which may require its own therapeutic strategy.

Indeed, it is becoming crucial the need of continuous assessment of each tumor evolution during the course of treatment not only to determine how it became resistant to therapy but also to allow us to design rational strategies to overcome resistance or to prevent acquired resistance in patients.

Since many patients do not undergo rebiopsy at progression, the lack of available resistant tumor tissue limits the molecular guided stratification of patients and negatively affects further investigation of acquired resistance. Of note, mechanisms of primary resistance are not usually analyzed in rebiopsy protocols in EGFR mutant NSCLC patients receiving EGFR TKI after the initiation of EGFR TKI which compromises a better understanding of how to prevent resistance to therapy.

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Footnote

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EGFR, EGFR TKI, and EMSI: a never-ending story

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Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib and erlotinib are the first generation of EGFR inhibitors that were developed more than a decade ago. Beginning with the disappointing results of phase III trials that combined EGFR TKIs with chemotherapy in unselected patients with non-small cell lung cancer (NSCLC) (IDEAL-I and IDEAL–II for gefitinib, TALENT and TRIBUTE for erlotinib), both TKIs had a history of ups (BR.21 for erlotinib, INTEREST for gefitinib) and downs (ISEL for gefitinib), until the recent success of pivotal studies comparing EGFR TKIs to doublet chemotherapy in patients with activating *EGFR* mutations (IPASS, NEJ002 and WJTOG3405 for gefitinib, OPTIMAL and EURTAC for erlotinib).

Nowadays, screening for *EGFR* mutations is mandatory prior to selecting a first-line treatment for stage IV adenocarcinoma of the lungs, as EGFR TKIs are the first choice of treatment for NSCLC with activating *EGFR* mutations. If activating mutations are detected on the *EGFR* gene, the disease and symptoms can be controlled by treatment with an EGFR TKI in more than 70% of cases. However, acquired resistance to EGFR TKIs is inevitable after a median response duration of 11 to 14 months.

Second generation EGFR TKIs (afatinib, dacomitinib) were developed to overcome the acquired resistance after the failure of 1st generation EGFR TKIs. However, the Lux lung 1 trial failed to demonstrate any improvement in the overall survival of patients in the afatinib arm compared to the placebo arm. As afatinib monotherapy was not sufficient to overcome resistance caused by *EGFR* T790M mutations in the clinical setting, trials combining afatinib with cetuximab are ongoing, although this combination has higher toxicity.

Subsequently, results of the BR.26 trial comparing dacomitinib—another second generation EGFR TKI—versus a placebo after the failure of prior EGFR TKI therapy were presented at the 2014 annual meeting of the American Society of Clinical Oncology (ASCO) (1). As with the afatinib therapy, there was no improvement in the overall survival of patients receiving dacomitinib, although the progression free survival improved.

Meanwhile, afatinib therapy was being studied as a potential first-line treatment for NSCLC with activating *EGFR* mutations. In the Lux lung 3 and 6 studies, afatinib was proved superior to doublet therapy with pemetrexed plus cisplatin or gemcitabine plus cisplatin in patients with NSCLC harboring activating *EGFR* mutations. As a result, gefitinib, erlotinib, and afatinib are currently the first-line treatment for NSCLC with activating *EGFR* mutations. The results of a study comparing gefitinib to afatinib therapy in patients with activating EGFR mutations are expected to be presented in next year. A similar study comparing the use of dacomitinib with gefitinib as first-line treatment is ongoing (ARCHER 1050).

No standard treatment exists for patients with lung cancer who experience disease progression after the use of 1st or 2nd generation EGFR TKIs. For this reason, the development of EGFR mutant selective inhibitors (EMSIs) effective against both EGFR TKI-sensitive and EGFR TKI-resistant (T790M) mutants is eagerly awaited. The EMSIs target not only *EGFR* T790M, the mutant form of EGFR that is associated with clinical resistance to EGFR TKIs, but also the initial activating EGFR mutants, including those with exon 19 deletions and L858R. They do so while sparing the wild-type EGFR, and may thus treat refractory NSCLC while minimizing side effects on skin

and mucosa. Because the EMSIs target both the sensitive activating mutations as well as the resistance mechanism (the T790M mutation), they have the potential to be used both as first-line treatment in NSCLC patients with EGFR activating mutations, and as second-line treatment in patients with acquired resistance.

There are various mechanisms of EGFR TKI resistance, such as the presence of the T790M mutation, c-Met amplification, activation of alternative pathways (Insulin-like growth factor 1, Hepatocyte growth factor, Phosphoinositide 3-kinase, AXL), and the transformation to mesenchymal cells or small cell features. Among these, the *EGFR* T790M mutation accounts for more than 60% of the EGFR TKI-resistant cases. As EMSIs have shown efficacy against *EGFR* T790M mutants in a selective manner, it has been suggested that EMSIs only have activity in T790M positive cases, while they have little efficacy against other resistance mechanisms such as the activation of alternative pathways or transformations.

In the 2014 ASCO meeting, three clinical studies examining the use of three different EMSIs were presented (2-4). As expected considering the mechanism of action, all three compounds showed that the therapeutic efficacy is particularly good in patients harboring the *EGFR* T790M mutation. Among the EMSIs presented, AZD-9291 showed the best response rate (64%) in T790M-positive cases when compared with CO-1686 (58%) and HM-61713 (30%).

The response rates of the EMSIs were much lower in T790M negative cases (HM-61713, 12%; AZD-9291, 23%), compared to T790M positive cases. These results suggest that resistance mechanisms that do not involve T790M mutations should be treated by using other strategies. For example, acquired resistance via bypass tract activation (i.e., the MET-HGF pathway) may be blocked in a better way by using a combination of monoclonal antibody targeting molecules of the bypass tract and EGFR TKIs.

It is important to note that there are few toxicities associated with the use of EMSIs compared to 1st and 2nd generation EGFR TKIs. While 1st and 2nd generation EGFR TKIs block both the mutant EGFR in the tumor and the wild-type EGFR in the skin and other organs, often leading to the appearance of debilitating skin rashes, acne, and diarrhea, EMSIs act mostly on the mutant EGFR within the tumor. In the case of AZD9291, no dose limiting toxicities were observed. The most common adverse events were diarrhea (30%), skin rashes (24%), and nausea (17%), all of which were classified as grade 1 under the Common Terminology Criteria for Adverse Events guidelines. Grade 3/4 adverse events occurred in 16% of patients. Six patients (3%) had dose reductions. Five cases of interstitial lung disease-like events are under investigation.

As EMSIs act mainly on T790M mutant cases, obtaining tumor DNA after the development of acquired resistance is going to be an essential prerequisite. However, a re-biopsy is not always easy to perform in those patients who have already been heavily treated for advanced NSCLC. Interestingly, liquid biopsy using circulating tumor DNA (ctDNA) is becoming available. In the IFUM study, the positive predictive value of mutations detected from ctDNA was very high (98.6%), although the sensitivity was 65.7% (5). Along with the clinical development of EMSIs, companion diagnostic methods are being investigated. For example, for the detection of *EGFR* T790M mutations from ctDNA, digital PCR-based (dPCR) approaches using BioRad ddPCR (MolecularMD) of BEAMing (Inostics) were superior to ARMS-based detection using Roche Cobas and Qiagen Therascreen *EGFR* mutation detection kits (6).

Further studies need to address several issues. Firstly, should we halt the use of EMSIs until after the development of resistance to 1st or 2nd generation EGFR TKIs? Alternatively, should we use the EMSIs as a first-line treatment for NSCLC with activating *EGFR* mutations? To address these questions, the efficacy of EMSIs in EGFR TKI naïve patients is currently under investigation. Furthermore, the direct comparison of EMSIs with 1st or 2nd generation EGFR TKIs as first-line treatment for NSCLC harboring activating *EGFR* mutations will answer this question. Secondly, the development of sensitive, specific diagnostic techniques to detect the mechanisms of acquired resistance should be accompanied by the development of EMSIs. Lastly, clinical resistance to EMSIs is likely to develop eventually. Thus, further research to combat the acquired resistance to EMSIs will be needed, which is why this is going to be a never-ending story.

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Footnote

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Advances on EGFR mutation for lung cancer

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Abstract: Patients with advanced non-small-cell lung cancer (NSCLC) and somatic activating mutations of the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR) gene represent a biologically distinct disease entity that shows exquisite sensitivity to the reversible EGFR-TK inhibitors (-TKIs) gefitinib or erlotinib. Phase III randomized studies have clearly demonstrated that a reversible EGFR-TKI is significantly superior in terms of response rate, progression-free survival and quality of life to platinum-based chemotherapy in advanced NSCLC patients who carry an activating EGFR mutation, thus resulting into a new standard of care for this biologically selected group of patients. Unfortunately, approximately one third of EGFR-mutated patients show primary resistance to gefitinib or erlotinib, whereas virtually all patients who initially benefit from treatment will eventually develop acquired resistance. Importantly, revealing the molecular mechanisms that underlie resistance to reversible EGFR-TKIs is key to the development of EGFR-targeting strategies with the potential to prevent, delay or overcome such resistance. Early results of clinical trials with irreversible EGFR-TKIs or dual combination strategies aiming to block EGFR-mediated signaling at different levels have shown encouraging results in EGFR-mutated patients pretreated or not with a reversible EGFR-TKI. Therefore, in the near future it is reasonable to hypothesize that EGFR-mutated NSCLCs could be treated with multiple lines of EGFR-targeting therapies beyond disease progression, limiting chemotherapy to selected cases of resistant disease. This evolving treatment scenario highlights once again how important is the identification of a single oncogenic "addiction" that functions as unique determinant of progression and survival of NSCLC.

Keywords: Epidermal growth factor receptor mutation; gefitinib-erlotinib; non-small cell lung cancer; tyrosine kinase inhibitors

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Introduction

Lung cancer is among the most commonly diagnosed cancers worldwide, representing the first cause of cancer-related death in both the U.S. and Europe (1,2). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers, being often diagnosed at an advanced stage when treatment options are limited. First-line chemotherapy for NSCLC patients with advanced disease is generally platinum-based, yielding a median overall survival of 8-11 months (3). Unfortunately, the addition of a targeted agent to a platinum-based chemotherapy backbone either in combination regimens and or as sequential treatment has only marginally improved overall prognosis of patients with advanced disease (4-6). Against this background, the recent recognition that certain genetic abnormalities play a major role in the oncogenic process of NSCLC, has allowed in some cases for appropriate selection of patients candidate to targeted therapies based on well-defined biological characteristics (7,8).

EGFR as a target in NSCLC

Since its identification in 1986, the epidermal growth factor receptor (EGFR) has emerged as a crucial factor for the development and growth of human malignancies, including lung cancer (9). In fact, EGFR signal transduction network

plays an important role in multiple tumorigenic processes such as proliferation of cancer cells, angiogenesis, and metastasization. Consistently, EGFR aberrant activation has been shown to be prognostic in NSCLC, which provided a solid rationale for the development of EGFR-targeting strategies for NSCLC (10).

EGFR belongs to the Erb family of transmembrane receptor tyrosine kinases which includes also HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Upon ligand binding, EGFR undergoes homo- or hetero-dimerization with other receptors of the same family with subsequent autophosphorylation and activation of the intracellular tyrosine-kinase (TK) domain, recruitment of second messengers and intensification of the anti-apoptotic signaling (11). Interestingly, no ligand has been identified for the HER2 orphan receptor while no kinase activity has been documented for HER3, which allow both HER2 and HER3 to be actively involved in EGFR-mediated signaling as preferred hetero-dimerization partners of EGFR itself. There are several ways through which EGFR can be aberrantly activated including receptor overexpression, gene amplification and gene mutation (10). However, because of its crucial role as oncogenic determinant, the presence of an activating (meaning ligand-independent activation of the TK) *EGFR* mutation in NSCLC carries major therapeutic implications. The present review will focus on the most recent acknowledgements on *EGFR* gene mutations in NSCLC, also discussing their potential applicabilities in the clinic.

EGFR gene mutations in NSCLC

In 2004, the identification of somatic mutations of the *EGFR* gene in NSCLC has led to the recognition of a biologically distinct disease entity which has been termed 'oncogene addicted' to reflect its dependence on EGFR-mediated pro-survival signalling (12-14). Consistently, *EGFR*-mutated NSCLC patients represents a subgroup which seems to experience a more indolent course of disease irrespective of treatment (15,16). However, the clinical relevance of detecting an activating *EGFR* mutation in NSCLC as assessed by DNA gene sequencing cannot be understated given the exquisite sensitivity that *EGFR*-mutated NSCLCs show to the 'reversible' EGFR-TK inhibitors (-TKIs) gefitinib or erlotinib (to which we will also refer to as 'first-generation' EGFR-TKIs) (7), which allows patients to experience a particularly extended survival in the presence of EGFR-TKI treatment, thus in contrast with the historical data reported for NSCLCs when considered as a single disease entity (3).

Importantly, alhough the incidence of *EGFR* mutations is higher in patients with certain clinical characteristics such as never smoking history, Asian ethnicity (where they can be found in up to 30% of advanced NSCLCs as opposed to 15% for the western population), female sex and adenocarcinoma histology (17), it is not possible to rule out the possibility of an *EGFR* mutation solely on the basis of clinical characteristics (18-22). This concept is the basis for testing for an *EGFR* mutation all NSCLC tissues (preferentially adenocarcinoma) irrespective of clinical chaacteristics in order not to exclude from a very active targeted treatment patients who are discovered to carry an *EGFR* mutation.

Specific activating *EGFR* mutations are either short, in-frame nucleotide deletions, in-frame duplications/insertions or single-nucleotide substitutions clustered around the adenosine triphosphate (ATP) binding pocket of the TK domain (23). To date, in-frame deletions in exon 19 around the LeuArgGluAla motif (del19) at residues 746-750 (the most common being del E746_A750) and exon 21 Leu858Arg (L858R) point mutation are the best characterized mutations, together representing 85-90% of all *EGFR* mutations in NSCLC (23). The frequency of classic *EGFR* mutations seem to differ according to ethnic backgrounds. In fact, EGFR genotyping from large prospective studies have shown a higher frequency of del19 mutation compared with L858R for European patients (18,22), whereas the incidence of del19 mutation appear to be only slightly superior in Asiatic patients (19-21). Interestingly, clinical data seem to indicate that patients harboring the del19 mutation are more susceptible to the activity of a reversible EGFR-TKI compared to those carrying the L858R mutation (24). However, the molecular mechanisms underlying this apparent inter-mutation discrepancy in drug sensitivity are not clearly understood, possibly being related to a higher EGFR-dependence of the tumor owing to common association of del 19 mutations with EGFR amplification (25). Moreover, it cannot be excluded that gefitinib or erlotinib possess a different inhibitory effect on del19 mutation favoring tha erlotinib, as suggested by biochemical studies (26).

Nevertheless, activating *EGFR* mutations other than del19 or L858R have been described, usually defined as 'other uncommon mutation'. However, their ability to predict sensitivity to a reversible EGFR-TKI is less striking compared with del 19 or L858R mutations. A recent report exploring the sensitivity of uncommon *EGFR* mutations to gefitinib or erlotinib showed that two types of uncommon *EGFR* mutations, namely point mutations in position Gly719 of exon 18 (G719) and Leu861Gln mutation in exon 21 (L861) may

Table 1 Phase III studies comparing gefitinib or erlotinib versus a standard platinum-based doublet in the first line treatment of advanced NSCLC patients selected based on the presence of EGFR mutation

Study	EGFR-TKI	No. of patients	Type of EGFR mutation	Population	RR (EGFR-TKI *vs.* chemotherapy)	PFS (EGFR-TKI *vs.* chemotherapy)	OS (EGFR-TKI *vs.* chemotherapy)	Quality of life (EGFR-TKI *vs.* chemotherapy)
WJTOG 3405 (19)	Gefitinib	172	del19 or L858R	Asiatic	62.1% *vs.* 32.2%[†] P<0.0001	9.2 *vs.* 6.3 months P<0.0001	Not available	Not assessed
NEJ002 (20,34)	Gefitinib	228	Any[‡]	Asiatic	73.7% *vs.* 30.7% P<0.001	10.4 *vs.* 5.5 months P<0.001	30.5 *vs.* 23.6 months P=0.31	Significant less deterioration[§]
OPTIMAL (21)	Erlotinib	154	del19 or L858R	Asiatic	83% *vs.* 36% P<0.0001	13.1 *vs.* 4.6 months P<0.0001	Not available	Significant improvement[¶]
EURTAC (22)	Erlotinib	173	del19 or L858R	Caucasian	58% *vs.* 15% P<0.05	9.7 *vs.* 5.2 months P<0.0001	Not available	Not available

EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; No., number; OS, overall survival; PFS, progression-free survival; RR, response rare. [†]Only patients with measurable disease considered. [‡]Excluded the T790M resistant mutation. [§]As assessed by a Care Notebook (QOL Res 2005, http://homepage3.nifty.com/care-notebook/) questionnaire. [¶]As assessed with the Functional Assessment of Cancer Therapy-Lung (FACT-L) questionnaire and the Lung Cancer Subscale (LCS)

have unaltered sensitivity to a reversible EGFR-TKI, being associated with clinical responses in apporximately half of cases (27). On the other hand, exon 20 insertions have been associated with primary resistance to EGFR-TKIs (28). However, owing to their rarity, it is not possible to draw definitive conclusions on the true relationship between uncommon *EGFR* mutations and sensitivity to gefitinib or erlotinib and even case reports may orientate in the decision making process of patients with uncommon activating mutations of the *EGFR* gene (29).

EGFR gene mutations and sensitivity to gefitinib or erlotinib

Gefitinib or erlotinib are orally bioavailable anilinoquinazoline small molecules that act by selectively and reversibly blocking the phosphorylation of the EGFR-TK domain through competition with ATP for binding at the active site of EGFR itself (30). Early phase III studies comparing gefitinib or erlotinib to placebo in chemotherapy pretreated NSCLCs showed a survival improvement for individuals receiving the EGFR-TKI (31,32) which, in case of gefitinib, was statistically significant only for patients with certain clinical characteristics such as never-smoking history and Asian ethnicity (31). However, only one of these two trials, namely the BR.21 study, showed for erlotinib a statistically significant improvement in overall survvial (OS) for the whole population (6.7 versus 4.7 months, respectively, HR=0.70, P<0.001). Therefore, based on these data, erlotinib was granted approval by American and European regulatory agencies for use as second or third-line therapy after failure of cytotoxic chemotherapy.

Nevertheless, since their identification, activating *EGFR* gene mutations have emerged as the most important predictor of response to reversible EGFR-TKIs (12-14). From that moment on, several retrospective and prospective studies confirmed that patients carrying an *EGFR* mutation were particularly sensitive to a first-generation EGFR-TKI, with responses observed in up to 90% of cases (33). Recently, four large phase III trials comparing a reversible EGFR-TKI to standard platinum-based chemotherapy in untreated advanced NSCLCs biologically selected for the presence of an activating *EGFR* mutation clearly stated the superiority of gefitinib or erlotinib over chemotherapy in terms of response rates (RR) and progression-free survival (PFS) (*Table 1*) (19-22). Also, as expected, gefitinib and erlotinib were associated with a significantly lower incidence of grade ≥3 adverse events. Notably, the fact that OS was not statistically in favor of gefitinib or erlotinib does not come as a surprise given the high rate of cross-over to an EGFR-TKI in the experimental arm at the time of disease progression. In addition, the particularly long median survival (>24 months) experienced by *EGFR*-mutated patients treated with a reversible EGFR-TKI might have led to miscalculation of the optimal sample size required to detect a statistically significant difference in survival (35).

At the present time, no head-to-head randomized comparison exists between gefitinib and erlotinib for *EGFR*-

Table 2 Known mechanisms of either primary or acquired resistance to gefitinib or erlotinib in advanced NSCLC patients with activating EGFR gene mutations

Mechanisms of resistance	
Primary	Acquired
Exon 20 insertions (42,43)	Secondary T790M mutation (44-47)
T790M mutation (51,52)	Non-T790M secondary EGFR mutation (46,53,54)
HGF overexpression (56)	MET gene amplification (47,55)
	PI3KCA mutation (57)
	Histologic change from NSCLC to SCLC (57)
	HGF overexpression (56,59)
	IGF-1R hyperphosphorylation (60)

EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; IGF-1R, nsulin-like growth factor-1; NSCLC, non-small cell lung cancer; PI3KCA, phosphatydil inositol 3-kinase catalytic subunit; SCLC, small cell lung cancer

mutated advanced NSCLCs. However, although preclinical studies have shown a differential sensitivity to gefitinib or erlotinib according to the type of activating *EGFR* mutation expressed by the tumor (del19 or L858R) (26), indirect evidence suggests that it is unlikely that this difference would translate into a clinically meaningful benefit in favor of one of the two agent (*Table 1*) (19-22). Interestingly, a recent randomized phase III study compared a new reversible EGFR-TKI, icotinib, to gefitinib in chemotherapy pretreated advanced NSCLCs showing comparable efficacy in the *EGFR*-mutated subgroup of patients (36).

Importantly, if *EGFR*-mutated patients benefit much from first-line treatment with gefitinib or erlotinib, the replacement of chemotherapy with a reversible EGFR-TKI as front-line therapy in biologically unselected patients with unknown *EGFR* mutation status is associated with a worse clinical outcome in terms of both PFS and OS (37,38). Moreover, selection of patients candidate to gefitinib or erlotinib according to clinical characteristics known to be associated with enrichment for the presence of an activating *EGFR* mutation is per se not sufficient to identify individuals who benefit the most from up-front therapy with a reversible EGFR-TKI (39,40). This question was matter of the IPASS and First-SIGNAL trials in which gefitinib was compared with standard chemotherapy in East-Asian advanced NSCLC patients with adenocarcinoma histology who were only (First-SIGNAL) or mostly (IPASS) never smokers. Although in both studies gefitinib was associated with a significant improvement in the primary PFS endpoint (HR=0.74, P<0.0001 and HR=0.81, P=0.044, for IPASS and First-Signal respectively), this benefit was shown to be driven by the high proportion of *EGFR*-mutated patients present in the studies population, since the analysis of EGFR wild type patients showed a significantly longer PFS in favor of chemotherapy.

These data strongly support the use of a reversible EGFR-TKI in *EGFR*-mutated advanced NSCLC patients and allowed recent approval of gefitinib by the European Medicines Agency with this indication. As for erlotinib, it is likely that its current indication will soon be extended to include also treatment-naïve patients with activating *EGFR* mutations.

Mechanisms of resistance to gefitinib or erlotinb

Unfortunately, approximately 20% to 30% of *EGFR*-mutated patients do not undergo tumor shrinkage on a first generation EGFR-TKI (19-22,39,40). Moreover, virtually all *EGFR*-mutated patients who initially benefit from gefitinib or erlotinib eventually develop progressive disease, usually after approximately a year since treatment initiation. Since no standard treatment exists for *EGFR*-mutated patients who progress while on a reversible EGFR-TKI, strict criteria for definition of acquired resistance have been proposed for better interpretation of clinical trials investigating novel agents in this setting (41). Against this background, the identification of the molecular mechanisms that underlie either primary or acquired resistance to gefitinib or erlotinib is of crucial importance in order to prevent, delay or overcome resistance to treatment. To date, a few mechanisms of resistance to reversible EGFR-TKIs have been identified (*Table 2*). Preclinically, primary resistance has been associated with in-

frame insertion mutations in exon 20 (42). Consistent with these data, most patients with tumors harboring exon 20 insertions have been shown to be resistant to gefitinib (43). As for acquired resistance, in approximately 50% of patients this can be attributed to the occurrence of a secondary threonine-to-metionine missense mutation in codon 790 (T790M) in exon 20 of the *EGFR* gene, which is located in the "critical" catalytic region of the ATP binding pocket of the EGFR-TK domain (44-47). The way through which the T790M mutation induce resistance to gefitinib or erlotinib is thought to be due to an increased binding affinity between EGFR and ATP rather than to a decreased affinity between EGFR and EGFR-TKI (48). Nevertheless, recent evidence suggests that the T790M mutation might pre-exist in minor clones in almost all reported cases of T790M-related acquired resistance, becoming evident during exposure to a reversible EGFR-TKIs as a result of evolutionary selection during treatment (49). Importantly, a poorer clinical outcome is usually experienced by patients with pre-treatment T790M compared with those without it (49). However, an interesting prospective clinical study suggested that *EGFR*-mutated patients with T790M-related acquired resistance may have a more favorable prognosis as opposed to non-T790M resistant patients, which might have important clinical implications for the design of clinical trials in this setting (50). Notably, although extremely rare, T790M mutations may exist as major clones irrespective of EGFR-TKI administration in certain patients, thus being implicated also in primary resistance (51,52). More recently, three other less common secondary mutations have been identified as 'de novo' alterations in patients with acquired resistance to first generation EGFR-TKIs, namely the D761Y (exon 19), L747S (exon 19) and T854A (exon 21) mutations (46,53,54).

On the other hand, amplification of the MET proto-oncogene, which encodes a transmembrane TK receptor for the hepatocyte growth factor (HGF) and is involved with invasion, metastasis and angiogenesis in tumors, has been implicated in approximately 20% of the cases of acquired resistance to gefitinib and erlotinib (47,55). MET amplification causes resistance through activation of HER3, which in turn sustains the activity of the phosphatydilinositol 3-kinase (PI3K)/Akt downstream signalling pathway (47). Therefore, even with gefitinib inhibiting the phosphorylation of HER3 by EGFR, the proliferation signal is not inhibited because of the maintenance of the phosphorylation of HER3 by MET. Interestingly, similarly to the T790M mutation, MET gene amplification might be the result of selection of minor clones of pre-existing MET amplified tumor cells becoming dominant during exposure to an EGFR-TKI (56). Occasionally, resistant tumors with MET amplification may have a concurrent secondary T790M mutation (50,55).

A recent study identified mutations in the catalytic subunit of PI3K and phenotipic change into small cell lung cancer (SCLC) as two other mechanism of acquired resistance to reversible EGFR-TKIs in *EGFR*-mutated patients (57). Intriguingly, the latter mechanism might have important clinical implications since it implies that a rebiopsy at the time of progression would result into significant change in disease management. However, it is still not known whether this phenotipic change reflects the selection of a population of SCLC from a histologically mixed tumor following eradication of the majority of NSCLC clones. Even more intriguingly, *EGFR* mutations are maintained in SCLCs arising in EGFR-TKIs resistant patients, although the relevance of this phenomenon is uncertain given that *EGFR*-mutated SCLCs do not seem to be addicted to EGFR pro-survival signalling (58).

Finally, HGF overexpression has been advocated as another possible mechanism of acquired resistance (56,59), probably acting by inducing downstream signal activation independently of HER3 or EGFR (59). Notably, HGF overexpression is likely to be implicated also in primary resistance to a reversible EGFR-TKI in patients with activating *EGFR* gene mutations (56).

In conclusion, these proposed mechanism of resistance, stronglly encorouge the use in the clinic of certain strategies to prevent/overcome resistance to reversible EGFR-TKIs in advanced NSCLC patients with activating *EGFR* gene mutations. Among these, the use of irreversible EGFR-TKIs or combination regimens of an EGFR-TKI with a MET-inhibitor appear to be the most appealling ones.

Irreversible EGFR-TKIs

Similarly to gefitinib or erlotinib, irreversible EGFR-TKIs are anilinoquinazoline inhibitors that, however, unlike them, irreversibly bind EGFR to the amino acid position 797 which enables blockade of EGFR kinase activity even in the presence of an EGFR T790M mutation (61-63). In addition to irreversible binding, simultaneous blockade of two or more members of the EGFR family represents another key feature through which these agents might prove clinically active in delaying/preventing resistance to first-generation EGFR-TKIs.

The dual irreversible EGFR/HER2 inhibitor afatinib (BIBW 2992) is among the most promising drugs for use in the setting of gefitinib- or erlotinib-resistant NSCLCs. Recently, a large randomized phase IIb/III trial comparing afatinib versus placebo was conducted in advanced

adenocarcinomas of the lung who had progressed after ≤2 lines of chemotherapy (including at least one platinum-based regimen) and ≥12 weeks of treatment with gefitinib or erlotinib (64). Interestingly, afatinib showed signs of activity by significantly prolonging PFS over placebo in this population of patients with clinically acquired resistance to a reversible EGFR-TKI (3.3 versus 1.1 months, respectively, HR=0.38, P<0.0001) (64). More importantly, this benefit was particularly evident when the analysis was restricted to key subgroup populations that were likely to be enriched for the presence of *EGFR* mutations such as those who had experienced prior response or treatment duration ≥48 weeks with a reversible EGFR-TKI (4.4 vsersus 1.0 month, respectively, HR=0.28) (65).

More recently, afatinib was tested in combination with the anti-EGFR monoclonal antibody cetuximab, based on the solid preclinical background that this combination would overcome resistance to gefitinib or erlotinib in *EGFR*-mutated NSCLCs (66,67). Crucial prerequsites for trial participation were the presence of *EGFR*-mutated tumors with clinically acquired resistance to gefitinib or erlotinib (stable disease ≥6 months or prior response to gefitinib or erlotinib) and acquisition of tumor tissue at baseline for molecular analysis. Of note, out of the 47 patients so far enrolled, the afatinib/cetuximab combination reported a RR of 40% with an overall disease control rate (RR + stable disease) of 92% (67). Improtantly treatment activity seemed to be independent of the presence of the T790M mutation.

Current areas of research of afatinib in advanced NSCLC include its use in *EGFR*-mutated and gefitinib or erlotinib-naïve patients where a RR of 61% with an outstanding median PFS of 14 months was observed in a recently conducted phase II study (68). Also, two relevant phase III studies are currently being run in order to compare afatinib with platinum-based chemotherapy in *EGFR*-mutated advanced adenocarcinomas of the lung (33).

Dacomitinib (PF-00299804) is another irreversible EGFR-TKI under clinical testing for advanced NSCLC, which acts also as inhibitor of other EGFR family memebers, namely HER2 and HER4. As monotherapy in clinically (adenocarcinoma, never or light smokers) or biologically (presence of *EGFR* mutation) selected treatment-naïve advanced NSCLC patients it showed a RR of 45% (69). More importantly, in a subset analysis of 29 evaluable patients with EGFR-mutation positive disease, 51% of responses were observed, including one case of exon 20 insertion, and some degree of tumor shrinkage was observed overall in >90% of *EGFR*-mutated patients (69).

Currently a double-blind, randomized phase III study is being conducted in chemotherapy-pretreated advanced NSCLC to compare dacomitinib to erlotinib, the primary endpoind being PFS (70). Notably, collection of tissue samples is mandatory for study inclusion, this in order to molecularly characterized whether exists a group of patients (iEGFR mutated or not) who derive more benefit from dacomitinib than from erlotinib.

MET-inhibitors

Importantly, because MET amplification and T790M mutation often occur in the same patient, probably the best strategy is to combine a second-generation irreversible EGFR-TKIs with MET inhibitors. Preclinically, in MET amplified NSCLC cell lines treatment resistance could be suppressed by the addition of erlotinib to a MET inhibitor (71). There are several ways to inhibit the MET signaling pathway, including anti-MET antibodies, inactivation of MET ligand, namely the hepatocyte growth factor (HGF) or inhibition of MET kinase activity. Currently, the anti-MET monoclonal antibody MetMab and the MET-TKI tivantinib have been tested in randomized phase II studies of chemotherapy pretreated advanced NSCLCs, which were hypothesis-generating for identifying biomarkers of sensitivity to MET inhibition such as MET expression by immunohistochemistry and MET gene copy number as assessed by fluorescence in situ hybridizaton (72,73). However none of the ongoing studies with these agents has been thought for the *EGFR*-mutated NSCLC population undergoing resistance to a reversible EGFR-TKI.

Conclusions

EGFR-mutated NSCLC is a totally distinct disease entity whose EGFR "addiction" is maintained despite progression and/or prior exposure to a first-generation EGFR-TKIs (67,74,75). Therefore, therapeutic advances beyond gefitinib and erlotinib should keep focusing on EGFR blockade, possibly by means of revealing novel mechanisms of EGFR-interference or biological combinations of EGFR-targeting agents. Future scenarios include the possibility to develop therapeutic strategies that can delay further the onset of treatment resistance to EGFR-TKIs such as covalent pyrimidine EGFR inhibitors (76). These agents are 30 to 100-fold more potent against EGFR T790M, and up to 100-fold less potent against EGFR wild type, thus possibly resulting in greater efficacy and better tolerability compared with quinazoline-based inhibitors such as gefitinib, erlotinib

or afatinib. To conclude, in recent years the rapid clinical development of EGFR targeting drugs for *EGFR*-mutated NSCLC represents a proof of concept of how important can be the discovery of a target to which the tumor is addicted for proliferation and survival. Against this background only rationally designed clinical trials can help research move faster toward a personalized therapeutic approach based on patinets biological characteristics.

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Footnote

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Kinase inhibitor-responsive genotypes in *EGFR* mutated lung adenocarcinomas: moving past common point mutations or indels into uncommon kinase domain duplications and rearrangements

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Abstract: The most frequent *epidermal growth factor receptor* (*EGFR*) mutations found by traditional or comprehensive molecular profiling of lung adenocarcinomas include indels of exon 19 (the exon 19 deletion delE746_A750 being the most common) and the exon 21 L858R point mutation. The current approval labels for first line palliative gefitinib 250 mg/day, erlotinib 150 mg/day and afatinib 40 mg/day for advanced lung cancers require the presence of the aforementioned classical/sensitizing *EGFR* mutations. Other gefitinib, erlotinib and afatinib sensitizing mutations include exon 18 indels, G719X, exon 19 insertions, A763_Y764insFQEA, S768I and L861Q; for which off-label EGFR kinase inhibitor use is generally agreed upon by thoracic oncologists. The main biological mechanism of resistance to approved first line EGFR inhibitors is the selection/acquisition of *EGFR*-T790M that in itself can be inhibited by osimertinib 80 mg/day, a 3rd generation EGFR inhibitor that is bypassed by *EGFR*-C797X mutations. Another class of *de novo* inhibitor insensitive mutation includes *EGFR* exon 20 insertions. More recently, the dichotomy of only point mutations or indels explaining aberrant kinase activation of EGFR plus inhibitor response has been shattered by the discovery of uncommon (<0.5% of all *EGFR* mutations) genomic events involving exon 18–25 kinase domain duplications (KDD) and rearrangements (*EGFR-RAD51* or *EGFR-PURB*). The latter lead to oncogene addiction, enhanced sensitivity to kinase inhibitors *in vitro* and clinical responses to approved EGFR inhibitors. The enhanced landscape of EGFR inhibitor-responsive genotypes highlights that comprehensive molecular profiling may be necessary to maximize the identification of all cases that can benefit from precision oncology.

Keywords: Epidermal growth factor receptor (EGFR); exon 18–25 duplication; rearrangement; exon 19; L858R; L861Q; G719X; exon 18; exon 20; T790M; C797S

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Epidermal growth factor receptor (*EGFR*) mutations were first identified as driver oncogenes in non-small-cell lung cancers (NSCLCs) in 2004 by three separate independent groups (1-3), and originally thought to consistent of only in-frame deletions, insertions (i.e., indels) or point mutations within exons 18 to 21 of the kinase domain of *EGFR* (4). The most abundant *EGFR* mutations are deletions/indels (around amino-acid residues 747 to 752) of exon 19 (these account for ~45% of all *EGFR* mutations, with the most common delE746_A750) and the exon 21 point mutation L858R mutation (~35% of all *EGFR* mutations). Inhibition of mutant EGFR in preclinical models through tyrosine kinase inhibitors (TKIs) unsettles the intracellular signaling cascade, generating cell cycle arrest and apoptosis (5). In the clinic, the 1st generation EGFR TKIs gefitinib and erlotinib, both reversible ATP mimetics with a favorable therapeutic window in relation to the wild-type (WT) EGFR (4,6), induce overall response rate (ORR),

progression-free survival (PFS) and quality of life (QoL) improvements that exceed platinum-doublet cytotoxic chemotherapies in advanced *EGFR* mutated NSCLCs (7,8). The 2nd generation irreversible EGFR TKI afatinib, with a narrower therapeutic window due to its exceedingly potent inhibition of WT EGFR, also improves ORR, PFS and QoL when compared to cytotoxic agents (9). Exceedingly high ORRs of >70% have been observed for *EGFR*-exon 19 deletion mutated NSCLCs treated with gefitinib 250 mg/day, erlotinib 150 mg/day or afatinib 40 mg/day (7-9). The ORR of *EGFR*-L858R mutated tumors seems to be slightly lower than 70% with afatinib 40 mg/day, while only at around 50–60% with gefitinib 250 mg/day and intermediate with erlotinib 150 mg/day (7-9). Indeed, a head-to-head phase II trial (LUX-Lung7) of afatinib 40 mg/day versus gefitinib 250 mg/day showed that the ORRs were 66% *vs*. 42% and median PFSs of 10.9 *vs*. 10.8 months (HR 0.71), respectively, for the 133 *EGFR*-L858R mutated NSCLCs (10). The ORRs were 73% *vs*. 66% and median PFSs of 12.7 *vs*. 11.0 months (HR 0.71), respectively, for the 186 *EGFR*-exon 19 deletion mutated NSCLCs (10). The improved predictive and prognostic impact of tumor *EGFR*-exon 19 deletions versus *EGFR*-L858R in TKI-treated patients are well known since 2006 (11,12) and confirmed in all randomized clinical trials of EGFR TKI versus chemotherapy (13). All three—gefitinib, erlotinib and afatinib—Food and Drug Administration (FDA) approved EGFR TKIs continue to be prescribed worldwide without a clear "go-to" drug in view of their different biological doses, toxicities (afatinib with higher rates of mucositis and diarrhea, erlotinib of rash, and gefitinib of liver dysfunction) and provider-patient preferences. As afatinib is the more toxic of the approved first line EGFR TKIs, one must take into consideration its reported higher ORR and PFS rates together with the increased rates of adverse events plus dose reductions required with this agent (9,10).

The third most common type of *EGFR* mutations (>7% of all *EGFR* mutations) consist of in-frame insertions and indels following/encompassing the regulatory C-helix amino-acids of exon 20 (14,15). In preclinical models, these mutations lead to auto-phosphorylation of EGFR and engagement of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinases (PI3K) cascades; concurrent with oncogene addiction (15). However, these mutant EGFRs at the structural and biological level do not have a favorable therapeutic window in relation to WT EGFR. The later realization explains why gefitinib (16), erlotinib (15) and afatinib (17) have limited activity (near 0% ORRs and short PFSs) in *EGFR* exon 20 insertion mutated NSCLCs (14). Grippingly, near identical exon 20 insertion mutations can be found on the *erb-b2 receptor tyrosine kinase 2* (*ERBB2*) gene and the resulting encoded proteins are also not particularly sensitive to standard dosing schemes of dual EGFR/ERBB2 TKIs (18). The development of TKIs for these recalcitrant variants in EGFR and ERBB2 continues to be an unmet medical need for the management of NSCLC.

Certain other clinically-relevant kinase domain *EGFR* mutations, named by others as uncommon or atypical mutations, seem to be EGFR TKI sensitive in preclinical models (where they are transforming and activate the MAPK/PI3K signaling cascades) and in available published clinical reports (4,16,17). These mutations encompass *EGFR*-exon 18 indels/E709X (<0.5% of *EGFR* mutations), exon 18 G719X (~3% of *EGFR* mutations), exon 19 insertions (<0.5% of *EGFR* mutations), exon 20 A763_Y764insFQEA (<0.5% of *EGFR* mutations), exon 20 S768I (<1.5% of *EGFR* mutations) and the exon 21 L861Q (~3% of *EGFR* mutations); either alone or compound with other *EGFR* mutations (19). It is interesting to note that in preclinical models, the inhibitory concentrations of 1st generations EGFR TKIs are usually 10–200 times higher for *EGFR*-exon 18 indels/E709X (20,21), exon 18 G719X (20), exon 19 insertions (22), exon 20 A763_Y764insFQEA (15), exon 20 S768I (23) and the exon 21 L861Q (23) when compared to EGFR-exon 19 deletion mutants. These observations may explain why the ORRs in the clinic seldom exceed 55% for tumors that harbor these mutations types in patients treated with gefitinib or erlotinib (15,16). The same preclinical models show slightly higher relative potency for the 2nd generation EGFR TKI afatinib, specifically for *EGFR* exon 18 mutations (20). Indeed, the ORRs to afatinib 40 mg/day seem to be higher than 55% for tumors harboring *EGFR*-G719X, L861Q or S768I mutations (17).

Despite initial rapid and sometimes prolonged responses to gefitinib, erlotinib and afatinib for lung cancers with the aforementioned EGFR TKI-sensitizing mutations, acquired resistance to EGFR TKIs is inevitable for most tumors due to biological (on-target mutations, bypass tracks or histological transformation) and pharmacokinetic mechanisms (24). The most common abnormality identified on rebiopsy specimens is the *EGFR*-T790M (within the gatekeeper position of exon 20) mutation in >50–60% of progressing lesions (6,25). *EGFR*-T790M is most commonly identified in *EGFR*-exon 19 deletion

Table 1 Types, frequency and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor sensitivity of *EGFR* kinase domain mutations in lung cancer

EGFR mutation	Approximate frequency (%)	EGFR TKI [*in vitro* sensitivity and expected overall response rate (ORR)]		
EGFR TKI sensitivity type		1st generation	2nd generation	3rd generation
		Gefitinib 250 mg Erlotinib 150 mg	Afatinib 40 mg	Osimertinib 80 mg
Sensitizing				
Exon 19 deletion	45.0	++++ (ORR >70%)	++++ (ORR >75%)	++++ (ORR >70%)
L858R	35.0	++++ (ORR >60%)	++++ (ORR >70%)	++++ (ORR >60%)
G719X	3.0	++ (ORR >55%)	+++ (ORR >65%)	++ (ORR ?)
L861Q	3.0	++ (ORR >55%)	++ (ORR >55%)	++ (ORR ?)
S768I	<1.5	+ (ORR >45%)	++ (ORR >55%)	? (ORR ?)
Exon 18 indel/E709X	<0.5	++ (ORR >55%)	+++ (ORR >65%)	++ (ORR ?)
Exon 19 insertion	<0.5	++ (ORR >55%)	++ (ORR ?)	++ (ORR ?)
A763_Y764insFQEA	<0.5	++ (ORR >55%)	++ (ORR ?)	++ (ORR ?)
Exon 18–25 duplication (*EGFR*-KDD)	<0.5	++ (ORR >55%)	+++ (ORR >65%)	++ (ORR ?)
Rearrangement (*EGFR-RAD51)*	<0.5	++ (ORR >55%)	+++ (ORR ?)	++ (ORR ?)
Insensitizing				
Exon 20 insertion	>7.0	– (ORR <5%)	– (ORR <10%)	– (ORR ?)
T790M inherited	<1.0	– (ORR ~0%)	– (ORR ~0%)	++++ (ORR >60%)
Others	>2.0	? (ORR ?)	? (ORR ?)	? (ORR ?)
Acquired resistance				
T790M + sens.	>50.0 (1st/2nd gen. TKI)	– (ORR ~0%)	– (ORR <5%)	++++ (ORR >60%)
C797X + T790M + sens.	<50.0 (osimertinib)	– (ORR ~0%)	– (ORR ~0%)	– (ORR ~0%)

++++, maximum inhibition; +++, moderate inhibition; ++, adequate inhibition; +, minimal inhibition; –, no significant inhibition beyond the therapeutic window of wild-type EGFR; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; ?, unknown; sens, sensitizing mutation; gen., generation.

mutated tumors but has also been reported in conjunction with L858R, L861Q, and S768I among others (26). Germline *EGFR*-T790M has also been described as a rare (<1%) high relative risk susceptibility allele in families with lung cancers independent of smoking risk (27,28). Eloquent structural and biochemical experiments have irrefutably defined that the addition of *EGFR*-T790M to a sensitizing mutant alters the kinetics of inhibitor binding of gefitinib, erlotinib and afatinib (29,30); leading to resistance to achievable clinical doses of these EGFR TKIs. However, 3rd generation EGFR TKIs that were selected on the basis of their covalent binding to *EGFR*-C797, plus their mutation over WT EGFR sensitivity, can inhibit *EGFR*-T790M bearing cancers (6,31). The most advanced of the clinical candidate 3rd generation EGFR TKIs is osimertinib given at 80 mg/day (32). The drug is exceedingly active against tumors with acquired resistance to gefitinib, erlotinib or afatinib when *EGFR*-T790M is present, with reported ORRs of >55% (26). Osimertinib was FDA-approved in 2015. Unfortunately, resistance to osimertinib monotherapy seems again to be inevitable with a predominance of on-target mutation events (including *EGFR*-C797S) in progressing tumors or circulating tumor DNA (33). The Thoracic Oncology community awaits a new generation of EGFR TKIs and of anti-cancer therapy combinations with EGFR TKIs to prevent and/or treat resistance to 3rd generation EGFR TKIs.

Just as the field of *EGFR* mutated NSCLC seemed to restricted to point mutations and indels that congregated in the kinase domain (as reviewed above and summarized in *Table 1*), two new reports led by investigators of the commercial comprehensive genomic profiling company Foundation Medicine and of Vanderbilt University School of Medicine have broadened our horizon to rare genomic events that also activate the kinase domain of EGFR: *EGFR*-exon 18–25 kinase domain duplication (*EGFR*-KDD)

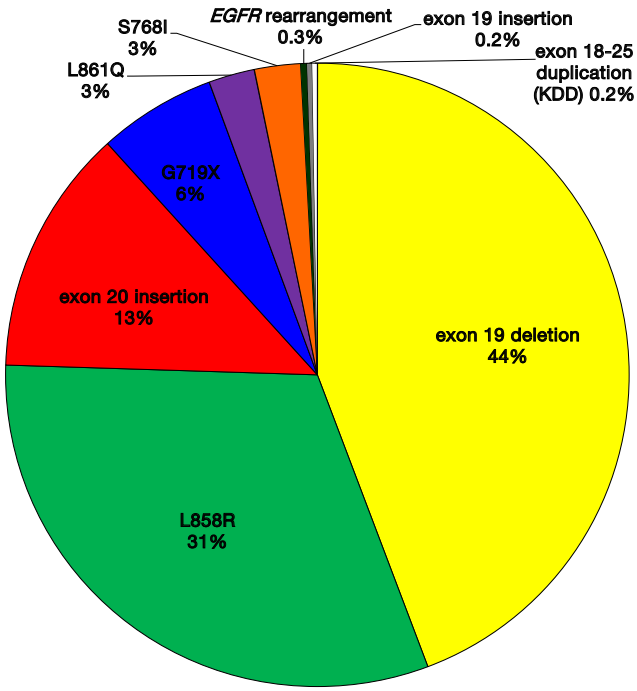

Figure 1 Pie chart display of epidermal growth factor receptor (*EGFR*) genomic aberrations identified by a single commercial vendor (Foundation Medicine) using the FoundationOne comprehensive genomic profiling that can be identify indels, point mutations, copy number changes, kinase domain duplications (KDD) and rearrangements. The data was obtained from (35).

and *EGFR* rearrangements (34,35). It seems the frequency of these changes does not exceed individually 0.5% of all *EGFR* mutation events (*Table 1*). In the 1,510 *EGFR* mutated tumor cohort described from 10,097 analyzed cases using FoundationOne's comprehensive genomic profiling (35), the frequency of *EGFR*-KDD was 0.2% and of *EGFR* rearrangements was 0.3% (*Figure 1*). These changes had not been reported previously because most traditional *EGFR* sequencing strategies used in day-to-day clinical care (Sanger sequencing, allele-specific PCR-based or focused next generation sequencing panels) are unable to identify these rare genomic variants.

The *EGFR*-KDD alteration consists of an intragenic alteration in *EGFR*, resulting in the tandem duplication of exons 18 to 25 (34). As these exons encompass the tyrosine kinase domain, this duplication generates an in-frame kinase domain duplication at the protein level. This type of *EGFR*-KDD had only been previously reported in rare cases of glioma (36) and was additionally found to occur in sarcomas, peritoneal carcinomas and Wilms' tumors (34). In preclinical and computational models, the resulting EGFR-KDD protein is transforming, may generate EGFR intramolecular asymmetric activated dimers, and is hypersensitive to 1st, 2nd and 3rd generation EGFR TKIs (34). The same report also describes a case of advanced chemotherapy-progressive *EGFR*-KDD mutated lung adenocarcinoma with a 7-month partial response to afatinib (doses not provided) and subsequent progression due to amplification of the *EGFR*-KDD allele (34). Another case report of a prolonged multi-year response to gefitinib and then erlotinib has been described for advanced *EGFR*-KDD mutated lung adenocarcinoma (37). Therefore, it seems these variants are responsive to 1st and 2nd generation EGFR TKIs in the clinic.

EGFR rearrangements were for the first time described in 2016, with rearrangements following the kinase domain of *EGFR* (at exon 25) with other partners. The two reported partners include the C-terminal portion of the *RAD51 recombinase* (RAD51) or *purine-rich element binding protein B* (*PURB*) genes (35). The resulting N-terminal EGFR-RAD51 C-terminal fusion protein retains an important regulatory auto-phosphorylation site (Y845) of EGFR (35). In preclinical models, EGFR-RAD51 is transforming, activates downstream signaling pathways, may form activation dimers, and is hypersensitive to 1st, 2nd and 3rd generation EGFR TKIs (35). Of most interest, three patients with *EGFR-RAD51* and one patient with *EGFR-PURB* rearranged NSCLCs had between 5- to 20-month periods of partial response to standard clinical doses of erlotinib (35); confirming that EGFR fusion proteins are TKI-sensitive variants. Other type of *EGFR* genomic aberrations outside the kinase domain of EGFR—including extracellular domain in-frame deletions (such as the truncated EGFR-vIII deletion), extracellular domain point mutations and C-terminal activating exon 25-26 deletions—have also been described in whole genome sequencing cohorts of lung adenocarcinoma (38). The prevalence and clinical significance of the latter genomic changes remains to be elucidated in the clinical care of NSCLC with off-label use of FDA-approved EGFR TKIs.

In summary, the enhanced landscape of EGFR TKI-responsive genotypes (including exon 19 deletions, L858R, exon 18 indels, G719X, exon 19 insertions, A763_Y764insFQEA, S768I, L861Q, KDD and rearrangements to gefitinib, erlotinib or afatinib; and T790M to osimertinib) highlights that comprehensive molecular profiling may be necessary to maximize the identification of all cases that can benefit from precision oncology when dealing with *EGFR*

mutated NSCLC. It also demonstrates that we have not yet identified all genomic variants that are actionable and/or clinically-relevant in NSCLC (39-50).

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Footnote

Conflicts of Interest: The author has received consulting fees from Pfizer Inc., Boehringer Ingelheim and Ariad. The author also conducts unremunerated clinical trials using afatinib (Boehringer Ingelheim), erlotinib (Astellas), osimertinib (AstraZeneca) and rociletinib (Clovis Oncology).

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

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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

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

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

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

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

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,

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

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.

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

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

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

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

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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Footnote

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

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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

(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

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

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

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

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)	Republic of 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)	Republic of 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

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.

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, 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 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

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	BEAMling	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.

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 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 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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Targeted therapy in NSCLC driven by HER2 insertions

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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

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

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

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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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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HER2 driven non-small cell lung cancer (NSCLC): potential therapeutic approaches

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Abstract: Oncogenic driver mutations identified in non-small cell lung cancer (NSCLC) have triggered the development of drugs capable of interfering in intracellular signaling pathways involved in tumorigenesis. Tyrosine kinase inhibitors, such as erlotinib or gefitinib, have demonstrated promising results in patients with advanced NSCLC that harbor EGFR mutations. Human epidermal growth factor 2 (HER2/ERBB2/neu) is a member of the ERBB family of tyrosine kinase receptors, and is activated by homodimerization or heterodimerization with other ERBB receptors. Deregulation of HER2 gene, by overexpression and/or gene amplification has been proved important in breast and gastric cancer, in which overexpression of HER2 confers greater response to specific anti-HER2 treatment, including trastuzumab. In lung carcinogenesis, HER2 mutations are thought to be more clinically relevant than overexpression or gene amplification. HER2 mutations in NSCLC, described exclusively in adenocarcinoma histology, are present in approximately 4% of this subset of lung cancer patients, suggesting that thousands of patients per year may possibly benefit from targeted therapy. Therefore, we conclude that systematic genotypic testing in this subgroup of NSCLC patients should include detection of HER2 mutations. In addition, clinical trials with standard antiHER2 agents and new investigational therapies are ongoing, with promising preliminary results, as illustrated in this review, although further research is warranted in this field.

Keywords: HER2; lung adenocarcinoma; mutation; targeted therapy

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Introduction

Lung cancer continues to be the leading cause of cancer-related death, as estimated by the American Cancer Society, responsible for 26% of all female cancer deaths and 29% of all male cancer deaths in the U.S. in 2012 (1). Considering that non-small cell lung cancer (NSCLC) accounts for 80-85% of cases of lung cancer (2) and that significant improvement in survival rates, approximately 17% at 5 years for recently diagnosed NSCLC and less than 4% if presenting with distant metastasis (3), has not been achieved in the last decade with conventional chemotherapy, novel therapeutic approaches are warranted in this field. As a result of these advances, systematic genomic testing for patients with NSCLC is becoming the new standard of care in clinical decision-making, due to the identification of driver mutations that have triggered the development of new molecules targeting these specific alterations in cancer cells. For example, somatic mutations in epidermal growth factor receptor (EGFR) confer greater response rates to tyrosine kinase inhibitors (TKIs) that target the catalytic domain of EGFR, such as erlotinib and gefitinib, compared to standard therapy in advanced NSCLC, 70% *vs.* 33.2% in first-line trials (4,5). In a similar manner, crizotinib, the anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitor, has demonstrated response rates of approximately 60% with progression-free survival greater than 10 months in those NSCLC characterized by ALK rearrangements (6). These studies have enabled to conclude that both EGFR-mutant and ALK-positive NSCLC constitute two defined

subgroups of oncogene-driven tumors with potentially effective targeted therapy. Furthermore, approximately 15-20% of NSCLC diagnosed in Europe and North America bear EGFR mutations or ALK rearrangements (7), enhancing the significance of the development of drugs capable of interfering with their intracellular effects.

Based on these results, the identification of other activating mutations has been pursued in hopes of improving survival in NSCLC by specifically treating these genomic alterations. These potential therapeutic targets include KRAS, BRAF, HER2 and PIK3CA, in addition to ROS1 fusions. KRAS mutations, in codons 12, 13 and 61, reported in approximately 20% of cases of lung adenocarcinomas, predict negative outcome in terms of response to EGFR TKIs. No targeted therapies have demonstrated an increase in overall survival in KRAS-mutant NSCLC, although selumetinib, an inhibitor of MAPK extracellular signal-regulated kinase (MEK) 1/2 (downstream of KRAS), in combination with docetaxel in previously treated advanced NSCLC has shown promising results in a recent phase 2 trial (8).

Regarding activating mutations in BRAF, HER2 and PIK3CA, incidence reported for each group ranges from 1-4%, a lower although significant frequency that is encouraging further investigation of these genetic alterations and consequent therapeutic implications. HER2 mutations in NSCLC constitute a clear molecular target, particularly in a subset of patients with distinct clinical features, including female non-smokers with adenocarcinomas, similar to those patients with EGFR-mutant lung cancer. Here, we seek to review the characteristics of HER2 mutations that enable interaction with molecules that specifically target these receptors in lung adenocarcinomas, as well as the results of preliminary studies that assess the efficacy of anti-HER2 therapy applied to NSCLC.

Tumorigenesis induced by HER2 mutations

HER2 [also known as epidermal growth factor receptor-2 (*EGFR2*), *ERBB2* or *NEU*] is a member of the *ERBB* receptor tyrosine kinase family, which includes 3 additional members; *EGFR* (HER1/*ERBB1*), HER3 (*ERBB3*) and HER4 (*ERBB4*). The binding of ligands to the extracellular domain of EGFR, HER3 and HER4 induces homo- and heterodimerization of these receptors, catalytically activating a cascade of intracellular pathways involved in cellular proliferation, differentiation and migration. These reactions are induced by cytoplasmic signal transducers such as PLC-γ1, Ras-Raf-MEK-MAPKs, phosphatidylinositol-3 kinase (PI3K), Src or the signal transducers and activators of transcription (STATs). However, no ligand has been described for HER2, regardless of structural resemblance between *ERBB* receptors. In fact, HER2 has been identified as the preferred binding partner of the other *ERBB* receptors, in particular, of EGFR with formation of HER2/EGFR heterodimers with increased potential for signaling than EGFR homodimers (9). This unique characteristic of HER2 has been partially attributed to its increased flexibility due to a glycine-rich region following the alpha-helix C of HER2, which explains its low intrinsic catalytic activity and less stable conformation when activated (10). Consequently, HER2 overexpression potentiates EGFR signaling which relates to the increased response in EGFR-positive NSCLC with HER2 overexpression to erlotinib or gefitinib (11), specific inhibitors of active EGFR, but not of HER2 or inactive EGFR.

HER2 gene, regulated by overexpression and/or gene amplification, has been proven important in many cancers, including breast and gastric cancer, in which overexpression of HER2 confers poor prognosis although it relates to possible benefit from specific anti-HER2 therapy. With the arrival of trastuzumab, a humanized monoclonal IgG1 that targets the extracellular domain of HER2, and its effect in combination with cytotoxic chemotherapy on survival rates of breast and gastric cancer with overexpression of HER2, a new door in molecular-targeted therapy was opened. However, 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 treated with trastuzumab in addition to gemcitabine-cisplatin or to docetaxel, failed to demonstrate benefit in survival in HER2 IHC-positive patients (12,13).

These findings triggered investigation of activating mutations in the tyrosine kinase domain of HER2 gene, first described in 2004. HER2 mutations have been reported to exist in up to 4% of NSCLC and are more common in Asians, never smokers, women and adenocarcinomas (14), characteristically similar to patients with EGFR mutations. These mutations occur in the first four exons of the tyrosine kinase domain (exons 18-21), including the most frequently observed alteration, a 12-bp duplication/insertion of the amino acid sequence YVMA in exon 20 at codon 776 (HER^{YVMA}). The mutated region of exon 20 in the HER2 gene corresponds to the nine codon region in exon 20 of the EGFR gene, where duplications and insertions have

Table 1 Frequency of HER2 mutations among lung adenocarcinoma samples in recently published studies

Study group	Total (No.)	HER2 mutation (No.)	%
Tomizawa K *et al.* (*Lung Cancer* 2011)	504	13	2.58
Li C *et al.* (*J Thor Oncol* 2012)	224	8	3.57
Sun Y *et al.* (*J Clin Oncol* 2010)	52[‡]	2	3.85
Arcila M *et al.* (*Clin Cancer Res* 2012)	560	25	4.46
Zhang Y *et al.* (*Clin Cancer Res* 2012)	349[ǂ]	16	4.58
Cardarella S *et al.* (*J Thor Oncol* 2012)	276	13	4.71
Li C *et al.* (*PLos One* 2011)	202[‡]	12	5.94

[‡]Inclusion of adenocarcinoma samples of never-smokers only; [ǂ]Inclusion of adenocarcinoma samples of female never-smokers only

also been described, resulting in conformational changes of the tyrosine kinase domain that lead to narrowing of the ATP binding cleft and, consequently, increased kinase activity compared to wild-type receptors (HER^{WT}). *In vitro* studies have demonstrated that HER^{YVMA} induces ligand-independent transphosphorylation and stronger association with signal transducers that mediate cell proliferation, motility and survival processes than HER^{WT} (15). In fact, HER^{YVMA} activates EGFR in absence of ERBB ligands and EGFR kinase activity, which explains that EGFR TKIs erlotinib and gefitinib have no effect on EGFR and HER2 phosphorylation in HER^{YVMA} cells. However, when the effect of trastuzumab in cell proliferation was tested in these *in vitro* studies, inhibition was achieved in presence of HER^{YVMA} but not cells overexpressing HER^{WT}, findings consistent with the reported inability of the IgG1 to bind with EGF and or EGFR/HER2 heterodimers (16). Therefore, authors concluded that tumor cells harboring HER2 mutations are resistant to EGFR inhibitors although remain sensitive to HER2 inhibitors and dual EGFR/HER2 inhibitors.

Epidemiology of HER2 mutations in lung cancer

Up to date, few studies regarding HER2 mutations in NSCLC have been published, primarily in Asian patient populations in which never smokers constitute a greater percentage of lung cancer patients (approximately 30%) compared to North American and European populations (10%). Incidence of HER2 mutations has been reported in 2-5% of NSCLC adenocarcinomas (*Table 1*). In a retrospective study of pulmonary resection samples obtained at the Fudan University Shanghai Cancer Centre (17), a total of 202 patients, never smokers, with lung adenocarcinoma that had not received neoadjuvant chemotherapy, were included. The median age at diagnosis was 57.3 years and no significant differences were observed in age, stage or degree of tumor differentiation between males and females. Of these samples, 89.1% harbored known oncogenic driver mutations in EGFR (75.25%), HER2 (5.94%), ALK fusion (4.95%), KRAS (1.98%), ROS1 fusion (0.99%). Patients with no identified driver mutation were diagnosed at a younger age. 12 samples with HER2 kinase domain mutations were detected, including 11 exon 20 insertions and 1 L775P point mutation.

Recently, the Memorial Sloan Kettering Cancer Centre (MSKCC) group published the largest assessment to date of HER2 mutations in predominantly Caucasian population (18). Of 560 lung adenocarcinoma samples that resulted negative for EGFR and KRAS major mutations tested previously, 26 HER2 mutations in 25 cases were identified (5%), all mutually exclusive with point mutations in EGFR, KRAS, BRAF, NRAS, PI3KCA, MEK1 and AKT mutations as well as ALK rearrangements. No HER2 mutations were detected among 104 squamous cell carcinomas and 6 small-cell carcinomas tested. 92% (24/26) of these HER2 mutations were in-frame insertions in exon 20 (from 3 to 12 bp) between codons 775 and 881, of which the most common (83%) was the 12-bp duplication/insertion of YVMA at codon 775. The other two cases were point mutations, L775S and G776C. Median follow-up after diagnosis of advanced disease was 19 months for all patients. No significant differences in overall survival were described between HER2 and other molecular subsets. Morphologically, 92% were moderately or poorly differentiated adenocarcinomas. An additional

analysis was performed to assess for HER2 gene copy number alterations by FISH in 11 HER2 mutated and 39 WT cases. None of HER2-mutant specimens were positive for HER2 amplification; 18% presented high polysomy (>4 copies of HER2 in >40% of cells) and 73% low polysomy. Amplification of HER2 was detected in one case, in the WT group, and interestingly this case was also found to harbor an EGFR exon 19 deletion. Therefore, HER2 mutation was not associated with concurrent HER2 amplification.

In this study, the overall prevalence of HER2 mutations was estimated to be approximately 2%, similar to statistics obtained in smaller European studies (19). In addition, HER2 mutations were most frequent among never-smokers ($P<0.0001$) although there were no associations with gender, race or stage of disease.

Therapeutic implications: HER2-targeted therapy in NSCLC

HER2 overexpression and gene amplification has been observed in breast, gastric and ovarian malignancies, inducing sensitivity to HER2-targeted drugs including trastuzumab, pertuzumab, lapatinib and T-DM1. Both amplification and high copy number gains have also been identified in NSCLC, although first clinical trials with anti-HER2 therapies in unselected patients failed to demonstrate survival benefit in HER2 positive NSCLC (defined by immunohistochemistry) (12,20). However, there is new hope that HER2 mutations may be more relevant in lung carcinogenesis than HER2 amplification or overexpression. Based on previous *in vitro* and *in vivo* studies, Cappuzzo *et al.* showed that lung cancer harboring the HER2 Gly776Leu mutation responded to treatment with trastuzumab and paclitaxel in a patient with chemotherapy-refractory lung adenocarcinoma (21).

Considering that HER2-mutant NSCLC may benefit from HER2 inhibition or dual EGFR/HER2 inhibition, but not single blockage of EGFR, novel TKIs simultaneously targeting EGFR/HER2 have been investigated. Transgenic mice models with induced expression in lung epithelium of the most common HER2 mutant, $HER2^{YVMA}$, developed lung adenosquamous carcinomas in distal and proximal bronchioles (22). In these models, treatment with erlotinib, trastuzumab, BIBW2992 and/or rapamycin revealed that the combination of BIBW2992 (afatinib), an irreversible dual TKI targeting both EGFR and HER2, and rapamycin, an inhibitor of the downstream effector protein mTOR, produced the most significant shrinkage (50.1±27.4% tumor regression measured by MRI) of tumor specimens. In addition, immunohistochemical analysis of these tumors treated with BIBW2992 and rapamycin proved this combination to be the most effective regimen for inhibition of upstream and downstream signaling of both the ERBB/PI3K/mTOR and the MAPK signaling pathways. Surprisingly, a relatively low effect was observed in $HER2^{YVMA}$ models treated with trastuzumab, with an average tumor regression of 13.59% (±10.89%), which was theoretically explained by postulating that trastuzumab is capable of inhibiting phosphorylation of membranous HER2 but unable to inhibit intracellular HER2 signaling associated with Golgi, endoplasmic reticulum, and other transport vesicles. Interestingly, continuous expression of $HER2^{YVMA}$ was proven necessary for tumor maintenance, indicating that HER2 is of great importance in lung adenosquamous tumorigenesis.

Case reports of afatinib in patients with HER2 mutant NSCLC have revealed promising results (23). Of patients who were included in an exploratory Phase II study of afatinib, five patients with non-smoking history and metastatic lung adenocarcinomas were identified to harbor HER2 mutations in cancer specimens. Three of these were evaluated, observing objective response to afatinib in all cases.

Neratinib, an irreversible pan *ERBB*-receptor family inhibitor, has been studied in a phase II trial in patients with advanced NSCLC who progressed following erlotinib or gefitinib (24). Three subgroups, EGFR mutant, wild-type EGFR and EGFR TKI naive- adenocarcinoma with light smoking history, were compared obtaining objective response rates of 3.4%, 0% and 0%, respectively. Only a small subgroup of patients with G719X mutation at exon 18 of EGFR-positive tumors, refractory to reversible TKIs, benefited from neratinib. Based on these results, neratinib is no longer in development for NSCLC although investigation in HER2-positive breast cancer continues.

PF00299804 (dacomitinib), another irreversible TKI targeting *ERBB* family members EGFR, HER2 and HER4, is being evaluated in patients with NSCLC. Preliminary data of dacomitinib in the HER2-mutant cohort reveal a 14% (3 of 22) partial response rate and 27% of these patients (6 of 22) have maintained stable disease to date (25).

In addition to TKIs, other molecules targeting EGFR and HER2 receptors have been developed. Considering that the heat shock protein 90 (Hsp90) chaperone stabilizes various oncogenic kinases necessarily involved in signal transduction and proliferation of lung carcinoma cells, when Hsp90 was demonstrated to interact with

mutant EGFR, inhibition of these chaperones became a new potential therapeutic approach (26). NSCLC with activating EGFR mutations that develop acquired resistance to EGFR TKI after treatment with erlotinib or gefitinib, have been proven sensitive to Hsp90 inhibitors both in NSCLC cell lines *in vitro* and *in vivo* (27). Other targets of Hsp90 include mutant HER2, mutant BRAF or mutant or overexpressed MET; therefore, adenocarcinomas harboring HER2 mutations may benefit from disruption of chaperone function. In fact, ganetespib, a novel non-geldanamycin potent Hsp90 inhibitor that impedes binding of Hsp90 to its co-chaperone, p23, has been proven effective in NSCLC cell lines in mice models driven by mutations in both EGFR and HER2^{YVMA} (28). These promising data support further investigation in clinical trials.

Conclusions

The discovery of oncogenic driver mutations in NSCLC is leading to the development of new therapies targeting specific molecular alterations. Detection of EGFR mutations and ALK rearrangements in tumor specimens of recently diagnosed NSCLC is currently standard of care, in order to identify subsets of patients that may respond to TKIs, such as erlotinib or gefitinib and crizotinib, respectively. Considering the prevalence of lung adenocarcinoma and clinical relevance of other mutations in NSCLC, including HER2, at diagnosis of this subgroup of lung cancer patients, we suggest expanding systematic genotype testing to include detection of these molecular alterations. In comparison with other types of cancer (i.e. breast, gastric) in which HER2 overexpression and gene amplification is associated to greater response to anti-HER2 drugs such as trastuzumab, first clinical trials in HER2 IHC-positive NSCLC failed to demonstrate benefit in the addition of trastuzumab to chemotherapy. However, HER2 mutations are thought to play a more significant role in lung cancerogenesis than overexpression or gene amplification, achieving promising results with trastuzumab in advanced HER2-mutant NSCLC. Therefore, identification of HER2 mutations, rather than HER2 IHQ-positive cancer specimens, should be studied in recently diagnosed stage IV NSCLC patients.

In addition, considering that cancer cells harboring HER2 mutations may respond to both HER2 inhibitors and dual EGFR/HER2 inhibitors, newer agents, including dacomitinib and afatinib, are currently under investigation in clinical trials specifically for this indication. Phase II studies have demonstrated promising initial results, although further investigation is necessary. Inhibition of chaperones to oncogenic kinases has revealed favorable results in preclinical models, constituting a new therapeutic strategy to be explored in both EGFR- and HER2-mutant NSCLC.

In summary, mutations in the tyrosine kinase domain of HER2 identify a subset of NSCLC adenocarcinomas, with a greater prevalence among never-smokers, which may respond to novel agents that specifically target this alteration. HER2 mutations are mutually exclusive with other driver mutations and are independent of HER2 gene amplification. Considering the prevalence of lung adenocarcinomas and given the availability of standard and investigational therapies targeting HER2, clinical genotyping of these tumors should include HER2.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Targeted therapy in lung cancer: IPASS and beyond, keeping abreast of the explosion of targeted therapies for lung cancer

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Abstract: Advances in the treatment of non-small cell lung cancer (NSCLC) over the last decade have predominantly involved the development of therapies directed at molecular targets such as mutations in the epidermal growth factor receptor (EGFR) or rearrangements in the anaplastic lymphoma kinase (ALK) gene. Other targets have been discovered at low frequency, with multiple agents approved or in development for treatment of these rare molecular subtypes. The tumour microenvironment has also provided opportunities for therapies targeting angiogenesis and the host immune response. This review will provide an overview of current targeted therapies in NSCLC and promising treatment approaches on the horizon.

Keywords: Non-small-cell lung carcinoma (NSCLC); molecular targeted therapy; immunotherapy; epidermal growth factor receptor (EGFR); anaplastic lymphoma kinase (ALK)

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Introduction

Delivering a high chance of benefit and avoiding futile treatment is crucial in the management of advanced lung cancer where quality of life is constantly at risk from disease progression or treatment toxicity. This ideal is now achievable with the realisation of targeted therapy in non-small cell lung cancer (NSCLC). Targeted therapy refers to pharmaceutical agents that affect a known molecular target in the cancer cell or tumour microenvironment. In some cases, the presence of the target is determined prior to treatment by interrogating tumour samples with a variety of histological and molecular techniques. In other cases, the presence of the target is assumed to be present in the majority of patients on the basis of prior analyses on large numbers of samples. Detectable targets that indicate a high chance of treatment benefit with a given therapy are termed predictive biomarkers. This is in contrast to prognostic biomarkers, which merely indicate an influence on prognosis rather than treatment response. Testing for mutations in the epidermal growth factor receptor (*EGFR*) gene and rearrangements of the anaplastic lymphoma kinase (*ALK*) gene in adenocarcinoma of the lung are now in routine clinical use as predictive genomic biomarkers in the management of advanced lung cancer. The group of patients with lung adenocarcinomas that harbour either of these genomic alterations (15-50% depending on the population studied) are already benefiting from targeted therapy with oral kinase inhibitors such as erlotinib and crizotinib. Other potential predictive genomic biomarkers in known oncogenes such as *BRAF*, *ROS1*, *MET* and *PIK3CA* have been identified in a systematic fashion and efforts are underway to target them with novel drug compounds.

It is clear now that lung cancer represents a constellation of diseases with distinct molecular profiles and sensitivity to treatment. This re-imagining of the classification of lung cancer has been paralleled by the discovery that squamous cell carcinoma and adenocarcinoma of the lung have very different molecular architectures, and distinguishing the two on histological grounds remains a crucial first step to guide subsequent molecular analyses. Determining the molecular subtypes of lung cancer in the clinic requires an ongoing effort to develop reliable molecular diagnostics,

as has occurred with testing for *EGFR* mutation and *ALK* rearrangement. Lung cancer therapy is also likely to benefit from the nascent field of cancer immunotherapy, with preliminary evidence that targeting the host immune response to lung cancer will be a successful and versatile treatment modality in the future. This review will summarise the current state of targeted therapy for lung cancer with a focus on NSCLC, and discuss promising agents in development.

Targeting oncogenic mutations and chromosomal aberrations in NSCLC

EGFR-mutant NSCLC

Mutations in the *EGFR* gene found in adenocarcinoma of the lung was the first biomarker predictive of benefit from a targeted therapy in NSCLC, and was exemplary of the impressive efficacy that could be expected from this paradigm. Small molecule inhibitors of EGFR were originally developed and tested in unselected lung cancer populations, where some patients were noted to have dramatic responses (1,2). Subsequent studies revealed that tumours with mutations in the intracellular tyrosine kinase domain that mediates downstream signalling of the *EGFR* gene product had substantial clinical responses to oral tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib (3-5).

Before *EGFR* mutation was known to be a predictive biomarker, certain patient populations were seen to benefit more from EGFR TKIs, namely those with lung adenocarcinomas, Asian ethnicity, females and never-smokers. It is now known that the enhanced efficacy in these populations is explained by the greater likelihood that their tumours harbour *EGFR* mutations (5-8) and that such mutations are almost exclusively found in adenocarcinoma of the lung (7-9). There is however no clinical characteristic that can be used in lieu of *EGFR* mutation testing.

The efficacy of EGFR TKIs in advanced *EGFR*-mutant lung cancer has now been established in eight randomised phase III clinical trials. The first of these was the pivotal IPASS study which evaluated the efficacy of gefitinib versus first line chemotherapy with carboplatin and paclitaxel in an Asian population of light or never smokers with advanced lung cancer (10). As part of this study which involved over 1,200 patients, 437 patients had tumour samples assayed for *EGFR* mutations. In the overall population, the study showed a non-inferior progression free survival for gefitinib compared to chemotherapy. It was also found that *EGFR* mutation was a very strong predictor of improved progression free survival with gefitinib, and that gefitinib was inferior to chemotherapy in patients without *EGFR* mutations. These results were confirmed in the phase III First-SIGNAL study which also compared gefitinib to chemotherapy in never-smokers with advanced lung cancer (11).

In addition to IPASS and First-SIGNAL, there have been six randomised controlled phase III trials comparing the EGFR TKIs gefitinib, erlotinib or afatinib to chemotherapy in patients with exclusively *EGFR*-mutant lung cancer, both in Asian and Caucasian populations. These studies which are summarised in *Table 1* (12-17), uniformly show superior response rates, progression free survival and quality of life with EGFR TKIs compared to cytotoxic chemotherapy. Despite mature follow up data (18-20), no trial of a first line EGFR TKI has shown an overall survival benefit, most likely explained by the large numbers of patients in the chemotherapy arms of these trials that crossed over to EGFR TKI treatment after progression. Although there has been no direct comparison, the second generation EGFR TKI afatinib appears to have more toxicity compared to gefitinib and erlotinib, with higher rates of severe diarrhoea and skin rash (16).

It is now recommended that all patients with advanced adenocarcinoma of the lung be tested for *EGFR* mutations (21), which is typically carried out using DNA sequencing of archival formalin fixed tumour tissue obtained at biopsy. The frequency of *EGFR* mutation in current or former smokers is approximately 10%, and in never smokers can be up to 40-50% (8,22). Due to the superior response rates and quality of life seen with erlotinib or gefitinib compared to chemotherapy, it is also recommended that all patients with *EGFR*-mutant NSCLC receive these treatments as first line therapy (23-25).

EGFR TKIs continue to have a role in NSCLC without *EGFR* mutations, where they may inhibit the overexpressed non-mutant protein, so-called wild-type *EGFR*. Erlotinib was found to improve overall survival in advanced NSCLC compared to placebo following progression on second or third line chemotherapy in the NCIC Clinical Trials Group BR.21 phase III study (26). This study was conducted before the link between *EGFR* mutation and EGFR TKI response was known, but subsequent subgroup analysis showed that the benefit was maintained in patients with wild-type *EGFR* and non-adenocarcinoma histology. A similar phase III study comparing gefitinib to placebo in a heavily pre-treated population failed to meet statistical significance, but there was a trend towards improved survival (27) with gefitinib.

Table 1 Phase III trials of EGFR TKIs in exclusively EGFR-mutant advanced NSCLC

Trial	Patients	Targeted agent	Comparator arm	Primary endpoint
Western Japan Thoracic Oncology Group 3405 (12)	172	Gefitinib	Cisplatin + Docetaxel	Median PFS 9.2 versus 6.3 months (HR 0.49, 95% CI: 0.34-0.71, P<0.0001)
North East Japan Study Group 002 (13)	230	Gefitinib	Carboplatin + Paclitaxel	Median PFS 10.8 versus 5.4 months (HR 0.3, 95% CI: 0.22-0.41, P<0.001)
OPTIMAL (14)	165	Erlotinib	Carboplatin + Gemcitabine	Median PFS 13.1 versus 4.6 months (HR 0.16, 95% CI: 0.1-0.26, P<0.0001)
EURTAC (15)	174	Erlotinib	Cisplatin + Docetaxel or Gemcitabine	Median PFS 9.7 versus 5.2 months (HR 0.37, 95% CI: 0.25-0.54, P<0.0001)
LUX-Lung 3 (16)	345	Afatinib	Cisplatin + Pemetrexed	Median PFS 11.1 versus 6.9 months (HR 0.58, 95% CI: 0.43-0.78, P=0.001)
LUX-Lung 6 (17)	364	Afatinib	Cisplatin + Gemcitabine	Median PFS 11 versus 5.6 months (HR 0.28, P<0.0001)

PFS, Pogression free survival; HR, Hazard ratio; CI, Confidence interval.

Only one phase III study has compared EGFR TKIs to chemotherapy as second line therapy in a population that is specifically *EGFR* wild-type (28). Although this study suggested that docetaxel was a superior treatment in this group, final publication of results is awaited. A variety of studies have been conducted in unselected populations, showing that EGFR TKIs are non-inferior to second line chemotherapy (29), have a role as maintenance therapy after first line chemotherapy (30), and have similar efficacy to second line chemotherapy in patients that have failed to respond to first line treatment (31). There are no data to suggest the use of EGFR TKIs as first line therapy in *EGFR* wild-type disease, and this strategy appeared to be detrimental in IPASS (10) and also in the phase III TORCH study of erlotinib followed by chemotherapy versus chemotherapy followed by erlotinib (32).

Second generation EGFR TKIs are irreversible inhibitors of mutant *EGFR*, and also inhibit other receptors in the epidermal growth factor family. Afatinib, an ErbB receptor family blocker, is one such drug that has progressed furthest in development. In a phase IIb/III study of afatinib versus best supportive care in an unselected population of patients who had progressed on two chemotherapy regimens as well as either erlotinib or gefitinib, there was a modest prolongation of progression free survival by 2 months, but no overall survival benefit (33). Afatinib has also been tested in two phase III randomised trials as first line therapy in patients with *EGFR*-mutant NSCLC (*Table 1*) where it showed superior progression free survival compared to chemotherapy (16,17). It has been approved by the United States Food and Drug Administration (FDA) for this indication. Another second generation EGFR TKI dacomitinib has shown superior progression free survival compared to erlotinib when given after failure of prior chemotherapy in a phase II study of 188 patients (34), and is currently under investigation in two phase III studies compared to erlotinib (ARCHER) or placebo (BR26).

An alternative approach to targeting EGFR in NSCLC has been the use of monoclonal antibodies engineered to have strong affinity for the EGFR protein, such as cetuximab (35). Two randomised phase III trials have been conducted comparing chemotherapy to chemotherapy plus cetuximab in advanced NSCLC. The FLEX study of 1,125 patients with advanced NSCLC showed a modest improvement in overall survival of around 1 month with the addition of cetuximab to chemotherapy (36). A similar study failed to show benefit in the primary endpoint of progression free survival (37). Data about the role of EGFR protein expression in predicting benefit have been conflicting, although a retrospective subgroup analysis showed high EGFR expression was predictive of longer survival with cetuximab in the FLEX study (38,39). The lack of clear benefit and uncertainty over an appropriate biomarker has limited the use of cetuximab.

Acquired treatment resistance to EGFR TKIs

There is now little doubt about the effectiveness of EGFR

TKIs in *EGFR*-mutant NSCLC. However, despite high initial response rates, drug resistance and clinical failure is inevitable with the use of these agents over the course of a patient's treatment, so-called acquired resistance. In contrast to cytotoxic chemotherapy, the well defined mechanism of action of EGFR TKIs means that treatment resistance is a potentially tractable problem. Serial biopsies of tumours before and after treatment with EGFR TKIs have provided insight into the mechanisms of treatment failure (40-43), and have now been performed in sufficient numbers of patients to give an overview of the most common resistance mechanisms. In approximately 60% of cases, treatment failure is mediated by the presence of the secondary *EGFR* mutation T790M that is resistant to inhibition by current EGFR TKIs (40,43). This is presumed to develop from a resistant population of cells already present in low numbers before treatment with EGFR TKIs (44). In another 5-15% of cases, activation of alternative pathways within the cell that free it from dependence on *EGFR* signalling occurs, most commonly involving amplification of the MET gene (40-42,45) and mutations in *PIK3CA* (41). Mutations in *BRAF* have also been seen, and confirmed to confer resistance in cell line models (46), as has amplification of *HER2* (47). Activation of the *AXL* kinase appears to be another mechanism of acquired resistance (48). Unexpectedly, transformation to small cell histology has been observed in approximately 5% of cases (41,42) and several of these patients responded to conventional chemotherapy regimens used for small cell lung cancer (41). It is of note that several mechanisms of resistance may co-exist in the same tumour (41-43), such as T790M mutation and *MET* amplification.

The great value in understanding the mechanism of acquired resistance is that it provides a pathway to developing improved therapeutic strategies. Given that T790M mutations are the most common mechanism of acquired resistance, developing EGFR TKIs that inhibit T790M mutant *EGFR* is a logical next step. There is *in vitro* evidence that second generation EGFR TKIs such as afatinib may have better efficacy against T790M mutations (49), although response rates in trials with populations expected to have significant numbers of T790M mutations have been poor (33). A phase II study of afatinib combined with cetuximab has however shown promising results, controlling disease in all 22 patients enrolled with 36% showing partial responses (50). Toxicity has been a problem with this combination however. Finally, third generation mutation-selective EGFR TKIs such as CO-1868 have been developed that specifically inhibit the T790M mutant EGFR protein. CO-1868 is currently being tested in a phase I trial in patients with advanced *EGFR*-mutant NSCLC that have progressed on other EGFR TKIs, where it has shown preliminary evidence of efficacy in resistant disease and a favourable toxicity profile (51). AP26113 is another third generation EGFR TKI with T790M activity that is in phase I/II testing (52).

Targeted therapies already exist or are in development for other molecular pathways that may mediate acquired resistance, such as those involving *HER2*, *BRAF*, *PIK3CA* and *MET*. Combining such therapies with EGFR TKIs may provide an avenue for preventing or delaying acquired resistance. This has been applied *in vitro* where EGFR TKI resistance was reversed by co-administration of a MET inhibitor (53,54). Challenges remain in designing trials of tailored drug combinations in this setting and managing the potential toxicities that arise.

ALK-positive NSCLC

ALK was first detected as a fusion oncogene in lung adenocarcinoma in 2007 (55,56), although it had previously been identified as a fusion oncogene arising from a translocation between chromosome 2p and 5q in a subset of anaplastic large cell lymphomas (57). In the context of NSCLC the most frequent *ALK* gene rearrangement arises due to a short inversion in chromosome 2p where the *ALK* gene is fused with the echinoderm microtubule-associated protein-like 4 gene (*EML4*). The aberrant fusion protein EML4-ALK promotes cell growth, and is sufficient to transform cells into a malignant phenotype *in vitro* (55). *ALK*-positive cells seem to rely almost exclusively on the fusion protein to drive cell growth and survival, a concept termed 'oncogene addiction' that also applies to *EGFR*-mutant NSCLC (58). In this context, inhibition of oncogene function in EML4-ALK addicted tumours should result in growth arrest and cell death, and this was observed in animal models using small molecule kinase inhibitors targeting *ALK* (59,60).

Although developed originally as a small molecule inhibitor of the oncogene *c-MET*, crizotinib was also found to inhibit the *ALK* kinase (61), and was already in phase I trials when *ALK* was discovered to play a role in lung cancer. A reliable diagnostic method was also developed to detect *ALK* fusions in archival lung tissue using fluorescence in situ hybridisation (FISH) with break-apart probes. This enabled patients with advanced *ALK*-positive lung cancer to be

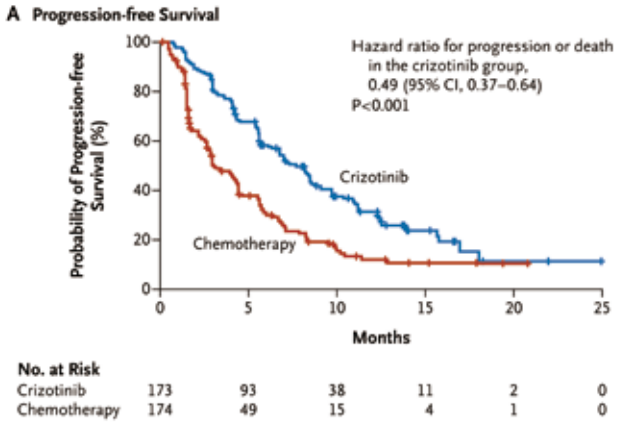

Figure 1 Progression free survival for second line crizotinib versus chemotherapy in ALK-positive NSCLC. From "Shaw AT, Kim DW, Nakagawa K, *et al*. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med 2013;368:2385-94. Copyright © 2013 Massachusetts Medical Society". Reprinted with permission.

enrolled rapidly into a phase I trial of crizotinib, where an impressive response rate of 60% was demonstrated (62,63). Most of these patients had received prior chemotherapy. A subsequent report with more mature data compared the overall survival of patients who received crizotinib in the phase I study to *ALK*-positive patients that were not enrolled and also *ALK* negative patients. Although not a randomised comparison, use of crizotinib was associated with improved survival compared to historical cohorts (64). It was also noted that the presence of an *ALK* fusion was not prognostic for survival in the absence of crizotinib.

Of the 1,500 patients screened for *ALK* fusions in the phase I study, only 5% were positive (62). In a similar fashion to *EGFR* mutations, some clinicopathologic characteristics predict a higher likelihood of *ALK* positivity, including young age, lack of smoking history and adenocarcinoma with solid, acinar or signet-ring histologic patterns. In an unselected population with NSCLC the frequency of *ALK* positivity is approximately 4% (62,65-68). *ALK* fusions are only very rarely found in lung cancers that have mutations in other oncogenes such as *EGFR* or *KRAS* (67).

Crizotinib has since been compared to standard second line chemotherapy in a multi-centre phase III randomised controlled trial in 342 patients with advanced *ALK*-positive lung cancer that had progressed after first line chemotherapy (69). Almost all of the patients in the standard arm received pemetrexed or docetaxel. The study was clearly positive for the primary endpoint with a median progression free survival of 7.7 months in the crizotinib arm and 3.0 months in the chemotherapy arm, shown in *Figure 1* (HR 0.49, 95% CI: 0.37-0.64, P<0.0001) (69). Crizotinib also improved baseline symptoms and delayed subsequent worsening to a greater degree than chemotherapy in quality of life analyses. There was no overall survival benefit seen, most likely because at least 64% of patients in the chemotherapy arm subsequently received crizotinib. A phase III trial of crizotinib as first line treatment for *ALK*-positive lung cancer has recently completed accrual. Crizotinib has received regulatory approval in Europe and the United States. It is recommended by international guidelines that testing for the presence of an *ALK* fusion be considered for all patients with adenocarcinoma of the lung (23,70).

Crizotinib and *ALK* positive lung cancer is a unique example of the promise of targeted therapy. It has taken only 4 years from the original discovery of the *EML4-ALK* fusion in lung cancer to the FDA approval of crizotinib and its widespread clinical use for this indication.

Acquired resistance to crizotinib

With time, resistance to *ALK* inhibition with crizotinib is inevitable. The median progression free survival in the largest study of crizotinib was 7.7 months (69). In a similar fashion to EGFR TKIs, biopsy of progressing lesions in patients treated with crizotinib has provided insight into resistance mechanisms (71-74). Mutations in the *ALK* gene appear to mediate resistance in around one third of patients, although there is a much wider spectrum of mutations than that seen in *EGFR*-mutant lung cancer where T790M dominates as discussed previously. Activation of alternate signalling pathways involving *EGFR* and *c-KIT* (an oncogene targeted by imatinib) may also play a role in mediating resistance (71). *In vitro* studies suggest that targeting the alternative pathway with existing agents such as gefitinib in the case of *EGFR* or imatinib for *c-KIT* may reverse resistance to crizotinib (71). The mechanism of crizotinib resistance in *ALK* positive tumours currently remains unknown in around one third of cases (75). Of concern, multiple different resistance mechanisms may occur simultaneously in the same patient (71).

Next generation ALK inhibitors with different properties to crizotinib have been developed to have greater potency and potentially target resistance mutations. One agent CH5424802, has been tested in phase I and phase II trials in crizotinib naïve *ALK*-positive NSCLC, and is notable for the

93% overall response rate seen (76). Another agent LDK378 has shown efficacy in a phase I trial which included both crizotinib resistant and naïve *ALK*-positive NSCLC (77), with a response rate of 70%. LDK378 also appeared effective in the presence of resistant *ALK* mutations.

KRAS-mutant NSCLC

KRAS mutations occur in around 30% of NSCLC (73), making them the most common driver mutation seen in an unselected population. Adenocarcinomas make up the majority of NSCLC with *KRAS* mutations (78), and there is a positive association with smoking history (79). *KRAS* mutations may predict a lack of benefit from EGFR TKIs in patient with wild-type *EGFR*, but data have been conflicting (80-82). Despite much research, it has not proved possible to directly target *KRAS*, although recent progress has been made (83). Alternative strategies have involved targeting the down stream signalling pathway of *KRAS* (84), a role fulfilled by the *MEK* inhibitor selumetinib (85). In a randomised phase II trial of second line therapy in *KRAS*-mutant advanced NSCLC, selumetinib plus docetaxel was superior to docetaxel in response rate and progression free survival (86). Other approaches to targeting *KRAS*-mutant NSCLC in early phase trials include PIK3CA/mTOR/AKT pathway inhibitors in combination with *MEK* inhibitors to effectively block downstream *KRAS* signalling (87).

Other oncogenes in NSCLC

With the advent of next generation sequencing technology, driver oncogenes beyond *EGFR*, *ALK* and *KRAS* have been characterised in NSCLC, often at frequencies of less than 5% (88). As targeted therapies already exist for several of these altered genes and are in use in other cancer types, there is currently a focus on identifying lung cancer patients with these alterations and matching them to appropriate therapies within early phase trials (89). There are clear differences between squamous cell and adenocarcinoma histologies in terms of driver oncogenes (9,90), so these will be discussed separately. The pattern and frequency of alterations are summarised in *Figure 2*.

Adenocarcinomas

ROS1 translocation

Fusion genes involving the receptor tyrosine kinase ROS1 have been found in 1-2% of NSCLC typically in never or light smokers with adenocarcinoma (91,92). This fusion is notable as it appears sensitive to inhibition with crizotinib (91,93), and defines a molecular subclass of lung cancers with clinical similarity to ALK-positive cancers.

MET amplification

MET is the gene for the hepatocyte growth factor receptor (HGFR). Activation of *MET* signalling is sufficient to transform cells to a malignant phenotype, and has effects on the cell cycle and survival. NSCLC cells commonly overexpress *MET*, and *MET* amplification is a defined pathway of resistance to EGFR TKIs (40-42,45). The monoclonal antibody onartuzumab (MetMAb) blocks binding of HGF to the *MET* receptor. It was combined with erlotinib in a randomised phase II trial in advanced NSCLC after failure of prior therapy. In patients with *MET* over-expression, combination therapy significantly prolonged overall survival from 4.6 to 12.6 months (HR 0.37, 95% CI: 0.2-0.71, P=0.002) compared to erlotinib alone. Tivantinib, a small molecule MET inhibitor was tested in a phase III trial in combination with erlotinib, but the study was closed early for futility (Press Release, ArQule Inc. and Daiichi Sankyo Co.).

BRAF mutations

BRAF is a well characterised driver mutation in metastatic melanoma, where it is treated with oral *BRAF* inhibitors such as vemurafenib or dabrafenib. A phase II trial of dabrafenib in *BRAF* mutant NSCLC is ongoing, with 7 out of the first 17 patients on trial demonstrating a partial response (94). The frequency of BRAF mutation in NSCLC is 1-5% (88,95,96), and appears to be at least equally as common in current or former smokers as non-smokers. The classic sensitising V600E mutation was only found in 50% of the *BRAF* mutant lung cancers, which may limit the use of currently available *BRAF* inhibitors (95).

HER2 amplification and mutations

HER2 amplification or mutation is known to exist in some lung cancers with a frequency of around 3% (97). Attempts at treating HER2 amplified NSCLC with the monoclonal anti-HER2 antibody trastuzumab were unsuccessful (98). *HER2* mutation in exon 20 is a more promising molecular subgroup, and there exist several small molecule inhibitors of the *HER2* tyrosine kinase such as afatinib or dacomitinib (99). There have been early reports of some responses to these drugs in patients with *HER2* mutations (100), and trials are ongoing.

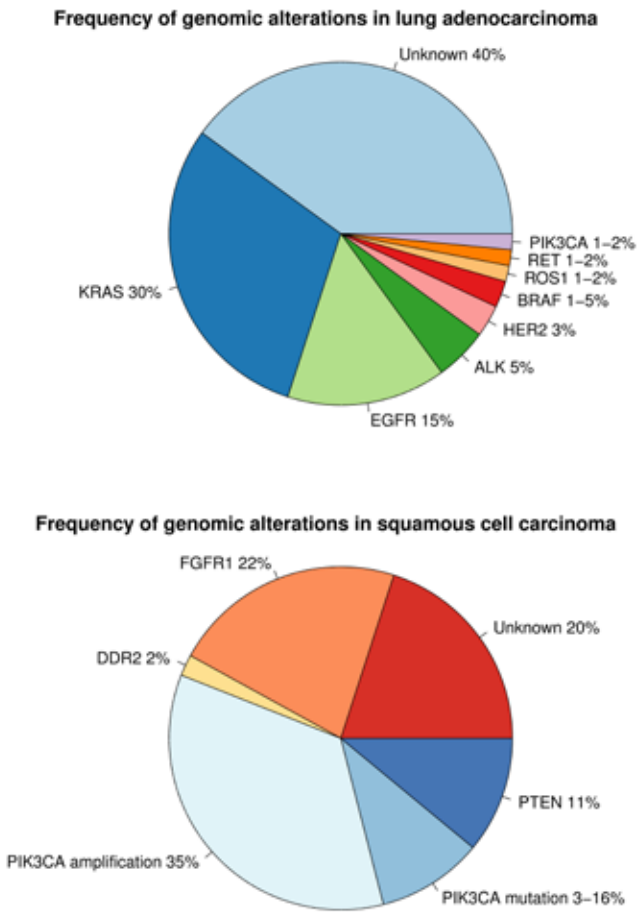

Figure 2 Relative frequency of genomic alterations in adenocarcinoma and squamous cell carcinoma. Data adapted from multiple references (see text) and are estimates only.

RET translocations

Fusions involving the receptor tyrosine kinase *RET* gene have recently been indentified in lung adenocarcinomas, and *in vitro* studies have confirmed the oncogenic potential of at least some of the identified fusions (101). The prevalence of *RET* rearrangements is estimated at between 1-2%, being higher in never or light smokers (92,101). The *RET* kinase inhibitor vandetanib (102) is a well established treatment for medullary thyroid carcinoma and may be a treatment option for *RET* positive adenocarcinoma of the lung.

PIK3CA mutation

PIK3CA is a known oncogene central to the phosphatidylinositide 3-kinase (PI3K) pathway that is deregulated in multiple cancer types (103). *PIK3CA* has been found altered in 1-2% of lung adenocarcinomas, and may co-exist with other mutant oncogenes (104-106). There is considerable effort to target this gene in other cancer types, and early phase trials are underway with *PIK3CA* targeted therapy for lung cancer both as monotherapy and in combination with other targeted agents and chemotherapy.

Squamous cell carcinomas

Recent progress has identified three potential therapeutic targets in squamous cell carcinoma of the lung. The fibroblast growth factor receptor 1 (FGFR1) is one such target, which is amplified in 21-22% of squamous cell carcinomas in recent studies (107,108). These studies also showed that *FGFR1* amplified cells underwent apoptosis when treated with a small molecule FGFR1 inhibitor, and *FGFR1* amplified tumours in mice shrank with inhibitor therapy, suggesting that FGFR1 is an important driver in some squamous cell carcinomas. Multiple small molecule inhibitors of FGFR1 are in development and entering early phase trials, with promising preliminary activity (109).

Mutations in the receptor tyrosine kinase *DDR2* gene have been seen in 2% of squamous cell carcinomas of the lung (9,110). TKIs widely used in treating chronic myeloid leukaemia such as dasatinib also have activity against *DDR2*. Dasatinib has produced partial responses in some squamous NSCLC patients in phase I trials (111,112). In one of the patients with a response, sequencing of a tumour biopsy revealed a *DDR2* mutation (110). Phase II trials of dasatinib specifically in squamous cell carcinoma of the lung are underway.

Alterations in genes playing a role in the PI3K pathway are present in 30-50% of squamous cell carcinomas, mostly comprising *PIK3CA* amplification and mutation, and deletion of the tumour suppressor gene *PTEN* (9,106). This pathway is important to maintaining cell survival and promoting growth (103), but the relationship between alterations in this pathway and response to inhibitors is complex. Phase I trials of *PIK3CA* inhibitors are underway in squamous NSCLC.

Targeting the tumour microenvironment

Angiogenesis in lung cancer

Angiogenesis has emerged as a broadly available target in multiple cancer types, as any sizeable tumour requires the

ability to form a new blood supply to survive (113,114). The most well studied pathway mediating angiogenesis involves the vascular endothelial growth factor (VEGF) family of ligands and associated receptors which have intracellular tyrosine kinase domains that mediate downstream signalling (115). Targeting VEGF receptor tyrosine kinase signalling using small molecule inhibitors has generally proven unsuccessful, despite multiple agents having been tested in phase III trials (116-122). The VEGF and FGF receptor inhibitor nintedanib combined with chemotherapy has shown a marginal benefit of less than one month in progression free survival over chemotherapy alone, as second line treatment of advanced NSCLC in two phase III trials (123,124).

Bevacizumab is the most widely used anti-angiogenic agent in routine practice. It is a recombinant humanised monoclonal antibody that binds to VEGF, specifically the VEGF-A isoform, and prevents activation of the VEGF receptor (125). The Eastern Cooperative Oncology Group E4599 trial was performed in 878 patients with advanced NSCLC, and compared bevacizumab plus chemotherapy with carboplatin and paclitaxel to chemotherapy alone (126). Bevacizumab was continued as maintenance therapy until progression after 6 cycles of chemotherapy. Median overall survival was superior with bevacizumab at 12.3 versus 10.3 months (HR 0.79, 95% CI: 0.67-0.92; P=0.003). Progression free survival and response rate were also superior with bevacizumab in a second phase III trial AVAiL, although overall survival was no different (127). Toxicities of bevacizumab include arterial thromboembolism, hypertension, augmented chemotherapy-related haematological toxicity and bleeding (126). Due to the higher risk of significant haemoptysis, bevacizumab should not be used for squamous cell histology. Bevacizumab has not had widespread uptake as standard first line therapy outside of the United States due to concerns about toxicity, cost and the lack of a biomarker predictive of benefit.

Immunotherapy

Recent advances in tumour immunology have revealed that the immune system plays an important role in controlling malignant growth, and shapes the characteristics of the tumour that eventually manifests clinically (128). Harnessing the immune system as a therapeutic modality has already shown success in advanced melanoma (129) and prostate cancer (130). Although traditionally not considered to be an immunogenic tumour type, there is evidence that markers of a host immune response to lung cancer have a significant prognostic impact in both the adjuvant setting and advanced disease (131-134). Enhancing the immune response may therefore represent a rational therapeutic target. Immunotherapy in lung cancer consists primarily of two approaches: vaccines derived from lung cancer cell lines or tumour associated antigens, and immuno-stimulatory checkpoint antibodies.

Vaccines

Several vaccines have shown promising results in phase II trials, and are currently being evaluated in randomised phase III trials. The largest trials will be discussed here.

Belagenpumatucel-L is an irradiated whole cell product consisting of multiple lung cancer cell lines reflecting adenocarcinoma, large cell carcinoma and squamous cell carcinoma histologies together with an immuno-adjuvant (135). A small single arm phase II trial conducted in a mixed population of early stage and advanced lung cancer demonstrated radiological responses in 15% of patients with measurable disease and a positive correlation between prolonged overall survival and higher vaccine dose (135). Belagenpumatucel-L is being further evaluated in a phase III trial recruiting patients with stage III-IV disease that is stable or responding after first line therapy.

Other vaccines consist of antigens expressed exclusively or predominantly in lung cancer cells. Melanoma-associated antigen-A3 (MAGE-A3) is expressed in 35% of NSCLC (136), and has been prepared as a mono-antigenic vaccine. This was tested in a randomised placebo-controlled phase II trial following resection of stage I-II NSCLC showing cellular expression of MAGE-A3 (137). Following surgery, the disease free survival and overall survival were no different between vaccine and placebo groups, but there were numerically fewer recurrences in the vaccine group after a median of 44 months post surgery (35% versus 43% in placebo group). 2,270 patients have been recruited to a phase III trial of the MAGE-A3 vaccine, with results awaited.

MUC-1 is an epithelial cell protein that is differentially glycosylated in malignant cells (138) and overexpressed in NSCLC (139,140). The BLP25 vaccine contains the MUC-1 peptide and an immuno-adjuvant encased in a liposomal delivery system (141). In a phase III randomised trial comparing BLP25 to placebo after concurrent or sequential chemoradiotherapy for stage III NSCLC, patients who had received concurrent treatment showed a median overall survival of 30.8 months compared to 20.6 months

with placebo (HR 0.78, 95% CI: 0.64-0.95; P=0.016) (142). BLP25 also prolonged survival in a phase II study in advanced NSCLC compared to best supportive care but this was not statistically significant (141). TG4010 is an alternative approach to MUC-1 vaccination, incorporating an attenuated but replication competent vaccinia virus that encodes for the MUC-1 protein and interleukin-2 (143). In a randomised phase II study, cisplatin and gemcitabine plus TG4010 was compared to cisplatin and gemcitabine alone in 148 patients with advanced NSCLC (144). Progression free survival at 6 months was 43% with the vaccine versus 35% without, but this difference was not statistically significant. Further studies with BLP25 and TG4010 are awaited.

Immune checkpoint blockade

Immune checkpoints refer to the molecular mechanisms that control T-cell responses to foreign antigens. Part of the immune checkpoint system encompasses stimulatory or suppressive co-receptors that modulate the interaction of the T-cell receptor (TCR) with human leukocyte antigen (HLA) expressed on the target cell. Two such receptors have emerged as important therapeutic targets in cancer. The cytotoxic T-lymphocyte antigen 4 (CTLA-4) receptor is expressed on T-cells following activation by antigen, and serves to dampen the T-cell response to promote self-tolerance and prevent autoimmune activation. Programmed cell death protein 1 (PD1) is also expressed on T-cells and similarly provides a mechanism for down-regulating the T-cell response if the ligand (programmed cell death 1 ligand 1 or PD-L1, also known as B7) is encountered. Preventing T-cell suppression at the tumour-immune interface by disrupting immunosuppressive signals forms a promising therapeutic strategy for advanced lung cancer that may also extend to adjuvant treatment.

The toxicities of the various immune checkpoint antibodies are similar and relate to autoimmune phenomena such as colitis, skin rash, pneumonitis and endocrinopathies. As these do not overlap with chemotherapy toxicity, combining these treatments with chemotherapy is a feasible approach. Ipilimumab is a humanised IgG1 anti-CTLA-4 receptor antibody, and is already an established therapy for advanced melanoma (129). A randomised placebo controlled trial was conducted comparing ipilimumab plus carboplatin and paclitaxel chemotherapy to placebo plus chemotherapy in 204 patients with advanced NSCLC (145). Ipilimumab was given in two schedules in the treatment arms: concurrent treatment starting from the first cycle of chemotherapy and phased treatment starting after two cycles of chemotherapy. In light of experience with melanoma that ipilimumab may cause an initial worsening in the radiological appearance of lesions used to assess progression free survival, modified immune-related radiological response criteria were used (146). The study was positive for the primary endpoint of immune-related progression free survival, which was 5.7 months in the phased treatment group compared to 4.6 months in the control group (HR 0.72, P=0.05). Efficacy was most pronounced in patients with squamous cell histology. A similar randomised phase II trial was carried out in 130 patients with extensive stage small cell lung cancer, and showed a trend towards improvement in immune-related progression free survival for the phased regimen in combination with chemotherapy compared to chemotherapy alone (6.4 versus 5.3 months; HR 0.64; 95% CI: 0.4-1.02; P=0.03) (147). Further trials for squamous cell lung cancer and small cell lung cancer are planned.

Multiple tumour types express the PD-L1 ligand on their cell surface, highlighting the role of the PD-1 receptor in suppressing anti-tumour T-cell responses (148). Monoclonal antibodies to both PD-1 and PD-L1 have been tested in several phase I trials that enrolled considerable numbers of patients with NSCLC (148,149). In one such trial the anti-PD-1 antibody nivolumab (formerly known as BMS-936558/MDX-1106) produced an unprecedented response rate of 18% amongst 129 NSCLC patients that were heavily pre-treated, with half of these patients having received three or more previous lines of therapy (148). In addition, the anti-PD-L1 antibody BMS-936559 produced response rates of 10% in a phase I trial that included 49 patients with NSCLC (149). The benefit was evident for both squamous cell carcinomas and adenocarcinomas. From these two trials there is early evidence that expression of the PD-L1 ligand in the tumour microenvironment, which can be evaluated with immunohistochemistry, may predict benefit from anti-PD-1/PD-L1 therapies. In addition to nivolumab, lambrolizumab is another anti-PD-1 antibody that has shown efficacy in melanoma and is being evaluated in lung cancer. Upcoming trials involving nivolumab and lambrolizumab are shown in *Table 2*.

Conclusions

The last ten years have seen a revolution in the way that lung cancer is conceptualised and treated, born out by advances in genomics, cell biology and drug development technologies. The same advances that facilitated this

Table 2 Upcoming trials of anti-PD-1 therapy in advanced NSCLC

Population	Treatment arms	Phase
Squamous cell carcinomas of the lung	Nivolumab versus Docetaxel	Phase III
Non-squamous carcinoma of the lung	Nivolumab versus Docetaxel	Phase III
All NSCLC, no previous therapy	Nivolumab monotherapy; Nivolumab + cisplatin/pemetrexed; Nivolumab + carboplatin/paclitaxel; Nivolumab + cisplatin/gemcitabine	Phase I
	Standard first line chemotherapy followed by nivolumab and bevacizumab maintenance	Phase I
	Ipilimumab + nivolumab	Phase I
EGFR-mutant NSCLC	Nivolumab + erlotinib	Phase I
All NSCLC	Lambrolizumab monotherapy; Lambrolizumab + standard chemotherapy; Lambrolizumab in NSCLC overexpressing PD-L1	Phase I

revolution will continue to provide a roadmap for ongoing improvements by identifying new targets and defining the mechanisms of treatment failure and resistance. The transition of crizotinib from an investigational compound to an approved therapy in a mere 4 years also provides hope that there will be a rapid expansion in therapeutic options available to patients in the near feature. Similarly, immunotherapy represents an entirely new class of agents with a promising efficacy and toxicity profile. With the arrival of targeted therapy come multiple challenges however. The development of targeted therapies is often at odds with the traditional clinical trial structure required by regulatory authorities, where phase III trials illustrating an overall survival benefit are considered the gold standard. In addition, targeted therapies carry high costs to the patient or funding agency, and the long term economic viability of the current drug development cycle is uncertain. Finally, it is still the case that the majority of patients with advanced lung cancer have no targeted therapy available to them at the current time, either due to a lack of known targets in their tumour or poor access to novel agents. Addressing both these issues will remain a priority if the successes of the past decade are to be maintained.

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Footnote

Conflicts of Interest: Brett Hughes has served on Advisory Boards for Roche, Pfizer and Boehringer Inglheim. Benjamin Solomon has served on Advisory Boards for Roche, Pfizer, Novartis, Astra Zeneca, Eli Lilly, Clovis Oncology and Boehringer Ingelheim. Peter Savas has no conflicts of interest to declare.

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Irreversible EGFR-TKIs: dreaming perfection

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Abstract: In the last few years, the treatment of Non-Small-Cell Lung Cancer (NSCLC) has dramatically changed. Presence of activating mutations in the *Epidermal Growth Factor Receptor (EGFR)* identified a particular group of NSCLC patients with different clinical characteristics and outcome. For *EGFR* mutant patients first-generation EGFR tyrosine-kinase inhibitors (TKIs), such as gefitinib and erlotinib, represent the best therapeutic option in first, second and maintenance setting. Unfortunately, all patients develop acquired resistance and despite an initial benefit, virtually all patients progress due to the development of resistance. Several molecular mechanisms are responsible for acquired resistance and the two prominent are the up-regulation of the downstream signal by mesenchymal-epidermal transition (MET) amplification and the emergence of T790M *EGFR* gatekeeper mutation. Preclinical and early clinical trials suggested a potential efficacy of a new class of panHER inhibitor, also called irreversible or covalent inhibitor, in overcome acquired resistance related to T790M. Afatinib, dacomitinib and neratinib, are currently in development in different setting and results from these trials are awaited in order to establish the role of these new compounds in the treatment of NSCLC.

Keywords: NSCLC (non-small-cell lung cancer); EGFR (epidermal growth factor receptor); afatinib; dacomitinib; neratnib; acquired resistance

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Introduction

It is hard to believe that only a decade ago the treatment of non-small-cell lung cancer (NSCLC) was based on simple exclusion of small-cell phenotype. In the last 10 years, steps toward a better knowledge of the mechanisms underlying this lethal disease moved researchers to investigate potential molecular alterations responsible for tumor growth and, consequently, for therapeutic approach. The discovery of mutations in the epidermal growth factor receptor (EGFR) has dramatically changed the treatment of NSCLC (1-3). For patients with lung adenocarcinoma and activating EGFR mutations who received first-generation EGFR-tyrosine kinase inhibitors (TKIs) - such as erlotinib or gefitinib - median overall survival (OS) ranges between 24 and 30 months (4-6), contrasting with the historical plateau of 10 months obtained with front line platinum-based chemotherapy in molecularly unselected populations (7).

Seven large phase III randomized trials conducted in more than 1,400 patients harboring classical EGFR mutations - such as deletion in exon 19 or the L858R substitution in exon 21 - have established a new standard of care (4,5,8-12). In fact, all of these studies demonstrated the superiority of gefitinib, erlotinib or, more recently, afatinib in terms of response rate (RR) and progression free-survival (PFS) when compared to conventional platinum-doublet chemotherapy (*Table 1*). Because the vast majority of subjects enrolled in chemotherapy arm received an EGFR-TKIs at progression, no formal advantage in overall survival has emerged from the aforementioned trials. Nevertheless, in all trials median survival was up to 2-3 years, indicating that EGFR-TKIs are changing natural history of EGFR mutated NSCLC. Finally, since TKI toxicity is generally less severe than the one observed with platinum-based chemotherapy, offering an EGFR-TKIs to a sensitive patient means delay toxic effects of chemotherapy and preserve quality of life (QoL). Similarly, a significant benefit

Table 1 Studies of EGFR TKIs versus chemotherapy as first-line therapy in NSCLC with typical *EGFR* mutations

Study	EGFR TKI	n	Median PFS in TKI arm (months)	P value	HR
OPTIMAL (11)	Erlotinib	154	13.1	<0.0001	0.16
First Signal (8)	Gefitinib	42	8.4	0.084	0.61
IPASS (4)	Gefitinib	261	9.5	<0.0001	0.48
WJTOG 3405 (9)	Gefitinib	177	9.2	<0.001	0.48
NEJSG 002 (10)	Gefitinib	200	10.8	<0.001	0.36
EURTAC (5)	Erlotinib	174	9.4	<0.0001	0.42
LUX-3 (12)	Afatinib	308	13.6	<0.0001	0.47

Table 2 Main criticisms reported with first-generation EGFR-TKIs

(I)	No response in near 30% of NSCLC with classical exon 19-21 mutation
(II)	No clear benefit in presence of uncommon mutations
(III)	Toxicity
(IV)	No patient is cured: median duration of response 9-12 months
(V)	Lack of efficacy in presence of "acquired" T790M mutation

was observed in those EGFR mutant patients treated with erlotinib or gefitinib as second- or third-line treatment (13,14) as well as in maintenance setting (15,16). Taken into account, all these data reinforced the conviction that patients carrying an activating EGFR mutation should never loose the opportunity of receiving an EGFR-TKI during the course of their disease.

However, the enthusiasm generated by these findings has been modulated by the awareness that, until now, no patient can be cured and inevitably all our patients progress and die for their disease. Aim of the present article is to briefly discuss the pitfalls of the first generation EGFR TKIs and to highlight the available data on a new class of inhibitors, also called irreversible or covalent, in the treatment of NSCLC.

Unmet needs with reversible EGFR-TKIs

Main criticisms related to first-generation EGFR-TKIs are listed in *Table 2*.

First, a consistent proportion of *EGFR* mutant patients, approximately 30%, never respond to anti-EGFR TKIs due to primary resistance and the mechanism of this phenomenon is poorly understood (17). On the other hand, we know that *EGFR* mutation does not mean sensitive mutation. *EGFR* mutations exist in exon 18-21 of the tyrosine-binding domain of the EGFR (1,2,18). As previously reported, deletion in exon 19 and L858R point mutation in exon 21 account for the 90% of EGFR mutations detected in NSCLC and are clearly associated with benefit to EGFR TKIs (4,5,8-12). Beside these classical or typical mutations, there is still a small group of "uncommon" mutations, as G719, S768, L861 and others, that can occur with or without a common mutation (19) and for which the clinical impact is poorly understood. Wu *et al.*, analyzed a large series of 1,261 lung cancer cases of which 627 were *EGFR* mutant, with the aim to evaluate the outcome to erlotinib or gefitinib according to the type of mutation (20). The authors confirmed that typical mutations derived the greatest benefit in terms of RR, PFS and OS (74%, 8.5 and 19.6 months respectively) from such treatment; nevertheless the absolute difference in outcome was not so huge when considering the less frequent G719 and L861 mutations (RR 53.3% and 60.0%, PFS 8.1 and 6.0 months, OS 16.4 and 15.2 months for G719 and L861 respectively); on the other hand, some rare uncommon mutations (i.e., V769M and A871E) failed to respond to EGFR TKIs (RR 20%, PFS 1.6 months and OS 11.1 months) with a clinical trend that was very similar to that observed for EGFR wild type population (RR 16.5%, PFS 2.0 months and OS 10.4). Although, the retrospective nature of the investigation and the low sample size of uncommon mutations in large phase III trials, only 6% and 3.8% in the NEJ002 and IPASS respectively (4,10), do not permit to drawn any definitive conclusion, at the present

time it is not recommended in clinical practice to treat in first-line a patient with uncommon mutation with erlotinib or gefitinib.

Second, treatment with reversible EGFR TKIs is generally defined as "overall well tolerated". Indeed in the large phase III trials comparing erlotinib and gefitinib versus standard platinum based chemotherapy, also the toxicity profile was significantly better in the "experimental" arms; the incidences of grade >3 skin rash, diarrhea and liver dysfunction, the three most common adverse events related to EGFR TKIs treatment, did not exceed 20% and the proportion of patients that discontinued therapy due to toxic effects is less than 10% (4,5,8-10). Nevertheless, this small amount of patients, even if molecularly-favored, no longer benefited from therapy. On the other hand, unlike conventional chemotherapy, treatment with targeted agents is continued until disease progression; as a consequence also a long-lasting grade 2 toxicity could became "psicologically serious" over the time mainly because, more often, treated patients are young and able to normal activities.

Last but not least, the most relevant problem related to EGFR TKI therapy is the emergence of acquired resistance (21-23). Indeed, despite an initial dramatic tumor regression in up to 80% of cases after a median time of 9-12 months, all patients progress and the possibility of further control tumor growth inevitably decreases.

Acquired resistance to EGFR TKIs: clinical, biological and therapeutic implications

From a clinical point of view, we refer to acquired resistance according to the criteria proposed by Jackman and coworkers (24) in 2010 considering as "resistant" those patients treated with single-agent erlotinib or gefitinib (I) who progressed while on treatment and (II) who harbored a sensitive EGFR mutation or (III) if *EGFR* status is wild type or unknown, who obtained partial or complete response or a significant and durable (>6 months) clinical benefit - according to RECIST or WHO criteria - after initiation of EGFR TKI therapy. Two important issues derived from this work: first, the utility of a relative simple criteria to correctly define and select for novel clinical trials a population otherwise too heterogeneous; second, the concept that a progression that occur *while on treatment* could be interpreted as a transitory clinical condition related to the type of therapy (i.e., reversible EGFR TKIs) rather than to a true EGFR-pathway-independent tumor growth. In other words, the sensitivity to an anti-EGFR TKIs could be restore after a break period (3,22,25); for this reason many trials with sequential use of chemo- and EGFR targeted therapies are ongoing (25).

From biological point of view, prolonged exposure to erlotinib or gefitinib provides selective pressure for the development of tumor clones able to growth irrespective of the drug inhibition. The mechanisms underlying the phenomenon of secondary resistance are object of extensive evaluation and some of these are so far elucidated (22,23,26). Several preclinical studies demonstrated that the two main mechanisms responsible for acquired resistance are the up-regulation of the downstream signal by mesenchymal-epidermal transition (MET) amplification and the emergence of T790M *EGFR* gatekeeper mutation (26-30). Other mechanisms include EGFR amplifications, PI3KCA mutations or a transition from epiyhelial to mesenchymal differentiation (26). More interestingly, for a little percentage of resistant tumors occurs transformation into SCLC (26).

MET amplification is found to be associated with acquired resistance in up to 20% of cases and inhibition of MET with the use of monoclonal antibodies (31-33) or small molecule TK inhibitor (34) alone or in combination with other targeted agents are currently under investigations. Anti-MET strategies have been extensively discussed elsewhere (35-37).

The "acquired" T790M mutation - a characteristic point mutation in the exon 20 of the *EGFR* gene - is associated with lack of activity of first generation EGFR TKI and is responsible for secondary resistance in at least 50% of patients exposed to erlotinib or gefitinib (22,23,26,38). Initial data showed that this event occur in less than 3% of mutated patients before starting and EGFR TKI therapy (30). More recently, using high sensitive methods, the EGFR T790M mutation was detected in up to 40% of previously untreated NSCLC, suggesting that what we call an "acquired resistance" is a pre-existing phenomenon (39). Retrospective data from Memorial Sloan Kettering Cancer Center suggested that this molecular event is largely underestimated, when assessed by low-sensitive technique (39). Whereas the vast majority of *EGFR* mutations are sensitive to TKIs because they decrease the affinity of the receptor for its natural substrate ATP, the presence of T790M, altering the conformation of the tyrosine kinase domain of the EGFR, restore its affinity for ATP at the levels similar than reported for *EGFR* wild type thus reducing the ability of reversible TKIs to effectively compete with ATP (40-41). *In vitro* studies demonstrated that gefitinib-resistant as well T790M mutation positive clones remain sensitive to irreversible

EGFR TKIs that are structurally similar to erlotinib and gefitinib (42); unlike reversible TKIs, this new class of inhibitor contain an acceptor-group that binds covalently with the Cys797 present at the ATP-binding site of mutant EGFR. As discussed above, due to their characteristics irreversible EGFR TKIs seemed to be the ideal compounds to test in order to overcome T790M acquired resistance (42).

A fascinating way to interfere with the signaling cascade of the EGFR, in order to overcome resistance, is to simultaneously inhibit both the extracellular and intracellular receptor domains. The clinical proof of the so-called "vertical inhibition" comes from previous experience in HER2-overexpressing trastuzumab-resistant metastatic breast cancer, in which the combination of trastuzumab and lapatinib was superior to lapatinib alone in terms of RR and PFS (43).

Similarly in NSCLC, the combination of afatinib and cetuximab induced nearly complete tumor regression in T790M transgenic murine models (44). On this base, a pivotal phase Ib study has been recently conducted in NSCLC patients with clinically defined acquired resistance with the aim to explore the safety and activity of the combination (45). In the initial cohort, 22 patients were exposed to afatinib at the oral daily dose of 40 mg and cetuximab 500 mg/m^2 intravenously every 2 weeks. Adverse events were consistent with the typical class-effects previously reported (i.e., diarrhea and skin rash) and were generally mild, with only 3 patients experiencing grade 3 skin toxicity. Every patient obtained disease control with a median reduction in tumor size of 76% and a promising activity of 36% (8/22 including 4/13 T790M positive cases), leading to enrollment of an additional cohort of 80 patients. Final results have been recently presented. Main grade 3 adverse events were skin rash (12%) and diarrhea (6%); 96 patients were evaluable for efficacy and treatment resulted in 75% of disease control rate with a response rate of 30%, without significant difference between T790M positive and T790M negative patients (32% versus 28% months); median PFS was 4.7 months (46). These encouraging results deserve further validation in large phase III trials.

New generations EGFR TKIs

The second generation of EGFR inhibitors, also-defined irreversible or covalent EGFR inhibitors, afatinib, dacomitinib and neratinib, are pan-ErbB inhibitors and their activity against both EGFR activating mutations and the T790M mutation has been demonstrated in *in vivo* models (47-49).

Afatinib

Afatinib (BIBW2992) binds irreversibly to EGFR, HER2, HER4 and also to EGFR receptors carrying the T790M mutation, suggesting a potential role in overcoming resistance. Multiple phase I studies identified in 50 mg once daily the maximum tolerated dose (MTD) with main toxicities represented by diarrhea and skin rash (50). On this basis, the LUX-Lung clinical trial program has been launched for testing this molecule in different setting in advanced NSCLC patients.

In the phase 2b/3 LUX-Lung 1 trial (51), a total of 585 adenocarcinoma patients who met criteria for acquired resistance to EGFR-TKIs as proposed by Jackman *et al.* (24), were randomized in a 2:1 fashion to receive daily oral afatinib 50 mg plus best supportive care (BSC) or placebo plus BSC as third or subsequent line of therapy. The primary end-point was overall survival. Interestingly, the trial did not need archival tumor tissue and the subjects were not screened for *EGFR* status, but the prior disease control for >3 months under TKIs treatment was used as surrogate criterion to increase probability of *EGFR* mutations. The treatment with afatinib resulted in better activity (RR 7% versus 0.5%) and longer PFS (3.3 months, 95% CI, 2.79-4.40 months) than it was in placebo group (1.1 months, 95% CI, 0.95-1.68 months, HR 0.38, P<0.0001). Surprisingly, the PFS benefit did not translate in survival benefit. Median overall survival was 10 and 12 months for the afatinib and placebo arm respectively; the reason behind this unusual finding could be the confounding effect of post-study therapies; indeed, a greater proportion in the placebo arm than in the afatinib arm receive subsequent treatment, including chemotherapy and EGFR TKI.

Similar activity was preliminary reported in the LUX-Lung 4, a phase II open label trial, in which 62 Japanese patients who progressed after 1 or 2 chemotherapy lines and prior erlotinib or gefitinib underwent therapy with afatinib at the dose 50 mg (52). Response rate was 8%, with DCR of 66%, while PFS resulted of 4.4 months.

Afatinib was also evaluated as first line and second line therapy in patients who had not received a first generation TKI. The LUX-Lung 2 trial was a single-arm, multicenter phase II study evaluating the efficacy of afatinib 40-50 mg daily in advanced adenocarcinoma with *EGFR* activating mutations (53). A total of 129 subjects (first line N=61; second line, N=68) were enrolled onto the study; notably 18% of patients presented an uncommon mutation. In

overall population objective RR, DCR and PFS were 59%, 83% and 14 months respectively, with a median overall survival of 24 months; no difference in outcome was noted between patients harbored L858R or deletion in exon 19 irrespective of line of therapy, while the efficacy in terms of RR, PFS and OS was lower in those patients with uncommon mutations (RR 39%; median PFS 3.7 months; OS 16.3 months).

The LUX-lung 3, the first phase III study using the combination of pemetrexed and cisplatin as a comparator arm, randomly assigned in a 2:1 fashion *EGFR* mutant adenocarcinoma patients to receive as front line therapy afatinib 40 mg daily or six cycles of chemotherapy (12). The study, which enrolled 345 patients, met its primary end point of PFS. Patients treated with afatinib had a 42% relative reduction in risk of progression compared with those receiving standard chemotherapy (11.1 versus 6.9 months, HR 0.58; 13.1 versus 6.9 months, HR 0.47 for patients with classical EGFR mutations). Treatment with afatinib was also associated with higher response rate (56% versus 23%, ITT population) and better toxicity profile than chemotherapy, although G3 diarrhea and skin rash occurred in 14% and 16% of cases receiving the study drug.

Dacomitinib

Dacomitinib (PF0299804), covalently binds the adenosine triphosphate domain of each of three kinase active members of the HER family: EGFR/HER1, HER2 and HER4. In preclinical experiences, dacomitinib showed greater antitumor activity in gefitinib-resistant NSCLC *in vitro* and *in vivo* models (49). In NSCLC clinical trials, Dacomitinib has been evaluated in three different setting: after EGFR TKI failure (54-56), in second line in patients not previously exposed to a reversible EGFR TKI and in front line in *EGFR* mutants patients (57,58).

In a phase I study (54), a disease control rate (PR + SD) of 34% was seen in 44 patients pretreated with first-generation EGFR TKIs (94%) and chemotherapy (79%); most frequently any-grade adverse events observed at the recommended daily dose of 45 mg were diarrhea (78%) and skin rash (65%). In another phase I/II trial conducted in 36 advanced NSCLC patients who progressed after one or two prior chemotherapy regimen and erlotinib (55), DCR was observed in 67% and 40 % of patients with adenocarcinoma and squamous cell carcinoma respectively. In another Korean phase II trial (56), enrolling 42 patients with similar characteristics, preliminary results demonstrated an activity of 15% with a DCR of 25%.

Ramalingam *et al*. published the results of the first randomized trial on irreversible EGFR TKI in lung cancer patients never exposed to TKI treatment (59). Subjects enrolled onto this phase II study were randomly assigned to receive as second line treatment erlotinib (N=94) or dacomitinib (N=94). The primary end point was PFS. In the dacomitinib arm there was a higher number of patients with ECOG performance status 2, *EGFR* mutant and treated with 2 or more prior chemotherapy than in the erlotinib arm. PFS resulted in favor of the experimental arm (median PFS 2.8 versus 1.91 months; HR 0.66); the improvement in PFS was reported across most of the subgroup considered and particularly in KRAS wild type/EGFR any status (median PFS 3.71 versus 1.91 months; HR 0.55), KRAS wild type/EGFR wild type (median PFS 2.21 versus 1.84 months; HR 0.61), while for EGFR mutant patients median PFS resulted of 7.44 in both arms. The objective RR was lower in the erlotinib arm than in dacomitinib arm (5.3% versus 17%), as DCR (14.9% versus 29.8%) did. However, grade diarrhea and skin rash were more frequent with dacomitinib than with erlotinib.

More recently, Kris *et al*. reported the results of the 1017 study of dacomitinib at the dose of 30-45 mg daily in NSCLC patients with *EGFR* mutations or *HER-2* mutations (i.e., exon 20 insertions or point mutations) or *HER-2* amplification (57). Endpoints included progression-free survival rate at 4 months (PFS at 4 M), PFS, partial response (PR) rate and safety. EGFR cohort included never or light-former smoker (<10 pack year) patients with metastatic non-pretreated adenocarcinoma or treatment-naïve patients with known *EGFR* mutations, while HER2 cohort enrolled subjects with *HER2* mutations or amplification who received any number of prior therapy. In the EGFR cohort (Cohort A, N=89), 46 of patients harbored a classical mutation (exon 19, N=25; exon 21, N=21); in this subgroup, RR rate was 76% while PFS at 4M and PFS were 95.5% (95% CI, 83.2-98.9%) and 18.2 months (95% CI, 12.8-23.8 months) respectively. As expected, common side effects were diarrhea, skin toxicity and nail changes. Cohort B is still recruiting and in the first 22 enrolled patients (*HER2* amplification, N=4; *HER2* mutation, N=18) an interesting activity of 14% was observed, but limited to those patients carrying a *HER-2* mutation.

Neratinib

Neratinib (HKI-272), an irreversible HER family inhibitor

Table 3 Comparison of best reported phase II results for EGFR TKIs in patients with *EGFR*-Mutant lung cancers (Exon 19 and Exon 21)

	Pts Enrolled, N	RR, %	mPFS, mos	mOS, mos
Dacomitinib (57)	46	74	17	NR
Afatinib (53)	129*	66	15	32-39
Erlotinib (61)	33	70	14	31
Gefitinib (62)	27	59	9.2	17.5

*51 treated first-line

targeting EGFR/HER-1, HER-2 and HER-4, was initially tested in a phase I trial of 72 patients with advanced ErbB2 or ErbB1/EGFR IHC positive tumors (58). Maximum tolerated dose (MTD) was determined to be 320 mg and the most common related adverse event at this dose was diarrhea. Strikingly, a long-lasting disease control (defined as stable disease for >24 weeks) was observed in 43% of refractory NSCLC patients.

A large non-randomized phase II tria l explored the activity of neratinib in three different cohorts of advanced pretreated NSCLC patients (60). Arm A included patients with activating EGFR mutation (N=91), arm B included *EGFR* wild-type patients (N=48) while arm C included EGFR TKI-naïve patients selected for adenocarcinoma histology and smoking history (N=28). Subjects in arms A and B had to have received at least 12 weeks of prior erlotinib/gefitinib treatment. In the overall population (N=158), the activity was lower than expected, with only 2% of responders (RR 3.4% arm A; 0% arm B; 0% arm C). Interestingly, the three responding patients harbored the rare G719X point mutation in exon 18, maybe suggesting that neratinib could be less effective in presence of classical *EGFR* mutations; on the contrary, the presence of T790M mutation did not seem guarantee any benefit from such treatment. Median PFS was 15.3 weeks in the entire cohort, without significant difference between the three arms (15.3, 16.1 and 9.3 weeks in arm A, B and C respectively). Nevertheless, in the first 39 patients receiving neratinib at the dose of 320 mg daily the occurrence of grade 3 diarrhea was unacceptably high (50%); as a consequence, a dose reduction to 240 mg was required in order to improve tolerability with the hypothetical disadvantage of negatively affect response. Anyway, this major limitation led to dissipate the interest to further explore neratinib in NSCLC.

Discussion

The ideal inhibitor might be equally effective irrespective of the type of *EGFR* mutations, highly similar to the binding site of the receptor, active even in presence of T790M clones and - from the patient point of view - at least with identical or better toxicity profile than older compounds. Have the irreversible EGFR TKIs met all this endpoints?

In front line setting, the efficacy of covalent inhibitors is comparable to the one reported for reversible TKIs. In the LUX Lung 3 trial median PFS for patients with typical *EGFR* mutations is more than 13 months, with an absolute improvement of nearly 7 months respect to chemotherapy arm (12). These results is quite similar to those reported in the OPTIMAL trial, in which an impressive HR of 0.16 for PFS in favour of erlotinib arm was observed (11); nevertheless, unlike OPTIMAL, in the LUX-3 the difference in outcome between *EGFR*-TKI therapy and chemotherapy appears to be real, considering the high performance of the comparator arm. In phase II trial, Dacomitinib showed an unexpected PFS of nearly 18 months, but this finding deserves further validation in prospective large phase III studies (57). In terms of activity, best response rate observed in phase II trials of first and second generation EGFR-TKIs seemed almost identical for both class of inhibitors (53,57,61,62) (*Table 3*). Large phase III trials comparing head-to-head irreversible versus reversible EGFR TKIs are urgently needed to define whether covalent inhibitors may improve outcomes and possibly delay the onset of resistance.

Once again, patients harboring a classical mutation gained the greatest benefit from such treatments. In the LUX Lung 2, in which 18% of patients presented uncommon mutations, the RR and PFS was lower for this population and in any case, were consistent with those reported for gefitinib and erlotinib (53). In the LUX Lung 3 study (12,63), 48 (10.6%) patients presented uncommon mutations that were were categorized into 5 groups: T790M, G719X, S768I, exon 20 insertions, L861Q; the first 3 groups included double mutant patients. Tumour response and prolonged PFS were noted in 2 double mutant patients (L858R + T790M; S768I + L858R) and in 2 with single uncommon mutation (G719X and S768I), while in the other cases SD was the best response. Nevertheless these results are inconclusive, as the effect of afatinib in doublet mutant patients could be in part referred to the presence of

Table 4 Grade >3 toxicity with EGFR-TKIs

	Gefitinib				Erlotinib		Afatinib
	NEJSG 002 (10) n=114	IPASS (4) n=607	First-SIGNAL (8) n=159	WJTOG3405 (9) n=87	OPTIMAL (11) n=83	EURTAC (5) n=84	LUX-3 (12) n=229
Rash	71.0 [5.3]	66.2 [3.1]	72.3 [1.3]	74 [2]	73.5 [2.4]	11 [13.0]	37 [16.2]
Diarrhea	34.2 [0.9]	46.6 [3.8]	NR	47[1]	25.3 [1.2]	4 [5.0]	33 [14.4]
Fatigue	10.5 [2.6]	NR	28.3 [0.6]	34 [2]	4.8 [0]	5 [6.0]	3 [1.3]
Anorexia	NR	21.9 [1.5]	44.7 [0]	NR	NR	0 [0]	7 [3.1]
Stomatitis	9.6 [0]	17.0 [0.2]	NR	19 [0]	13.3 [1.2]	NR	20 [8.7]
Paronychia	NR	13.5 [0.3]	NR	28 [1]	3.6 [0]	NR	26 [11.4]
Vomiting	6.1 [0.9]	12.9 [0.2]	NR	NR	NR	NR	7 [3.1]

the L858R mutation. As previously reported (60), neratinib seemed to be more effective in presence of the rare G719X mutation; this might simply reflect a different sensitivity of specific mutations to an EGFR TKI. Furthermore, is it not possible to exclude that this result was obtained by chance because of the very small number of patients.

Irreversible TKIs have been developed with a specific focus on patients with acquired resistance to erlotinib or gefitinib. LUX-Lung 1 (51) and LUX-Lung 4 (52) trials failed to demonstrate a clear benefit in terms of RR in patients with acquired resistance and particularly in those cancers with T790M; the activity reported in the 2 studies was only 7% and 8%, lower than expected. We recently presented a retrospective analysis of 68 advanced lung adenocarcinoma patients with acquired resistance to reversible EGFR TKIs treated with afatinib and we reported a response rate of 10.6% with a disease control rate of 65%. Four of the five responding patients harbored a classical mutation including 1 patient with T790M; in 9 patients in which tumor biopsy was repeated before starting afatinib, only 2 patients had T790M mutation, with no evidence of response (64). All these results are disappointing and suggest that the ability of covalent inhibitor in overcome acquired resistance may have limitations unpredicted in preclinical experiences; a possible explanation could be the different drug concentration achieved in humans respect to preclinical models.

Another critical issue concerns the toxicity profile of the irreversible inhibitors. In metastatic setting, the preservation of QoL still remains one of the goals of therapy, mainly when considering second and subsequent line of treatment. In the case of neratinib, an unacceptable incidence of 50% of grade diarrhea required a dose reduction in the Sequist's phase II trial (60). Grade 3 adverse events reported in LUX 1 and 2 trials (51,52), led the clinicians to consider 40 mg as the "optimal" tolerated dose, instead of 50 mg defined in phase I trial (50). Anyway, indirect comparison of phase III trials showed higher incidences of diarrhea, skin rash and stomatitis for afatinib respect to erlotinib or gefitinib (4,5,8). Main grade >3 toxicities with EGFR-TKIs are listed in *Table 4*. Taken into account, all these data suggested that toxicities of covalent inhibitors are probably higher than those observed with first-generation compounds.

Conclusions

Irreversible EGFR TKIs could represent a promising therapeutic option in the treatment of NSCLC. Although in absence of trials directly comparing reversible versus irreversible TKIs, available data failed to demonstrated a superior efficacy respect to first-generation inhibitors. Furthermore, the activity reported in patients harbouring an EGFR uncommon mutation is consistent with the one observed for gefitinib and erlotinib. Although the clinical development of covalent inhibitors focused on T790M-dependent acquired resistance, activity observed in this particular subgroup was only modest. The high affinity for ATP binding site could in part explain the prevalence of typical class-effects observed with afatinib, neratinib and dacomitinib. Results from ongoing and planned clinical trials, will help us to define the role of second generation TKIs in our clinical practice.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Targeted therapy in non-small cell lung cancer: a focus on epidermal growth factor receptor mutations

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Abstract: The main molecular targeting of lung cancer [non-small cell lung cancer (NSCLC)] concerns mutations of epidermal growth factor receptor (EGFR). The awaited responsiveness of tumors carrying these mutations is high with for instance 60% to 80% with tyrosine kinase inhibitors hitting EGFR mutations. The EGFR T790M as a secondary mutation is responsible for the occurrence of a resistance phenomenon. A multitude of drugs have been produced and tested with the property of a specific binding at the EGFR T790M site. There is currently an evolution oriented to a robust genotyping methods allowing the identification of given molecular anomalies (pyrosequencing for instance) towards the consideration of a much larger set of molecular anomalies under the form of a global genotyping realized with the use of next-generation sequencing (NGS). This phase of whole genome analysis necessitates the introduction of a specialized staff for data treatment. A possible substitution plasma/tumor for the mutation analyses is perceptible in lung cancer, a preference being however given to the intratumoral direct investigation when

this is feasible. EGFR mutations as targetable anomalies are illustrative examples, that the management of NSCLC is currently drawing a significant benefit from personalized therapy.

Keywords: Targeted therapy; epidermal growth factor receptor pathway (EGFR pathway); tyrosine kinase inhibitors (TKIs); lung cancer; tumor mutations

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Lung cancer represents the main tumoral pathology with a high mortality (1). The last ten years have seen the emergence of histology (squamous cell vs. non squamous cell) as a determining factor for the management of lung cancer. But, above all, an important proportion of patients may now benefit of a molecular characterisation of their tumoral lesions which can be treated with targeted therapy (on mutations, on fusion genes). Currently, the majority of the molecular targets concerned by this therapeutic strategy are found in tumors which are of adenocarcinoma type.

Background

The main molecular targeting of lung cancer [non-small cell lung cancer (NSCLC)] concerns mutations of epidermal growth factor receptor (EGFR). The first applied tyrosine kinase inhibitors (TKIs) like erlotinib and gefitinib have a preferential activity against activating EGFR mutations of lung cancer, these agents have been the first to open the era of targeted therapy of lung cancer in the beginning of 2000 (2). Of note, the presence of these mutations is globally at relatively low frequency in NSCLC with the occurrence in 17% of Caucasian patients and 40% of Asian patients of targetable EGFR mutations and around 6% of patients with the ALK translocation. The awaited responsiveness of tumors carrying these mutations is high with for instance 60% to 80% to TKIs hitting EGFR mutations (3). After an initial and satisfactory response to EGFR TKIs, almost all patients present a phenomenon of resistance manifested by tumoral progression evident after 9 to 12 months (4). Focused genotyping analyses performed on biopsy samples of resistant patients with acquired resistance have put the light on the EGFR T790M as a secondary mutation as responsible for the occurrence of this resistance phenomenon. This secondary mutation is occurring in almost 60% of resistant tumors (4). The mechanism of action by which the resistance is playing involves a conformational modification in the ATP pocket of the EGFR itself giving to the active site more affinity towards ATP than gefitinib or erlotinib. As a primary site of acquired resistance EGFR T790M was an evident tempting target for drug developers facing an important medical need. In principle, a drug which would impact preferentially the mutant EGFR would spare adverse events carried by the presence of WT-EGFR. Not surprisingly a multitude of drugs have been produced and tested with this property of a specific binding at the EGFR T790M site. Afatinib is among these emerging drugs showing activity on this specific form of EGFR (4). More recently (5), a 3rd generation of drugs targeting specifically T790M were made available (AZD9291, CO1686…). To summarize at this stage, most EGFR mutations concern exon 19 deletions (Del 19) and L858R mutation in exon 21, they represent globally 90% of all mutations and are linked with sensitivity to EGFR TKIs. At the opposite, lung cancers exhibiting exon 20 insertions or T790 M in exon 20 are shown to be resistant to these drugs (5).

ALK targeting with crizotinib is offering 50% to 60% of objective response rate in patients whose tumor is carrying the ALK anomaly (3). A new generation of ALK TKIs are now of clinical use with ceritinib and alectinib. These drugs allow a new phase of therapeutic gain to be obtained in cases of resistance to crizotinib (6). Work is in progress in order to identify predictive factors for a resistance to crizotinib with candidates being numerous including growth factors, kinases, interacting proteins, transcription factors but no one among this large list is emerging currently with sufficient evidence (6). A second-generation of ALK inhibitors, with ceritinib as a concrete example, can overcome several crizotinib-resistant mutations and has shown efficacy both *in vitro* and *in vivo* with the use of pertinent laboratory models of acquired

Table 1 Lung cancer—druggable targets (from INCa data 2012)

Target	Function of the marker	Drug	Activity of drug
EGFR activating mutations	Molecular target	Gefitinib Erlotinib	Reversible inhibitors of EGFR
		BIBW 2992	Irreversible inhibitor of EGFR and HER2
		PF00299804/PF299	Irreversible inhibitor of EGFR and HER2
Primary resistance to EGFR targeting	Molecular target + (resistance TKI-EGFR)	BIBW 2992	Irreversible inhibitor of EGFR and HER2
		PF00299804/PF299	Irreversible inhibitor of EGFR and HER2
Mutations in exon 20 of HER2	Molecular target + (resistance TKI-EGFR)	BIBW 2992	Irreversible inhibitor of EGFR and HER2
EML4-ALK translocations	Molecular target + (resistance TKI-EGFR)	PF-02341066	Double inhibitor MET/ALK
KRAS mutations	Prediction of response + (resistance TKI-EGFR and TKI EGFR irreversibles)	AZD6244/ARRY-142886	MEK inhibitor
	Prediction of response + (resistance TKI-EGFR irreversibles)	GSK1120212	MEK inhibitor
	Prediction of response + (resistance TKI-EGFR irreversibles)	Ridaforolimus (AP 23573, Deforolimus)	mTOR inhibitor

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitors.

resistance to crizotinib. This is consistent with recent clinical data showing an evident activity of ceritinib in patients with crizotinib-resistant disease (7).

Mutation analyses

In France, the National Cancer Institute (INCa) is playing a preponderant role for putting at disposal and unifying the methods for the practice of molecular testing with clinical applications for the larger number possible of patients (see *Table 1*). The French territory is covered with regional platforms dedicated to the practice of molecular biology testing under the auspices of the INCa. Thus, the analytical need for the determination of molecular anomalies of therapeutic interest is taken into consideration and this is particularly true for lung cancer. The analysis is to be considered in its totality including not only the analytical aspect with a specific equipment but also the biological sample itself on which the analysis is applied. There is currently an evolution from the use of robust genotyping methods allowing the identification of given molecular anomalies (pyrosequencing for instance) towards the consideration of a much larger set of molecular anomalies under the form of a global genotyping realized with the use of next-generation sequencing (NGS) necessitating in the whole analysis the introduction of specialized step for data treatment. Currently the precise field of utilization of NGS between research and routine use remains to be elucidated. As said above another consideration to be paid to these molecular analyses concerns the tumoral material itself. This is particularly true in the domain of lung cancer where it is often difficult to obtain a tumoral sample in adequate conditions (access, optimal volume) when keeping also in mind the inherent problem of the intra-tumoral heterogeneity. In this context a perspective of amelioration is perceptible. This ray of hope is brought by the use of so-called "liquid biopsies" which is, practically speaking, the possibility to get tumoral DNA isolated from a blood sample. A recent work by Douillard *et al.* (8) is particularly illustrative on these aspects. The authors have compared, on the basis of almost one thousand of patients, the results of the analysis of EGFR mutations classically performed on the solid tumor in place (deletions exon 19 and point mutation L858R, as the most frequent ones) with those arising from tumoral DNA extracted from blood in parallel in the same patient. The authors reported an interesting high level of concordance higher than 90% for the cases in comparison (652 in total). These data

Table 2 Gene mutations of clinical interest in NSCLC [2015]

Gene	Exon	Method	Type of analysis	Molecular analysis
EGFR	18	Pyrosequencage	Targeted	p.E709*, p.G719*
EGFR	19	Pyrosequencage	Targeted	Del19
EGFR	20	Pyrosequencage	Targeted	p.T790*, pS.S768*
EGFR	21	Pyrosequencage	Targeted	p.L858*, p.L861*
KRAS	2	Pyrosequencage	Targeted	p.G12*, p.G13*
KRAS	3	Pyrosequencage	Targeted	p.Q61*
KRAS	4	NR		NR
BRAF	15	Pyrosequencage	Targeted	p.V600*, p.G464E, p.G466v, p.G469A
PI3KCA	9	Direct sequencage	Global	
PI3KCA	20	Direct sequencage	Global	
HER2	20	Direct sequencage	Global	
ALK		FISH		Translocation ALK
		IHC		Translocation ALK
		RT-PCR		ALK-EML4 (V1,2,3a,3b,5)

NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction.

have led the authors to conclude to a possible substitution plasma/tumor for the mutation analyses in lung cancer, a preference being however given to the intratumoral direct investigation if this one is feasible. Following the publication of these results, European health authorities have confirmed this possibility for the delivery of Iressa and Tarceva (EGFR TKIs on the market).

Conclusions

In total one can consider EGFR mutations in NSCLC as an illustrative example for targeted therapy in cancer care. In France this personalized treatment is made possible to a large number of patients thanks to the concrete and constant implication of the INCa. *Table 2* is providing a complete list of gene mutations, all validated by the INCa, of concerns for the management of NSCLC with targeted therapy.

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EGFR inhibition and more: a new generation growing up

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The discovery of activating epidermal growth factor receptor (*EGFR*) mutations in non-small cell lung cancer (NSCLC) has led to a shift in treatment paradigm for some patients with advanced disease. Mutations in exons 18-21 in the tyrosine kinase domain are associated with improved clinical outcomes following treatment with tyrosine kinase inhibitors (TKIs). The first-generation EGFR TKIs erlotinib and gefitinib are most effective in the presence of *EGFR* mutations (1). However, despite the fact that the majority of patients with *EGFR* mutations benefit from these drugs, in excess of 20% of patients experience *de novo* resistance, and all tumours will ultimately develop resistance following initial response (2). This has driven research into the mechanisms of EGFR TKI resistance and the development of new approaches to overcome this. The study by Ramalingham *et al*. recently published in the Journal of Clinical Oncology is the first trial directly comparing a first generation EGFR TKI with one of the more potent and broadly-specific new generation of drugs in this class (3). It is useful to review EGFR TKI resistance mechanisms to understand some of the rationale driving the development of these newer drugs.

Both primary and *de novo* resistance to TKIs can occur even in the presence of activating *EGFR* mutations. A variety of molecular events is responsible for this, many of which can now be targeted using new agents in development. While many mutations in exons 18, 19 and 21 are predictive of response to TKIs, insertions, duplications or point mutations in exon 20 are observed in around 5% of all NSCLCs which result in a low response rates to first generation TKIs (4). The commonest of these is T790M. Although this mutation is more commonly seen in acquired resistance, varying allele frequencies can be detected prior to TKI exposure in some patients. Besides T790M mutation, alterations in parallel signalling pathways explain a significant further proportion of primary resistant tumours, which are often mutually exclusive with EGFR activation. Around 25% of lung adenocarcinomas harbour activating *KRAS* mutations, and are associated with lack of sensitivity to TKIs presumably because the driving oncogenic molecular event is acting downstream from the EGFR protein (5). Another 5% of tumours harbour a translocation of anaplastic lymphoma kinase (*ALK*) resulting in a fusion kinase (6). This rearrangement results in constitutive fusion activity contributing to carcinogenesis and resulting in resistance to drugs targeting other kinases. Other tumour genomic alterations driving *de novo* resistance to EGFR TKIs include *BRAF*, *PI3K* mutations and amplification of *MET* (2).

The clinical definition of acquired resistance to erlotinib or gefitinib includes patients with known sensitising *EGFR* mutations, and/or with objective clinical benefit from these drugs, progressing despite at least 30 days' continuous therapy. This definition is required to facilitate accurate reporting and the development of potential new agents that might overcome this problem. In contrast to *de novo* resistance, acquired resistance to EGFR TKIs is most often due to T790M mutations, which abrogate the inhibitory effect of first generation TKIs. This secondary *EGFR* mutation (exon 20) was found in nearly 50% of repeat tumour biopsies obtained from patients who developed acquired resistance against first generation TKIs (7). This T790M mutation results in the substitution of a bulky methionine side chain, which affects drug binding in the ATP pocket of *EGFR*.

Alterations in parallel signalling pathways, rather than *EGFR* mutations, can also play an important role in acquired resistance. Independent of T790M mutations,

amplification of the *MET* oncogene can be observed in up to 20% of *EGFR*-mutant tumours following TKI failure (2). Amplification of this receptor tyrosine kinase activates *PI3K* signalling via HER3, independent of *EGFR* activity. In addition mutations in *PIK3CA*, encoding PI3K, can result in tumour resistance to EGFR TKIs (8). Surprisingly, other cases of acquired resistance can be explained by dramatic phenotypic change within tumours. Repeat tumour biopsies upon TKI failure showed transformation to small cell lung cancer (SCLC) in 14% of patients in one series, and a smaller proportion showed evidence of epithelial-to-mesenchymal transition (EMT) (7). The molecular genetic mechanism of this is poorly understood.

New-generation EGFR TKIs such as dacomitinib and afatinib have superior potency *in vitro* and broader specificity, with low nanomolar inhibitory concentrations against HER2 and HER4 as well as EGFR. Irreversible binding by these newer pan-HER TKIs can overcome T790M-induced resistance in preclinical models through covalent binding at Cys-797 of EGFR (9,10). Afatinib was shown to improve progression-free survival (PFS) compared with placebo in the second or third line setting after gefitinib or erlotinib failure in the LUX-Lung 1 trial (11). Although no benefit was recorded in terms of overall survival, significant post-study crossover from the control arm to treatment with with TKIs occurred. These results suggest meaningful clinical activity, and afatinib is likely to become a treatment option for patients with acquired resistance to first generation drugs.

Dacomitinib is another example of a new generation EGFR TKI, which also irreversibly targets EGFR, HER2 and HER4 and has in vitro activity in T790M-mutated cells (9). The phase II study reported by Ramalingham *et al*. is the first trial to directly compare an irreversible pan-HER TKI with a first generation TKI in advanced NSCLC (3). Patients with one or two prior chemotherapy regimens were included, but no previous HER-directed therapy was allowed. Despite randomization, there were imbalances in baseline characteristics with higher numbers of ECOG performance status 2, *EGFR* mutations, and patients receiving two prior chemotherapy regimens in the dacomitinib arm. The primary end point was met with median PFS of 2.9 months for dacomitinib and 1.9 months for erlotinib (hazard ratio =0.66, 95% CI, 0.47 to 0.91, P=0.012), and this effect was seen across all molecular subtypes. These results could possibly have been confounded by an imbalance in baseline characteristics including differences in the number of patients with *KRAS* wild-type/*EGFR*-any status tumours. Nevertheless, after correcting for this a stratified log-rank test favoured superiority of dacomitinib over erlotinib for PFS. No significant difference was seen in overall survival. Treatment-related side effects were as expected, with frequently reported adverse events including diarrhoea, acneiform rash and mucositis. Although treatment withdrawal due to toxicity was uncommon for both arms, treatment-related dose reductions were significantly higher in the dacomitinib group (41%) compared to erlotinib group (17%).

Both this latest study and LUX-Lung 1 suggest clinical benefit from this new generation of irreversible pan-HER TKIs. Their proposed role in deferring or counteracting the most common mechanism of resistance to EGFR TKI therapy is supported by pre-clinical data, although clinical confirmation of this hypothesis is so far inconclusive. Ramalingham *et al*. do not provide data on mechanisms of acquired resistance to either drug in their randomised study. Ongoing phase III studies should clarify the position of the newer agents in the treatment algorithm for NSCLC, and molecular analysis continues to play an increasingly important part in guiding treatment decisions.

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Footnote

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Update on third-generation EGFR tyrosine kinase inhibitors

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Among patients with non-small cell lung cancer (NSCLC), *EGFR* mutations, 90% of which present as an exon 19 deletion or exon 21 point mutation L858R, have been detected in Western and Asian populations at a rate of ~15% and ~40%, respectively. To date, numerous trials have established the efficacy and toxicity profile of single-agent oral EGFR-tyrosine kinase inhibitor (TKI) therapies for EGFR-TKI-naïve NSCLC patients harboring *EGFR* mutations. These trials include IPASS for gefitinib (1), Optimal for erlotinib (2), and LUX-Lung 3 for afatinib (3). Still, the majority of patients will eventually develop resistance.

In the LUX-Lung 4 (4) trial, 61 Japanese patients with lung adenocarcinoma who progressed following gefitinib and/or erlotinib treatment were treated with afatinib 50 mg daily; however, minimal benefit was shown [8.2% confirmed partial response (95% CI, 2.7-18.1%); median progression-free survival (PFS) was 4.4 months (95% CI, 2.8-4.6 months), and median overall survival (OS) was 19.0 months (95% CI, 14.9 months to not achieved)]. Only one patient with a T790M achieved a meaningful outcome (stable disease for 9 months). In both the LUX-Lung 3 and LUX-Lung 4 trials, afatinib showed a higher rate of TKI-related toxicity than has been previously described with gefitinib (1) or erlotinib (2). Toxicities included diarrhea and rash/acne rates of >90% in the LUX-Lung 3, which can impact the ability to safely maintain patients on afatinib treatments and highlights the need for close monitoring and prophylactic medications.

For the AZD9291 trial, a third-generation EGFR-TKI, Janne *et al.* (5) enrolled *EGFR* mutation-positive NSCLC patients with acquired resistance to EGFR-TKI therapy. This trial demonstrated an efficacy benefit with a more amenable side effect profile (AURA; NCT01802632). These findings are likely due to AZD9291 being relatively sparing and selective against wild-type *EGFR* while having better potent activity against mutant *EGFR*, including T790M mutations. More specifically, for all evaluable patients, the overall response rate (ORR) was 51% (91/177), whereas T790M-positive patients (n=89) yielded a 66% ORR (95% CI: 53-74%). The observed ORR of 23% (95% CI: 12-39%) in 43 NSCLC patients whose biopsies tested negative for T790M may have been due to tumor heterogeneity, re-treatment effects (57% of enrolled patients had immediate prior EGFR-TKI), or off-target effects. Age of tumor tissue did not appear to play a role in the results observed in the T790M-negative group as fresh biopsies were required for enrollment to the expansion cohorts. The initial hints of duration of response appear intriguing, but further confirmation is awaited as the trial results continue to mature.

A key aspect of the AZD9291 trial is the improved toxicity profile, which compares favorably with earlier-generation EGFR-TKIs. As expected, the most common EGFR-related adverse events were rash (24%) and diarrhea (30%), both dose dependent and mainly grade 1. Other adverse events included anorexia, dry skin, and nausea. While no dose-limiting toxicities occurred, it is important to note that, in this population previously treated with an EGFR-TKI, side effects also included interstitial lung disease, most of which were grade 1 (n=5), and hyperglycemia, also grade 1 (n=4). Overall, the AZD9291 trial by Janne *et al.* (5) presented at ASCO 2014 demonstrated true clinical significance as there are no FDA-approved drugs for patients who progress after EGFR-TKI resistance, whether or not an acquired resistance molecular

abnormality is identified.

While limitations exist with performing cross trial comparisons, results from this study must be compared to the first-in-human study of CO1686. Similar to AZD9291, CO1686 is an irreversible, third-generation EGFR-TKI therapy that also targets EGFR mutations, including T790M. In the trial, presented by Sequist *et al.* at ASCO 2014 (6) (NCT01526928), 40 T790M-mutant patients with history of progression while on prior EGFR-directed therapy were enrolled. An ORR of 58% was observed, with nausea, fatigue, and impaired glucose tolerance/hyperglycemia as the most common adverse events. The estimated median PFS was >12 months but was ultimately not reached at time of the ASCO presentation. Due to improved bioavailability, the formulation was changed from the free-base capsule to hydrogen bromide salt tablets, with comparable responses reported to date but affecting drug development. Toxicity profile differences between AZD9291 and CO1686 include incidence of hyperglycemia (1% versus 55%), rash (24% versus 4%), and diarrhea (30% versus 23%) (5,6), respectively. These rates are comparable to those shown with erlotinib (25% and 73% for diarrhea and rash, respectively) (2). When choosing between these agents, PFS and OS benefits as well as co-morbidities such as diabetes and patient concerns such as skin toxicity will play a role in the decision-making process. Similar to AZD9291, CO1686 has been granted breakthrough status by the US FDA.

AZD9291 and CO1686 represent very promising therapeutic options for NSCLC patients with resistance to EGFR-TKIs and T790M mutations as well as those limited by severe uncontrolled diarrhea and rash due to targeting of *EGFR* wild-type by earlier generation EGFR TKIs. Still, even with clear demonstration of efficacy and tolerability, alternate treatment options should be evaluated. While a phase I/II trial of erlotinib plus cetuximab failed to reveal any significant clinical benefit in patients with erlotinib resistance (7), preliminary results from Janjigian *et al.* (8,9) (NCT01090011) showed that afatinib 40 mg/m^2 plus cetuximab 500 mg/m^2 in the first 96 patients with defined acquired resistance [Jackman criteria (10)] was efficacious (objective response rate of 30%). In the T790M-positive population, confirmed partial response was 32% versus 28% in the T790M-negative group. With rash and diarrhea occurring in 97%, and 71%, respectively, patients on this combination need to be followed closely. A phase III trial is being planned by SWOG. Other options include intercalating chemotherapy, as is being evaluated in the ongoing trial presented at ASCO 2014 by Schuler *et al.* (11) (NCT01085136). In this trial, 202 patients who had failed prior erlotinib, gefitinib, and afatinib were randomized in a 2:1 ratio of afatinib plus paclitaxel versus investigator choice chemotherapy. Results showed PFS of 5.6 versus 2.8 months (P=0.003), ORR of 32.1% versus 13.2% (P=0.005), and OS of 12.2 versus 12.2 months (P=0.994), along with notable increases in diarrhea and alopecia in the treatment arm. Furthermore, another third-generation EGFR-TKI, HM61713, is under clinical development and may represent another potential option (12) (NCT01588145).

With these promising agents, questions still remain about optimal sequencing, combination strategies, and central nervous system (CNS) penetration. The ongoing trials should provide clarifications. A randomized phase II/III trial of CO1686 versus erlotinib in *EGFR*-mutant NSCLC patients is planned (TIGER 1; NCT02186301), while evaluations of AZD9291 in the EGFR-TKI-naïve population are underway as part of the AURA trial. Combination studies have been initiated such as the trial of AZD9291 plus MEDI4736 (PDL-1 inhibitor), AZD6094 (c-Met inhibitor), or selumetinib led by Astra-Zeneca (NCT02143466), with hopes of further delaying the development of resistance.

CNS relapse remains a risk for patients with NSCLC regardless of *EGFR* mutation status. CNS response with AZD9291 (5) and CO1686 (6) has been reported per their respective ASCO 2014 presentations. Beyond these examples, to our knowledge no data exist specifically detailing the CNS effects of these third-generation EGFR-TKIs. For this class of medications, CNS activity remains uncertain and requires further elucidation.

Findings from the AZD9291 trial along with the CO1686 trial have true clinical significance as there are no FDA-approved drugs for patients who progress on an EGFR-TKI, whether or not a specific acquired resistance molecular abnormality is identified. Moving forward, in the interest of providing more opportunities to our NSCLC patients, all efforts toward rapid and safe clinical development of this compound is imperative. The future of targeting mutant-*EGFR* appears quite promising.

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The continuing role of epidermal growth factor receptor tyrosine kinase inhibitors in advanced squamous cell carcinoma of the lung

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Abstract: Squamous cell carcinoma (SCC) of the lung represents about 20-30% of non-small cell lung cancers (NSCLC) and is associated with a poorer prognosis with limited treatment options. Erlotinib is an approved, standard second-line therapy in this setting, besides docetaxel. The LUX-Lung 8 study has shown superior overall survival (OS), progression-free survival (PFS), as well as disease control rates for treatment with afatinib compared to erlotinib in this head-to-head trial in patients with previously treated advanced SCC of the lung, with manageable side effect profile. This is the first and largest prospective phase III trial comparing two different tyrosine kinase inhibitors in patients with advanced SCC of the lung. Whether the results would be practice-changing remains to be seen, especially with the advent of novel immunotherapeutic agents such as nivolumab, which is recently approved for advanced lung SCC.

Keywords: Non-small cell lung cancer (NSCLC); squamous cell cancer; epidermal growth factor receptor (EGFR); tyrosine kinase inhibitor

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Squamous cell carcinoma (SCC) is the second most common histology in non-small cell lung cancer (NSCLC), and account for 20-30% of NSCLC (1). Compared to advanced lung adenocarcinoma for which targeted therapeutics are available for those harbouring actionable mutations, including epidermal growth factor receptor (EGFR) mutations and ALK-rearrangement, treatment options for advanced lung SCC beyond first-line remain limited. Erlotinib and docetaxel were the only standard second-line treatment options for lung SCC (erlotinib being the only EGFR TKI approved for this setting), until the recent approval of ramucirumab (in combination with docetaxel) for NSCLC (2), and the PD-1 checkpoint inhibitor nivolumab (3).

Although EGFR mutations are rare (<5%) in lung SCC (4), EGFR overexpression and gene amplification tend to be common in these cancers and may play a role in their pathobiology (5). This is supported by phase III studies showing improved overall survival (OS) with the addition of anti-EGFR monoclonal antibodies to platinum doublet chemotherapy in NSCLC—cetuximab in the FLEX study (6), and necitumumab in the SQUIRE study (7). The higher proportion of high-level EGFR expression in lung SCC may also explain why erlotinib has shown efficacy and survival benefit in unselected non-small-cell lung carcinoma including SCC in the BR.21 trial (8,9).

Compared to erlotinib (a reversible EGFR TKI), afatinib is a second-generation EGFR TKI that is an oral, irreversible inhibitor of the ErbB family, blocking signalling from EGFR (ErbB1), HER2 (ErbB2) and HER4 (ErbB4). It has improved progression-free survival (PFS) compared to standard first-line platinum-based doublet

chemotherapy in the two phase III LUX-Lung 3 and 6 studies for EGFR mutant NSCLC (10,11). LUX-Lung 8 is the largest phase III trial for second-line treatment of lung SCC comparing two established EGFR TKIs, afatinib and erlotinib, based on the hypothesis that afatinib would be superior to erlotinib in pre-treated lung SCC, due to its broader mechanism of action and favourable activity seen for squamous histology cancers (12).

In LUX-Lung 8, Dr. Soria and colleagues looked at patients with pre-treated stage IIIB or IV lung SCC who had failed previous platinum-based chemotherapy, stratified by ethnic origin (eastern Asian *vs.* non-eastern Asian), and randomised to receive oral afatinib (40 mg per day) or erlotinib (150 mg per day), until disease progression (12). The patients were not pre-selected for presence of EGFR mutational status at baseline, as testing for EGFR is not standard practice for lung squamous cell cancers. The primary objective was PFS assessed by independent central review for intention-to-treat population, and the key secondary study endpoint being OS. The toxicity profiles were similar in each group (57% of at least grade 3 adverse events); most common adverse events were diarrhoea, rash or acne, fatigue, and stomatitis for afatinib; and rash or acne, diarrhoea, fatigue, and pruritus for erlotinib. There were more grade 3 diarrhoea and stomatitis for afatinib compared to erlotinib which caused more significant rash and acne than afatinib. Notably, there were fatal events from both groups: six treatment-related deaths from afatinib group *vs.* five cases from erlotinib group; causes include interstitial lung disease, pneumonia, pneumonitis, and acute renal failure.

This study had met its primary and secondary end-points. After a median follow-up period of 18.4 months at primary analysis of OS, treatment with afatinib demonstrated significantly longer PFS over erlotinib (median PFS 2.6 *vs.* 1.9 months, HR 0.81, P=0.0103); as well as longer OS (median OS 7.9 months for afatinib *vs.* 6.8 months for erlotinib, HR 0.81, P=0.0077). The effect of afatinib on OS was consistent across all the subgroups, but noted to be most significant and favourable for patients of Eastern Asian ethnicity. Afatinib also resulted in better disease control rate and objective response rate (ORR), as well as improved patient-reported outcomes and disease-related symptoms compared to erlotinib. A similar proportion of patients in both treatment groups went on to receive at least one line of subsequent treatment, docetaxel being the most common post-progression treatment, suggesting that the improvement in survival with afatinib was not due to difference in post-progression treatment.

Does the LUX-Lung 8 study establish EGFR TKI as standard second line therapy for patients with SCC of the lung? The use of erlotinib is still not widely practised for SCC in many institutions. Studies like TAILOR by Garassino *et al.* and DELTA by Kawaguchi *et al.* have not shown superiority of EGFR TKIs over chemotherapy in treatment of advanced NSCLC (unselected and EGFR wildtype) (13,14). In fact, docetaxel was more effective than erlotinib for EGFR wild type NSCLC in the TAILOR study, with slight improved PFS (2.9 *vs.* 2.4 months, HR 0.71, P=0.02); and median OS was 8.2 months for docetaxel *vs.* 5.4 months for erlotinib (HR 0.73, P=0.05). So perhaps it may have been preferable to compare using docetaxel as the control arm, instead of erlotinib. It is therefore uncertain whether the 1.1 month difference in OS in this head-to-head comparison of afatinib *vs.* erlotinib is clinically relevant and would translate into routine clinical practice.

Moreover, the advent of immunotherapeutic agents may possibly soften the appeal for TKIs. In the CheckMate 017 study, which led to the approval of nivolumab in advanced or metastatic squamous cell lung cancer by the FDA in March 2015, nivolumab demonstrated improved ORR, PFS and OS benefit (median OS 9.2 months) over docetaxel (median OS 6.0 months), with 41% lower risk of death with nivolumab than with docetaxel (3). However, there remain several unanswered questions on the use of immune checkpoint inhibitors, including the lack of a robust predictive biomarker, and uncertainty regarding the ideal schedule and duration of therapy (15).

Survival outcomes in patients with advanced SCC of the lung have largely plateaued in the last decade, in part due to the inability to identify actionable mutations that translate to new drug development. Recent data suggest that a detailed understanding of the possible targets in lung SCCs may identify targeted therapeutic approaches. The study on comprehensive genomic characterisation of lung SCC by The Cancer Genome Atlas (TCCA) Research Network has revealed the complex genomic landscape of lung SCC, with a higher mean somatic mutation rate [8.1 mutations per megabase (Mb)] than observed in other tumours including for acute myelogenous leukaemia (0.56 per Mb), breast carcinoma (1.0 per Mb) and colorectal carcinoma (3.2 per Mb) (16). A mean of 360 exonic mutations, 165 genomic rearrangements, and 323 segments of copy number alteration per tumour is found in lung SCC; and significantly altered pathways included NFE2L2 and KEAP1 (34%), squamous differentiation genes

(44%), phosphatidylinositol-3-OH kinase pathway genes (47%), and CDKN2A and RB1 (72%) of the 178 advanced untreated lung SCC profiled in the same study (16). The several molecular alterations found in lung SCC can be classified by their respective therapeutic targets: those involving the membrane receptors (e.g., FGFR1, MET, ERBB2/Her2); the signalling pathways (EML4-ALK, PIK3CA, PTEN, BRAF); and the transcription factors (p53, SOX2) (17). Of these, agents that target FGFR1 and MET amplification appear promising, with several orally available FGFR1 TKIs (BGJ398, AZD4547, TKI258, and E-3810) as well as MET inhibitors (crizotinib, XL 184, MetMAb, and ARQ 197), being developed and investigated in clinical trials. Whether the discovery of all these potential therapeutic targets in lung SCC will translate into corresponding therapeutic success in clinical practice is yet to be established, but it certainly highlights the increasing importance of molecular testing in patients with lung SCC.

In summary, EGFR TKI will continue to play an important but limited role in the treatment of patients with advanced and metastatic SCC of the lung, in part due to its ease of oral administration and acceptable toxicity profile. There is a need to develop predictive and specific molecular biomarkers that might identify subgroups of patients with SCC of the lung that are most likely to benefit from EGFR TKI treatment. Finally, as more treatment options become available for patients, what would be most important is to tailor the various therapeutic options to the patient's own preferences, tolerability, as well as affordability, especially in the era of rising healthcare costs and longer lifespan of patients with advanced lung cancers.

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Footnote

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Lung cancer biomarkers, targeted therapies and clinical assays

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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

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 a cytoplasmic chimeric protein with constitutive kinase activity allowing activation of the *RAS-MEK-ERK*, janus

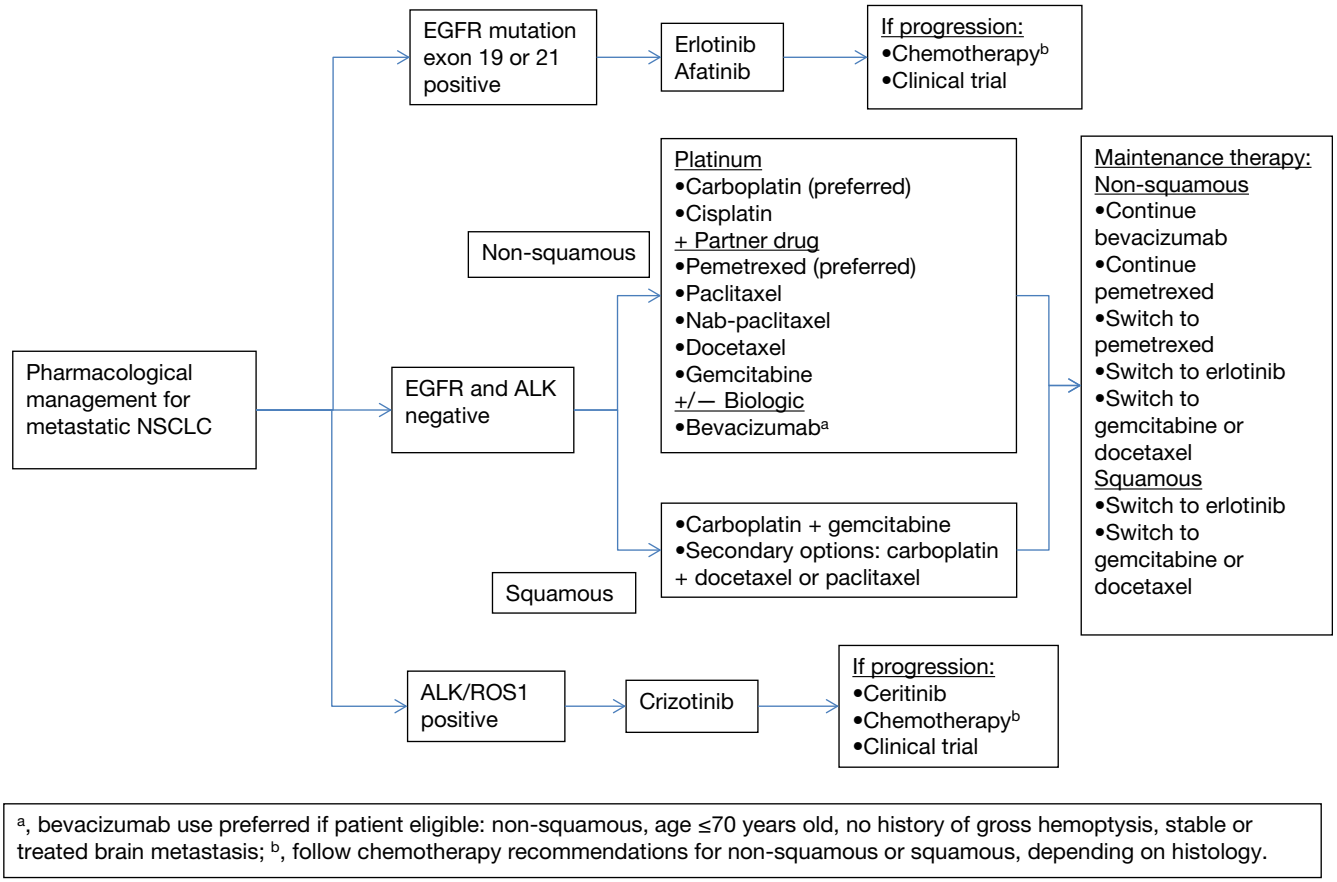

Figure 1 Example of a biomarker-driven treatment pathway for NSCLC, whereby mutations in EGFR or ALK drive targeted therapy selection, while patients with tumors negative for these biomarkers have therapy guided by histology and other clinical factors. NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase.

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

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

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

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

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

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

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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Targeted therapy for non-small cell lung cancer: current standards and the promise of the future

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Abstract: In recent years, there has been a major paradigm shift in the management of non-small cell lung cancer (NSCLC). NSCLC should now be further sub-classified by histology and driver mutation if one is known or present. Translational research advances now allow such mutations to be inhibited by either receptor monoclonal antibodies (mAb) or small molecule tyrosine kinase inhibitors (TKI). Whilst empirical chemotherapy with a platinum-doublet remains the gold standard for advanced NSCLC without a known driver mutation, targeted therapy is pushing the boundary to significantly improve patient outcomes and quality of life. In this review, we will examine the major subtypes of oncogenic drivers behind NSCLC as well as the development of targeted agents available to treat them both now and in the foreseeable future.

Keywords: Non-small cell lung carcinoma; targeted therapy; epidermal growth factor receptor (EGFR); anaplastic lymphoma kinase (ALK)

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Introduction

Lung cancer remains by far the single most common cause of cancer-related mortality with nearly 1.6 million deaths worldwide in 2012 or nearly 20% of cancer mortality as a whole (1). Over the last decade, molecular translational research advances have heralded major breakthroughs in the understanding, diagnosis and management of lung cancer, particularly for the more common (~80%) non-small cell lung cancer (NSCLC). Conversely, treatment for small cell lung cancer remains chemotherapy-based and whilst there are promising results with novel cytotoxics, its platinum-etoposide backbone holds strong (2).

The term '*Theranostics*' whereby therapeutics and diagnostics have been meaningfully combined to achieve personalised pharmacotherapy has now become commonplace in oncology. Sequencing of the human genome has permitted more efficient identification of epigenetic mutations, tumour-suppressor-gene inactivation as well as oncogene driver mutations that are potential targets for therapy (3-8). Such examples include trastuzumab for HER-2 over-expressing breast cancer and vemurafenib for BRAF-mutant melanoma (9,10).

It is now accepted that NSCLC is not a singular entity but is in fact multiple pathologies with unique molecular signatures that we are only beginning to unravel and understand (11-13). Broadly speaking, the main subtypes are pulmonary adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma. This distinction alone allows for a more tailored selection of cytotoxic chemotherapy in advanced NSCLC without a driver mutation, as seen with enhanced efficacy with pemetrexed in adenocarcinoma (14,15) or the toxicity concerns of bevacizumab in patients with squamous histology (16).

Optimal management of NSCLC now requires that tumours be screened for a range of predictive and prognostic biomarkers that help to predict sensitivity to targeted therapy and estimate prognosis respectively (17). For NSCLC, much of the work in the last decade has been focussed on mutations of the epidermal growth factor receptor (EGFR) and on the abnormal fusion of the anaplastic lymphoma kinase

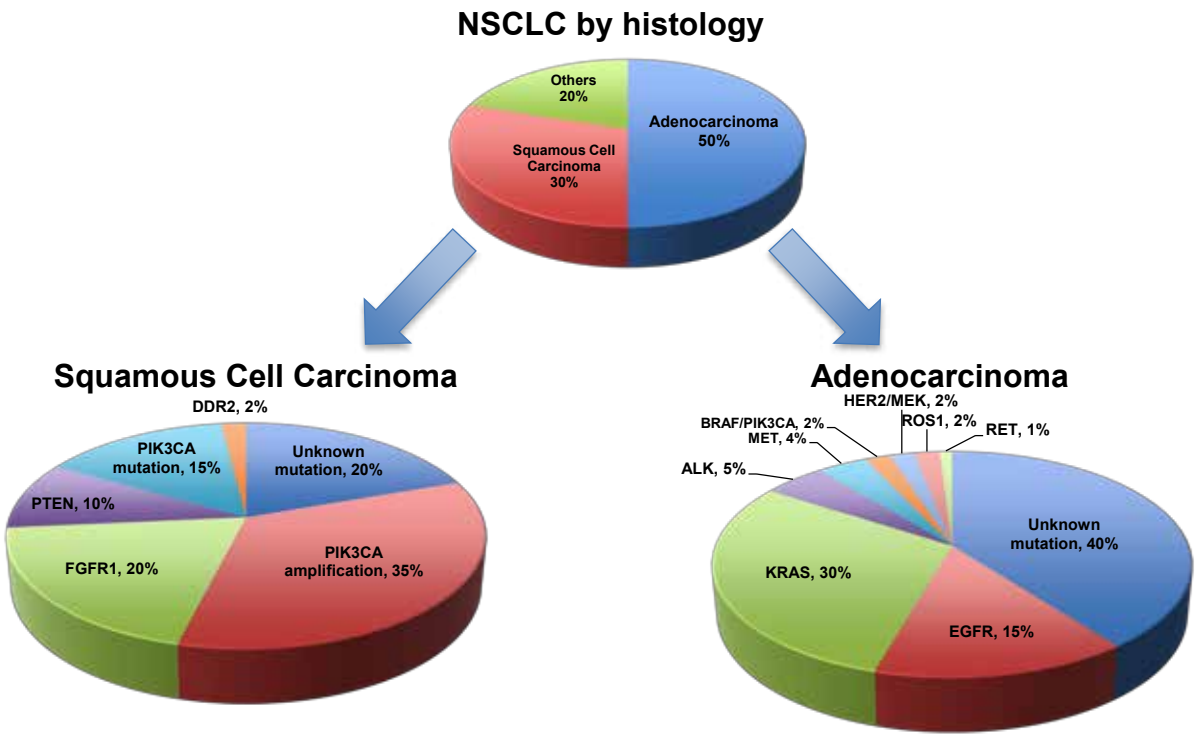

Figure 1 NSCLC by histology and mutations. NSCLC, non-small cell lung cancer.

(ALK) being inhibited successfully with EGFR tyrosine kinase inhibitors (TKI) and crizotinib respectively. Targeted agents are now being rationally designed to inhibit particular mutations leading to a more streamlined clinical trial process. In this review, we will examine the major subtypes of driver mutations that have been identified in NSCLC and relevant targeted therapies available both now, and in the foreseeable future.

Signalling pathway targets in NSCLC

The traditional and now over-simplified histological distinctions within NSCLC include adenocarcinoma, SCC and large cell carcinoma (*Figure 1*). Up to 60% of lung adenocarcinoma and up to 50-80% of SCC have a known oncogenic driver mutation (*Figure 1*) (18,19). These mutations in receptors or protein kinases can stimulate a complex cascade of cross signalling pathways such as the RAS-RAF-MEK-ERK or MAPK, PI3K-AKT-mTOR or JAK-STAT pathways (*Figure 2*) (3,4,7,18,20). Ultimately these lead to uncontrolled growth, proliferation and survival. Successful targeted therapy involves the identification and inhibition of these up-regulated pathways by either small molecule inhibitors or receptor monoclonal antibodies (mAb). The best studied in NSCLC is the interaction between EGFR and its downstream pathways.

Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR or ErbB1 or HER1) belongs to a family of receptor tyrosine kinases that can trigger a vast array of signalling pathways leading to cell growth, proliferation and survival (20,21). Such flow-on pathways include the RAS-RAF-MEK-ERK or MAPK pathway and the PI3K-AKT-mTOR pathways.

There are three main mechanisms leading to EGFR activation: increased expression of EGFR on malignant cells; enhanced ligand production by malignant cells; and activating mutations of EGFR within malignant cells. EGFR is overexpressed in up to 40-80% of NSCLC and was a promising translational therapeutic target however it was subsequently discovered that activating mutations rather than overexpression of EGFR was the prime therapeutic target. The two most common mutations are exon 19 deletions (60%) and L858R missense substitutions at position 858 (35%) where leucine is replaced by arginine

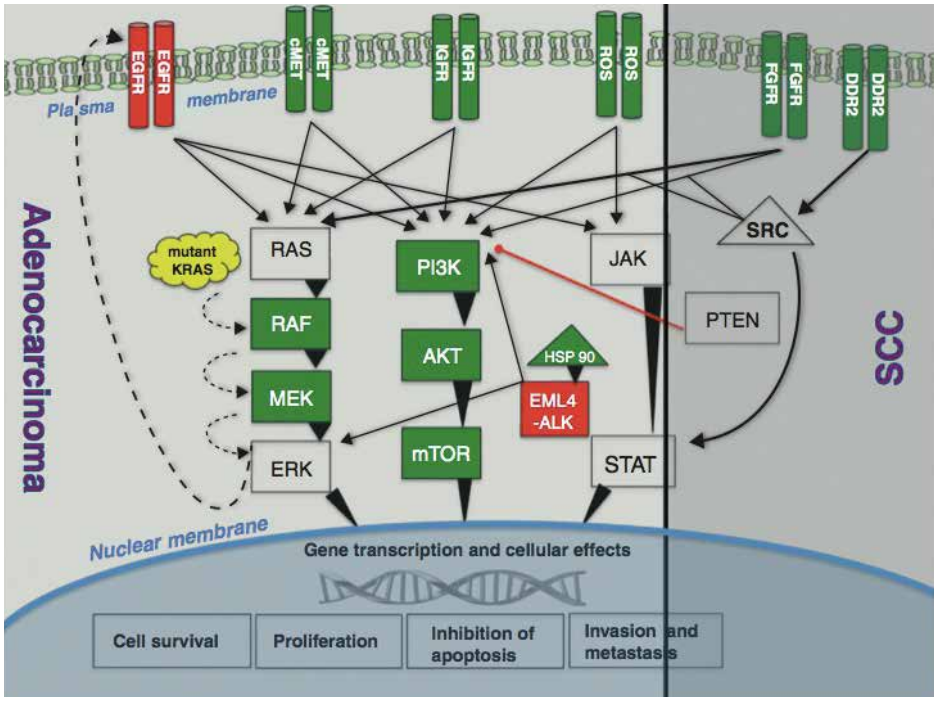

Figure 2 Overview of molecular pathways and potential targets in non-small cell lung cancer (NSCLC) [(from Alamgeer *et al*. (18)].

resulting in constitutive activation of the receptor without ligand binding (21-23). Mutant EGFR can be inhibited either by small molecule TKI (such as gefitinib and erlotinib) or mAb (such as cetuximab).

Gefitinib and erlotinib were the first EGFR TKIs to be developed. Both are reversible competitive inhibitors of ATP for the tyrosine kinase domain of EGFR resulting in blockade of downstream pathways. Early trials used EGFR TKIs in an unselected population as these predated the now known clinical and molecular predictive biomarkers (24-28). As trials matured, subgroup analyses identified characteristics that correlated with response such as adenocarcinoma histology, Asian ethnicity and minimal smoking history (24-26,29-34). Molecular testing of tissue samples from those who had responded to TKIs revealed that somatic activating mutations in EGFR underpinned the responses seen (29,30,35-37). The incidence of EGFR mutation varies with ethnicity, with Asian populations having up to 50% of adenocarcinomas driven by activating EGFR mutations compared to only 10% to 15% in Caucasians (37). Unfortunately, there are no reliable clinical phenotypes or characteristics that allow for accurate prediction of an EGFR mutation, thus all tumours must undergo specific mutational testing (38).

EGFR-mutant NSCLC

The most significant paradigm change in the last 10 years for NSCLC management was heralded by the use of EGFR TKIs as first-line therapy for patients with a targetable EGFR driver mutation. The landmark Iressa Pan-Asia Study (IPASS) randomised 1,217 patients from several East Asian countries with untreated stage IIIB or IV adenocarcinoma to gefitinib or carboplatin and paclitaxel chemotherapy (*Table 1*) (39). Subjects were clinically selected with no or minimal smoking history and EGFR was explored as a potential biomarker. IPASS met its primary endpoint with a 12-month progression-free survival (PFS) of 24.9% with gefitinib versus 6.7% with chemotherapy (39). EGFR status was known in approximately a third of patients, and of these, 60% harboured an activating mutation. For these patients, PFS was significantly prolonged with gefitinib compared to chemotherapy [HR 0.48 (95% CI, 0.36-0.64); P<0.001]. Conversely, patients with wild-type EGFR did better with chemotherapy [HR 2.85; (95% CI, 2.05-3.98); P<0.001]. The First-SIGNAL study (41) verified these findings by clinically selecting never smokers with adenocarcinoma then comparing chemotherapy to gefitinib first-line (*Table 1*). Overall PFS was not significantly different but

Table 1 Phase III Trials comparing EGFR-inhibitors to chemotherapy in advanced stage IIIB/IV NSCLC

Trial [year] (Ref)	Patient selection	Targeted therapy (TT)	Comparator (C)	Median PFS TT *vs.* C (mo.)	HR	P value
First-line EGFR TKI versus chemotherapy						
IPASS [2009] (39,40)	n=1,217, clinical, non/light smokers, Adc, 60% EGFR mutant (Asia)	Gefitinib	Carboplatin; Paclitaxel	9.8 *vs.* 6.4	0.48	≤0.001
First-SIGNAL [2012] (41)	n=309, clinical, never smokers, Adc, 44% EGFR mutant	Gefitinib	Cisplatin; Gemcitabine	5.8 *vs.* 6.4	1.198	0.138
WJTOG3405 [2010] (42)	n=172, molecular EGFR mutant	Gefitinib	Cisplatin; Docetaxel	9.2 *vs.* 6.3	0.489	<0.0001
NEJSG [2010] (43)	n=230, molecular EGFR mutant	Gefitinib	Carboplatin; Paclitaxel	10.8 *vs.* 5.4	0.30	<0.001
OPTIMAL [2011] (44)	n=154, molecular EGFR mutant, 88% Adc	Erlotinib	Carboplatin; Gemcitabine	13.1 *vs.* 4.6	0.16	<0.0001
EURTAC [2012] (45)	n=174, molecular EGFR mutant	Erlotinib	Platinum doublet	9.7 *vs.* 5.2	0.37	<0.0001
LUX-Lung3 [2013] (46)	n=345, molecular EGFR mutant Adc	Afatinib	Cisplatin; Pemetrexed	11.1 *vs.* 6.9	0.58	0.001
LUX-Lung6 [2014] (47)	n=364, molecular EGFR mutant Adc	Afatinib	Cisplatin; Gemcitabine	11.0 *vs.* 5.6	0.28	<0.0001
First-line EGFR therapy plus chemotherapy						
INTACT-1 [2004] (48)	n=1,093, unselected, Adc + SCC	Gefitinib 500 mg/250 mg or placebo + chemotherapy	Cisplatin + Gemcitabine (chemotherapy alone)	5.5 (500 mg), 5.8 (250 mg) *vs.* 6.0	NR	0.7633
INTACT-2 [2004] (49)	n=1,037, unselected, Adc + SCC	Gefitinib 500 mg/250 mg or placebo + chemotherapy	Carboplatin + Paclitaxel (chemotherapy alone)	4.6 (500 mg), 5.3 (250 mg) *vs.* 5.0	NR	0.0562
TRIBUTE [2005] (50)	n=1,059, unselected, Adc + SCC	Erlotinib + chemotherapy then maintenance Erlotinib	Carboplatin; Paclitaxel	5.1 *vs.* 4.9	0.937	0.36
TALENT [2007] (51)	n=1,172, unselected, Adc + SCC	Erlotinib + chemotherapy	Cisplatin; Gemcitabine	5.5 *vs.* 5.7 (23.7 *vs.* 24.6 wks.)	0.98	0.74
FLEX [2009] (52)	n=1,125, Adc + SCC, EGFR expression	Cetuximab + chemotherapy	Cisplatin; Vinorelbine	4.8 *vs.* 4.8	0.943	0.39
BMS099 [2010] (53)	n=676, unselected, Adc + SCC	Cetuximab + chemotherapy	Carboplatin; Paclitaxel or Docetaxel	4.40 *vs.* 4.24	0.902	0.2358
TORCH [2012] (54, 55)	n=760, unselected, Adc + SCC	Erlotinib (followed by Cisplatin Gemcitabine)	Cisplatin Gemcitabine (followed by Erlotinib)	6.4 *vs.* 8.9	1.21	NR

Table 1 (*continued*)

Table 1 (*continued*)

Trial [year] (Ref)	Patient selection	Targeted therapy (TT)	Comparator (C)	Median PFS TT *vs*. C (mo.)	HR	P value
Second- or third-line EGFR TKI versus placebo						
BR.21 [2005] (56)	n=731, unselected, Adc + SCC	Erlotinib	Placebo	2.2 *vs*. 1.8 (OS 6.7 *vs*. 4.7, P<0.001)	0.61	<0.001
ISEL [2005] (57)	n=1,692, unselected, Adc + SCC	Gefitinib	Placebo	3.0 *vs*. 2.6	0.82	0.0006
Second or third-line EGFR TKI versus chemotherapy						
INTEREST [2008] (31)	n=1,433, unselected, Adc + SCC, EGFR mutant subgroup	Gefitinib	Docetaxel	2.2 *vs*. 2.7	1.04	0.47
V-15-32 [2008] (32)	n=489, unselected, Adc + SCC	Gefitinib	Docetaxel	2.0 *vs*. 2.0	0.9	0.335
ISTANA [2010] (33)	n=161, unselected, Adc + SCC	Gefitinib	Docetaxel	3.3 *vs*. 3.4 (6 months PFS 32% *vs*. 13%)	0.729	0.04
TITAN [2012] (34)	n=424, unselected, Adc + SCC	Erlotinib	Docetaxel or Pemetrexed (physician's choice)	1.4 *vs*. 1.9 (6.3 *vs*. 8.6 wks.)	1.19	0.089
TAILOR [2013] (58)	n=222, molecular EGFR wild type, KRAS testing, Adc + SCC	Erlotinib	Docetaxel	2.9 (Docetaxel) *vs*. 2.4 (Erlotinib) in EGFR wild type	0.71	0.02

Adc, adenocarcinoma; SCC, squamous cell carcinoma; PFS, progression-free survival; HR, hazard ratio; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mo, months; wks, weeks.

upon review of patients treated with gefitinib, an activating EGFR mutation did predict for superior overall response rate (ORR) (84.6% *vs*. 25.9%, P<0.001) and significantly longer PFS (HR 0.377; 95% CI, 0.21-0.67; P<0.001) (41).

Further confirmatory trials (*Table 1*) compared gefitinib (42,43), erlotinib (44,45) or afatinib (46,47) to chemotherapy specifically in EGFR-mutated NSCLC rather than simply by the clinical enrichment criteria of earlier studies. All found that first-line EGFR TKIs afforded superior ORR, PFS and quality of life compared to chemotherapy. Thus upfront tumour interrogation for predictive biomarkers has now become standard and if EGFR demonstrates an activating mutation, then EGFR TKIs should be given as first-line therapy. However, despite mature follow up data for IPASS (40) and other studies, no EGFR TKI in first-line has demonstrated an overall survival benefit most likely due to extensive crossover after progression (59).

Currently, there are no published head to head trials directly comparing the efficacy of first-line EGFR TKIs. In general, these agents all demonstrate similar efficacy so the choice of agent depends on toxicity or clinician preference at the present time (60). Results of the phase IIb LUX-Lung 7 study directly comparing afatinib to gefitinib as first-line treatment for EGFR-mutant adenocarcinoma are eagerly anticipated and may address this (ClinicalTrials.gov Identifier: NCT01466660).

The role of adjuvant EGFR TKIs for resected stage I to III NSCLC remains uncertain (*Table 2*). Adjuvant erlotinib after surgery, specifically in EGFR-mutants, is currently being investigated in the RADIANT trial, with or without chemotherapy and is expected to complete in 2016 (ClinicalTrials.gov identifier: NCT00373425). Data from this study will be particularly interesting as a previous trial, NCIC BR19 (66), in an unselected patient population using adjuvant gefitinib, proved negative.

EGFR wild type and EGFR-unknown advanced NSCLC

Most tumours do not harbour an activating EGFR mutation (known as EGFR wild-type) and the role of TKIs in this specific population is contentious. With regards to first-line therapy, guidelines discourage the use of first-line TKIs based on the IPASS (39,40) and TORCH (54,55) trials which both demonstrated inferior survival compared to up-front chemotherapy (67,68). For second-line therapy (*Table 1*), the TAILOR trial (58) compared erlotinib to docetaxel specifically in EGFR wild-type tumours. All endpoints of ORR, PFS and overall survival (OS), were significantly better with docetaxel compared to erlotinib (58). This supports the continuing role for cytotoxic chemotherapy as the preferred therapeutic option in NSCLC without targetable driver mutations (69).

Four trials investigated whether adding EGFR TKIs to standard platinum doublet chemotherapy could improve outcomes (*Table 1*). The INTACT 1 (48) and INTACT 2 (49) looked at gefitinib whereas the TRIBUTE (50) and TALENT (51) trials used erlotinib. All proved to be negative trials with no improvement in efficacy or survival compared to standard chemotherapy alone.

The prognosis for patients remains poor for those who progress after initial platinum doublet chemotherapy. Both docetaxel (70) and pemetrexed (71) are approved active agents in the second-line setting, but more therapeutic options were needed, especially for those unable to have further chemotherapy. The INTEREST study was a multinational phase III randomised trial that compared gefitinib to docetaxel in unselected second-line patients (*Table 1*) (31). Gefitinib was non-inferior with respect to median OS of 7.6 months with gefitinib and 8.0 months with docetaxel, HR 1.02 (95% CI , 0.905-1.150). Further trials with second-line gefitinib (32,33) and erlotinib (34) showed superior response rates, PFS and quality of life without significant differences in OS compared to chemotherapy.

For patients with unknown EGFR status who are unfit for chemotherapy, the phase III TOPICAL study (72) found a significant survival benefit with first-line erlotinib over placebo but only in those who developed a rash within 28 days. It should be noted that those who failed to develop a rash with erlotinib had inferior survival compared to placebo. Two early phase III trials investigated EGFR TKIs versus placebo in second- or third-line in unselected patients, prior to knowledge of predictive biomarkers (*Table 1*) (56,57). The BR.21 trial (56) was the first, and still the only phase III trial to show an overall survival benefit from an EGFR TKI (59). Survival with erlotinib was 6.7 months compared to 4.7 months with placebo (HR 0.70; 95% CI, 0.58-0.85; P<0.001) (56). In contrast, gefitinib failed to show a significant survival benefit in the ISEL trial (57). Icotinib, a novel EGFR TKI has also demonstrated non-inferiority in a head to head trial compared to gefitinib in previously treated, unselected advanced NSCLC (73). Therefore in patients with unknown or wild-type EGFR status, who have no further chemotherapy options, erlotinib may be beneficial as second- or third-line therapy after platinum-based chemotherapy.

Switch maintenance therapy to EGFR TKIs after initial induction chemotherapy has shown a modest but statistically

significant benefit (*Table 2*). The WJTOG0203 (61) and INFORM (63) trials used gefitinib whereas SATURN (62) and IFCT-GFPC 0502 (64) showed similar benefits for erlotinib. However the SWOG S0023 study (65) demonstrated no benefit with gefitinib compared to placebo following definitive chemoradiation. In fact, there appeared to potentially be harm from gefitinib in this setting as placebo paradoxically demonstrated a superior PFS and OS.

Anti-EGFR monoclonal antibodies

Monoclonal antibodies represent an alternative way to inhibit EGFR activation and signalling. Apart from competitive inhibition of ligands binding to the extracellular domain, they can also form antibody-receptor complexes that are endocytosed and degraded. Available anti-EGFR mAbs now include cetuximab, necitumumab, panitumumab and matuzumab.Two phase III studies, FLEX (52) and BMS099 (53) have examined the combination of cetuximab with platinum doublet chemotherapy in advanced NSCLC (*Table 1*). Whilst the FLEX trial demonstrated a marginal improvement in median overall survival (11.3 months with cetuximab versus 10.1 months with chemotherapy alone), the smaller BMS099 trial was negative (52,53). Necitumumab is currently being investigated in two phase III studies. The ongoing INSPIRE study in non-squamous NSCLC (ClinicalTrials.gov identifier: NCT00982111) and the recently completed SQUIRE study for squamous NSCLC investigating cisplatin-gemcitabine with necitumumab. The SQUIRE study reportedly demonstrated an improved OS and formal publication of these results are eagerly anticipated (ClinicalTrials.gov identifier: NCT00981058). Other mAbs currently in phase II trials include panitumumab (ClinicalTrials.gov identifiers: NCT01038037 and NCT01088620) and matuzumab (ClinicalTrials.gov identifier: NCT00111839).

Resistance to EGFR targeted therapy

Although EGFR TKIs have revolutionised treatment of EGFR-mutant NSCLC, most responses have not proved to be durable with many patients progressing after 7-12 months. Resistance can occur primarily (that is, *de novo*) or develop after exposure to targeted agents, and can exist as resistant clones within a tumour or in different tumours within the same patient. Most will develop 'acquired resistance', either through secondary EGFR mutations or activation of EGFR-independent pathways. Clinicians should therefore consider re-biopsy at progression to assess contemporaneous tumour biology (74-77). The most frequent mechanism (~50%) is via concurrent acquisition of a mutation in exon 20 of EGFR, encoding for T790M (74-80). Threonine is replaced by methionine, altering the configuration of the kinase domain and enhancing its affinity (over wild-type) for ATP, with corresponding decreased affinity for first-generation reversible TKIs (81). The second most common mechanism (in 5-20%) involves amplification of MET to circumvent EGFR inhibition via PI3K-AKT-mTOR signalling (74-76). Other resistance mechanisms include mutations in PIK3CA (75), HER2 (79,82), BRAF (83), STAT3 (84), AXL kinase (85), CRKL amplification (86) and in 5%,the unexpected transformation into small cell lung cancer (75,76). Despite significant advances in our understanding of the mechanisms of acquired resistance, up to 30% of resistance is mediated via an unknown mechanism and hence empirical cytotoxic chemotherapy remains the treatment of choice (75).

In contrast to chemotherapy, resistance to targeted therapy can be approached rationally once aberrant pathways are identified. Second-generation irreversible ErbB-family TKIs such as afatinib, which covalently binds to EGFR/HER1 and HER2, can overcome the T790M mutation as seen in LUX-Lung1 with 7% ORR and PFS improved from 1.1 months with placebo to 3.3 months (HR 0.38; 95% CI, 0.31-0.48, P<0.0001) (87,88). Dual EGFR blockade with EGFR TKIs and cetuximab are now being tested after success in murine models (89-91). Combined inhibition of MET and T790M has also shown promise in murine models (92) and is now undergoing clinical trials in humans with a MET/ALK inhibitor (crizotinib) plus a pan-HER inhibitor (dacomitinib) (ClinicalTrials.gov identifier: NCT01121575). Third generation EGFR TKIs such as CO-1868 and AP26113 that specifically target T790M have preliminary evidence of efficacy in acquired resistance with reasonable toxicity (93,94). Although addressing resistance to targeted therapy appears possible, the challenge for the future will be rationally choosing combinations and whether upfront combination therapy will be more effective than first-line single-agents whilst balancing toxicity and costs.

EML4-ALK positive NSCLC

The *ALK* gene was first discovered in 1994 in the context of a subtype of Non-Hodgkin lymphoma where ALK was fused to nucleophosmin (NPM) as a result of a chromosomal translocation (95). In 2007, Soda *et al.* screened NSCLC tumours and found the same ALK

Table 2 Phase III Trials comparing EGFR-inhibitors to chemotherapy in maintenance and adjuvant settings

Trial [year] (Ref)	Patient selection	Targeted therapy (TT)	Comparator (C)	Median PFS TT *vs*. C (mo.)	HR	P value
Maintenance						
WJTOG0203 [2010] (61)	n=604, unselected, Adc + SCC (EGFR predictive biomarker not known)	Gefitinib (in those without PD after 3× cycles platinum doublet)	Platinum doublet (up to 6 cycles)	4.6 (Gefitinib) *vs*. 4.3 (chemo)	0.68	<0.001
SATURN [2010] (62)	n=884, unselected for entry, Adc + SCC, 7% EGFR mutant	Erlotinib (in those without PD after 4× cycles platinum doublet)	Placebo	2.8 *vs*. 2.6 (12.3 *vs*. 11.1 wks.)	0.71	<0.0001
INFORM [2012] (63)	n=296, unselected, Adc + SCC (known EGFR status excluded)	Gefitinib (in those without PD after 4× cycles platinum doublet)	Placebo	4.8 *vs*. 2.6	0.42	<0.0001
IFCT-GFPC 0502 [2012] (64)	n=464, unselected, Adc + SCC	Erlotinib or Gemcitabine maintenance (in those without PD after 4× cycles cisplatin gemcitabine)	Observation	2.9 *vs*. 1.9 (Erlotinib)	0.69	0.003
				3.8 *vs*. 1.9 (Gemcitabine)	0.56	<0.001
SWOG S0023 [2008] (65)	n=243, unselected, Adc + SCC, closed after unplanned interim analysis after ISEL trial	Gefitinib (after chemoradiation and docetaxel in inoperable stage III)	Placebo	8.3 *vs*. 11.7	0.80	0.17
Adjuvant						
BR.19 [2013] (66)	n=503, unselected, Adc + SCC, closed after unplanned interim analysis after ISEL trial	Gefitinib (after completely resected stage IB, II or IIIA NSCLC)	Placebo	50.4 *vs*. not yet reached (4.2 years *vs*. not yet reached)	1.22	0.15

Adc, adenocarcinoma; SCC, squamous cell carcinoma; PFS, progression-free survival; HR, hazard ratio; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mo, months; wks, weeks.

gene but this time fused to Echinoderm Microtubule-associated protein-like protein 4 (EML4) as a result of a small inversion within chromosome 2p (96). The EML4-ALK fused oncogene is present in up to 3-7% of NSCLC and promotes malignant growth and proliferation (96). As with EGFR, ALK rearrangements are more likely to be seen in specific populations; younger patients who are light or never-smokers with adenocarcinoma and frequent signet ring cells seen on histology (97-101). Tumours carrying ALK rearrangements are mutually exclusive from those harbouring EGFR or KRAS mutations and represent the prototype for 'oncogene addiction' where a single gene product can result in malignancy (97,102,103).

Unlike the history of EGFR, lessons learnt since have allowed a more logical approach for ALK as a therapeutic target; from discovery, prospective tumour genotyping and specifically designed trials to test inhibitors and achieve positive patient outcomes. Crizotinib is an oral small molecule inhibitor of the ALK, MET and ROS tyrosine kinases (104). It was granted FDA approval in 2011 after only phase I/II studies showed impressive response rates (ORR 57%, including one complete response) in pre-treated patients (98). Final results revealed a PFS of 9.7 months (95% CI, 7.7-12.8 months) (105). Median OS data are awaited but a retrospective analysis of ALK-positive NSCLC suggests that crizotinib is associated with a survival advantage compared to those who did not have crizotinib available (106). Importantly, ALK-positivity itself is not a favourable prognostic factor as those without treatment have similar poor outcomes to the general population of NSCLC (106).

Crizotinib has also proved its superiority over second-line chemotherapy in those who had previously received a platinum doublet (101). Median PFS was 7.7 months with crizotinib versus 3.0 months with pemetrexed or docetaxel chemotherapy (HR 0.49; 95% CI, 0.37-0.64, P<0.001) (101). Overall survival was no different, likely due to extensive crossover and immature follow up for survival. This was all achieved with relatively few adverse effects, mainly mild visual disturbances (photopsia, blurred vision) and gastrointestinal side effects. Elevations in liver aminotransferases were severe in 16%, and one progressed to fatal hepatic failure. Interstitial lung disease was seen in 2% with two fatalities. Overall patients still reported superior reduction of symptoms and improvements in overall quality of life with crizotinib (101).

The phase III PROFILE 1014 study is currently investigating crizotinib as first-line therapy compared to platinum-pemetrexed chemotherapy in untreated patients and has now completed recruitment (ClinicalTrials.gov Identifier: NCT01154140). Results are expected shortly and if positive, will cement crizotinib as the gold standard treatment for all lines of therapy for ALK-positive NSCLC.

As with EGFR TKIs, resistance can also develop to crizotinib for ALK rearranged NSCLC. Unfortunately a wide variety of mechanisms are being discovered including; ALK amplification, EGFR/HER1, HER2 and HER3 up-regulation, cKIT amplification and various ALK mutations including L1196M (analogous to T790M for EGFR) (107-110). In those with acquired resistance to crizotinib, a phase I trial has just shown that a second-generation ALK inhibitor, ceritinib (LDK378), had an ORR of 56% (95% CI, 45-67%) (111). It is up to 20 times a more potent ALK inhibitor than crizotinib, explaining its potential to overcome the L1196M mutation (111-113). Particularly encouraging is that response rates were similar for patients with various known resistance mechanisms as well as those without an identifiable mutation (114). Other similar second generation ALK inhibitors such as alectinib are under investigation but, as is the case with EGFR, a rational approach to overcoming ALK-resistance holds promise for the future (115-117).

K-RAS mutation in NSCLC

K-RAS (Kirsten rat sarcoma 2 viral oncogene homolog) belongs to a family of GTPases that transduce growth signals from multiple tyrosine kinases including EGFR and MET (*Figure 2*) (18). Activating mutations in KRAS leading to constitutive signalling are present in about 30% of adenocarcinoma and 4% of SCC (118,119). KRAS mutations are more likely to be found in Caucasians, former or current smokers and are mutually exclusive from EGFR or ALK mutations (103,119-121). They have also been associated with a poorer prognosis as well as resistance to chemotherapy and EGFR TKIs (122-125). Despite KRAS being one of the earliest known oncogenic drivers in NSCLC (126), effective targeting remains a therapeutic challenge. Direct RAS inhibition with salirasib was unsuccessful (127), so novel approaches are currently attempting to inhibit downstream molecules in the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways (119). Other approaches include targeting the heat shock protein (HSP90) which KRAS mutant cells have increased dependence upon (92,119). Selumetinib (AZD6244; ARRY-142866) a MEK1/MEK2 inhibitor showed a PFS advantage when combined with docetaxel in a recent phase II trial in advanced KRAS-mutant NSCLC (128). It

is now being investigated in a confirmatory phase III study, SELECT-1 (ClinicalTrials.gov Identifier: NCT01933932), in addition to preclinical combinations with AKT inhibitors (129).

MET amplification in NSCLC

Amplification of mesenchymal-epithelial transition (MET) factor is found in about 5% of lung adenocarcinoma and results in overexpression of its gene product—hepatocyte growth factor receptor (HGFR)—which is involved in cell proliferation, migration, invasion and metastasis (130). Various strategies to inhibit MET/HGFR mediated growth are in development including: HGF antagonists, anti-HGFR mAb, anti-MET mAb and MET TKIs such as tivantinib (ARQ197), cabozantinib (XL184) and of course crizotinib (131).

MET and EGFR appear to be synergistic for growth and MET amplification is also the second most common cause of acquired EGFR TKI resistance. Dual EGFR and MET inhibition, with erlotinib and tivantinib respectively, was tested in non-squamous NSCLC in the much anticipated global phase III trial MARQUEE (132), after phase II data (133) suggested improved PFS for KRAS-mutants. Onartuzumab, a monoclonal antibody against MET also showed promise in a phase II trial (134) so was brought to phase III in the MetLung study where it was combined with erlotinib for MET-positive NSCLC (ClinicalTrials.gov Identifier: NCT01456325) (135).

Despite these early promising results, confirmatory studies using MET TKIs and MET mAb have yielded disappointing results and early trial closures for both phase III trials. MARQUEE (132) was closed in late 2012 due to an interim analysis declaring futility in its primary outcome of overall survival (136). MetLung was also terminated early due to lack of efficacy (137). Interestingly, subset analyses from MARQUEE were presented at the European Cancer Conference 2013, which suggested that in tumours with strong MET immunostaining, there was a PFS and OS benefit (138). Only 40% of tumours in MARQUEE had tissue for MET expression analysis and it appears that the future progress with MET inhibition is likely to require a clear predictive biomarker to enhance appropriate patient selection moving into the future.

ROS1 rearrangements in NSCLC

ROS1 rearrangements were first seen in 2007 with around 1-2% of NSCLC harbouring different ROS1 fusion variants (139,140). Whilst its function in humans is yet unknown, its highest expression is seen in normal lung tissue (141). Like many other receptor tyrosine kinases, ROS1 feeds into multiple downstream pathways such as the RAS/RAF/MEK or MAPK, JAK/STAT3 and PI3K/AKT/mTOR pathways (*Figure 2*) (141,142). Both rearrangements share similar clinical phenotypes: younger, non-smokers with adenocarcinomas (141,143). There also appears to be ~50% sequence homology between ROS1 and ALK, and fortunately ALK inhibitors such as crizotinib can and do inhibit both kinases (139,141). Indeed crizotinib has shown some early activity in the phase I setting (144), but again, acquired resistance appears to limit the long-term efficacy of kinase inhibition (ClinicalTrials.gov Identifiers: NCT01449461, NCT01284192) and specific ROS1 inhibitors, such as foretinib are currently under investigation (145).

RET fusions in NSCLC

The RET (rearranged during transfection) is a novel fusion gene with various partners including KIF5B (kinesin family member 5B) and others such as CCDC6, NCOA4, and TRIM33 (146). It is found in around 1-2% of lung adenocarcinomas and predominantly in non-smokers (143,147). No specific RET inhibitors are currently available but multi-kinase inhibitors such as vandetanib (phase II) and cabozantinib (phase III) are being trialled in RET fusion-positive NSCLC (ClinicalTrials.gov Identifiers: NCT01823068 and NCT01639508).

HER2 overexpression and mutations in NSCLC

Human epidermal growth factor 2 (HER2/ErbB2/neu), like EGFR/HER1, is a member of the ErbB family of tyrosine kinase receptors that are activated by homo- or hetero-dimerisation with other ErbB receptors (21). HER2 overexpression is seen in up to 20% of NSCLC (148,149) but HER2 mutation rates occur less frequently in up to 3-4% (149,150). Rationale for blockade in NSCLC was borrowed from successes seen in HER2-positive breast cancer (9), however phase II trials combining trastuzumab with chemotherapy in NSCLC have so far been negative to date (148,149).

BRAF mutations in NSCLC

BRAF mutations in NSCLC are uncommon and seen in less than 5% of cases (151). As an important part of the RAS/RAF/MEK/ERK or MAPK pathway, BRAF inhibition

seemed logical, especially since TKIs were already available for melanoma (10). However, only around half of those identified harbour the specific V600E mutation for which effective therapies exist (151). Currently a phase II trial is looking at the combination of a BRAF and MEK inhibitor, dabrafenib and trametinib respectively, in stage IV NSCLC (ClinicalTrials.gov Identifier: NCT01336634).

Squamous cell carcinomas (SCC)

Although many of the pathways and targeted agents described thus far apply primarily to adenocarcinoma, targeted therapy for SCC is now a focus of current research. Recent discoveries from the cancer genome atlas about the molecular pathology of SCC have identified several important signalling pathways (152). Although these pathways can be inhibited, clinically meaningful benefits are currently lacking but ongoing work should hopefully see the realisation of targeted agents for SCC in the near future.

The phosphatidylinositol 3-kinase (PIK3CA) pathway is one of the most commonly altered in SCC with PIK3CA mutation and amplification as well as loss of the PTEN tumour suppressor gene (4,153). Ongoing phase II trials of the PI3K inhibitor, buparlisib (BKM120) are underway in squamous NSCLC in combination with chemotherapy (ClinicalTrials.gov Identifiers: NCT01911325, NCT01820325).

The fibroblast growth factor receptor 1 (FGFR1) is another exploitable pathway with overexpression in up to 20% of SCC compared to only 3% of adenocarcinoma (154). FGFR inhibitors, such as brivanib (BMS-582664) and other multi-kinase inhibitors showed positive signals *in vitro* (154) and are now in early phase trials (ClinicalTrials.gov Identifier: NCT00633789) (155).

DDR2 (discoidin domain receptor 2) is a tyrosine kinase receptor seen in up to 4% of SCC (156). Again DDR2, with collagen as its ligand, is involved in cell migration, proliferation and survival (156). Early promise was seen *in vitro* and in murine models of DDR2 inhibition with dasatinib, a multi-TKI targeting BCR-Abl and the Src family of tyrosine kinases (156). The phase II trial was negative (157) but further research on DDR2 inhibition is ongoing.

Angiogenesis inhibition in NSCLC

Disrupting tumour blood supply and angiogenesis has been a enticing target for many years now (158) with some successes in other malignancies such as colorectal cancer (159), ovarian (160) and now cervical cancer (161). Complex signalling pathways with multiple growth factors and cytokines are thought to regulate angiogenesis (162,163). Two key growth factors include vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (162,163).

Two pivotal phase III trials provide evidence for targeting angiogenesis in NSCLC with both utilising the anti-VEGF monoclonal antibody, bevacizumab in combination with standard platinum chemotherapy doublets (164-166). The Eastern Cooperative Oncology Group ECOG 4599 study (164) reported a median OS advantage from 10.3 months with chemotherapy alone to 12.3 months with the addition of bevacizumab to chemotherapy and as maintenance (HR 0.79; 95% CI, 0.67-0.91; P=0.003). The AVAiL study (165) demonstrated an improved ORR and longer PFS although failed to demonstrate an improvement in overall survival. Toxicities with bevacizumab include bleeding, thromoboembolism, and hypertension (164,165). Major bleeding and haemoptysis was associated with squamous histology and cavitation, thus limiting its clinical use to non-squamous NSCLC after fatal pulmonary haemorrhagic events were noted in earlier phase II studies (164,167,168). A further phase III study (AVAPERL) in non-squamous NSCLC suggests that perhaps maintenance therapy with pemetrexed is improved by the addition of bevacizumab (169,170).

Small molecule TKI can also be utilised to inhibit the VEGF pathway. To date, several multi-TKIs have failed to demonstrate a clinically significant survival benefit in phase III trials (171-175). Nintedanib combined with second-line chemotherapy (LUME-Lung1) resulted in a very modest benefit in PFS without a benefit in OS, however, planned subgroup analyses suggest that patients with adenocarcinoma histology may benefit most (12.6 months with nintedanib plus docetaxel versus 10.3 months with docetaxel alone (HR 0.83; 95% CI, 0.70-0.99; P=0.0359) (176).

A novel class of anti-angiogenesis drugs known as tumour vascular disrupting agents did show some promise in pre-clinical trials. However vadimezan (ASA404) failed to show a benefit in phase III trials (177) and so further development has been abandoned. Further research is needed to elucidate appropriate predictive biomarkers for anti-angiogenic therapies in the future.

Conclusions

Within the last decade, significant advances in molecular pathology have afforded an improved understanding of the underlying pathology and significant heterogeneity

of NSCLC. Multiple signalling pathways have now been identified as well as specific oncogenic driver mutations that lead to malignant transformations. Indeed in clinical practice, reflex molecular interrogation of tumour tissue for such driver mutations has now become commonplace. For the vast majority at present, no known drivers are detected and such patients are still empirically treated with standard cytotoxic chemotherapy. Whilst impressive clinical benefits have been observed for NSCLC with a known driver mutation, acquired resistance is frequently seen and presents us with the next challenge in the goal to deliver unique personalised medicine.

Building on past experience is helping to improve the approach to targeted therapy. For example, it took just over six years to progress from initiation of phase I to positive phase III trials of crizotinib in ALK-positive patients and just four years to achieve FDA approval with only phase II data—a truly remarkable achievement. The key to the future success of theranostics and truly personalised oncological management will be to ensure appropriate patient selection using predictive biomarkers to optimise limited resources and minimise harm. Addressing resistance, utilising the correct inhibitor, or combination of inhibitors, whilst minimising adverse effects will hopefully lead to the realisation of ongoing improvements in survival for patients in the future. Further to this, the real challenge will be bringing these agents into the management of patients with earlier stage disease with the hope of truly improving rates of cure for the devastating illness that is lung cancer.

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Footnote

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Treatment of advanced squamous cell carcinoma of the lung: a review

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Abstract: Lung cancer remains the single deadliest cancer both in the US and worldwide. The great majority of squamous cell carcinoma (SCC) is attributed to cigarette smoking, which fortunately is declining alongside cancer incidence. While we have been at a therapeutic plateau for advanced squamous cell lung cancer patients for several decades, recent observations suggest that we are on the verge of seeing incremental survival improvements for this relatively large group of patients. Current studies have confirmed an expanding role for immunotherapy [including programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibition], a potential opportunity for VEGFR inhibition, and even future targets in fibroblast growth factor receptor (FGFR) and PI3K-AKT that collectively should improve survival as well as quality of life for those affected by squamous cell lung cancer over the next decade.

Keywords: Non-small cell lung carcinoma; squamous cell carcinoma of the lung; epidermal growth factor receptor (EGFR); angiogenesis inhibitors; immunotherapy

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Not only is lung cancer the most commonly diagnosed cancer internationally, representing approximately 17% of new cancer diagnoses worldwide, but it also bears the highest mortality rate among all cancers (24% of cancer-related mortality worldwide) (1). In the United States (US), lung cancer is the second most commonly diagnosed cancer with an estimated 224,000 new cases in 2014 and remains the leading cause of cancer death in the US (2,3). Of these lung cancer cases, over 85% of them are classified as non-small cell lung cancer (NSCLC), with squamous cell carcinoma (SCC) of the lung comprising approximately 30% (4).

Nearly 80% of all lung cancer cases in men and 90% of cases in women are associated with smoking (5,6). SCC is most strongly associated with smoking in a dose-dependent manner, with one study finding that 91% of SCC was attributed to cigarette smoking (7-9).

With the exception of the newly approved nivolumab, there have been no other US Food and Drug Administration (FDA) approvals specifically for SCC of the lung. Moreover, driver mutations/rearrangements connected with FDA-approved agents in the epidermal growth factor receptor (*EGFR*) and echinoderm microtubule associated protein like 4—anaplastic lymphoma kinase (*EML4-ALK*) are very rarely associated with squamous cell histology. Recently, however, molecular genotyping has led to the application of targeted agents for mutations prevalent in SCC. This overview of the treatment of squamous cell lung carcinoma highlights these recent molecular advances and discusses applications of newer cytotoxic and targeted agents evaluated for the treatment of advanced SCC (*Figure 1*).

Cytotoxic chemotherapy

Cytotoxic chemotherapy for NSCLC has reached a therapeutic plateau as evidenced by the published data from Eastern Cooperative Oncology Group (EGOG)

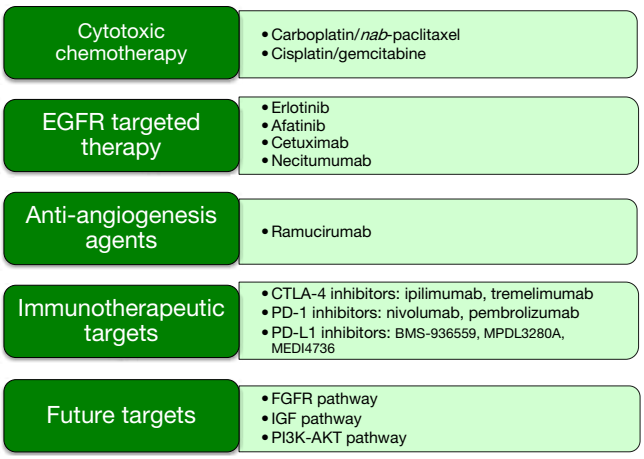

Figure 1 Review of current and potential future therapies for squamous cell carcinoma (SCC) of the lung. EGFR, epidermal growth factor receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; FGFR, fibroblast growth factor receptor; IGF, insulin-like growth factor.

1594 showing equivalent survivals among four different platinum doublet chemotherapies, with outcomes not analyzed by histology (10). Subsequent published data of a large phase III trial of cisplatin/pemetrexed versus cisplatin/gemcitabine, however, did indicate a difference in outcome based on histology (11). In this non-inferiority trial, patients with squamous cell histology received a relative benefit with the treatment of gemcitabine/cisplatin versus pemetrexed/cisplatin. Additional studies identified outcome discrepancies based on histology; a retrospective analysis of a phase III second-line trial revealed inferior survival in squamous cell cancer patients receiving pemetrexed compared with docetaxel and a phase III pemetrexed maintenance trial showed no benefit with pemetrexed maintenance in the squamous cell histologic subset (12,13). Based on the consistency of results across multiple trials indicating shorter survival in those with squamous histology, pemetrexed is not recommended for the treatment of patients with SCC (14).

Recently a large phase III trial comparing carboplatin/paclitaxel (solvent-based) to carboplatin/*nab*-paclitaxel (albumin bound) in stage IIIB and IV NSCLC also found a difference in efficacy based on histology. Though the two arms of the trial had similar survival outcomes, the *nab*-paclitaxel arm had an improved response (the primary endpoint of the trial) compared to the solvent-based paclitaxel arm; however, this benefit was limited to the SCC subset. The SCC subset exhibited a 41% radiologic response in the *nab*-paclitaxel arm compared to a 24% radiologic response in the solvent-based paclitaxel arm. Compared to the solvent-based paclitaxel group, the *nab*-paclitaxel group exhibited a numerically higher median overall survival in SCC (10.7 *vs.* 9.5 months) yet this was not statistically significant (HR 0.89, 95% CI, 0.719-1.101, P=0.284). In addition, the side effect profile in the *nab*-paclitaxel arm was more favorable, with less myalgias, neuropathy, and cytopenias (15). Ongoing studies should clarify the role of *nab*-paclitaxel in the treatment of squamous cell lung cancer patients (NCT identifier 02328105) (16). The lower toxicity profile has also bolstered its role as a potential agent in the maintenance setting (NCT identifier 02027428) (17).

EGFR targeted therapy

In patients with an EGFR activating gene mutation, there is ample evidence to offer first line EGFR tyrosine kinase inhibition (TKI) based on improved progression free survival and overall survival compared with cytotoxic chemotherapy (18-27). EGFR activating gene mutations are found in approximately 20% of adenocarcinomas but the prevalence in squamous cell cancers is considerably less (28). A study from Rekhtman *et al.* in 2012 illustrated that EGFR mutations do not occur in pure SCCs but appear only in mixed adeno-squamous carcinomas (29).

Though the response rate in patients without EGFR activating mutations is low, recent data may support the use of EGFR TKIs for later lines of therapy in wild type patients, including those with SCC (18). A retrospective study examining erlotinib in patients with advanced SCC found that of the 92 patients analyzed (74 of whom were current or former smokers), 16 achieved a partial response and 9 had stable disease. However, only 27 patients actually had molecular analysis performed on tumor specimens, and 2 were found to have EGFR complex mutations (30). The SATURN trial examining the efficacy of erlotinib as maintenance treatment in advanced NSCLC revealed that erlotinib prolonged progression free survival compared to placebo in both EGFR mutation-positive and EGFR mutation-negative tumors. The squamous cell subset analysis failed to reach statistical significance (31). The TAILOR trial comparing erlotinib to docetaxel as second-line treatment of patients with wild-type EGFR stage IV NSCLC showed that docetaxel was more effective than erlotinib (median overall survival was 8.2 months

for docetaxel versus 5.4 months for erlotinib, and results trended in a similar direction for the SCC subset) (32).

It is possible that with a favorable proteomic signature, patients with wild-type EGFR tumors may have similar overall survival when treated with second-line chemotherapy or erlotinib as presented in the PROSE study using the VeriStrat test. Squamous cell patients were equally represented in both arms of the study (33). The ongoing LUX-Lung 8 trial is a prospective phase III trial comparing EGFR TKIs (afatinib *vs.* erlotinib) in patients with relapsed/refractory stage IIIB or IV SCC with ECOG performance status of 0-1 who had progressed after at least four cycles of platinum-based doublet chemotherapy and had not received prior EGFR TKI. Preliminary data suggest that the median progression free survival and disease control rate are higher for afatinib compared to erlotinib (2.7 *vs.* 1.9 months; 45.7% *vs.* 36.8%, respectively). This is tempered by higher incidences of diarrhea and stomatitis with afatinib (34).

Monoclonal antibodies against EGFR have shown moderate activity in NSCLC. Cetuximab, a recombinant human/mouse chimeric monoclonal antibody against EGFR, showed only minimal survival benefit when combined with cisplatin and vinorelbine (*vs.* chemotherapy alone) in a subset of patients with SCC (9 *vs.* 8.2 months), but this subgroup analysis did not reach statistical significance (35). Necitumumab, an IgG1 monoclonal antibody against EGFR, did not show any evidence that its addition to cisplatin/pemetrexed increased survival in first-line treatment of metastatic non-squamous NSCLC (36). However, outcomes were different when necitumumab was combined with different chemotherapy in a different histologic subset. The addition of necitumumab statistically improved overall survival, progression free survival, and disease control rate when added to cisplatin/gemcitabine in a trial conducted in SCC patients with a median overall survival improvement of 11.5 *vs.* 9.9 months (HR =0.84, P=0.012) (37).

Anti-angiogenesis agents

Bevacizumab, a VEGF inhibitor, has shown efficacy in NSCLC but is not recommended for SCC as it has been associated with life-threatening hemoptysis when used in SCC (38,39). Ramucirumab, a VEGFR2 inhibitor, was recently approved for second-line therapy for stage IV NSCLC based on results from the REVEL trial. The study compared ramucirumab/docetaxel to placebo/docetaxel in patients who progressed on platinum-based chemotherapy. Median overall survival was better (HR 0.86, 95% CI, 0.75-0.98, P=0.023) in the ramucirumab arm (10.5 months) compared to the placebo arm (9.1 months). Median progression free survival was also superior in the ramucirumab arm (4.5 *vs.* 3 months, P<0.0001). The study was not powered for subgroup analysis, though SCC patients made up approximately 25% of the trial and experienced a numeric improvement in median overall survival in the ramucirumab arm (9.5 *vs.* 8.2 months in placebo arm, HR 0.88, 95% CI, 0.69-1.13) (40). Phase II data investigating ramucirumab with paclitaxel/carboplatin as first-line therapy for stage IIIB/IV NSCLC revealed 6-month progression free survival rate of 59%, though 85% of patients had adenocarcinoma and phase II randomized data in the front-line squamous cell population has not been presented (41).

Immunotherapeutic targets

Another potential avenue within the field of targeted therapy for SCC involves immune-checkpoint inhibition. Aberrancies in the HLA-A gene were frequently noted in SCC from the Cancer Genome Atlas Project, suggesting a prominent role for immune evasion for these cancers (34). Pathways further along in study include the programmed cell death ligand 1 (PD-L1) and programmed cell death-1 (PD-1) and the CTLA-4 pathway. Tumors attempt to escape surveillance and detection by expressing PD-L1, which in turn interacts with the PD-1 on T-cells. This interaction leads to suppression of the antitumor T-cell response. Novel therapies are being developed to disrupt this PD-1/PD-L1 checkpoint (*Figure 2*). Two such therapies are nivolumab and pembrolizumab, which are monoclonal antibodies against the PD-1 receptor on T-cells so as to unmask the dormant T-cell antitumor response (42-44). PD-L1 inhibitors (BMS-936559, MPDL3280A, and MEDI4736) are also in development. While PD-1 inhibitors have been most extensively tested in patients with melanoma, new data suggest efficacy in NSCLC as well (45,46). As of October 2014, pembrolizumab has achieved breakthrough therapy designation for EGFR- and ALK- rearrangement-negative NSCLC following platinum-based chemotherapy, based on phase I results from the KEYNOTE-001 study. A total of 282 patients with treatment-naïve or previously treated advanced NSCLC were treated with pembrolizumab once every 3 weeks. The overall response rate (ORR) in the squamous histology group was 18-25% compared to 23% for the non-squamous histology group. At the time of publication of the data, only half of the patients had PD-L1 staining performed; of these, the ORR was 39-47% in patients with strong PD-L1

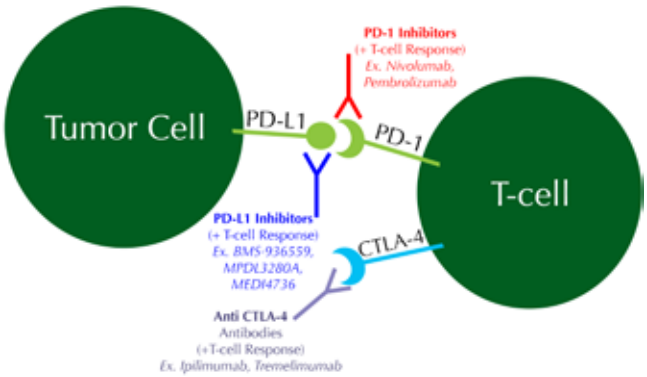

Figure 2 Schematic of immune checkpoint mechanisms. Tumors can express PD-L1, which interacts with PD-1 on T-cells, leading to suppression of the antitumor T-cell response. PD-L1 and PD-1 inhibitors prevent this interaction, unleashing the T-cell antitumor response. Anti-CTLA-4 antibodies bind to CTLA-4 to increase the ratio of effector T-cells to negative regulatory T-cells to achieve the same effect. PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4.

expression but only 9-16% in patients with weak/negative PD-L1 expression. The progression free survival and overall survival were also longer in patients with PD-L1 strong-positive patients. The median overall survival was found to be 8.2 months, while the median overall survival had not yet been reached in the treatment-naïve group (47).

Nivolumab is still undergoing active trials. A prior phase II open-label, single-arm trial investigating the use of nivolumab in heavily pretreated patients with advanced squamous cell NSCLC (CheckMate-063) showed an 11-month ORR of 15% (95% CI, 9-22%), and all were partial responses. At the time of analysis, 10 of the 17 responding patients had response durations exceeding 6 months. This marks a key advancement over the previously demonstrated 1-year survival rates of 5.5-18% for third-line squamous cell NSCLC (48). A recent phase III trial of nivolumab compared to docetaxel as second-line therapy in patients with squamous cell NSCLC (CheckMate-017) was stopped early because of superior overall survival in the nivolumab arm (49). The 272 patients with advanced or metastatic SCC were randomized to either nivolumab or docetaxel after having progressed on prior platinum-based chemotherapy. The nivolumab arm experienced a 41% overall survival advantage over the docetaxel arm (9.2 *vs.* 6.0 months; HR 0.59, 95% CI, 0.44-0.79, P=0.00025). In contrast to the available pembrolizumab data, nivolumab exhibited improved overall survival compared with second line docetaxel regardless of PD-L1 immunohistochemistry expression (50). These milestone data were responsible for the recent expedited FDA approval of nivolumab specifically for the treatment of patients with advanced SCC who have progressed after platinum-based chemotherapy (51).

CTLA-4 inhibition has also been a topic of research in NSCLC. CTLA-4 is expressed by active cytotoxic T-cells, which acts as a negative regulatory molecule against T-cell response. These T-cells are silenced through interaction with ligands on antigen presenting cells. Anti-CTLA4 antibodies such as ipilimumab and tremelimumab bind to CTLA-4 thereby unleashing the antitumor effect of T-cells and increasing the ratio of effector T-cells to negative regulatory T-cells (52). In a phase II trial comparing the efficacy of paclitaxel/carboplatin alone (control arm) versus paclitaxel/carboplatin with ipilimumab (phased or concurrent) in stage IIIB and IV NSCLC, phased ipilimumab improved immune-related progression free survival (5.7 months for the phased ipilimumab arm *vs.* 4.6 months for the control arm). In comparison to non-squamous NSCLC, the SCC subgroup exhibited an even greater improvement in progression free survival with phased ipilimumab (53).

Future targets

Recent work by the Cancer Genomic Access Research Network has confirmed the complexity of SCC with a somatic mutation rate of 8.1 mutations per megabase, higher than other tumors studied including breast, glioblastoma, colorectal (54). There were only three cases of activating EGFR or KRAS mutations of 178 cases analyzed but the frequency of mutations predicted to have functional effect was over 50%. Targetable pathways such as PI3K/AKT, receptor tyrosine kinase and RAS had frequent alterations with at least one of those pathways altered in 69% of cases. The work also found previously identified targets such as fibroblast growth factor receptor (FGFR) 1 and PIK3CA (amplified in 20%), EPHA2 (mutated in 7%), MET (amplified in 6%), PDGFR (amplified in 8-10%), EGFR and AKT (mutated in 2-5%), some of which are highlighted below (42,55,56).

Fibroblast growth factor receptor (FGFR)

FGFR1 is a member of the FGFR tyrosine kinases, and

activation is responsible for igniting the PI3K/AKT and RAS/MAPK pathways that stimulate growth and angiogenesis in several cancers (including SCC). FGFR1 is amplified in approximately 20% of SCC, and has shown to be associated with cigarette smoking in a dose-dependent fashion. There is some discordance as to whether FGFR1 amplification serves as a negative prognostic factor in surgically resected SCC with Kim *et al.* and a recent meta-analysis by Chang *et al.* supporting this assertion (55,57-60). Several FGFR inhibitors exist, including cediranib, nintedanib, pazopanib, and ponatinib (46). Cediranib is no longer under investigation given lack of efficacy in an early randomized trial (61). Nintedanib was studied with docetaxel (*vs.* docetaxel and placebo) in advanced NSCLC; overall survival in the nintedanib arm was only significantly improved in the adenocarcinoma patients but not in the total study population (62). Pazopanib (a dual FGFR and VFGFR inhibitor) was under investigation (NCT01208064, recently terminated early) but it has been limited by its heavy toxicity profile (63,64). Ponatinib is still undergoing trials (NCT01935336) but prior studies with head and neck cancer (NCT01761747) have been terminated due to toxicity (65). Novel non-ATP competitive FGFR1 inhibitors derived from nordihydroguaiaretic acid (NDGA) have shown promise in FGFR1 amplified SCC (66).

Insulin-like growth factor (IGF) pathway

The IGF pathway was recently a subject of interest, most notably with the IGF1R monoclonal antibody figitumumab. Initial phase II studies had suggested a benefit in SCC specifically, but two different phase III studies with figitumumab with either chemotherapy or erlotinib were prematurely ended due to excess toxicity and a lack of improvement in overall survival. Though this toxicity seemed to be correlated with low levels of circulating IGF, further progress in this pathway has been slow (55,67,68).

PI3-AKT signaling pathway

The PI3K-AKT signaling pathway is another potential candidate for targeted therapy. PIK3CA copy-number gains occur in 20% of all lung cancers, and frequency is even higher in SCC. PIK3CA mutations occur in approximately 6.5% of SCC. There are several PI3K inhibitors that are being actively developed; these include dual PI3K/MTOR inhibitors, isoform-selective PI3K inhibitors, and pan-PI3K inhibitors (55,69,70).

Conclusions

Lung cancer remains the single deadliest cancer both in the US and worldwide. The great majority of SCC is attributed to cigarette smoking, which fortunately is declining alongside cancer incidence. While we have been at a therapeutic plateau for advanced squamous cell lung cancer patients for several decades, recent observations suggest that we are on the verge of seeing incremental survival improvements for this relatively large group of patients. Current studies have confirmed an expanding role for immunotherapy, a potential opportunity for VEGFR inhibition, and even future targets in FGFR and PI3K-AKT that collectively should improve survival as well as quality of life for those affected by squamous cell lung cancer over the next decade.

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Footnote

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Epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer: a decade of progress and hopeful future

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Nearly 50% of patients with non-small cell lung cancer (NSCLC) are found to have metastatic disease at presentation (1). Platinum doublet chemotherapy remains the standard initial treatment for the vast majority of patients with advanced NSCLC who have a good performance status. Approximately 10% of patients with advanced NSCLC have activating mutations in the epidermal growth factor receptor tyrosine kinase (*EGFR* TK) in the tumor tissues (2). Significant progress has been made with molecularly targeted therapies in lung cancer since the initial discovery linking the presence of certain *EGFR* TK mutations with exquisite responsiveness to *EGFR* tyrosine kinase inhibitor (TKI) gefitinib (3,4). Although erlotinib, another *EGFR* TKI, has been approved for use in patients with advanced NSCLC who have progressive disease after platinum doublet therapy based on the randomized study sponsored by the National Cancer Institute (NCI)-Canada, it is evident now that the impressive clinical benefit from EGFR TKIs is seen almost exclusively in patients whose tumor cells demonstrate specific mutations in the *EGFR* TK domain (5).

The IPASS trial first established the superiority of gefitinib in significantly prolonging progression free survival (PFS) over standard chemotherapy when used as a first line therapy in patients with *EGFR* mutant lung adenocarcinoma (6). The phase III EURTAC trial conducted in Europe was the first trial to demonstrate the superiority of erlotinib over chemotherapy in previously untreated patients with advanced NSCLC with either the exon 19 deletion or exon 21 L858R mutation (7). The median PFS was 9.7 months for erlotinib versus 5.2 months for platinum based chemotherapy. Two studies have reported improvements in median PFS with an irreversible *EGFR* TK inhibitor, afatinib compared to chemotherapy in patients with *EGFR* mutant lung adenocarcinoma (8,9). Furthermore, initial therapy with afatinib improved overall survival (OS) compared to platinum based doublets in the subset of patients with exon 19 deletion in both these studies.

On the other hand *EGFR* TK inhibitors have consistently been found not to be superior to chemotherapy in patients with advanced NSCLC with *EGFR* wild type or when the *EGFR* mutation status is unknown. INTEREST trial showed gefitinib to be non-inferior to docetaxel (HR: 1.020, 96% CI, 0.905-1.150) with a median OS (7.6 *vs.* 8.0 months, respectively) (10,11). The DELTA study published recently once again confirms the lack of superiority of erlotinib over docetaxel in patients without known *EGF*R activating mutations (12). Of 301 patients enrolled from Japan, 151 were assigned to erlotinib 150 mg/day or docetaxel 60 mg/m^2 every 3 weeks. Patients with advanced NSCLC who had received one or two prior chemotherapy regimens were enrolled in this study. Majority of patients enrolled in this study had EGFR wild type. Not surprisingly, the median PFS for erlotinib was 2.0 months compared to 3.2 months for docetaxel [hazard ratio (HR) 1.22; 95% CI, 0.97-1.55; P=0.09]. The median OS was 14.8 months for erlotinib and 12.2 months for docetaxel (HR, 0.91; 95% CI, 0.68-1.22; P=0.53). Other investigators have reported similar findings (13-15). As we move forward, significant progress in the treatment of lung cancer can only be made with a better understanding of the molecular alterations underlying tumor evolution particularly in response to targeted therapies, improved drug development process and effective use of

immunotherapy. Finally we should evaluate the potential benefits of using molecularly targeted agents in early stage and locally advanced NSCLC in order to improve the cure rates.

Advances in genomic sequencing have now made it possible to discover molecular alterations present in malignant cells in great detail and precision (16-18). It is now clear that lung cancer associated with tobacco smoking results in complex genomic alterations including a number of single nucleotide variations, insertions, deletions, copy number alterations and structural rearrangements. Several institutional studies and The Cancer Genome Atlas (TCGA) project have reported novel potentially actionable alterations in lung adenocarcinoma.

On a very encouraging note, several large-scale innovative studies are currently ongoing to define the role of targeted agents in molecularly selected groups of patients with early and locally advanced NSCLC. The adjuvant lung cancer enrichment marker identification and sequencing trials (ALCHEMIST) will screen nearly 8,000 patients with completely resected lung adenocarcinoma for *EGFR* mutations and EML4-*ALK* rearrangement in a central laboratory (NCT02194738). Patients with *EGFR* mutations or *ALK* rearrangement will be randomized to specific molecularly targeted therapy (erlotinib or crizotinib) or placebo following standard post-operative therapy (NCT02193282; NCT02201992). The primary endpoint of the study is OS. Comprehensive genomic analyses will be performed on tumor specimens from patients enrolled in this trial. The role of molecularly targeted agents in patients with unresectable locally advanced NSCLC is being studied in an ongoing multi-center study (NCT01822496). In this study, patients with *EGFR* mutant lung adenocarcinoma will receive either induction therapy for three months with erlotinib followed by definitive chemoradiation or chemoradiation alone. Similarly patients with *ALK* positive locally advanced NSCLC will receive either induction therapy with crizotinib followed by chemoradiation or chemoradiation alone.

It is likely that a number of novel treatment options will soon be available for patients with *EGFR* mutant and *ALK* positive NSCLC. Promising results have been reported now in patients with acquired resistance to EGFR inhibitors and ALK inhibitors (19,20). AZD 9291, a third generation EGFR TKI produced an impressive response rate of 64% among 107 patients with centrally confirmed *EGFR* T790M. A similar study using a different compound, CO-1686 reported a response rate of 58% in 40 patients with centrally confirmed *EGFR* T790M. The median PFS had not been reached at the time of presentation and was estimated to exceed one year. Several ongoing clinical trials are now available for patients with *EGFR* mutant NSCLC prior to and after therapy with first generation EGFR TKIs.

Finally, genomic analyses of multiple regions from the primary tumor reveal significant intra-tumoral heterogeneity in lung cancer (21-23). Tumor clones evolve either in a linear fashion by acquiring progressively fitter clones, or follow a branched pattern where multiple sub clones thrive simultaneously, resulting in a complex heterogeneous tumor. A better understanding of clonal evolution in response to therapy is critical to optimally treat acquired resistance. Studies with AZD 9291 and CO 1086 underscore the importance of genotyping growing lesions following targeted therapy in the salvage setting. Hopefully genotyping of cell free DNA from plasma would make the process of serial molecular evaluation easier in the coming years. While much work remains to be done, it is heartening to see the pace of progress in cancer therapy that we have witnessed over the past few years.

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A potential new therapeutic option for patients with advanced EGFR mutation-positive non-small cell lung cancer in first-line setting

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Provenance: This is an invited Commentary commissioned by the Section Editor Ming-Hui Zhang (Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, China).

Comment on: Cheng Y, Murakami H, Yang PC, *et al.* Randomized Phase II Trial of Gefitinib With and Without Pemetrexed as First-Line Therapy in Patients With Advanced Nonsquamous Non-Small-Cell Lung Cancer With Activating Epidermal Growth Factor Receptor Mutations. J Clin Oncol 2016;34:3258-66.

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EGFR mutation-positive non-small cell lung cancer (NSCLC) is a well-defined molecular subtype of lung cancer. We already know data on frequency and characteristics of EGFR mutations among patients with NSCLC and their response to tyrosine kinase inhibitors (TKIs) (1). Actually these small molecules represent the standard first-line treatments for this setting of patients, while platinum-based doublet chemotherapy is the standard first-line treatment for patients with wild type EGFR NSCLC (2).

Gefitinib, an orally active, selective and reversible EGFR-TKI, had been largely studied and developed for treatment in first-line setting of patients with advanced EGFR mutation-positive NSCLC compared with chemotherapy (3,4) both in Caucasian and non-Caucasian patients (5-7).

Pemetrexed is a potent inhibitor of folate-dependent enzymes involved in the *de novo* biosynthesis of thymidine and purine nucleotides, essential for cell replication. *In-vitro* studies had shown that pemetrexed inhibits glycinamide ribonucleotide formyltransferase (GARFT), dihydrofolate reductase (DHFR), thymidylate synthase (TS) (8).

Pemetrexed was firstly approved for second-line treatment as a single agent (9) then in first-line setting in association to cisplatin for the treatment of NSCLC patients with non-squamous histology, on the basis of the JMDB study (10).

We also know that low TS expression is a predictive factor for pemetrexed efficacy and that gefitinib suppresses the expression of TS in NSCLC cell lines, independently from *EGFR* status. Thus the addition of pemetrexed to first-line treatment with gefitinib may increase its efficacy (11,12).

On this basis, Cheng and colleagues (13) conducted a randomised phase II trial to determine whether in first-line setting the addition of pemetrexed to gefitinib could provide a clinical benefit compared with gefitinib alone for patients with advanced EGFR mutation-positive non-squamous NSCLC. All patients were from East Asia with a histologically or cytologically confirmed diagnosis of NSCLC in advanced-stage with a common EGFR mutation (*exon 19* deletion or *exon 21 Leu858Arg* point mutation). They were randomised at a ratio of 2:1 to receive pemetrexed 500 mg/m^2 in intravenous infusion on day 1 every 3 weeks and oral gefitinib (250 mg) once per day continuously or gefitinib alone. Patients received treatment until disease progression, unacceptable toxicity, or other study discontinuation criteria.

Primary endpoint of the trial was progression-free survival (PFS), while secondary endpoints were time to progressive disease (TtPD), overall survival (OS), tumor response rates, duration of response (DoR), and safety.

One hundred and twenty-nine patients were enrolled in pemetrexed plus gefitinib arm and 66 patients in gefitinib

alone arm. Sixty-five percent of patients in pemetrexed plus gefitinib arm and 63% of patients in gefitinib arm were women. The majority of patients were younger than 65 years and never-smokers. In each study arm patients with *exon 19* deletion were more represented than those with *exon 21 Leu858Arg* point mutation. In particular in pemetrexed plus gefitinib arm patients with *exon 19* deletion were 52% and those with *exon 21 Leu858Arg* mutation were 41% respectively, while in gefitinib arm they were 62% and 35% respectively.

All patients receiving at least one administration of study drug composed the intention to treat (ITT) population and they were included in the efficacy and safety analyses.

Median PFS in pemetrexed plus gefitinib arm was significantly higher compared with that in gefitinib arm (15.8 *vs.* 10.9 months; HR, 0.68; P=0.029), and the advantage of combined therapy was reported both for patient with EGFR *exon 19* deletion and *exon 21 Leu858Arg* mutation (median PFS 17.1 *vs.* 11.1 months in *exon 19* deletion subgroup and 12.6 *vs.* 10.9 months in *Leu858Arg* point mutation subgroup). This finding confirmed the evidence of previous literature supporting a better outcome with first generation TKIs for patients with NSCLC harbouring an *exon 19* deletion as EGFR mutation (14,15), suggesting that *exon 19* deletion and *exon 21 Leu858Arg* point mutation define two distinct forms of NSCLC.

TtPD was longer with pemetrexed plus gefitinib than with gefitinib alone too. TtPD was 16.2 versus 10.9 months, respectively (HR, 0.66; P=0.018). Data about OS were immature at time of analysis.

The objective response rates (ORRs) were 80% in pemetrexed plus gefitinib arm and 74% in gefitinib arm, with no statistical significant difference. The disease control rates (DCRs) were similar between the two study arms too (93% and 94% respectively), with a greater number of stable disease in gefitinib arm.

The median DoR was analysed in the ITT population that reached a complete or a partial response. It was 15.4 months for pemetrexed plus gefitinib arm and 11.3 months for gefitinib arm.

Similar findings were reported in a small Japanese phase II trial including 26 patients with advanced EGFR mutation-positive NSCLC who received in first-line setting treatment with pemetrexed and gefitinib (16). Patients' characteristics deviated from the typical ones of similar studies. In effect the majority of patients were *Leu858Arg* mutation-positive, 50% of patients were women and 54% were current or ex-smokers. In this study the authors reported an ORR of 84.6% and a DCR of 96.2%, with a median PFS of 18.0 months. The advantage was reported both for patients with *exon 19* deletion positive and patients with *Leu858Arg* mutation-positive NSCLC, with a tendency to be more effective in tumor with *exon 19* deletion, similarly to the results of a recent meta-analysis (15).

Moreover several studies investigated whether the addition of a TKI to chemotherapy both in first and second line of treatment could provide an efficacy advantage. INTACT-1 and INTACT-2 trials evaluated the addition of gefitinib to first-line cisplatin plus gemcitabine and carboplatin plus paclitaxel, respectively. Both studies concluded that gefitinib did not provide any advantage in terms of survival (17,18). Other studies on TKIs in first-line setting in addition to a platinum based chemotherapy demonstrated no benefit both in PFS (19) and survival (20).

These negative findings were explained by the action of EGFR-TKIs and chemotherapeutic agent in different cell cycle phases. In effect EGFR-TKIs cause G1 cell cycle arrest, while cytotoxic chemotherapies act on dividing cells. So the arrest of cell cycle in G1-phase protects cells from the cytotoxic effects of cell cycle phase-dependent chemotherapeutic agents (21,22).

On the contrary, sequential administration of EGFR-TKIs following chemotherapy has been shown to provide greater efficacy than concurrent administration (23,24).

On this basis several studies of different and sequential combinations of drugs were conducted, as the FAST-ACT phase II study (25) and the subsequent FASTACT-2 (26), a multicentre, randomised, placebo-controlled, double-blind, phase III study of intercalated erlotinib or placebo with gemcitabine and carboplatin or cisplatin for six cycles, followed by maintenance with erlotinib or placebo in Asian patients with advanced NSCLC. In this trial PFS was significantly higher with chemotherapy plus erlotinib compared with chemotherapy plus placebo (7.6 *vs.* 6.0 months; HR, 0.57; P<0.0001). OS was longer too (18.3 *vs.* 15.2 months, respectively). The benefit was more evident among patients with an EGFR mutation-positive NSCLC, with a median PFS of 16.8 *vs.* 6.9 months (HR, 0.25; P<0.0001) and a median OS of 31.4 *vs.* 20.6 months (HR, 0.48; P=0.0092). The investigators concluded that this intercalated treatment is an option for EGFR mutation-positive NSCLC and for patients with unknown EGFR status too, but this trial was conducted among Asian patients, who presented a higher rate of EGFR mutations (27).

It should be stressed that all these mentioned trials were conducted in a population of patients unselected *a*

priori for EGFR mutational status. Furthermore no benefit was reported also in IMPRESS trial, where patients who progressed after first-line treatment with gefitinib received cisplatin plus pemetrexed associated to gefitinib or placebo, to overcome the acquired resistance to EGFR-TKI (28).

Therefore the study of Cheng and colleagues (13) is the first randomised trial evaluating concurrent pemetrexed and gefitinib as first-line treatment in NSCLC patients selected for histology and EGFR status.

The significant better PFS in pemetrexed plus gefitinib group increases with time as demonstrated by the progressive separation of curves with time in the ITT population. Regarding clinical characteristics, the PFS advantage with pemetrexed plus gefitinib was better among women and never smokers as expected for the efficacy of a TKI. Moreover patients who had received a prior adjuvant or neoadjuvant treatment showed a higher PFS too.

Considering the adverse events (AEs) the majority of them were of grade 1 or 2. However 42% of patients in pemetrexed plus gefitinib arm experienced AEs of grade ≥3 compared with 19% of patients in gefitinib arm. The most commonly reported AEs were diarrhea, increased serum level of ALT and AST and dermatitis acneiform in pemetrexed plus gefitinib arm, diarrhea, dermatitis acneiform and dry skin in gefitinib arm. Two patients in pemetrexed plus gefitinib arm and one patient in gefitinib arm reported interstitial lung disease.

The trial presented the limitation due to the immature data on OS precluding robust analysis.

Although no benefit in OS was reported in this trial in first-line setting, the association of pemetrexed and gefitinib might be more effective than gefitinib alone, in terms of PFS.

The study reported very prolonged PFS. Until now in patients with EGFR mutation-positive advanced NSCLC previous trials had reported median PFS of 9.6 months for gefitinib alone (29) and recently 11 months for the irreversible ErbB family blocker afatinib (30).

However the trial showed also an increased but manageable toxicity profile for pemetrexed plus gefitinib arm, similar response rates and DCR between the two arms. So it is to be evaluated the risk-benefit ratio considering the findings of the trial and all the clinical relevant endpoints such as disease control, survival prolongation, tolerability and quality of life. These factors are to be taken into account to choose the most appropriate treatment for every patient.

Moreover this trial included only East Asian patients. It could be investigated if the advantage in PFS remains in EGFR mutation-positive Caucasian patients too.

It would be interesting to study whether the association of pemetrexed and gefitinib could delay the onset of the acquired resistance to TKIs, designing future trial about combination approaches and/or sequence strategy.

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Keeping our fingers crossed on 2nd generation EGFR TKIs: is better good enough?

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It has been almost a decade since the first generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) has been approved for use in non-small cell lung cancer (NSCLC). When EGFR TKIs (gefitinib, erlotinib) were approved, it was based on response rates (gefitinib) or significant improvement in overall survival when compared to placebo (erlotinib) in 2nd line or 3rd line treatment in an unselected NSCLC patient population regardless of histology, gender, or smoking status (1,2). With the advent of the discoveries of activating EGFR mutations (EGFRm), six randomized clinical trials have now unequivocally demonstrated 1st generation EGFR TKIs achieved significant prolongation of progression-free survival (PFS) over standard doublet chemotherapy as 1st line treatment of NSCLC EGFRm patients (3-8).

However, despite the significant PFS prolongation achieved by 1st generation EGFR TKIs in EGFRm patients, the median PFS on average is only about 10-15 months. One of the major resistance mechanisms to 1st generation EGFR TKIs is the generation of T790M gate keeper mutation (9). Thus there is a need for 2nd generation "irreversible" EGFR TKIs that can inhibit the T790M mutation. Currently there are two lead 2nd generation EGFR TKI candidates, afatinib (BIBW2992) and dacomitinib (PF0299804) (10). Afatinib inhibits both EGFR and human epidermal receptor 2 (HER2) while dacomitinib is a pan-HER inhibitor (EGFR, HER2, HER4). However different strategies are being employed by the manufacturers of afatinib (Boehringer Ingelheim) and dacomitinib (Pfizer) in gaining regulatory approval.

Afatinib has successfully demonstrated significant PFS prolongation as 1st line treatment when compared to platinum/pemetrexed doublet combination chemotherapy in NSCLC EGFRm patients from the recently presented LUX Lung 3 trial (11). LUX Lung 6 employs the same design but compares afatinib to cisplatin/gemcitabine doublet chemotherapy in NSCLC EGFRm patients in China, Republic of Korea and Thailand. The LUX Lung 3 (and likely positive LUX Lung 6) results will likely lead to the approval of afatinib as 1st line treatment of NSCLC EGFRm patients worldwide. Nonetheless, the median PFS (13.6 months) (11) achieved by afatinib in EGFRm patients with common (del19/L858R) in the LUX Lung 3 trial is similar to the PFS (13.1 months) achieved by erlotinib in the same patient population in the OPTIMAL trial (8). In addition, the gatekeeper T790M mutation can also develop on progression from afatinib (12). Furthermore, in LUX Lung 1 where advanced NSCLC patients who had failed either erlotinib or gefitinib were randomized to afatinib or placebo, afatinib generated a statistical significant but only an absolute increase in median PFS of about 2.2 months when compared to placebo but no overall survival (OS) benefit [Hazard Ratios (HR) =1.08; 95% confidence interval (CI): 0.86-1.35; P=0.74] (13). Even among EGFRm patients the absolute increase in median PFS is only 2.3 months from afatinib over placebo. Taken together, afatinib may not offer any therapeutic advantage over erlotinib in the 1st line treatment of EGFRm NSCLC patients and offers only modest PFS but no OS benefit in EGFRm patients who failed 1st generation EGFR TKIs regardless of EGFR mutational

status thus limiting its therapeutic benefit in NSCLC.

As the recognition of the efficacy of EGFR TKIs is best for EGFRm patients, the use of erlotinib in the US has been waning for the vast majority of NSCLC patients who did not harbor activating EGFRm. Cetuximab, an antibody against EGFR when added to cisplatin/vinorelbine achieved statistically significant improved overall survival than cisplatin/vinorelbine alone in unselected NSCLC (FLEX trial) (14). However, cetuximab has yet to receive US Food and Drug administration (FDA) approval for use in combination with chemotherapy as 1st line treatment of NSCLC. The recently presented TAILOR trial comparing erlotinib to docetaxel in EGFR wildtype (wt) patients demonstrated docetaxel had superior response rate (RR) [13.9% (docetaxel) versus 2.2% (erlotinib); P=0.004] and PFS [3.4 months (docetaxel) versus 2.4 months (erlotinib); HR=0.69, 95% CI: 0.52-0.93; P=0.014] than erlotinib (15). Take together TAILOR has sown further doubts about the efficacy of EGFR blockade as a therapeutic strategy in EGFR wt NSCLC.

Theoretically, if EGFR pathway blockade is important in the management of EGFR wt NSCLC then a more potent EGFR pathway inhibitor should result in better clinical outcome when compared to a less potent EGFR TKI. Indeed this is the case. Ramalingam *et al.* published a randomized phase II trial comparing dacomitinib to erlotinib as 2nd line treatment in unselected NSCLC patients (16). Dacomitinib achieved significant better PFS among all patients [2.86 months (dacomitinib) versus 1.91 months (erlotinib), HR=0.66; 95% CI: 0.47-0.91; P=0.012], among KRAS wt patients [3.71 months (dacomitinib) versus 1.91 months (erlotinib), HR=0.55; 95% CI: 0.35-0.85; P=0.006], and more importantly among KRAS wt/EGFR wt patients [2.21 months (dacomitinib) versus 1.84 months (erlotinib), HR=0.61; 95% CI: 0.37-0.99; P=0.043]. Overall survival was better but not significant with dacomitinib than erlotinib [9.53 months (dacomitinib) versus 7.44 months (erlotinib), HR=0.80; 95% CI: 0.56-1.13; P=0.205]. Dacomitinib had more frequent treatment related adverse events such as diarrhea (73.1% versus 47.9%), dermatitis acneiform (64.5% versus 57.4%), and stomatitis (29.0% versus 10.6%) than erlotinib (16). The results of this phase II trial results implies that EGFR blockade remains an important therapeutic strategy among in EGFR wt/KRAS wt NSCLC as evidenced that tight or more comprehensive blockade of EGFR signaling pathway resulted in better PFS and OS.

Dacomitinib is being now compared to erlotinib in a global phase III randomized registration trial as 2nd/3rd line treatment in unselected advanced NSCLC patients with improvement in PFS as the primary endpoints in two co-primary populations: all patients with advanced NSCLC and KRAS wt NSCLC (ARCHER 1009, www.clinicaltrials.gov number: NCT01360554). Stratification factors include histology (adenocarcinoma versus non-adenocarcinoma), race (Asian versus non-Asians), Eastern Cooperative Oncology Group (ECOG) performance status (0-1 versus 2), and smoking status (never-smoker versus ever-smoker). Sample size calculations are powered to allow detection of 33% improvement of PFS among all patients receiving dacomitinib over erlotinib and 45% improvement in PFS among KRAS wt patients receiving dacomitinib over erlotinib which were exactly what was achieved by the phase II trial reported by Ramalingam *et al.* (16). A total of 800 patients will be enrolled. Given that the survival benefit in randomized phase III trials is usually less pronounced than in randomized phase II trials it remain to be seen if the PFS improvement observed in dacomitinib-treated patients will hold true. Given there was numerical but no statistical improvement in OS observed by Ramalingam *et al.*, it will be interesting to observe if there is any significant improvement in OS will be achieved in ARCHER 1009. If ARCHER 1009 achieves its primary endpoint, dacomitinib as a 2nd generation EGFR TKI should be available to all NSCLC patients as 2nd line treatment regardless of histology or EGFR mutation status. Interestingly afatinib is also pursuing a similar trial design comparing afatinib to erlotinib as 2nd line treatment in squamous cell carcinoma patients (LUX Lung 8, www.clinicaltrials.gov number NCT01523587).

Finally, subgroup analysis of the 16 patients (8 on dacomitinib arm and 8 on erlotinib arm) harboring EGFR exon 19 deletion on the Ramalingam *et al.* study seemed to indicate dacomitinib may confer significant better PFS [77 weeks (dacomitnib) versus 24 weeks (erlotinib), HR=0.27; 95% CI: 0.076-0.94] on (17). Therefore a direct comparison between dacomitinib and a 1st generation EGFR TKI is warranted to confirm the subgroup analysis of Ramalingam *et al.* (17) so as to provide better therapeutic option for NSCLC EGFRm patients and to provide an alternative option for the regulatory approval of dacomitinib in case ARCHER 1009 fails to achieve its primary endpoints. Thus while the phase II data on dacomitnib is promising, we have to keep our fingers crossed to see if better PFS is good enough.

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Footnote

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Improved overall survival following tyrosine kinase inhibitor (TKI) treatment in NSCLC—are we making progress?

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Non-small cell lung cancer (NSCLC; 80–85% of all lung cancers) continues to be one of the major causes of cancer related deaths around the world (1). The development of molecularly targeted therapies (small molecules and monoclonal antibodies) has, however, significantly improved outcomes in the metastatic setting for NSCLC patients harbouring activated oncogenes such as epidermal growth factor receptor (EGFR) and translocated anaplastic lymphoma kinase (ALK) (2). By targeting the main pathways of NSCLC signal transduction, these drugs dramatically improved progression-free survival (PFS) and quality of life (QoL) in this highly selected subgroup of NSCLC patients sparing them from toxic chemotherapy approaches (del16) (3).

The development EGFR tyrosine kinase inhibitors (TKIs) changed dramatically the history of NSCLC patients harbouring EGFR sensitive mutations. Several randomised prospective trials confirmed the superiority of these target agents about survival and response rate when comparing with platinum-based chemotherapy (4-6). Our knowledge about EGFR mutations increased gradually during the development of target agents and different clinical trials. EGFR mutations cannot be considered all equal, but different entities should be considered in our clinical practice: exon 19 deletions (del19), exon 21 mutation (L858R) and uncommon mutation (exon 20, exon 18 and double mutations) (7). Currently, of three different EGFR TKIs (afatinib, erlotinib, and gefitinib) approved for the treatment of NSCLC patients harbouring activating EGFR mutations, only results generated by indirect meta-analyses have been reported which were not always clear and convincing (7,8). In patients harbouring EGFR mutations, different randomised trials confirmed the significant superiority of EGFR TKIs *vs.* standard platinum-based chemotherapy in first-line settings in terms of PFS, QoL and safety profile. No randomised clinical trials evaluating erlotinib, gefitinib, or afatinib showed a statistical improving in overall survival (OS) for patients treated with EGFR TKIs, when considered individually and based on overall population (4-6). Although these trials seems to be very similar, exploring the same indications and end-points with different EGFR TKIs revealed many differences about study design, patient population and statistical analysis.

Recently, targeted therapies administered to patients selected by reliable and biologically relevant biomarkers (e.g., EGFR mutations, ALK rearrangement, PD-L1 expression) have produced substantial improvements in outcomes that have rapidly transformed patient care for several types of NSCLC (2).

Most recently, results from the first head-to-head comparison of two different TKIs (afatinib *vs.* gefitinib) have been reported (9). This multicentre, international, open-label, exploratory, randomised controlled phase 2B trial (LUX-Lung 7, NCT01466660) enrolled treatment-naive patients (N=319) with stage IIIB or IV NSCLC and a common EGFR mutation (del19 or L858R). Patients were randomly assigned (1:1) to receive afatinib (40 mg/d) or gefitinib (250 mg/d) until disease progression, or beyond if deemed beneficial by the investigator. Clinicians and patients were not masked to treatment allocation; independent review of tumour response was done in a blinded manner. Co-primary endpoints were PFD by independent central review, time-to-treatment failure (TTF), and OS. Efficacy analyses were done in the

intention-to-treat population and safety analyses were done in patients who received at least one dose of study drug.

PFS [median 11.0 months (95% CI: 10.6–12.9) with afatinib *vs.* 10.9 months (95% CI: 9.1–11.5) with gefitinib; HR 0.73 (95% CI: 0.57–0.95), P=0.017] and TTF [median 13.7 months (95% CI: 11.9–15.0) with afatinib *vs.* 11.5 months (95% CI: 10.1–13.1) with gefitinib; HR 0.73 (95% CI: 0.58–0.92), P=0.0073] were significantly longer with afatinib than with gefitinib. OS data are not yet mature. The most common treatment-related grade 3 or 4 adverse events were diarrhoea [20 (13%) of 160 patients given afatinib *vs.* two (1%) of 159 given gefitinib] and rash or acne [15 (9%) patients given afatinib *vs.* five (3%) of those given gefitinib] and liver enzyme elevations [no patients given afatinib *vs.* 14 (9%) of those given gefitinib]. Serious treatment-related adverse events occurred in 17 (11%) patients in the afatinib group and seven (4%) in the gefitinib group. Ten (6%) patients in each group discontinued treatment due to drug-related adverse events. Fifteen (9%) fatal adverse events occurred in the afatinib group and ten (6%) in the gefitinib group. All but one of these deaths were considered unrelated to treatment; one patient in the gefitinib group died from drug-related hepatic and renal failure. Overall, the frequency of severe adverse events was similar in both arms with slightly different toxicity profiles. The adverse events observed with both treatments were predictable and manageable, leading to an equally low rate of treatment discontinuation in both arms (6.3%).

Moreover, first-line afatinib treatment significantly reduced the risk of NSCLC progression by 27% *vs.* gefitinib. Interestingly, the improvement in PFS became more pronounced over time with a significantly higher proportion of patients alive and progression-free at 18 months (27% *vs.* 15%; P=0.018) and 24 months (18% *vs.* 8%; P=0.018), showing a greater long-term benefit for afatinib (9).

From this study it was concluded that afatinib significantly improved outcomes in treatment-naive NSCLC patients with activating EGFR mutations with gefitinib, with a manageable tolerability profile and may become the new first-line therapy of choice. However, tolerability also plays a determining role in the selection and dosing of a TKI. The tolerability profiles between gefitinib and afatinib are different and the selection of the therapy will still be based on the individual clinical decision.

Dacomitinib is another small molecule targeting EGFR (erbB1, erbB2, and erbB4) that had been tested in a head-to-head comparison with gefitinib (10). The drug binds irreversibly to cysteine-797. In a multinational, multicentre, randomized, open-labeled, phase III trial (ARCHER1050; NCT01774721) the efficacy and safety of treatment with dacomitinib (45 mg/d) *vs.* gefitinib (250 mg/d) in patients (N=440) with locally advanced or metastatic NSCLC with EGFR activating mutations was investigated. Primary endpoint is PFS, secondary endpoints include OS and safety. The study is ongoing, but not recruiting patients. Results are expected early 2017.

All large previous randomized phase III trials so far assessing first-line treatment demonstrated a significantly higher response rate and longer PFS in patients treated with EGFR TKIs, including gefitinib, erlotinib, and afatinib (4-6) than in patients treated with standard platinum-based combination chemotherapy. Although these trials met their primary endpoint with significantly longer PFS, no significant difference was observed in terms of OS. However, no restrictions were imposed on treatment after the end of protocol therapy in any of these trials and the majority of patients in the control arm received EGFR TKI therapy at least once. None of these randomized trials had demonstrated a statistically significant improvement with these TKIs in terms of OS, which is of course the strongest endpoint for clinical research in oncology, in a condition of no effective treatment afterwards. When effective treatment is given as post therapy, it will be difficult to distinguish the treatment effect of original and subsequent treatments because differences in OS are potentially confounded by crossover, and a relevant number of patients assigned to chemotherapy arms received TKIs as second- or third-line treatment after disease progression. Intuitively, the high proportion of crossover may extend the benefit associated with the administration of TKIs to patients assigned to the control arm, and its 'salvage'-effect may compensate for the relevant differences in PFS of first-line treatment consistently demonstrated in all TKI trials.

Considering individually the OS data coming out from all randomised clinical trials with erlotinib, gefitinib and afatinib so far it was not possible to found a statistically significant superiority of one drug on the other. The was mainly due to the facts that (I) no randomized head-to-head comparisons were available; and (II) indirect comparisons were derived from several meta-analyses (7,8).

Frankly, the goals of any new cancer treatment are to allow the patient to live longer and to live better. Therefore, clinical trials in NSCLC have two important endpoints: OS and the QoL of that survival. All other endpoints should be considered intermediate, becoming surrogates to those

Table 1 Overall survival (OS) of advanced or metastatic NSCLC patients following treatment with TKIs (phase IIB/III trials)

Drug	Study design	ΔOS (months)	Reference
Nintedanib	Docetaxel *vs*. docetaxel plus nintedanib (N=1,314; LUME-Lung 1)*	2.3 (HR =0.83)	Reck *et al*. (12)
Gefitinib	Platinium-based doublet chemotherapy, followed by either placebo or gefitinib (N=296; INFORM)	15.9 (HR =0.39)	Zhang *et al*. (13)
Afatinib	Cisplatin plus pemetrexate *vs*. cisplatin, pemetrexate plus afatinib (N=345; LUX-Lung 3)**	12.2 (HR =0.54)	Yang *et al*. (8)
Afatinib	Cisplatin plus gemcitabine *vs*. cisplatin, gemcitabine plus afatinib (N=364; LUX-Lung 6)**	13.0 (HR =0.64)	Yang *et al*. (8)
Afatinib	Afatinib *vs*. erlotinib (N=795; LUX-Lung 8)***	1.1 (HR =0.81)	Soria *et al*. (14)
Afatinib	Afatinib *vs*. gefitinib (N=319; LUX-Lung 7)****	Alive at 24 months: 18% *vs*. 8% (P=0.018)	Park *et al*. (9)
Dacomitinib	Dacomitinib *vs*. gefitinib (N=440; ARCHER1050)	Awaited Q1/2017	www.clinicaltrials.gov (10)

*, adenocarcinoma only; **, meta-analysis for del19 patients; ***, squamous histology only; ****, OS data not yet mature.

important two endpoints only if formally validated. Clinical trials in NSCLC have typically investigated agents or regimens in patients selected for study based primarily on histology, molecular biology (e.g., EGFR, ALK, c-MET, PD-1/PD-L1) and clinical characteristics (11). In the many of these cases this approach has resulted in only small incremental improvements in OS (*Table 1*) that probably reflect the impact of agents with modest efficacy in a subset of the study population that appears not to be readily identifiable. Although this work has certainly improved the lives of many patients with NSCLC, appears to be slow, costly, and empiric (15).

However, the results of pooled analysis showed that a significant improvement in OS with afatinib was achieved in NSCLC patients harboring the EGFR del19 mutations adding weight to the proposal that exon 19 deletions and L8585R mutations are two different disease entities (8).

While waiting for the results of the first randomised phase III trial, comparing two different EGFR TKIs (dacomitinib vs. gefitinib; ARCHER-1050), the LUX-Lung 7 study (phase IIb) may open the door towards a new era of clinical trials evaluating two different EGFR agents, and thereby reducing statistical issue developed from indirect comparison analyses. Moreover, it is conceivable that the choice of first-line EGFR-TKI has no effect on the subsequent therapy, considering that the development of EGFR T790M mutations (and c-MET amplifications) is one the major causes of resistance to first-generation TKIs (16) and also in patients treated with afatinib. In the era of precision medicine, it will be very interesting to understand the T790M rate in patients treated with afatinib as front-line therapy. Indeed, the only preliminary results of a prospective trial that evaluated the presence of T790M in TKI-naïve patients that progressing to afatinib, showed that the presence of T790M mutation was less common (33%) then is expected with first generation EGFR TKIs, however, these data are based on a small group of patients (17).

In addition, it remains to be seen whether combinations of TKIs with newly developed immune checkpoint inhibitors, targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PD1) receptor and programmed cell death 1 ligand (PD- L1) might change current treatment paradigms in all NSCLCs (18). Only the identification of prognostic or predictive markers of response could help oncologists in choosing the most effective treatment (TKIs *vs*. chemotherapy *vs*. immunotherapy *vs*. combinations) for NSCLC patients.

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Footnote

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Is there a third line option after chemotherapy and TKI failure in advanced non-small cell lung cancer?

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The EGFR gene is a major therapeutic target in advanced Non-small cell lung cancer (NSCLC). Two reversible tyrosine kinase inhibitors, Erlotinib and Gefitinib, have been validated and registered for the treatment of NSCLC. Gefitinib has a label that is limited to NSCLC carrying mutations in the kinase domain of the EGFR gene, while the label of Erlotinib also includes second line treatment of patients with undefined EGFR status in their tumor, based on an early randomized study that showed a small benefit in such unselected population (1). Today there is a strong evidence based consensus that the best first-line treatment for patients carrying sensitizing mutations in the EGFR gene in their tumor, is with reversible EGFR TKI inhibitors Erlotinib or Gefitinib. These treatments yield impressive and durable responses, prolonged progression free survival (PFS) and improved quality of life when compared to first-line chemotherapy, with an acceptable tolerance profile due to a significant lesser toxicity than first-line chemotherapy (2,3). If the diagnosis of a mutation was missed in the first-line, these patients should be offered these treatments in second-line, as early as possible. There is also a growing consensus and data supporting that these treatments should not be used in patients with a wild-type EGFR in their tumor (4,5).

Unfortunately all patients ultimately develop resistance to EGFR TKI and become eligible for standard chemotherapy. The resistance mechanisms so far identified at baseline or at progression of the disease are: the outgrowth of a subclone of cancer cells with a T790M secondary resistance mutation, activation of the MET pathway, Pi3kinase and other downstream mutations, heterogeneity in EGFR mutation status in multifocal disease or outgrowth of a small cell lung cancer (6-9).

Upon progression, second-line chemotherapy leads to an appreciable, albeit lesser, response rate in this population. When however ultimately also chemotherapy fails, these patients are confronted with a high unmet medical need for which several strategies are being explored (6).

Afatinib, a covalent EGFR/HER2/HER4 inhibitor ("pan-HER" inhibitor), has higher potency in inhibiting EGFR in preclinical testing (10), has the potential to interfere more effectively with HER heterodimerisation signals (11) and is able to block EGFR carrying the T790M mutation, albeit at much higher concentration than what is needed to inhibit EGFR sensitizing mutations only (12).

In the LUX-Lung 1 study (13), afatinib was compared with placebo (double blind 2:1 randomization in favor of active drug), with all 585 patients also getting concomitant supportive care. The trial was open to patients with advanced lung adenocarcinoma who had previously received at least one line of prior chemotherapy, and had not progressed for at least 12 weeks on another EGFR inhibitor, either gefitinib or erlotinib. This is a true third-line setting. The patient selection criteria strongly enriched for an EGFR TKI sensitive population carrying sensitizing mutations in EGFR (which was confirmed in a retrospective mutation analysis on a fraction of the patients). Most patients were never-smokers, the majority (62%) of East-Asian ethnicity; almost half had been pretreated for 48 weeks or more with a first-line TKI and 46% had experienced a prior objective remission on TKI. The study failed to meet its primary endpoint of improved overall survival (OS). There was even a numerical trend for inferior OS with afatinib compared to placebo: the median OS was 10.8 months (95%

CI, 10.0-12.0 months) in the afatinib group and 12.0 months (95% CI ,10.2-14.3 months) in the placebo group (hazard ratio 1.08, 95% CI, 0.86-1.35; P=0.74). The median overall survival (OS) in both arms of the study was better than anticipated by the authors in a more general population of lung cancer such as included in the BR 21 study (1), but this can be attributed to the strong selection of patients in the current study. The response rate was low (7%). Median PFS was longer in the afatinib group (3.3 months, 95% CI, 2.79-4.40 months) than it was in the placebo group (1.1 months, , 95% CI, 0.95-1.68 months; hazard ratio 0.38, 95% CI, 0.31-0.48, P<0.0001) and afatinib treated patients had decreased lung cancer related symptoms. On the other hand, afatinib came with significant toxicity: diarrhea (87% all grades), rash (78% all grades), stomatitis, nail changes (mainly paronychia), diminished appetite, and less commonly epistaxis and pruritus. As a consequence, 36% of the patients needed a dose reduction although only 5% discontinued treatment because of these toxicities. Drug-related serious adverse events (SAE's) occurred in 39 (10%) patients in the afatinib group with two possibly treatment-related deaths.

It should also be noted that the placebo treated patients might have experienced a shortened PFS, simply because they were weaned from TKI upon inclusion in the study. It is becoming evident that even in disease progression under TKI treatment, the TKI retain some activity and stopping the treatment might lead to an accelerated disease progression or "flare" (14). For such patients there are now several options: continue the TKI (Erlotinib or Gefitinib) with local therapy of focal progressive disease sites, switching to chemotherapy or even continuation of the EGFR TKI with chemotherapy, which might be superior to chemotherapy alone (15). Subsequent progression might even be temporarily responsive to a rechallenge or cross-over with a reversible TKI (e.g., Erlotinib if Gefitinib was given in the first line).

The main conclusion of the Lux-Lung 1 study is that afatinib is not a solution for patients with advanced NSCLC failing prior EGFR TKI and at least one line of chemotherapy. In fact, the low response rate, the significant toxicity and the OS data argue against using afatinib in such a third line setting.

In contrast, Afatinib is a valuable drug in the first line treatment of adenocarcinoma of the lung carrying EGFR mutations and was recently shown to be strongly superior over doublet chemotherapy with cisplatinum and pemetrexed in that population with an impressive PFS of 11.1 months, and even 13.6 months with the common exon 19/21 mutations, and improved symptom control compared to chemotherapy (16). The OS data are not yet available. Dacomitinib, a drug with a similar profile, is in an earlier stage of development and also has a long PFS in phase 2 (17). Whether these two pan HER inhibitors will have an increased therapeutic ratio in the first-line setting compared to the first generation TKI's Erlotinib and Gefitinib remains to be determined. Cross trial comparisons suggest that the PFS might be longer with the pan HER inhibitors, but at the expense of increased toxicity.

Afatinib is also the first targeted drug that has shown activity in lung cancer patients with HER2 mutations in their tumor, a mutation that is tenfold less prevalent than EGFR mutations (18).

So, is there a third-line option after chemotherapy and TKI failure in advanced non-small cell lung cancer? The answer today is negative. For the patients that have a baseline or an acquired true resistance to currently available EGFR TKI's, we need the exploration of better strategies to overcome or prevent such resistance. Possible strategies are the concomitant inhibition of c-MET, the development of effective inhibitors of T790M and other specific mechanisms of resistance (e.g., Pi3kinase mutations) and the discovery of additional, currently unknown, driver mutations that cooperate with EGFR mutations in the pathogenesis of the disease that subsequently could be examined for (combined) therapeutic targeting.

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Is the third generation EGFR TKIs the solution for making EGFR mutant NSCLC a curable disease?

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Much promise and encouragement has been linked to the treatment of patients with advanced NSCLC harboring EGFR mutations. The first generation EGFR TKIs (e.g., erlotinib/gefitinib) gave promise as single agent therapy in the first-line setting (1). The second generation EGFR TKI with covalent irreversible binding to the receptor and with the potential to target heterodimers of the Erb-B receptors gave further promise regarding response, progression-free survival and overall survival, particularly in patients with exon 19 deletions (2-4). However, while significant improvement in outcome was achieved with these agents, no reports on cure have yet been seen! The main reason for that is the development of acquired resistant abnormalities with the most common resistant mechanism the development of T790M mutations (5). Most recently we learned about the third generation EGFR TKIs, which are designed to target the activating EGFR mutations as well as the resistance T790M mutation. AZ 9291 is one of these third generation EGFR TKIs and the results from the phase I/II study in patients with advanced NSCLC with EGFR Mutation and acquired resistance was presented at ASCO Annual Meeting 2014 by Dr. Janne *et al.* with very promising efficacy results in patients with T790M mutations (RSP: 64% and DCR: 94%) (6). The drug was well tolerated without any serious side effects. As a matter of fact, the new generation EGFR TKIs spares the EGFR wild type and, therefore, the patients will not suffer from the "traditional" EGFR side effects such as skin rash, diarrhea, hypomagnesemia, etc. Thus, much improvement has been achieved in this particular subgroup of advanced NSCLC patients. The current question is whether this therapy is enough to achieve long-term remissions and eventually cure by itself? Another question is of course whether the new generation EGFR TKIs is better than the previous generations in first-line therapy? A crucial element in this discussion is the fact that T790M mutations are not the only resistant mechanism. Several other mechanisms have been identified and more mechanisms for resistance to EGFR TKIs are expected to be learned in the future. Among already well known resistant mechanisms are activation of the MET pathway, transition to small cell carcinoma morphology, and based on preclinical data a possible role of FGFR, Mer and Axl as part of the acquired resistance to EGFR TKIs (5,7). Thus, while the development of the new generation EGFR TKIs represent a significant achievement in the fight for "curable" EGFR mutant tumors, most likely a partnership with other agents will be needed in order to achieve the goal.

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Is epidermal growth factor receptor tyrosine kinase inhibitor in combination with cytotoxic chemotherapy a better treatment option for patients with *EGFR*-mutated non-small-cell lung cancer?

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Provenance: This is a Guest Editorial commissioned by the Section Editor Hongbing Liu (Department of Respiratory Medicine, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China).

Comment on: Sugawara S, Oizumi S, Minato K, *et al*. Randomized phase II study of concurrent versus sequential alternating gefitinib and chemotherapy in previously untreated non-small cell lung cancer with sensitive EGFR mutations: NEJ005/TCOG0902. Ann Oncol 2015;26:888-94.

Abstract: Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) combined with cytotoxic chemotherapy achieved a high disease control rate and favorable progression-free survival (PFS) for *EGFR*-mutated non-small-cell lung cancer (NSCLC) patients. This combination therapy might circumvent *de novo* resistance to EGFR-TKI. Randomized phase III studies are required to confirm the survival benefit of this combination therapy in NSCLC patients. In addition, there are some other promising strategies including the combination of EGFR-TKI plus bevacizumab, third-generation EGFR-TKIs, and immune checkpoint inhibitors that remain a future challenge for lung cancer treatment.

Keywords: Combination drug therapy; epidermal growth factor receptor (EGFR); non-small-cell lung cancer (NSCLC)

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Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) is demonstrated to have a dramatic response to non-small-cell lung cancer (NSCLC) harboring activating *EGFR* mutation (1-3). Therefore, it is considered as a standard treatment for patients with *EGFR*-mutated NSCLC. For further efficacy, the combination therapy with EGFR-TKI and cytotoxic agents has also been considered. However, recent studies on this combination therapy failed to demonstrate any further benefit for patients with NSCLC in comparison to chemotherapy (4-7). Two main reasons have been proposed for these failures. Firstly, the patients recruited in these studies were not selected by analyzing the *EGFR* mutation; thus, the efficacy of EGFR-TKI was diluted. Secondly, the preclinical studies indicated that the G1 phase arrest induced by EGFR-TKI may have interfered with the cell cycle-dependent cytotoxic chemotherapy (8). However, the second reason is not definitive. Two days of gefitinib treatment before paclitaxel was found to be more effective than the reverse treatment pattern in tumor xenografts (9). In contrast, paclitaxel treatment followed by gefitinib produced a more anti-proliferative effect than the reverse pattern in NSCLC cell lines (10).

Sugawara *et al*. reported a randomized phase II study that evaluated the safety and efficacy of concurrent or sequential alternating regimen with gefitinib and carboplatin/pemetrexed in patients with *EGFR*-mutated NSCLC (11). The median progression-free survival (PFS) obtained in this study was 18.3 and 15.3 months for the concurrent and sequential alternating regimens, respectively. The PFS,

especially in the concurrent group, is more favorable in comparison to the PFS in previous studies, which was 9.2 to 10.8 months with first-line gefitinib monotherapy for *EGFR*-mutated NSCLC patients (2,3). Clinically, *EGFR* mutant patients having disease progression after the first-line treatment of gefitinib or erlotinib, are administered with platinum-based chemotherapy. Since the median PFS of carboplatin and pemetrexed was 5.7 months (12), the total PFS of the first-line gefitinib and the second-line carboplatin/pemetrexed treatment added up to about 15 to 16 months. Although the PFS of 18.3 months of the concurrent arm in the present study was longer than the added PFS, the difference was not substantial. A longer PFS benefit is expected from the concurrent regimen of the combination therapy that would outweigh the increased adverse events and cost of the treatment than the sum of PFS of each treatment given sequentially.

It should be noted that the disease control rate in this study is 100%, and the median overall survival (OS) time in the concurrent arm is 41.9 months. In general, approximately 10% of patients treated with first-line EGFR-TKI exhibit initial progression (2,3). Several mechanisms for the *de novo* resistance have been reported, and the early concurrent use of cytotoxic agents might be one of the countermeasures. OS must be interpreted with caution because of this being a randomized phase II study with immature survival data. The prolongation of the survival time is partly due to the long PFS of the first-line treatment and partly due to the long post-progression survival time. The efficacy of the second-line or third-line therapies as well as the improvement in the supportive care throughout the treatment might be partly responsible for the favorable post-progression survival time. Treating *EGFR*-mutated patients with EGFR-TKI has been shown to improve their OS. Updated median OS for the first-line gefitinib monotherapy in the NEJ002 and WJTOG3405 studies were 27.7 and 34.8 months, respectively (13,14). Moreover, median OS of the Japanese patients treated with the first-line afatinib was 46.9 months (15).

The most common grade 3 or greater adverse events in the study were neutropenia and thrombocytopenia. These hematological toxicities occurred most frequently in the concurrent group than in the sequential alternating group. The occurrence of non-hematologic toxicities like vomiting, appetite loss and diarrhea were also frequent in the concurrent group. Although almost half of the patients experienced grade 3 or greater adverse events, these events were still predictable and manageable. One of the greatest concerns in this combination therapy including EGFR-TKI is the increase of interstitial lung disease (ILD). However, in this study, only 5% of the total patients were observed to have ILD, which is comparable to that in EGFR-TKI monotherapy (2,3).

In addition to the combination of EGFR-TKI and cytotoxic chemotherapy, there are some other promising combination therapies including the combination of EGFR-TKI plus bevacizumab, third-generation EGFR-TKI, and anti-programmed cell death-1 (PD-1) monoclonal antibodies.

Erlotinib combined with bevacizumab demonstrated a median PFS of 16.0 months, which was significantly better in comparison to erlotinib monotherapy (16). Almost all the patients (99%) in the erlotinib plus bevacizumab arm achieved disease control. While hypertension and proteinuria were commonly found in this combination therapy, serious adverse events also occurred at a similar frequency in both the groups. As this is a randomized phase II study, further evaluation is required to confirm the efficacy of such combination therapies.

The most common resistance mechanism after gefitinib or erlotinib is the acquisition of the second mutation in *EGFR*, which result in the substitution of threonine with methionine at the amino acid position 790 (T790M). AZD9291 is a selective third-generation inhibitor of both *EGFR* sensitizing and T790M resistance mutation. This inhibitor was reportedly administered to patients who had disease progression after being treated with EGFR-TKI (17,18). Its antitumor activity depended on the T790M status. The response rate was 61% and 21% and median PFS was 9.6 and 2.8 months in T790M-positive patients and T790M-negative patients, respectively. It was highly active in patients with NSCLC with T790M mutation who had disease progression during the initial EGFR-TKI therapy.

Nivolumab, a fully human anti-PD-1 immune checkpoint inhibitor antibody, was compared to docetaxel in patients with advanced non-squamous NSCLC after the failure of the platinum-based doublet chemotherapy (19). Nivolumab demonstrated superior OS of 12.2 months and improved response rate of 19.2%. Although the response rate was not remarkable, the median response duration of 17.1 months attracted more attention. This monoclonal antibody had durable responses in the limited subset of patients. Although high PD-L1 expression correlated with positive treatment outcomes, this association was not conclusive. Search for new reliable predictive markers is essential to spare non-

responders from unnecessary toxicities and financial burden of the treatment.

In conclusion, EGFR-TKI plays an essential role in the treatment of *EGFR*-mutated NSCLC patients. Combination therapy of EGFR-TKI and cytotoxic chemotherapy is attractive in the light of favorable PFS and high disease control rate. As described above, with the development of new promising drugs, further prolongation of OS might be achievable. The main challenge is how to combine the first- to third-generation EGFR-TKIs, cytotoxic chemotherapies, bevacizumab, and immune checkpoint inhibitors, either concurrently or sequentially, for the treatment of *EGFR*-mutated NSCLC patients.

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Improved overall survival following tyrosine kinase inhibitor treatment in advanced or metastatic non-small-cell lung cancer—the Holy Grail in cancer treatment?

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Abstract: Advanced or metastatic non-small-cell lung cancer (NSCLC) is characterized by a poor prognosis and few second- or third-line treatments. First-generation epidermal growth factor receptor tyrosine kinase inhibition has paved the way for targeted therapies in lung cancer. Although these drugs result in excellent responses [and significantly improved progression-free survival (PFS)] in patients with activating EGFR mutations, none of these randomized studies has yet demonstrated a statistically significant improvement of overall survival (OS). PFS is often used as a predictor for improved OS since it is independent of subsequent treatment, but OS is acknowledged as the key clinical outcome in the treatment of advanced NSCLC. When effective treatment is given as post therapy, it will be difficult to distinguish the treatment effect of original and subsequent treatments because differences in OS are potentially confounded by crossover, and a relevant number of patients assigned to chemotherapy arms received tyrosine kinase inhibitors (TKIs) as second- or third-line treatment after disease progression. The high proportion of crossover may extend the benefit associated with the administration of TKIs to patients assigned to the control arm, and its "salvage"-effect may compensate for the relevant differences in PFS of first-line treatment consistently demonstrated in all TKI trials. Results for the INFORM trial (maintenance therapy with gefitinib following platinum-based chemotherapy) provided evidence that maintenance therapy with gefitinib significantly improved PFS, with greatest benefit in patients harboring EGFR mutation. Despite a high crossover rate (53%) final OS results of this study have now demonstrated a significant survival benefit for the gefitinib-treated EGFR mutation-positive patients (46.9 *vs.* 21.0 months, P=0.036). This is the first randomized clinical trial that showed a significant and clinical meaningful OS benefit in EGFR mutation-positive NSCLC patients following maintenance therapy with gefitinib as compared to placebo. It remains to be seen whether further exploration of this treatment strategy will confirm these promising results.

Keywords: Gefitinib; non-small-cell lung cancer (NSCLC); maintenance therapy; overall survival (OS); editorial

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The introduction of the epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) gefitinib (Iressa®, AstraZeneca, UK), erlotinib (Tarceva®, Roche, Switzerland), and afatinib (Giotrif®, Boehringer Ingelheim, Germany) and the anaplastic lymphoma kinase (ALK) inhibitors crizotinib (Xalkori®, Pfizer, USA) and ceritinib (Zykladia®, Novartis, Switzerland) represent the most important innovations in non-small-cell lung cancer (NSCLC) treatment over the past ten years (1). By targeting the main pathways of NSCLC signal transduction, these drugs significantly improved progression-free survival (PFS) and quality of life in a highly selected subgroup of NSCLC (harbouring EGFR mutations), sparing them from toxic chemotherapy approaches. However, for the vast majority of patients platinum-based chemotherapy remains the only potential treatment and has led to significantly improved survival outcomes with a "plateau" of about 10-11 months median survival (2). Subsequently, significant advances have been made with the introduction of pemetrexed, especially against the non-squamous cell subtype. The addition of this agent led to a further improvement in survival to 12-13 months (3) and up to 14 months with the introduction of maintenance therapy (4).

Maintenance therapy is a treatment strategy that has been investigated extensively in NSCLC and has been the subject of considerable recent debate. Options for maintenance include continuing the initial combination chemotherapy regimen, continuing only single agent chemotherapy ('continuation maintenance') or introducing a new agent ('switch' maintenance therapy). Therapies that have been studied in this setting in randomized trials to date include chemotherapy, molecularly targeted agents and immunotherapy approaches (5).

The outstanding results of the JMEN study proved that maintenance of pemetrexed (for patients with tumours of non-squamous histology) significantly improved the overall survival (OS) in advanced NSCLC patients was a proof of principle (6). Subsequently, the results of the SATURN study also showed a significant prolongation of PFS and OS with maintenance erlotinib (for patients with stable disease) compared with placebo (7). Despite considerable controversy, it has become an acceptable treatment paradigm and both drugs are approved for maintenance therapy of advanced NSCLC patients in Europe (EMA) and the USA (FDA) and this has certainly shifted the pendulum towards maintenance therapy.

Zhang and colleagues (8) first presented results from the INFORM trial evaluating gefitinib in the maintenance setting in 2012 (8). In this large phase III multicentre, double-blind trial patients (Asian ethnic origin, n=296) with stage IIIb or IV NSCLC after four cycles of platinum-based doublet chemotherapy were randomized either to placebo or maintenance therapy with gefitinib (250 mg/d) until progression or unacceptable toxic effects. Primary endpoint was PFS as assessed in the intent-to-treat population, whereas OS was a secondary endpoint. Assessment of PFS according to the tumour EGFR mutation status was also a pre-planned exploratory objective [highlighted in a previous editorial in this journal by Dempke (9)].

Median duration of treatment was 148 [49-467] days with gefitinib and 73 [42-127] days with placebo. PFS was significantly longer with gefitinib than that with placebo [median PFS 4.8 (95% CI: 3.2-8.5) *vs.* 2.6 (1.6-2.8) months; hazard ratio 0.42; 95% CI: 0.33-0.55; P<0.0001]. OS did not differ between both treatment groups [hazard ratio 0.84; 95% CI: 0.62-1.14; P=0.26; median OS 18.7 (95% CI: 15.6-22.2) *vs.* 16.9 (14.5-19.0) months]. Moreover, the greatest PFS benefit with gefitinib was found in the subgroup positive for EGFR mutations [hazard ratio 0.17; 95% CI: 0.07-0.42; median PFS 16.6 (9.4-22.7) *vs.* 2.8 (1.3-4.1) months].

In a most recently published update of the INFORM trial OS results were detailed (10). The median duration of follow-up for OS was 17.83 months (95% CI: 15.43-20.23). At the time of data cut-off for OS (June 17, 2014), 230 patients (78%) had died. In the subgroup positive for EGFR mutation, a higher OS was observed in patients treated with gefitinib than the placebo arm (HR 0.39; 95% CI: 0.15-0.97; P=0.036; median OS 46.87 *vs.* 20.97 months). In contrast, there was no significant difference in OS for gefitinib *vs.* placebo in patients negative for EGFR mutations (HR 1.27; 95% CI: 0.7-2.3; P=0.431; median OS 10.9 *vs.* 14.0 months). In the subgroup with unknown EGFR mutation, OS was numerically but not statistically longer with gefitinib *vs.* placebo (HR 0.92; 95% CI: 0.68-1.25; P=0.603; median OS 20.6 *vs.* 16.8 months). However, it is worth noting that a large proportion of patients (73%) had insufficient tumour samples to perform a mutation analysis.

Targeted therapies are currently being evaluated in a variety of treatment settings in NSCLC and novel strategies of disrupting tyrosine kinase-controlled pathways have been investigated. However, almost all of the recently reported trials have failed to improve OS for which there may be several key reasons.

Firstly, without a validated biomarker, specific subgroups of patients who are more likely to respond cannot be selected. Furthermore, the redundancy in tyrosine kinase-triggered

Table 1 Crossover rates (control → TKI) and median OS for selected clinical trials with gefitinib, erlotinib, and afatinib in EGFR mutation-positive NSCLC

Study	Design	Cross-over rate (%)	Median OS	References
SATURN	Platinum-based chemotherapy followed by erlotinib or placebo	67	12.0 *vs*. 11.0 months (P=0.0088)	Cappuzzo *et al*. (7)
EURTAC	Erlotinib *vs*. platinum-based chemotherapy	76	19.3 *vs*. 19.5 months (NS)	Rosell *et al*. (13)
OPTIMAL	Erlotinib *vs*. carboplatin/gemcitabine	68	PFS: 13.1 *vs*. 4.6 months (P<0.0001); OS: no differences	Zhou *et al*. (14)
IPASS	Gefitinib *vs*. carboplatin/paclitaxel	64	18.6 *vs*. 17.3 months (NS)	Mok *et al*. (15)
NEJ002	Gefitinib *vs*. carboplatin/paclitaxel	95	27.7 *vs*. 26.6 months (NS)	Inoue *et al*. (16)
FIRST-SIGNAL	Gefitinib *vs*. cisplatin/gemcitabine	75	22.3 *vs*. 22.9 months	Han *et al*. (17)
WJTOG3405	Gefitinib *vs*. cisplatin/docetaxel	91	34.8 *vs*. 37.3 months (NS)	Yoshioka *et al*. (18)
INFORM	Platinum-based chemotherapy followed by gefitinib or placebo	53	46.9 *vs*. 21.0 months (P=0.036)	Zhao *et al*. (10)
LUX-Lung 3 (LL-3)	Afatinb *vs*. cisplatin/pemetrexed	65	28.2 *vs*. 28.2 months (NS)	Sequist *et al*. (19)
LUX-Lung 6 (LL-6)	Afatinib *vs*. cisplatin/gemcitabine	48	23.1 *vs*. 23.5 months (NS)	Wu *et al*. (20)
LL3 and LL-6	Pooled analysis	–	27.2 *vs*. 24.3 months (del19 only, P=0.037)	Yang *et al*. (21)

TKI, tyrosine kinase inhibitor; EGFR, epidermal growth factor receptor; NS, not significant; PFS, progression-free survival; OS, overall survival; NSCLC, non-small-cell lung cancer.

pathways leads to primary and secondary resistance to an agent that targets a specific signal transduction cascade; as a result, agents that target multiple pathways are currently under investigation. Finally, it is unlikely that any TKI could achieve complete inhibition of its target(s), which may result in reduced but not completely abrogated signalling (11). Moreover, the reasons that TKIs have failed to improve survival when added to chemotherapy remain far from clear. A possible potential mechanism for the lack of synergy between these agents and chemotherapy may be the G_1 phase cell-cycle arrest caused by TKIs, which then may interfere with the cell cycle-dependent cytotoxicity of chemotherapy (12).

The question remains whether the benefit of targeted therapy for NSCLC may be best defined by PFS since in this regard published data are still inconclusive. Truly, PFS is regarded as a good predictor for improved OS (and is independent of subsequent treatment), but OS is acknowledged as the key clinical outcome in the treatment of advanced NSCLC. All large previous randomized phase III trials assessing first-line treatment demonstrated a significantly higher response rate and longer PFS in patients treated with first- and second-generation EGFR-TKIs, including gefitinib, erlotinib, and afaftinib than in patients treated with standard platinum-based combination chemotherapy. Although these trials met their primary endpoint with significantly longer PFS, no significant difference was observed in terms of OS. However, no restrictions were imposed on treatment after the end of protocol therapy in any of these trials and the majority of patients in the control arm received EGFR-TKI therapy at least once (*Table 1*).

None of these randomized trials has yet demonstrated a statistically significant improvement with these TKIs in terms of OS, which is of course the strongest endpoint for clinical research in oncology, in a condition of no effective treatment afterwards. When effective treatment is given as post therapy, it will be difficult to distinguish the treatment effect of original and subsequent treatments because differences in OS are potentially confounded by crossover, and a relevant number of patients assigned to chemotherapy arms received TKIs as second- or third-line treatment after disease progression (*Table 1*). Intuitively, the high proportion of crossover may extend the benefit associated with the administration of TKIs to patients assigned to

the control arm, and its "salvage"-effect may compensate for the relevant differences in PFS of first-line treatment consistently demonstrated in all TKI trials.

However, a most recently published joint analysis of the LUX-Lung trials 3 and 6 revealed that afatinib prolonged survival of patients with NSCLC with common EGFR mutations compared with standard chemotherapy by a median of 3 (27.3-24.3) months, significantly reducing the risk of death by 19% (HR =0.81, CI =0.66-0.99; P=0.037). The most pronounced reduction in risk of death, by 41% (HR =0.59, CI =0.45-0.77; P<0.001), was noted for patients whose tumors have the most common type of EGFR mutation (namely deletion in exon 19), which is present in approximately 48% with an EGFR mutation. For patients with the exon 21 (L8585R) mutation, there was no impact on OS (HR =1.25, CI =0.92-1.71; P=0.160) (21). From a methodological point of view, subgroup and post-hoc analyses can be informative, but should be interpreted with caution since PFS was chosen as the primary endpoint in both trials

Moreover, crossover was high for afatinib and erlotinib, and very high for gefitinib in all studies (*Table 1*) making the statistical power for analysis of OS very low (22,23).

In conclusion, the updated results of the INFORM trial clearly do not support the routine use of gefitinib for maintenance therapy as standard of care in NSCLC patients with advanced or metastatic NSCLC following treatment with platinum-based chemotherapy. However, to our knowledge the INFORM study is the first randomized clinical trial that shows a significant OS benefit in the EGFR mutation-positive population following maintenance therapy with gefitinib as compared to placebo. It remains to be seen whether further exploration of this treatment strategy will confirm these promising data.

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Footnote

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Novel radiotherapy approaches for lung cancer: combining radiation therapy with targeted and immunotherapies

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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.

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

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.

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 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 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).

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 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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Epidermal growth factor receptor tyrosine kinase inhibitors for the treatment of central nervous system metastases from non-small cell lung cancer: the present and the future

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Abstract: Lung cancer is one of the major causes of cancer related mortality worldwide. Brain metastases (BM) complicate clinical evolution of non-small cell lung cancer (NSCLC) in approximately 25–40% of cases, adversely influencing quality of life (QoL) and overall survival (OS). Systemic therapy remains the standard strategy for metastatic disease. Nevertheless, the blood-brain barrier (BBB) makes central nervous system (CNS) a sanctuary site. To date, the combination of chemotherapy with whole brain radiation therapy (WBRT), surgery and/or stereotactic radiosurgery (SRS) represents the most used treatment for patients (pts) with intracranial involvement. However, due to their clinical conditions, many pts are not able to undergo local treatments. Targeted therapies directed against epidermal growth factor receptor (EGFR), such as gefitinib, erlotinib and afatinib, achieved important improvements in EGFR mutated NSCLC with favorable toxicity profile. Although their role is not well defined, the reported objective response rate (ORR) and the good tolerance make EGFR-tyrosine kinase inhibitors (TKIs) an interesting valid alternative for NSCLC pts with BM, especially for those harboring EGFR mutations. Furthermore, new-generation TKIs, such as osimertinib and rociletinib, have already shown important activity on intracranial disease and several trials are still ongoing to evaluate their efficacy. In this review we want to highlight literature data about the use and the effectiveness of EGFR-TKIs in pts with BM from NSCLC.

Keywords: Brain metastases (BM); epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs); non-small cell lung cancer (NSCLC); whole brain radiation therapy (WBRT)

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Introduction

Lung cancer is one of the major causes of cancer related mortality worldwide accounting for approximately 1.4 million deaths per year (1). In approximately 25–40% of non-small cell lung cancer (NSCLC), brain metastases (BM) complicate clinical evolution of disease causing the onset of neurological symptoms, the deterioration in quality of life (QoL) and reducing overall survival (OS) (2,3). About 10–20% of patients (pts) show BM at diagnosis whilst another 20% experience brain progression during the course of disease, often within the first 2 years from diagnosis (2-6). Central nervous system (CNS) represents the first site of

relapse after radical treatments for loco-regional disease (7). Furthermore, the prolongation of survival of NSCLC pts, due to the therapeutic advances of the last decades, is likely to explain the increased incidence of BM over time. Unfortunately, for pts with BM the prognosis remains poor with a median OS equal or less than 3 months without any treatment (8). To date, systemic therapy is the standard strategy for metastatic disease. Nevertheless, the blood-brain barrier (BBB) presence with its continuous endothelium, tight junctions, basal membrane, efflux membrane transporters and absence of fenestrations, makes CNS a sanctuary site. Most chemotherapeutic agents do not cross BBB and only the crossing of small lipid-soluble molecules is allowed (9-12). For this reason the role of systemic chemotherapy in the treatment of CNS secondary lesions is controversial (13,14). In the case of macroscopically evident BM, both tumor neoangiogenesis and BBB destruction due to tumor growth, seem to favor intracranial penetration of chemotherapeutic drugs (15,16). This phenomenon could support the use of upfront chemotherapy for BM that damage the integrity of the barrier (15,16). First line upfront platinum based chemotherapy has been evaluated in different prospective trials and an objective response rate (ORR) of 23–50% was reported (5,17-24). Pemetrexed and temozolomide showed some activity (25-29) while 5-FU, topotecan and vinorelbine, did not show any improvement in ORR and OS (23,30,31).

To date local treatments, including whole brain radiation therapy (WBRT), surgery and/or stereotactic radiosurgery (SRS) represent the most used approaches in pts with BM (32). WBRT, in association with corticosteroids, showed a median OS that ranges from 2.4 to 4.8 months (33-35). In some cases, considering the site and the number of lesions, surgery or SRS can be used (32,36-38). Usually SRS is applied when few or small volume isolated lesions (maximum diameter 4 cm) are present (32). WBRT significantly improves brain tumor control after SRS but the role of adjuvant WBRT remains undefined because of the increased risk of neurocognitive toxicity (36). If surgery does not seem useful for multiple BM, prospective trials documented an advantage in terms of survival and local control with surgery and WBRT compared with WBRT alone in oligometastatic brain disease (37,38). Moreover the combination of the three options can be evaluated in selected cases as well as their association with chemotherapy and targeted therapy (32,36-38).

In particular targeted treatments directed against epidermal growth factor receptor (EGFR), such as gefitinib, erlotinib and afatinib, achieved important results in NSCLC, in particular in pts harboring activating EGFR mutations. Considering their favorable safety profile, tyrosine kinase inhibitors (TKIs) may represent a valid alternative in pts with BM but to date the role of TKIs, and their correct place within the therapeutic strategy in this setting, are still debated. Furthermore other new-generation TKIs, such as osimertinib and rociletinib, have already shown important activity on intracranial disease and several trials are still ongoing to evaluate their activity and efficacy.

Here, we review literature data about EGFR-TKIs use in pts with BM from NSCLC, analyzing the most relevant aspects concerning their role and effectiveness compared to current standard treatments.

EGFR mutated NSCLC metastatic to the brain

Approximately 10–15% of NSCLC Caucasian pts show *EGFR* gene somatic activating mutations (39). Exon 19 in-frame deletion and exon 21 point mutation L858R are the most frequent aberrations, representing about 90% of cases (39). Mutations in *EGFR* gene cause the expression of a structurally altered receptor that, through the activation of different signaling pathways, promotes cell proliferation and survival (40). In recent years EGFR-TKIs (erlotinib, gefitinib and afatinib) specifically directed against EGFR, and in particular against its mutated form, changed the paradigm of care for a subgroup of NSCLC. Their superiority in terms of efficacy and toxicity in comparison to standard chemotherapy has led to EGFR-TKIs approval for first line treatment of EGFR mutated NSCLC (41-44). Several studies suggested a significant association between EGFR mutation and risk of developing BM, with a reported higher incidence of BM, both at the time of diagnosis and during the course of disease, in EGFR mutated compared with EGFR wild-type (WT) pts (45-48). Generally pts with EGFR mutations had longer OS after BM diagnosis than EGFR WT pts (47,48). However, these data were not confirmed by all studies (49-52).

For this reason more effective agents are needed in order to prolong survival, maintain neurocognitive functions and prevent neurologic deterioration. The high rates of durable response and the good safety profile make EGFR-TKIs an attractive therapeutic option also in these pts, especially considering that standard local approaches in pts metastatic to the brain are associated with a high rate of adverse events (36).

First generation EGFR-TKIs

Erlotinib and gefitinib are reversible TKIs targeting

EGFR, the first to enter into clinical practice. Initially, they reported an improvement in progression-free survival (PFS) and OS compared to placebo when used as second line therapy in unselected NSCLC pts, especially never-smokers, females or Asian pts (53,54). Later EGFR mutational status became the most accurate predictor of response to EGFR-TKIs in NSCLC (39,55). Today erlotinib and gefitinib, together with the second generation TKI afatinib, are recognized as the standard first line therapy in NSCLC pts with activating EGFR mutations, instead of conventional cytotoxic chemotherapy. Randomized studies showed that in this setting they were able to obtain an ORR of 60–80%, a PFS ranging from 10 to 13 months and an OS of 13–20 months (41-43,56-65).

CNS penetration

Evidences suggest that EGFR-TKIs can cross the BBB (66,67). Nevertheless, despite their small molecular weight, both erlotinib and gefitinib, seem to reach limited concentrations into cerebrospinal fluid (CSF). In fact, at standard dose CSF levels are lower than plasma levels (68-72). Available data do not favor one EGFR-TKI over another but the concentration and the penetration in CSF are significantly higher with erlotinib than gefitinib (73-75). Moreover, P-glycoprotein (P-gp) efflux pump, that is associated with multiple drug resistance in brain tumor, has gefitinib as one of its substrates (76).

The limited CNS exposure to TKIs can explain the high incidence of BM in EGFR mutated NSCLC despite the good control of extracranial disease during EGFR-TKIs therapy. However, BM occurrence can damage the integrity of BBB and favor TKIs penetration (77). So, while erlotinib and gefitinib at the standard dose do not sufficiently penetrate BBB in absence of CNS involvement, when BM are evident, they probably improve their CNS concentration with a consequent improvement in central activity (67). Furthermore the inadequate TKIs penetration across the intact BBB, could explain the frequent absence of secondary resistance mutations in BM also when they are present in extra-cranial disease sites (70,77,78).

Alternative schedules

Literature data report that dose escalation, pulsate dose or switching TKIs, seem to improve TKIs concentration in CNS and to relieve resistance to standard TKIs treatment in pts with BM from EGFR mutated NSCLC (70-73,76,78-83) (*Table 1*).

Progressively increasing doses of erlotinib or gefitinib are able to control BM progression or relapse in NSCLC pts (70-73,79,80). The greater penetration through the BBB when plasma concentrations are higher, also thanks to P-gp saturation, allows EGFR-TKIs to exert greater activity in CNS (76). However, dose escalation is inevitably related to more frequent and significant side effects including high grade fatigue, nausea and liver damage (70-73,79,80).

Pulsate high dose erlotinib, with a median dose of 1,500 mg weekly, appears to provide a significant advantage with reduced toxicity (81). In a small retrospective analysis of nine NSCLC pts, higher pulsate erlotinib dosage (1,500 mg once a week) achieved 67% partial response (PR) after progression to conventional dose (78). In contrast, another retrospective evaluation of ten NSCLC pts who received pulsate dose erlotinib for CNS progression, reported an ORR of 10% with a very limited median OS (1.7 months) (82). To date, there is no prospective trial comparing pulsate high dose *vs.* standard dose TKIs, but pulsed high doses of EGFR-TKIs could be considered in NSCLC pts with brain progression after standard EGFR-TKI therapy.

Switching to different EGFR-TKIs may represent another valid therapeutic alternative. In a small trial (83), seven lung cancer pts with good response to gefitinib, showed interesting results receiving erlotinib at the time of brain progression: three PR, three stable disease (SD) and one progressive disease (PD) with improvement in PS and neurological symptoms control.

All these results are very preliminary. Further larger prospective studies are needed to validate these approaches in clinical practice.

Standard schedules

Today although their emerging role, the specific indication of EGFR-TKIs in the management of BM from NSCLC, with or without radiotherapy, remains not well defined. Literature data suggest that TKIs alone are able to obtain a high intracranial ORR (99-101) (*Table 1*). In preclinical mouse model of EGFR mutated NSCLC with BM, gefitinib has proven effective (102). Complete and sustained responses following BM treatment with erlotinib and gefitinib have been reported in several case reports (103-106). Several small phase II trials, have shown that TKIs alone can obtain 75–88% of intracranial ORR in pts with EGFR mutated NSCLC who have not received any prior local therapy

Table 1 First and second generation EGFR-TKIs use in NSCLC pts with BM (selection of studies)

Authors	Years	Study	Patients	Treatment	Results
Grommes *et al.* (78)	2011	Retrospective study	9 EGFRm NSCLC with BM progressed after standard treatment with TKIs	Pulsatile erlotinib 1,500 mg once weekly	CNS response: PR 67% (6/9 pts), SD 11% (1/9 pts), PD 22% (2/9 pts); median CNS TTP: 2.7 months (0.8–14.5 months); median OS: 12 months (2.5 months–not reached)
Jackman *et al.* (82)	2013	Retrospective study	10 EGFRm NSCLC with BM progressed after treatment with TKIs	Pulsatile erlotinib 1,000–1,500 mg once weekly	CNS response: PR 10% (1/10 pts); SD 20% (2/10 pts), PD 70% (7/10 pts); median OS: 1.7 months (0.6–7.0 months)
Katayama *et al.* (83)	2009	Retrospective study	7 NSCLC with BM developed after an initial response to gefitinib (6 EGFRm pts)	High dose erlotinib	CNS response: PR 42.5% (3/7 pts), SD 42.5% (3/7 pts), PD 15% (1/7 pts)
Park *et al.* (84)	2012	Prospective open-label, single-institution, phase II study	28 naive EGFRm NSCLC with BM	Erlotinib 150 mg or gefitinib 250 mg once a day	CNS response: PR 83% (23/28 pts); SD 11% (3/28 pts), PD 7% (2/28 pts); median PFS: 6.6 months (3.8–9.3 months); median OS: 15.9 months (7.2–24.6 months); no difference in PFS and OS according to erlotinib or gefitinib use
Wu *et al.* (85)	2013	Prospective open-label, phase II study	48 NSCLC pts (8 EGFRm) after first-line platinum-doublet CHT, with asymptomatic BM without extracranial progression	Erlotinib 150 mg once a day	Median CNS PFS: 10.1 months (7.1–12.3 months); median CNS and systemic PFS: 9.7 months (2.5–17.8 months); EGFRm pts had significantly longer median PFS *vs*. EGFR WT pts 15.2 months (8.3–22.2 months) *vs*. 4.4 months (0.0–11.6 months) Median OS: 18.9 months (14.4–23.4 months); overall ORR: 58.3%
Iuchi *et al.* (86)	2013	Prospective open-label, phase II study	41 naive EGFRm NSCLC with BM	Gefitinib 250 mg once a day	CNS response: CR 31.7% (13/41 pts), PR 56.1% (23/41 pts), SD 9.8% (4/41 pts), PD 2.4% (1/41 pts), ORR: 87.8%; median PFS: 14.5 months (10.2–18.3 months); median OS: 21.9 months (18.5–30.3 months)
Hotta *et al.* (87)	2004	Monocentric retrospective study	14 pretreated NSCLC with BM unselected for EGFR mutational status	Gefitinib 250 mg once a day	CNS response: CR 7% (1/14), PR 42.5% (5/14), SD 42.5% (8/14)
Porta *et al.* (88)	2011	Retrospective study	69 NSCLC pts with BM, (17 EGFRm, 26 naive for CHT and TKIs, 55 treated previously with WBRT)	Erlotinib 150 mg once a day	CNS response: CR 15.1% (8/53 pts all EGFRm), PR 11.3% (6/53 pts all EGFRm), SD 58.5% (31/53 pts), PD 15.1% (8/53 pts); median CNS TTP: 11.7 months (7.9–15.5 months) in EGFRm pts *vs*. 5.8 months (5.2–6.4 months) in WT or unknown EGFR pts (P<0.05); ORR: 82.4% in EGFRm pts *vs*. 0% in unselected pts; median OS: 12.9 months (6.2–19.7 months) in EGFRm pts *vs*. 3.1 months (2.5–3.9 months) in WT or unknown EGFR pts (P<0.001)
Kim *et al.* (89)	2009	Retrospective study	23 naive Asian never-smoking NSCLC pts with synchronous asymptomatic BM	Erlotinib 150 mg or gefitinib 250 mg once a day	CNS response: 73.9%; ORR: 69.6% (16 PR, 3 SD, 4 PD); DCR: 82.6%; median PFS: 7.1 months (1.08–12.87 months); median OS: 18.8 months (0.64–27.0 months)
Ceresoli *et al*. (90)	2004	Prospective phase II study	41 NSCLC pts with BM, 37/41 pts already treated with CHT, 18/41 pts previously treated with WBRT, 23/41 radio naive pts	Gefitinib 250 mg once a day	CNS response: PR 10% (4/41 pts); SD 17% (7/41 pts), DCR 30%; DCR WBRT + gefitinib sequential: 56% (10/18); DCR gefitinib alone: 9% (2/23 pts); median PFS whole population: 3 months (0.0–14 months)

Table 1 (*continued*)

Table 1 (*continued*)

Authors	Years	Study	Patients	Treatment	Results
Wu *et al.* (91)	2007	Prospective phase II study	40 lung ADK pts with BM, 40/40 pts already treated with CHT, 23/40 pts previously treated with WBRT, 9/40 previously treated with SRS	Gefitinib 250 mg once a day	CNS response: CR 2.5% (1/40 pts), PR 35% (14/40 pts), SD 45% (18/40 pts), PD 17.5% (7/40 pts), ORR 38%, DCR 83%; overall ORR: 32%; overall DCR: 77%; median PFS: 9.0 months (4.5–13.5 months); median OS: 15.0 months (11.1–18.8 months)
Zhuang *et al*. (92)	2013	Prospective phase II study	WBRT group: 31 lung ADK pts; concurrent WBRT + erlotinib group: lung 23 ADK pts	WBRT group: 30 Gy/10 f; concurrent WBRT + erlotinib group: 30 Gy/10 f + 150 mg once a day	In the WBRT arm *vs*. WBRT + erlotinib respectively: median CNS PFS: 6.8 *vs*. 10.6 months (P=0.003); ORR: 54.84% *vs*. 95.65% (P=0.001); median general PFS: 5.2 *vs*. 6.8 months (P=0.009); median OS of 8.9 *vs*. 10.7 months (P=0.020)
Welsh *et al.* (93)	2013	Prospective phase II study	40 NSCLC with BM (9 EGFRm, 8 EGFR WT, 23 unknown)	Concurrent WBRT (2.5 Gy per day 5 days per week, to 35 Gy) + erlotinib 150 mg once a day	CNS response: CR 31% (11 pts), PR 56% (20 pts), MR 6% (2 pts), SD 3% (1 pts), PD 6% (2 pts), ORR 86%, PFS 8.2 months; median OS: 11.8 months (7.4–19.1 months); 9.3 *vs*. 19.1 months in EGFR WT *vs*. EGFRm pts respectively
Hoffknecht *et al.* (94)	2015	Report of compassionate use	100 NSCLC with BM (74% EGFRm pts) progressing after at least one line of CHT and one line of EGFR-TKIs treatment	Afatinib 50 mg once a day	CNS response: median TTP 3.6 months, ORR 35% (11/31 pts), DCR 66% (21/32 pts)
Lind *et al.* (95)	2009	Prospective phase I study	11 NSCLC with BM	Concurrent WBRT (30 Gy/10 f) + erlotinib 150 mg once a day	CNS response: PR 5 pts, SD 2 pts, PD 1 pt
Ma *et al.* (96)	2009	Prospective phase II study	21 NSCLC with BM	Concurrent WBRT (40 Gy/20 f) + gefitinib 250 mg once a day	CNS response: CR 19% (4/21 pts), PR 62% (13/21 pts), SD 14.3% (3/21 pts), PD 4.7% (1/21 pts), ORR 81%, DCR 95.2%; median PFS: 10.0 months (7.5–12.5 months); median OS: 13.0 months (8.2–17.8 months)
Zeng *et al*. (97)	2012	Retrospective study	90 NSCLC with BM gefitinib alone group: 45 pts; concurrent WBRT + gefitinib group: 45 pts	Gefitinib alone group: 250 mg once a day; concurrent WBRT + gefitinib group: 40 Gy/20 f + 250 mg once a day	In the WBRT + gefitinib arm *vs*. gefitinib alone arm respectively: CNS ORR: 64.4% *vs*. 26.7% (P<0.001); CNS DCR: 71.1% *vs*. 42.2% (P=0.006); median CNS TTP: 10.6 *vs*. 6.57 months (P<0.001); median OS: 23.40 *vs*. 14.83 months (P=0.002)
Lee *et al.* (98)	2014	Prospective randomized phase II study	80 NSCLC with BM (1/35 evaluable pts EGFRm); concurrent WBRT + placebo group: 40 pts; concurrent WBRT + erlotinib group: 40 pts	Concurrent WBRT + placebo group: 20 Gy/5 f; concurrent WBRT + erlotinib group: 20 Gy/5 f + 100 mg once a day	Median CNS PFS: 1.6 months in both arms (P=0.84); median CNS OS: 2.9 *vs*. 3.4 months in the placebo *vs*. erlotinib arm (P=0.83)

EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors; NSCLC, non-small cell lung cancer; pts, patients; BM, brain metastases; EGFRm, epidermal growth factor receptor mutated; CNS, central nervous system; PR, partial response; SD, stable disease; PD, progressive disease; TTP, time to treatment progression; OS, overall survival; PFS, progression-free survival; WT, wild-type; ORR, objective response rate; CR, complete response; DCR, disease control rate; CHT, chemotherapy; WBRT, whole brain radiation therapy; ADK, adenocarcinoma; SRS, stereotactic radiosurgery; MR, mixed response.

for BM (84-86). An open-label, single-institution, phase II study (84) prospectively evaluated the efficacy of EGFR-TKIs, erlotinib or gefitinib, in pts with BM from NSCLC harboring EGFR mutations. Pts did not receive any prior therapy for existing BM. Twenty-three (83%) out of 28 enrolled pts showed PR, 3 (11%) had SD with a disease control rate (DCR) of 93%. Median PFS and OS were 6.6 months (95% CI, 3.8–9.3 months) and 15.9 months (95% CI, 7.2–24.6 months), respectively. There were no differences in PFS and OS between the different TKIs. After progression, 14 pts (50%) received local therapy, either WBRT or SRS, with a local therapy-free interval of 12.6 months (95% CI, 7.6–17.6 months). An Asian phase II, open-label study (85) evaluated the efficacy and safety of erlotinib in NSCLC with BM after first line platinum-based chemotherapy. Forty-eight NSCLC pts with adenocarcinoma histology or activating EGFR mutation and asymptomatic BM, without extra-cranial progression after first-line therapy, were enrolled. The ORR, both intra and extra-cranial, was 58.3%. The median PFS was 10.1 months (95% CI, 7.1–12.3 months) for intracranial progression and 9.7 months (95% CI, 2.5–17.8 months) for both intracranial and systemic progression. Median PFS was significantly longer in pts with EGFR mutated disease than in those with EGFR wild-type disease, 15.2 months (95% CI, 8.3–22.2 months) *vs.* 4.4 months (95% CI, 0.0–11.6 months; P=0.02), respectively. Most common adverse events were predominantly of grade 1/2. In this trial erlotinib given alone was active and well tolerated also as second line treatment in NSCLC pts with BM. The BM responses to gefitinib, even without irradiation, were reported in a third phase II trial (86) in which 41 pts with BM from EGFR mutated lung adenocarcinoma were enrolled. The ORR was 87.8%, median PFS and OS were 14.5 months (95% CI, 10.2–18.3 months) and 21.9 months (95% CI, 18.5–30.3 months), respectively. Exon 19 deletion was associated with better outcome in both PFS (P=0.003) and OS (P=0.025) compared with L858R. No pts experienced grade ≥4 toxicity.

Several retrospective analyses confirmed the efficacy of TKIs used alone in BM, in particular in EGFR mutated NSCLC (87-89). Gefitinib was evaluated in a Japanese monocentric retrospective study (87) of 57 pts with advanced NSCLC unselected for EGFR mutational status. Fourteen pts had BM. Six of them experienced objective responses to brain lesions [one complete response (CR) and five PR] and eight had SD. Objective responses in extracranial disease were reported in 7 of 14 pts with BM and, interestingly, intracranial objective responses were documented in 6 (86%) of these pts. Porta *et al.* (88) retrospectively evaluated erlotinib therapy in 69 pts with BM from NSCLC, 17 of whom harboring EGFR activating mutations. Overall ORR in mutated pts was 82.4%, while no responses were observed in unselected ones. The median time to treatment progression (TTP) for intracranial disease in mutated group was 11.7 months (95% CI, 7.9–15.5 months) compared with 5.8 months (95% CI, 5.2–6.4 months) in WT or unknown EGFR pts (P<0.05). The OS was 12.9 *vs.* 3.1 months in the two groups, respectively (P<0.001). Erlotinib was equally tolerated. Finally, in another retrospective analysis (89), 23 Korean never-smoking pts with lung adenocarcinoma and synchronous asymptomatic BM, treated with either gefitinib or erlotinib as first-line, were considered. They had received no prior treatment, nor chemotherapy nor any kind of radiotherapy. Out of 23 pts, 16 achieved PR, 3 SD and only 4 pts experienced PD, resulting in an ORR of 69.6% and a DCR of 82.6%. Seventeen pts (73.9%) showed intracranial tumor response. The median PFS and OS were 7.1 (95% CI, 1.08–12.87 months) and 18.8 months (95% CI, 0.64–27.0 months), respectively. According with these results clinical benefit from EGFR-TKIs seems to be mainly associated with the presence in the *EGFR* gene of activating mutations or with those clinical features (sex, ethnicity, smoking status) strongly related to this genotype.

Promising results were also reported in other prospective trials (90,91). In the study by Ceresoli *et al.* (90) gefitinib was prospectively evaluated in 41 NSCLC pts with BM, of which 37 had already received chemotherapy while 18 had been previously treated with WBRT. Gefitinib proved active in both WBRT-treated and WBRT-naive pts. Four pts (10%) reported PR with an overall DCR of 27%. The median duration of response was 13.5 months. In another prospective study (91) in 40 unselected pts, all previously treated with chemotherapy, gefitinib showed an ORR of 32%, a median PFS of 9.0 months (95% CI, 4.5–13.5 months) and an OS of 15.0 months (95% CI, 11.1–18.8 months).

Recently Soon *et al.* (107), in a systematic review and meta-analysis of 12 prospective and retrospective studies, compared the effects of brain radiotherapy *vs.* TKIs alone on intracranial disease, in EGFR mutated NSCLC with BM. In contrast with previous data, this meta-analysis showed an advantage in the 2-year OS for the upfront cranial radiotherapy, either WBRT or SRS, compared with TKIs alone (WBRT: 60%, SRS: 93%, TKIs alone: 45%). Nevertheless radiotherapy did not improve disease response

and no significant differences in ORR were documented. In general, cranial irradiation caused a rate of neurological adverse events higher than that reported in studies with TKIs alone (84-86), but lower than that of the concurrent upfront WBRT/TKIs studies (92,93). By limiting the analysis to prospective studies, there was no significant difference in intracranial disease control and survival outcomes between concurrent upfront WBRT plus TKIs and TKIs alone. Thus, considering the high intracranial ORR, consistent with results from other reviews (99-101), TKIs alone may be used upfront before WBRT in those pts with EGFR mutated NSCLC and asymptomatic BM. With a similar strategy the side effects of WBRT may be potentially avoided as long as intracranial disease is well controlled by TKIs alone.

Finally, a pooled analysis of published data (108), including 464 pts from 16 different prospective and retrospective trials, was performed. The primary endpoint was to evaluate the effectiveness of EGFR-TKIs in NSCLC pts with BM, particularly in EGFR mutated ones. Out of 464 enrolled pts, 102 had activating EGFR mutations, while in 362 pts the EGFR mutational status was unknown (unselected group). In this analysis EGFR-TKIs yielded significant results, with an intracranial ORR of 51.8%, a DCR of 75.7%, a median PFS of 7.4 months (95% CI, 4.9–9.9 months) and an OS of 11.9 months (95% CI, 7.7–16.2 months). Better results were reported in the mutated group compared to the unselected one: higher ORR (85.0% *vs.* 45.1%), a trend of greater benefit in DCR (94.6% *vs.* 71.3%), longer PFS (12.3 *vs.* 5.9 months) and OS (16.2 *vs.* 10.3 months). In 12 of the 16 pooled studies EGFR-TKIs were administered alone, while in four studies they were used in combination with WBRT. Subgroup analysis indicated a greater advantage with WBRT and EGFR-TKIs concurrent administration in unselected pts, with a ORR of 66.2% *vs.* 45.2% and a DCR of 94.4% *vs.* 73.1%, respectively.

These studies globally showed EGFR-TKIs promising antitumor activity against both intra and extra-cranial disease in pts with NSCLC, supporting their use as treatment of choice also in pts with CNS asymptomatic metastases. In general, the selection of NSCLC pts based on EGFR mutational status or, as surrogate, demographic features, resulted in greater benefit than in unselected pts. So EGFR-TKIs therapy may be the first treatment option for NSCLC metastatic to the brain in pts harboring activating EGFR mutations. Surely, further studies are warranted.

Second generation EGFR-TKIs

Afatinib is an oral irreversible second-generation EGFR-TKI that acts as a pan-HER inhibitor blocking all members of ErbB family. Analogously to first generation TKIs erlotinib and gefitinib, also afatinib today is approved for the treatment of EGFR mutated TKIs-naive NSCLC pts (109,110). It showed preclinical activity in models with EGFR mutations that confer resistance to EGFR-TKIs (111). Its higher binding affinity and broader target could enhance therapeutic efficacy and delay the development of resistance mutations in EGFR-mutated pts (112). Despite the effectiveness in NSCLC with BM, there are evidences that pts treated with first generation EGFR-TKIs over a period of many months may have an increased risk of developing BM (113). In fact the concentration of TKIs in the CSF seems sufficient to inhibit treatment naive but non-TKIs-resistant cells. Moreover the lower drug concentration could select for resistant clones over time (112,113).

Due to its potency at relatively low concentration, afatinib can be effective in the CSF also in the case of resistance to other TKIs. In preclinical studies, afatinib demonstrated high potency and *in vitro*, the median inhibitory concentration of afatinib was lower than other EGFR-TKIs (109,110). This suggests that afatinib has the potential to treat BM effectively, despite incomplete BBB penetration. Just before clinical approval, Li *et al.* (114), reported three cases of EGFR mutated NSCLC with BM in which afatinib, with or without combination with local treatment (WBRT or surgery), showed efficacy as first line therapy. In the LUX-Lung 1 study (115) pts already treated with platinum-based chemotherapy and first generation TKIs were randomized to receive afatinib or placebo. Although no benefit in terms of OS was recorded, afatinib achieved a prolonged PFS in comparison with best supportive care (median PFS 3.3 *vs.* 1.1 months). In two large randomized trials, LUX-Lung 3 (116) and LUX-Lung 6 (117), afatinib was compared to standard chemotherapy as first line therapy in EGFR mutated NSCLC pts showing a statistically significant advantage in PFS (median PFS 13.6 months). The enrollment of pts with stable BM was allowed in all LUX-Lung studies. In May 2010 the afatinib compassionate use program started with the aim to provide drug access after progression with erlotinib or gefitinib. Recently an efficacy analysis, in pts with BM who were treated with afatinib after chemotherapy and an EGFR-TKI within the compassionate use program, has been published (94). In particular 42% of pts reported

PR, 39% SD and only 19% PD. Brain responses were documented in 35% of pts. The safety profile of afatinib reflected that of previous experiences. The most important adverse events were diarrhea, dermatological toxicity, nausea, vomiting, and fatigue. The OS was 9.8 months and TTF did not differ in pts with or without BM. Over 70% of pts with BM had either PR or SD and 76% of pts did not develop new metastases. Considering that in the compassionate use pts received afatinib as third line treatment or greater, these data are very outstanding, especially for pts with BM and for pts who developed resistance to reversible EGFR-TKIs. The observed BM responses provide clinical evidence that afatinib concentration in CSF is sufficient to inhibit tumor growth. The subgroup analysis of LUX-Lung 3 trial (116) have further confirmed the effectiveness of first-line afatinib in CNS metastatic setting, with a median PFS of 11.1 *vs.* 5.4 months in pts who received afatinib or chemotherapy, respectively [hazard ratio (HR), 0.52; P=0.13].

Radiotherapy and EGFR-TKIs

Different data about the association of TKIs and WBRT exist (*Table 1*). In a preclinical study a synergistic effect of the combination EGFR-TKIs/radiation therapy has been documented (118). This possible synergism may derive from the radio-sensitizing effect of TKIs and from the damage of BBB created by radiation. *In vitro* radiation caused increased expression of EGFR and the EGFR blockade, both from gefitinib and erlotinib, enhanced sensitization to radiation in different human carcinoma cell lines and tumor xenografts (118,119). Several trials showed that brain irradiation can cause the opening of BBB, playing an important role in increasing TKIs concentrations in CSF (120-122).

A phase I trial, in which NSCLC pts with BM were enrolled, evaluated the toxicity of WBRT with concurrent and maintenance erlotinib showing that erlotinib was well tolerated and the combination did not cause any significant increase in treatment related toxicity (95). Moreover different phase II studies evaluated the efficacy and toxicity of the concurrent approach (93,96). The phase II trial by Ma *et al.* (96) studied the concomitant treatment with WBRT and gefitinib in 21 Chinese pts with BM from NSCLC to assess its impact on pts QoL and post-treatment survival. All pts received 40 Gy WBRT in 20 fractions. Gefitinib was administered during the radiation course and was continued until progression or unacceptable toxicity. Four (19%) pts had CR, 13 pts showed (62%) PR, 3 pts had SD and only 1 pt showed PD. The ORR was 81%. Median PFS and OS were 10.0 months (95% CI, 7.5–12.5 months) and 13.0 months (95% CI, 8.2–17.8 months), respectively. The great majority of toxicities were grade 2 and QoL was significantly improved following treatment. Erlotinib achieved similar results in a single-arm phase II trial (93) in which 40 NSCLC pts with BM, not selected for EGFR mutations, were treated with standard dose TKIs and concurrent WBRT. The ORR was 86%, median OS was 11.8 months (95% CI, 7.4–19.1 months) and the combination resulted well tolerated with no grade 4 toxicity, limited neurotoxicity and only 3 cases of grade 3 rash (3%). EGFR status was known in 17 pts and median OS was 9.3 *vs.* 19.1 months in EGFR WT *vs.* mutated pts respectively. These data are promising and concomitant treatment was well tolerated, with important activity and improvement in QoL.

Concomitant therapy was also compared both to EGFR-TKI alone and WBRT alone (90,92,97,98,123). Ceresoli *et al.* (90), in a previously mentioned study, evaluated 41 NSCLC pts with BM. Eighteen pts received gefitinib after previous WBRT, 23 pts were radio-naive and 37 pts received previous chemotherapy. Four PR (10%) were observed, SD was reported in seven cases and nearly 30% of pts achieved DCR, showing an interesting activity of gefitinib both in previously irradiated and non-irradiated pts. The median PFS of the whole population was 3 months (95% CI, 0.0–14 months). Neurological improvement was also observed in four of nine symptomatic pts. Combination treatment showed a significant prognostic advantage at the univariate analysis (P=0.0006) obtaining disease control in 10/18 pts (56%) compared to 2/23 (9%) in radio naive pts. These data were confirmed by a retrospective analysis (97) that compared the efficacy of gefitinib alone with gefitinib plus concomitant WBRT. Ninety pts were divided in two groups: the gefitinib group and the gefitinib-WBRT group. The combination group showed higher ORR (64.4% *vs.* 26.7%, P<0.001) and higher DCR (71.1% *vs.* 42.2%, P=0.006) with nearly doubled median PFS and OS (10.6 *vs.* 6.57 months, P<0.001 and 23.40 *vs.* 14.83 months, P=0.02, respectively). In a recent randomized phase II trial (98) concurrent WBRT and erlotinib compared to WBRT alone failed to demonstrate any advantage in intracranial disease control. The 80 enrolled NSCLC pts metastatic to the brain were predominantly EGFR WT (only 1/35 evaluable pts was mutated). Median PFS was 1.6 months in both arms and median OS was 2.9 and 3.4 months in the placebo compared with erlotinib arm respectively (HR, 0.95; 95%

CI, 0.58–1.55; P=0.83). The Radiation Therapy Oncology Group (RTOG) designed a phase III study (123) to test if erlotinib and temozolomide in association to WBRT and SRS could improve OS in NSCLC pts with one to three BM and unknown EGFR mutational status. Unfortunately the combination showed higher percentage of grade 3–5 toxicities without any statistically significant efficacy result and the study was closed early for poor accrual. Finally in another previously cited study (92), 54 NSCLC pts with multiple BM, receiving WBRT with or without concurrent erlotinib, reported an advantage with additional erlotinib regardless of EGFR-mutational status. The ORR was 54.84% *vs.* 95.65% (P=0.001), with a median brain PFS of 6.8 *vs.* 10.6 months (P=0.003), a median general PFS of 5.2 *vs.* 6.8 months (P=0.009) and a median OS of 8.9 *vs.* 10.7 months (P=0.020) in the WBRT arm and the concurrent arm, respectively. Furthermore erlotinib resulted the most important prognostic factor for prolonged survival at the multivariate analysis. In contrast with literature data, in the combination group there were no differences in brain PFS, general PFS and OS between EGFR-mutated and EGFR WT pts. Thus the EGFR-TKIs radiosensitizing effect in this trial doesn't seem to be dependent on EGFR-mutations. Nevertheless, in the management of BM, the addiction of TKIs to WBRT as radiosensitizing agents also in WT NSCLC pts, should be confirmed by other specific studies.

To date no prospective study exists that has really compared the use of cranial irradiation alone *vs.* TKIs alone *vs.* combination of the two modalities.

Third generation EGFR-TKIs

Although NSCLC pts harboring EGFR sensitizing mutations derive significant clinical advantage from EGFR-TKIs therapy, invariably, after about 9–13 months from the beginning of treatment, disease progression occurs. Several mechanisms of acquired resistance exist: the onset of secondary mutations in EGFR (50–60%), the activation of alternative pathways (1–25%) and the histologic transformation (5–10%). In the remaining 20–30% of cases resistance mechanisms are not known yet (124,125). Surely, the development of EGFR T790M mutation is the most common cause of acquired resistance. The substitution of methionine with threonine at position 790 in the exon 20 blocks the binding of first generation EGFR-TKIs to the ATP pocket and increases its affinity to ATP rather than to EGFR-TKIs (126,127). Third generation EGFR-TKIs (osimertinib, rociletinib, HM61713 and others) have been developed as T790M mutant-specific inhibitors. First data support their effectiveness and safety also in NSCLC pts with BM.

AZD9291 (osimertinib), a novel TKI that specifically and irreversibly binds the cysteine-797 residue in the ATP binding site of EGFR, has recently obtained the accelerated Food and Drugs Administration (FDA) approval in EGFR mutated NSCLC with documented T790M resistance mutation, on the basis of important results of phase I and II trials (128-130). Its activity has been also evaluated in pts with BM from NSCLC. A combined analysis of AURA and AURA 2 (131) studies reported that 39% of enrolled pts (162 of 411 pts) had BM. The systemic ORR of overall population was 61%, and it became 56% and 64% in pts with or without BM respectively. Cases of shrinkage of brain lesions were reported. Currently the Real World Treatment Study of AZD9291 for Advanced/Metastatic EGFR T790M Mutation NSCLC (ASTRIS) is ongoing, to assess the efficacy and safety of single agent AZD9291 in a real world setting in EGFR T790M mutation-positive NSCLC, who have received prior EGFR-TKIs therapy. Also pts with stable BM can be enrolled.

CO1886 (rociletinib) is another irreversible third generation mutant selective EGFR-TKI, specifically directed against common sensitizing EGFR mutations and T790M (132,133). Also rociletinib showed to be effective in BM from NSCLC. Out of 401 pts who received rociletinib within clinical trials 42% (170 of 401 pts) had BM. At the interim analysis pts with BM reached an ORR of 41% (134). At an indirect comparison the ORR of NSCLC pts with or without BM resulted equal to 45% and 55%, respectively (135).

AZD3759 is the first EGFR-TKI designed to penetrate BBB and to achieve high free drug exposure inside the brain, CSF and plasma, with the aim to treat BM and leptomeningeal disease in pts with EGFR mutated NSCLC. In a recent phase I, open-label, multicentre study (136), in pts with advanced stage EGFR mutated NSCLC who progressed after at least one EGFR-TKI and one line of chemotherapy, AZD3759 was well tolerated, achieved sufficient CNS concentration and showed promising antitumor activity in the dose escalation phase. Among 20 pts with measurable BM, 8 had tumor shrinkage in the brain, with 3 confirmed and 3 un-confirmed PR. The most common adverse events were skin rash and diarrhea.

Results of activity in BM of other third generation TKIs, such as ASP8273, EGF816 and HM61713 are still awaited.

Conclusions

First and second generation EGFR-TKIs represent a valid therapeutic option in NSCLC pts with BM (*Table 1*), especially in pts with activating EGFR mutations. In many studies they are able to obtain similar activity to local treatments, with a beneficial toxicity profile. Probably EGFR-TKIs effectiveness is conditioned by the heterogeneity of the EGFR mutational status between CNS metastases and extracranial disease. Thus, their combination with other treatment options, such as surgery, radiotherapy, chemotherapy, monoclonal antibodies and immunotherapy, may further improve results. The use of biopsy at the time of progression should be always evaluated. Considering the inevitable development of drug resistance, the identification of third generation EGFR-TKIs, able to overcome secondary resistance, is of major importance and is very promising especially in pts with BM. At the same time prospective studies focused on the use of TKIs with or without concurrent WBRT in pts specifically selected on the basis of the EGFR mutational status are needed.

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Footnote

Conflicts of Interest: MC Garassino declares consultancies from AstraZeneca, Roche, Boehringer. The other authors have no conflicts of interest to declare.

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Are immune checkpoint blockade monoclonal antibodies active against CNS metastases from NSCLC?—current evidence and future perspectives

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Abstract: Brain metastases occur in approximately half of patients with non-small cell lung cancer (NSCLC) and are associated with a poor prognosis and an inferior quality of life. Historically systemic therapy has had a limited role in CNS disease with a reliance placed on local treatments. The emergence of targeted therapies and immune checkpoint inhibitors (ICIs) in recent years has dramatically changed the treatment landscape of NSCLC. Programmed cell death-1 (PD-1) inhibitors have demonstrated efficacy in three randomized trials and now represent standard second line therapy after platinum failure. Trials have largely excluded patients with symptomatic or untreated CNS disease as the brain has been considered an 'immune-privileged' organ. We review the evidence and future prospects of ICIs in treating brain metastases in NSCLC.

Keywords: Brain metastases; immune checkpoint inhibitors (ICIs); non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer remains the leading cause of cancer mortality in men and women worldwide (1). Non-small cell lung cancer (NSCLC) is the most common subtype accounting for approximately 85% of all lung cancers (2). The 5-year survival in unselected NSCLC at all stages of diagnosis remains less than 20% and for stage IV disease is less than 5% (3,4). In advanced NSCLC, testing for distinct molecular genotypes has led to a personalized approach to treatment, which has improved outcomes when compared to standard platinum chemotherapy (5-13). Maintenance chemotherapy and other targeted agents have had a modest impact on survival (14-16). Immune checkpoint inhibitors (ICIs) are negative regulators of T cells and include anti cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibodies and anti-programmed cell death-1 (anti-PD-1)/programmed cell death receptor ligand-1 (PD-L1) antibodies. These drugs have demonstrated efficacy in NSCLC, melanoma and renal cell cancer, three cancer types with a predilection to brain metastases. Approximately 30–50% of patients with NSCLC can expect to develop CNS disease at some point (17,18). The number of patients with brain metastases is rising and can be explained by the more frequent use of sensitive imaging techniques such as magnetic resonance incidence (MRI) and by the improved survival seen in patients owing to new systemic treatments (19,20). Patients with molecular subtypes such as epidermal growth factor receptor positive (*EGFR*+) and anaplastic lymphoma kinase positive (*ALK*+) lung cancers may have an increased risk of CNS disease at diagnosis compared with *EGFR/ALK* wild-type (WT) NSCLC however this risk may also be explained by a potential lag in diagnosis in this patient population (21-23). The overall survival (OS) in patients with brain metastases is variable and ranges from 3 to 15 months (24). Prognostic

factors such as number of lesions, performance status and extra-cranial control are important determinants (24). In the *EGFR*+ and *ALK*+ subgroups a superior survival of 34 and 38 months respectively has been reported (25).

Historically standard treatments for brain metastases in NSCLC focused on achieving local control with mixed results. Dependent on size, number, symptoms, site and histology of lesions, patients may have been offered surgery and or whole brain radiation (WBRT). WBRT is associated with cognitive decline and inferior quality of life (26-28). While stereotactic radiosurgery (SRS) has the advantage of less cognitive impairment and shorter treatment times, the number of metastases is thought to limit SRS (28). Systemic treatment has inferior CNS disease control due to variable penetration across the blood brain barrier (BBB) (29). Platinum regimens have however demonstrated response rates between 23–50%, which approximated extra-cranial responses (30). Guidelines have suggested that chemotherapy could have a role in patients with asymptomatic disease where local therapies are not possible (31). Bevacizumab in combination with carboplatin/paclitaxel has demonstrated efficacy and early results of a phase II study of 67 patients with non-squamous histology and brain metastases, revealed a 61.2% overall response rate (ORR) in intracranial lesions and a 6-month progression-free survival (PFS) of 56.5% (32). Oral *EGFR*-tyrosine kinase inhibitors (TKIs) and *ALK* inhibitors can gain access to the CNS and response rates, especially in *ALK*+ NSCLC are promising (33-38).

ICIs in NSCLC

The evasion of immune destruction is now recognized as a hallmark of cancer (39). Immune checkpoints are crucial to this and under normal physiological conditions control immune homeostasis and prevent autoimmunity (40). Immune checkpoints belong to a large diverse family of receptors that can negatively impact the efferent immune response by impairing T cell clonal expansion, repressing function and activation and by preventing immune attack against tumor antigens (41). The PD-1/PD-L1 and CTLA-4 axes are the most common checkpoints studied with monoclonal antibodies that can inhibit ligand binding. CTLA-4 is expressed on T cells and appears to primarily inhibit the early activation of effector T cells within lymphoid organs and can enhance the immunosuppressive FOXP3+ regulatory T (Treg) cell population (42). PD-1 counterattacks the T cell response foremost at the tumor or inflammatory site and is upregulated on activated T-cells and other immune cells within the tumor microenvironment. Binding of PD-1 to its ligands (PD-L1 and PD-L2) promotes tumor immune escape by initiating a signaling cascade that inhibits T cell proliferation and limits cytotoxic function (41,43). PD-L1 can be found on a spectrum of cells including endothelial and epithelial cells together with T and B cells, mast and dendritic cells and the high expression of PD-L1 in NSCLC may correlate with inferior prognosis (44). Nivolumab and pembrolizumab are IgG4 monoclonal antibodies targeting PD-1 with early efficacy data presented in phase I studies (45,46). Three large randomized trials have recently confirmed the activity and improved survival of PD-1 inhibitors after failure of first line platinum chemotherapy in unselected NSCLC as well as those selected by tumor PD-L1 expression (47-49). Durable responses across trials are reported in approximately 20% of patients, 30% of those with PD-L1 tumor expression (45,48-50). PD-1 inhibitors now represent a standard option in NSCLC patients with metastatic disease. The efficacy of PD-L1 inhibitors post platinum doublet chemotherapy (POPLAR) and the combination of CTLA-4 inhibitors and PD-L1 inhibitors has also been established (51,52). Trials comparing ICIs to chemotherapy in the first-line setting are expected to report in 2016, with ongoing trials of combination ICI plus chemotherapy regimens versus standard first-line chemotherapy (53,54). The only biomarker known to predict response to PD-1 axis inhibitors in NSCLC is the percentage of PD-L1 positive tumor cells. In KEYNOTE-010, untreated patients who had a tumor proportion score ≥50% (membranous PD-L1 expression in at least 50% of tumor cells) demonstrated higher response rates of 50% (47). This is however far from an ideal biomarker and the lack of PD-L1 expression does not preclude a response (48,49,53,55,56). There has been a growing interest in mutation load as a predictive marker for immune checkpoint inhibition; determining this however, may be costly and impractical on a global scale (57,58). Most of the published studies of ICIs in NSCLC required local CNS control and stability prior to study entry, thus the value of ICIs in patients with brain metastases is understudied.

The immunogenicity of the CNS

Until recently the brain was considered an immune-privileged organ, a term first coined by Billingham and Boswell in the 1950s (59,60). The limited regenerative capacity of neural cells means that strict control must be

in place to prevent autoimmunity. Over the past century foreign tissues and pathogens have been shown to evade the immune system when transplanted into brain parenchyma (61-63). Anatomical barriers such as the BBB and an absent lymphatic system were thought responsible for poor CNS immunogenicity. The latter has now been refuted since the discovery of an intact CNS lymphatic system, which questions our traditional understanding of CSF flow and explains how peripheral immune responses can be generated (64,65). CNS-specific immune cells have also been shown to traverse the cribriform plate in order to reach deep cervical nodes (66). Although the BBB restricts access and flow of peripheral innate and adaptive immune cells, other interfaces such as the CSF and choroid plexus can provide mechanisms of entry (67).

The various compartments of the CNS are complex and heterogeneous in immune cell composition. Microglia are the only immune cells within brain parenchyma and are considered poor antigen presenting cells (68). However within the ventricles, leptomeninges and perivascular spaces are cells of the innate immune system, predominantly macrophages, as well as of the adaptive immune system with a relatively high density of CD4+ memory T cells (67,69). These resident cells are important for ongoing immunesurveillance. Once the CNS becomes inflamed or tumourigenesis initiates, the BBB becomes more permeable and the production of cytokines and chemokines may perpetuate immune cell infiltration (60). Despite this theory, primary CNS tumors do not appear to have a high density of tumor infiltrating lymphocytes (TILs) whereas renal cell carcinomas and melanomas have a higher TIL burden in the microenvironment in CNS metastases (70,71). Similar to systemic disease, the reasons for immune cell heterogeneity within the tumor environment have not been fully explained.

A number of studies have evaluated the prognostic impact of TILs in systemic cancers (72). Within the CNS, the association of TILs with survival has been conflicting. Harter *et al*. investigated a large cohort of patients with CNS tumors including NSCLC metastasis (n=62) and could not find a correlation between TIL burden and patient survival. This group also reported low TIL levels in lung cancer brain metastases, with highest density of TILs in RCC and melanoma (73). Similarly Berghoff reported increased TILs in RCC and melanoma brain metastases but also reported high density in NSCLC samples (n=57), and correlated survival with density of TILs and the 'immunoscore' (71). Both studies were retrospective and the latter only included patients with a single brain metastasis. The median number of lesions in the study by Harter *et al*. was also one. Lung cancer genotype was not available in either study.

An analysis of PD-L1 and TIL densities in NSCLC primary tumor and matched brain metastases revealed higher PD-L1 expression in brain metastases (52% *vs*. 32%) but denser TILs in primary tumors (74). The density of TILs in tumor may be a predictive marker for immune checkpoint inhibition. Given that the non-synonymous mutational burden may represent a predictive marker in NSCLC, the differences in mutational load in systemic disease versus brain metastases may be a contributing factor in TIL differences but this theory has not been explored (57).

Immunotherapy in NSCLC CNS disease—clinical evidence

Clinical evidence to support the efficacy of ICIs in CNS disease is limited. Early data from a phase II study has been reported by Goldberg *et al*. and represents the first report of PD-1 inhibitors in untreated or progressive NSCLC brain metastases (75). This single institution study enrolled 18 patients with melanoma and 18 patients with NSCLC including one *EGFR*+ and one *ALK*+ lung cancer patient. Patients were required to have asymptomatic intracranial disease with at least one brain metastasis measuring between 5 and 20 mm that was untreated. Primary NSCLC tumors had to have at least 1% PD-L1 staining. In the lung group, 10/18 patients had received previous local therapy for brain metastases but evidence of progressive disease. All patients received pembrolizumab 10 mg/kg every 2 weeks until disease progression. Among the patients with NSCLC, 33% of patients (n=6) had a response (four with complete response, one each with confirmed and unconfirmed partial response) with a median response duration of more than 6 months. The numbers of CNS responders in both cohorts correlated with patients achieving a systemic response. Responses in the CNS lasted from 3 to 7 months. It is unknown if responders included specific molecular subtypes. Another third (n=6) of NSCLC patients had confirmed progressive disease intra-cranially and an additional four (22%) could not be evaluated due to rapid systemic progression. The median OS in the NSCLC cohort was 7.7 months but had not been reached in the melanoma group. Neurological toxicities were predominantly grade 1–2, such as seizures, headache and dizziness, and did not result in treatment cessation. Cognitive dysfunction and stroke were less common although a melanoma patient experienced a

Table 1 Ongoing studies including untreated brain metastases in NSCLC

Group or institution trial	Phase	Study	Status
Yale University, NCT02681549	II	Pembrolizumab plus bevacizumab for treatment of brain metastases in metastatic melanoma or NSCLC	Recruiting
BMS, CheckMate 012	I	Study of nivolumab (BMS-936558) in combination with gemcitabine/cisplatin, pemetrexed/cisplatin, carboplatin/paclitaxel, bevacizumab maintenance, erlotinib, ipilimumab or as monotherapy in subjects with stage IIIB/IV NSCLC (CheckMate 012)	Ongoing but not accruing
MD Anderson, NCT02444741	I/II	MK-3475 and hypofractionated stereotactic radiation therapy in patients with NSCLC	Recruiting
Medimmune, D4190C00006	I	A phase Ib study of MEDI4736 in combination with tremelimumab in subjects with advanced NSCLC (52)	Recruiting
AstraZeneca, NCT02179671	II	Immune-modulated study of selected small molecules (gefitinib, AZD9291, or selumetinib + docetaxel) or a 1st immune-mediated therapy (IMT; tremelimumab) with a sequential switch to a 2nd IMT (MEDI4736) in patients with locally advanced or metastatic non-small-cell lung cancer	Completed

NSCLC, non-small cell lung cancer.

transient but severe episode of cognitive dysfunction.

In a phase II study (CheckMate 063) of nivolumab, lung cancer patients with squamous cell cancer who had received at least two lines of systemic treatment were treated with nivolumab. Of two patients with evaluable CNS disease, both had a response (55). Neurotoxicity was again uncommon. A further retrospective review of five patients with NSCLC and new or progressing brain metastases not requiring corticosteroids were treated with nivolumab. Two patients had an intracranial response, including one partial response and one complete response both sustained for over 24 weeks (76). A number of early phase immunotherapy trials are now including patients with untreated asymptomatic CNS disease; however as yet there are no phase III studies that allow enrolment of patients with untreated brain metastases from NSCLC (*Table 1*).

In patients with brain metastases from melanoma, the role of ICIs has been more extensively investigated. Ipilimumab, a CTLA-4 inhibitor, was evaluated in both patients with asymptomatic brain metastases and those with symptomatic disease requiring steroids. The response rates were 18% and 5% respectively (77). It should be noted that 76% of patients with asymptomatic disease had progressive brain metastases at 12 weeks, likely requiring local interventions (78). A retrospective study of ipilimumab reported similar responses (79).

Updated analysis from a phase II study of ipilimumab and fotemustine in metastatic melanoma (NIBIT-M1) has confirmed that 7 of 20 patients enrolled with brain metastases were alive over 2 years from study entry (80). The NIBIT 3 phase III study includes a cohort of patients with untreated asymptomatic brain metastases (81).

Nivolumab has also demonstrated activity in hypermutated glioblastoma and may have a role in primary neurodegenerative disorders such as Alzheimer's disease which reinforces the potential application of ICIs in select populations with intracranial pathology (58,82).

While limited data suggest that intracranial response rates to ICIs are similar to response rates with platinum doublet therapy, ICI therapy has the distinct advantage of producing durable responses in select patients. As yet there is no definitive biomarker to enrich this population. The role of ICIs in *EGFR*+ and *ALK*+ NSCLC has been controversial, with subgroup analyses of phase III trials suggesting no significant survival advantage over second-line chemotherapy (47,48). Gettinger *et al*. on the other hand did report responses in *EGFR*+ patients and a recent study has shown that *EGFR/ALK*+ lung cancer may upregulate PD-L1 expression through activation of PI3K-AKT and MEK-ERK signaling pathways (53,83). In these molecular subgroups where the incidence of brain metastases is high, further clarification of response to ICIs will be important. When brain metastases develop, the cost of patient care rises significantly (84). It is unlikely that use of ICIs without better patient selection will be cost effective in treating an overall poor prognostic cohort of patients.

Future prospects

A number of studies are now investigating the role of ICIs in patients with untreated brain metastases and it is likely that this will expand following the recent report of Goldberg and colleagues. For example, CheckMate 012, a phase I study of combination nivolumab and ipilimumab in NSCLC, includes an arm of patients with asymptomatic brain metastases (*Table 1*). The role of combination radiation and immunotherapy is a rapidly evolving field. Specifically in the brain metastases population, combinations of ipilimumab/SRS and nivolumab/SRS have demonstrated safety and feasibility in retrospective analyses of melanoma patients (85-87). Kniesley reported a series of melanoma patients with brain metastases and found an improvement in median survival of 21.3 *vs.* 4.9 months when ipilimumab was added to SRS. Radiation necrosis is however, thought to occur with a higher frequency when immunotherapy is used (88). Also the potential for an abscopal effect in malignancy is a subject of great interest, with case reports in NSCLC (89,90). Radiation is thought to repair aberrant vasculature and attract tumor specific T cells into the tumor microenvironment therefore enhancing the immune response (91). Recently it has been shown in mouse models that there is a persistent influx of bone marrow-derived immune cells into the CNS after radiation, suggesting that the physiologic effects of radiation may unleash restraints on the regulation of immune homeostasis (92). The diagnosis of pseudoprogression can be a challenge and case reports of surgical resections have revealed necrotic tissue with inflammatory cells and only scattered tumor cells (93,94).

Given that patients with small asymptomatic brain lesions seem to respond best to ICIs, and that brain metastases have a lower TIL infiltrate compared to primary lung tumors, immunotherapy in the adjuvant setting may be more efficacious in delaying time to development of CNS disease. The adjuvant studies of immunotherapy versus placebo post resection or radical chemoradiation in stage III disease (NCT02273375, NCT02595944, NCT02125461) will help address this question.

Conclusions

A select group of patients with brain metastases from NSCLC may have durable responses to immune checkpoint blockade. More data are needed for better patient selection but this cohort is likely to reflect extra-cranial responders. Combination treatments including radiotherapy may enhance outcomes. In a historically poor prognostic patient population, ICIs offer a promising systemic approach to intracranial disease without major toxicity.

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Footnote

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Non-small cell lung cancer (NSCLC) and central nervous system (CNS) metastases: role of tyrosine kinase inhibitors (TKIs) and evidence in favor or against their use with concurrent cranial radiotherapy

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Abstract: Central nervous system (CNS) metastases, including brain metastases (BM) and leptomeningeal metastases (LM) represent a frequent complication of non-small cell lung cancer (NSCLC). Patients with BM comprise a heterogeneous group, with a median survival that ranges from 3 to 14 months. However, in the majority of patients, the occurrence of CNS metastases is usually accompanied by severe morbidity and substantial deterioration in quality of life. Local therapies, such as whole brain radiotherapy (WBRT), stereotactic radiosurgery (SRS) or surgical resection, either alone or as part of a multimodality treatment are available treatment strategies for BM and the choice of therapy varies depending on patient group and prognosis. Meanwhile, introduction of tyrosine kinase inhibitors (TKIs) in clinical practice has led to individualization of therapy based upon the presence of the exact abnormality, resulting in a major therapeutic improvement in patients with NSCLC who harbor epidermal growth factor receptor (EGFR) activating mutations or anaplastic lymphoma kinase (ALK) gene rearrangements, respectively. Based on their clinical activity in systemic disease, such molecular agents could offer the promise of improved BM control without substantial toxicity; however, their role in combination with radiotherapy is controversial. In this review, we discuss the controversy regarding the use of TKIs in combination with radiotherapy and illustrate future perspectives in the treatment of BM in NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); central nervous system metastases (CNS metastases); tyrosine kinase inhibitors (TKIs); concurrent radiotherapy

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Introduction

Non-small cell lung cancer (NSCLC), which accounts for 84% of lung cancer cases in the US, is one of the major causes of cancer-related deaths worldwide (1). Central nervous system (CNS) metastases, including brain metastases (BM) and leptomeningeal metastases (LM) represent a frequent complication; it has been postulated that approximately 40% and 5% of NSCLC patients will develop BM and LM respectively during the course of the disease (2). Patients with BM comprise a heterogeneous group, with a median survival that ranges from 3 to 14 months (3). However, in the majority of patients, the occurrence of CNS metastases is usually accompanied by severe morbidity and decrease in quality of life.

Through the years, advances in evaluation of BM, such as the development of the Diagnosis-Specific Graded Prognostic Assessment (GPA) score enabled quantification of prognosis and assessment of patient survival (4). Local therapies, such as whole brain radiotherapy (WBRT), stereotactic radiosurgery (SRS) or surgical resection, either alone or as part of multimodality treatment are available treatment strategies for BM and the choice of therapy varies depending on patient group and prognosis. On the other hand, the role of systemic therapy in the treatment of patients with BM is less well-defined. Recent studies assessing the efficacy of chemotherapeutic agents, such as temozolomide, in combination with radiotherapy in patients with NSCLC and BM have failed to demonstrate any benefit compared to radiotherapy alone, possibly as a result of low blood brain barrier (BBB) penetration (5,6). However, several prospective trials in NSCLC patients with asymptomatic BM haves shown substantial activity of first line chemotherapy for BM, with intracranial response rates (RR) comparable to systemic RR, warranting further research on the role of chemotherapy in CNS disease from NSCLC (7-12).

Most recently, an improved understanding of the molecular pathways that drive malignancy in NSCLC triggered the development of agents that act against specific molecular targets in cancer cells, such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK). Introduction of tyrosine kinase inhibitors (TKIs) in clinical practice has led to individualization of therapy based upon the presence of the exact abnormality, resulting in a major therapeutic improvement in patients with NSCLC who harbor EGFR or ALK activating mutations. Based on their clinical activity in systemic disease, such molecular agents could offer the promise of improved BM control without substantial toxicity; however, their role in combination with radiotherapy is controversial.

In this review, we will discuss the controversy regarding the use of TKIs in combination with radiotherapy and illustrate future perspectives in the treatment of BM in NSCLC.

CNS metastases in NSCLC: current clinical practice

CNS metastases are present at initial diagnosis in approximately 10–20% of patients with NSCLC. Furthermore, it has been estimated that they develop as site of first recurrence following successful locoregional treatment for non-metastasized locally advanced NSCLC in approximately 18% of NSCLC patients (13). Traditionally, systemic therapies have a limited role in the treatment of CNS metastases, due to presence of a BBB that prevents systemic drugs from reaching brain parenchyma. The BBB is formed by brain endothelial cells connected by tight junctions with high electrical resistivity and acts as a selective barrier between the systemic circulation and cerebrospinal fluid (CSF) (14). BBB is surrounded by a basement membrane covered by podocytes and astrocytes. It permits the passage of lipid-soluble molecules by passive diffusion, in addition to molecules essential for neural function. Selective chemotherapeutic drugs that are able to achieve good BBB penetration are those that are not substrates of efflux transporters, such as P-glycoprotein, which is high expressed by the BBB and carries the majority of drugs outside the intracranial region (15). Nevertheless, the integrity of BBB is usually disrupted following the occurrence of BM at later stages, albeit permeability is inhomogeneous (16). More specifically, when BM reach a size more than 5 mm, the BBB is disrupted, as demonstrated by enhancement upon intravenous contrast medium injection during imaging techniques (12). In addition, WBRT commonly disrupts the BBB. The disruption of the BBB might explain the activity of first line chemotherapy in NSCLC BM (12). However, BM is frequently the site of relapse after curative treatment in NSCLC; this indicates that chemotherapeutic drugs might not sufficiently cross the BBB.

Initial therapy for symptomatic BM includes the administration of corticosteroids to reduce peri-tumoral edema and anticonvulsant therapies in case of seizures (17). Subsequently, treatment depends on the location, number of BM and prognosis. Patients with a single brain metastasis who are good surgical candidates should be offered surgical resection or SRS, as several studies have shown a survival advantage with the addition of surgery or SRS to WBRT compared to WBRT alone (18-20). Patients with 1–4 cerebral metastases should be treated with SRS with or without WBRT. The combination of SRS and WBRT has been shown to improve intracranial control but not overall survival (OS) in patients with oligo metastatic or oligo progressive disease (21,22). On the other hand, the vast majority of patients are not eligible for invasive strategies due to multiple metastases or poor performance status. WBRT represents the only therapeutic option for these patients; it results in improvement of neurological deficits in approximately 30% of patients (23). However,

in the recent randomized QUARTZ trial, that assessed the efficacy of WBRT compared to best supportive care in patients with BM and NSCLC, no clear survival advantage or improvement in quality of life was shown for patients that were treated with WBRT (24).

There is currently no standard of care for the treatment of LM; this is mainly due to the fact that LM occurs relatively rarely. Consequently, there is a lack of randomized studies; available therapeutic options, such as intravenous or intrathecal chemotherapy and radiation of the brain or affected neuro-axis are somewhat based on the treatment of patients with LM and hematological malignancies. In either case, patients with LM carry a dismal prognosis that ranges from 4 to 22 weeks (25,26).

TKIs and NSCLC-associated BM

EGFR TKIs

EGFR TKIs, such as erlotinib, gefitinib and afatinib are the standard therapy for advanced NSCLC patients with EGFR-activating mutations, having shown superiority in progression free survival (PFS) compared to chemotherapy as first line treatment (27-29). There is relative controversy regarding the change of EGFR mutational status during the metastatic process; several studies suggest a poor correlation (30,31), while others have shown consistency between EGFR mutations found in the primary tumor and corresponding BM (32). At present, there is some retrospective evidence supporting a higher incidence of BM in EGFR mutant tumors (33); however, it is unclear whether there is a difference at initial diagnosis. Most importantly, EGFR mutant tumors are more likely to develop BM during the course of the disease mainly due to longer life expectancy. On the other hand, it has been postulated that approximately 14–17% of patients with EGFR mutant NSCLC present with isolated CNS progression after front line treatment with EGFR TKIs (34-37). However, others have demonstrated a lower incidence of BM in the same population (38). In a retrospective report by Heon *et al*., patients with EGFR mutant NSCLC treated with front line erlotinib and gefitinib had a lower rate of CNS progression compared with patients treated with chemotherapy [21% *vs*. 32% at 1 year, HR =0.56; 95% confidence interval (CI), 0.34–0.94] (39).

In contrast to cytotoxic agents, EGFR TKIs have been shown to cross the BBB. This might be attributed to their low molecular weight; however, concentration in the CSF is generally much lower than in blood circulation, which partially hampers their ability to reach the brain parenchyma (40,41). Interestingly, higher concentrations are achieved with erlotinib than gefitinib, suggesting an increased efficacy of erlotinib in treating BM (40).

Several case reports have postulated complete and continuous responses following treatment of BM with gefitinib or erlotinib (42-44). Furthermore, gefitinib has clinical activity as monotherapy in unselected patients with NSCLC and BM after failure of standard therapy (45). In patients with EGFR mutant tumors, retrospective data suggest an overall intracranial response of 89% for gefitinib and 82% for erlotinib (2,14,46). Interestingly, erlotinib has been investigated as monotherapy in the management of BM. Gerber *et al*. retrospectively analyzed data from 222 patients with EGFR mutant tumors and newly diagnosed BM who were treated with either erlotinib, WBRT or SRS. WBRT was associated with better intracranial control, albeit similar OS compared to erlotinib. In this study, the authors underlined the importance of WBRT in achieving local control of BM (47). In another phase II trial, erlotinib was evaluated as second line therapy in NSCLC patients with asymptomatic BM and no extracranial progressive disease following first line platinum-based chemotherapy treatment. The median intracranial PFS was 15.2 months for patients with EGFR positive tumors, albeit only 4.4 months for EGFR unselected patients. It is important to note that a series of phase I/II studies using high dose erlotinib for the treatment of LM in patients with NSCLC has shown both efficacy and tolerability (48,49). On the other hand, second generation TKI afatinib has also shown clinical activity against BM. In a study by Hoffknecht *et al*., afatinib demonstrated a disease control rate (DCR) of 66% in NSCLC patients with BM pretreated with chemotherapy and first generation TKIs (50).

Finally, third generation irreversible EGFR TKI osimertinib, which has been proven effective against EGFR-mutant tumors with acquired T790M resistance, has shown substantial CNS penetration and remarkable CNS activity both at preclinical and clinical level (phase II data) (51-53). Furthermore, in the recent I BLOOM study that was presented in the 2016 ASCO annual meeting and included 21 patients with LM from NSCLC, osimertinib provided LM disease control in 76% of patients, among which 33% had radiologic improvement (54). The majority of patients were heavily pretreated.

Of note, there is a question whether there is a potential role of prophylactic cranial irradiation (PCI) in patients

with EGFR-mutant tumors that are characterized by a higher incidence of BM. In a recent report, patients with L858R mutations have been found to have a greater risk of developing BM (55). There are no randomized studies addressing this issue. A recent study has shown a potential benefit of PCI in patients with surgically resected stage IIIA-N2 NSCLC and high risk of BM after adjuvant chemotherapy (56); however, this study does not provide data on EGFR mutations.

ALK-TKIs

Rearrangement of ALK is seen in approximately 2–7% of patients with NSCLC and is a therapeutic target in advanced NSCLC. It is not clear whether patients with ALK positive tumors present more frequently with BM at initial diagnosis; however, it has been estimated that 60% of patients develop CNS metastases during treatment with first generation TKI crizotinib (57). Several reports suggest a very low CSF to plasma concentration ratio for crizotinib (58,59). In a retrospective analysis of patients with BM included in the pivotal trials PROFILE 1005 and PROFILE 1007 that let to approval of crizotinib as first and second line treatment in ALK positive NSCLC, crizotinib showed an intracranial RR of 18% in untreated BM and 33% in pretreated BM, compared to 50% overall response rate (ORR) in systematic disease (60). Furthermore, patients with no preexisting CNS disease developed BM in 20% of cases, while progressive disease in the CNS occurred in 71.1% of patients with known BM at baseline. Based on data of poor CNS activity of crizotinib, it is suggested that patients experiencing CNS progression on crizotinib should be offered local CNS therapies whereas the administration of crizotinib should be continued.

Novel ALK-TKIs such as ceritinib and alectinib have shown promising activity against BM. In a recent report, efficacy and safety of ceritinib was assessed in a subset of patients with BM in the phase I ASCEND-1 trial. Among 14 patients with BM, 7 had intracranial response, 4 of which have been previously treated with crizotinib (61). On the other hand, alectinib has been designated by the FDA as breakthrough therapy, following the high RR it demonstrated in the phase I/II trial in crizotinib-naïve ALK positive NSCLC patients (62). Alectinib has a better BBB penetration than crizotinib because it is not expelled by P-glycoprotein from the intracranial environment (57). In a phase II trial conducted in crizotinib-resistant or intolerant patients, 21 patients had BM; alectinib achieved a 52% RR (63). Furthermore, among for patients who have not received WBRT, CNS control was 100% with alectinib. This trial provides evidence that alectinib is active in BM after failure of crizotinib. However, prospective comparison across ALK-TKIs regarding CNS activity is hampered by lack of CSF pharmacokinetic measurements. The randomized phase III ALEX trial is currently assessing the efficacy of alectinib *vs.* crizotinib as front line treatment in ALK positive NSCLC; its design will allow discriminate between intracranial and extracranial failure. Finally, activity of ALK-TKIs in LM is anecdotal (63,64); results are eagerly expected from ongoing phase III trials ALEX and ASCEND-7, which include patients with LM (NCT02075840, NCT02336451).

TKIs with concurrent radiotherapy

Rationale and clinical data

The management of BM continues to pose a major challenge in oncology and current therapeutic options have modest results in achieving good or long intracranial responses. WBRT is the mainstay of treatment for patients with multiple metastases. According to NCCN guidelines, patients with poor performance status should receive a shorter course of WBRT. EGFR and ALK TKIs have demonstrated good clinical activity in systemic disease and might delay CNS progression in patients with EGFR mutant and ALK positive tumors respectively. However, in patients with driver mutations, whether EGFR-TKIs can enhance or replace cranial irradiation in the initial treatment of BM remains unclear. In a recent meta-analysis, upfront radiation therapy was shown to improve intracranial disease control and survival compared to TKI monotherapy in patients with EGFR mutant tumors (65). In this meta-analysis, a small proportion of patients received a combination of WBRT and EGFR TKI. On the other hand, there is evidence that sequential use of TKIs can delay administration of WBRT in EGFR mutant tumors (66). An intriguing question in clinical practice is whether a TKI could be safely combined with WBRT and in which patient population.

Preclinical data support the combined use of radiotherapy and EGFR inhibitors as a strategy for cancer treatment. In the clinical setting, anti-EGFR monoclonal antibody cetuximab has been suggested as a radiosensitizer, demonstrating improved OS in conjunction with radiation compared to radiation alone in patients with squamous cell carcinoma of the head and neck (67), albeit having failed to show any

benefit in combination with chemoradiation in locally advanced NSCLC (68). On the other hand, EGFR TKIs have shown to potentiate radiotherapy response in human carcinoma cell lines *in vivo* and *in vitro* (69,70). Potential mechanisms of synergism include cell cycle arrest, induction of apoptosis, inhibition of radiation-induced DNA repair mechanisms and increased EGFR expression in radioresistant clones (69-71). In addition, radiotherapy might disrupt the BBB, facilitating passage of drugs into the brain (72).

A dose-escalation phase I trial reported by Lind *et al*. evaluated the tolerability of WBRT with concurrent and maintenance erlotinib in an unselected population of patients with NSCLC and BM (73). Patients in cohort 1 received erlotinib at a dose of 100 mg/d before and during WBRT, whereas in cohort 2, erlotinib was administered at a dose of 150 mg/d before and during WBRT; patients in both subgroups received maintenance erlotinib at a dose of 150 mg/d. Out of 11 patients, no serious treatment related toxicity was observed in cohort 1; however, in cohort 2, one patient developed grade 3 rash, one had grade 3 fatigue and two patients died of interstitial lung disease attributed to erlotinib. No neurotoxicity was reported. Interestingly, only one patient experienced intracranial progression, suggesting a high intracranial disease control (73).

Following the results of the phase I study, a phase II study was conducted in patients with NSCLC and newly diagnosed BM regardless of EGFR status (74). Erlotinib was given at a dose of 150 mg/d one week before and concurrently with WBRT followed by maintenance. ORR was 86% in the whole population and median survival was 11.8 months, significantly longer than historical controls. No neurotoxicity was noted. As expected, median PFS and OS were longer in patients with EGFR mutant tumors [PFS: 12.3 *vs*. 5.2 months and OS: 19.1 *vs*. 9.3 months in EGFR wild type (WT) tumors]. This is in concordance with a recent retrospective study that showed an excellent intracranial control and a median OS of 26 months in patients with EGFR mutant tumors treated with WBRT plus EGFR-TKIs (75).

A phase III trial was subsequently performed by the Radiation Therapy Oncology Group (RTOG) evaluating the addition of temozolomide or erlotinib in combination with WBRT and SRS in patients with 1–3 BM and unselected EGFR status (76). The study closed early due to accrual limitations. Median survival was numerically longer with WBRT + SRS compared to WBRT + SRS and temozolomide, or WBRT + SRS and erlotinib (13.1 *vs*. 6.3 *vs*. 6.1 months respectively) albeit not statistically significant. This deleterious effect in survival was possibly attributed to increased grade 3 to 5 toxicity in the combination arms, which reached 49% with the addition of erlotinib (P<0.001) (76).

In a subsequent randomized, placebo controlled phase II study, patients were treated with WBRT with or without erlotinib in a population of predominantly EGFR-WT patients (77). In this study, only 37.5% of patients were alive and without neurological progression following WBRT and no advantage in neurological PFS or OS was observed with the addition of erlotinib (PFS and OS HR =0.95). This is the only study demonstrating an absence of efficacy of erlotinib in combination with WBRT in EGFR WT patients. This was confirmed in a recent meta-analysis presented in the 2015 ASCO meeting; in an unselected population of patients with BM, the addition of EGFR-TKIs to WBRT did not provide significant benefit (78).

Gefitinib has also been evaluated in combination with WBRT in phase II trials. In a phase II study conducted in a Chinese population, gefitinib was administered in combination with WBRT, followed by maintenance therapy (79). The study showed promising results; ORR was 86% and OS was 13 months. Most side effects were grade II (rash, diarrhea) and well tolerated. In another randomized phase II trial, patients with NSCLC and BM were treated with WBRT in combination with either gefitinib or temozolomide (80). Median OS was 6.3 months in the gefitinib-WBRT group compared to 4.9 months in the temozolomide-WBRT group. No significant toxicity was observed. Concomitant use of gefitinib and WBRT is further supported by a retrospective analysis that included Chinese patients with BM who were treated with gefitinib with or without WBRT (81). Patients in the combination group demonstrated a superior intracranial DCR, median time to progression of BM and median OS (71.1%, 10.6 and 23.40 months respectively in the gefitinib-WBRT *vs*. 42.2%, 6.57 and 14.83 months respectively in the gefitinib-only group). Nevertheless, these two studies both involve a Chinese population with known intrinsic sensitivity to gefitinib; it is unclear whether results can be generalized in the European population. Of note, no studies assessing the efficacy of afatinib with WBRT have been performed.

The results of those trials were assessed in two recent meta-analyses, designed to evaluate the efficacy and safety of the use of EGFR TKIs with concurrent intracranial radiotherapy in patients with NSCLC and BM. The first meta-analysis, which included 8 studies, demonstrated a superior ORR (HR =1.56, P=0.0008) and time to CNS progression (HR =0.58, P=0.03) in patients treated with

WBRT in combination with an EGFR-TKI (TKI-group) compared with patients treated with WBRT without an EGFR-TKI (non-TKI group) (82). Furthermore, no difference in severe adverse events was shown (HR =1.49, P=0.14). The second meta-analysis that included 15 studies had similar results; radiotherapy plus an EGFR TKI resulted in improved RR and DCR (RR =1.48; 95% CI, 1.12–1.96; P=0.005; and DCR =1.29; 95% CI, 1.02–1.60; P=0.035; respectively) than radiotherapy without an EGFR-TKI (83). Moreover, time to CNS progression and median OS were both prolonged (HR =0.56; 95% CI, 0.33–0.80; P=0.000 and HR =0.58; 95% CI, 0.42–0.74; P=0.000 respectively), albeit with an increased rate of any grade adverse events (RR =1.25; 95% CI, 1.01–1.57; P=0.009), especially rash and dry skin. The results of these meta-analyses should be interpreted with caution, due to heterogeneity of the included studies and different treatment modalities combined.

With regards to ALK-TKIs, there is currently no evidence in favor or against their concomitant use with radiotherapy. However, concurrent use should be applied with caution, as it is possible that concurrent radiotherapy could exacerbate ocular toxicity of crizotinib (84).

Clinical trials of radiotherapy plus TKIs in patients with NSCLC and BM are summarized in *Table 1*.

Expert opinion

The paradigm shift occurring in NSCLC is encapsulated by the management of patients harboring activating mutations. In patients with EGFR mutant or ALK positive tumors, front line treatment with EGFR or ALK inhibitors results in high systemic RRs and a lower risk of CNS progression. However, isolated or predominant CNS progression represents a major issue in patients treated with EGFR or ALK TKIs, regardless of impressive initial response. In an attempt to increase intracerebral efficacy, concurrent use of TKIs and radiotherapy is undoubtedly a tempting approach. Advantages would be the possible synergistic antitumor effect against BM, as suggested in preclinical studies, as well as prevention of disease flare, which refers to accelerated progression of disease and subsequent worsening of symptoms following TKI discontinuation (85).

At present, several clinical studies and meta-analyses have shown superior clinical activity in BM with the combination of WBRT and TKIs. However, there are many limitations that need to be addressed. First, most of the studies have been performed in an unselected population. Second, a phase III trial has demonstrated unacceptable toxicity of the combination of WBRT, SRS and erlotinib (76). Furthermore, in a recent randomized study, WBRT has been shown to impair cognitive function when added to SRS (86). Preservation of cognitive function is of major importance in these patients considering their younger age. In addition, studies evaluating the efficacy of gefitinib are mainly preformed in Asian populations, and it is unknown whether results can be globally generalized.

At this time, concurrent use of TKIs with radiotherapy is not recommended outside of a clinical trial. Interestingly, the data in EGFR mutant patients treated with erlotinib alone (47) prompt the question whether this could be a front-line approach in patients with asymptomatic BM, reserving WBRT for symptomatic cases. However, this should probably not be considered in ALK positive tumors, since patients with BM have been shown to have significantly better survival when treated with radiotherapy compared to patients with ALK WT tumors (87). These patients display prolonged survival and interventions to control intracranial disease is crucial (88). Therefore, radiotherapy should be a part of multimodality treatment somewhere in the course of their disease; it has been also suggested that the role of PCI could be reconsidered (89). In clinical practice, burden of extracranial disease and therefore concerns regarding disease flare might also guide treatment decisions; physicians might select not to discontinue a TKI during WBRT in case of extended extracranial disease.

Ongoing clinical trials are currently evaluating the effectiveness of concomitant use of radiotherapy and TKIs. Among them, ENTER is a phase III trial evaluating the addition of erlotinib to WBRT as front line treatment in patients with multiple BM from NSCLC (NCT01887795). Similarly, another study is assessing concurrent use of erlotinib and IMRT (NCT02556593), with the view to reduce neurotoxicity.

Conclusions

In conclusion, the incidence of BM from all cancers is increasing. Current research is focusing on improving management of BM based on genetic background of malignancies. In NSCLC, agents targeting EGFR and ALK have shown very promising results in systemic disease and delay of CNS progression. However, resistance to these agents commonly manifests as isolated CNS recurrence. In an attempt to improve management of BM, combining WBRT with TKIs is a promising approach. Because all these agents are relatively new, their role

Table 1 Summary of trials of radiotherapy plus TKIs in patients with NSCLC and BM

Author/year	Phase	No of pts	EGFR mutation status	Treatment groups	Control group	Outcomes
Lind *et al*., 2009	I	11	NA	Cohort 1: erlotinib 100 mg + WBRT; cohort 2: erlotinib 150 mg + WBRT	–	Grade 3–5 toxicity in cohort 2; high IDCR
Welsh *et al*., 2013	II	40	EGFR mutant: 9 of 17 pts tested	Erlotinib 150 mg + WBRT	–	ORR 86%; median OS 11.8 months; median OS 19.1 months in EGFR mutant
Sperduto *et al*., 2013	III	126 (closed early)	NA	Arm 2: TMZ + WBRT + SRS; arm 3: erlotinib 150 mg + WBRT + SRS	Arm 1: WBRT + SRS	OS not improved with addition of drugs; no difference in CNS-TTP between the three arms; 49% grade 3-5 toxicity in arm 3
Lee *et al*., 2014	II	80	EGFR mutant: 1 out of 35 tested	WBRT + erlotinib	WBRT	No difference in OS
Ma *et al*., 2009	II	21	NA	WBRT + gefitinib	–	ORR 86%; median OS 13 months; no significant grade 3 toxicity
Pesce *et al*., 2012	II	59	NA	WBRT + gefitinib *vs*. WBRT + TMZ	–	Median OS 6.3 months (gefitinib arm), 4.9 months (TMZ arm); no relevant toxicity
Zeng *et al*., 2012	Retrospective	90	NA	WBRT + gefitinib	Gefitinib	Higher ORR and OS with WBRT + gefitinib
Luo *et al*., 2015	Meta-analysis	980 (8 trials)	NA	Radiotherapy + TKI (TKI group)	Radiotherapy or radiotherapy + chemotherapy (non-TKI group)	Higher RR, CNS-TTP and OS in radiotherapy + TKI group; no difference is serious AEs
Jiang *et al*., 2016	Meta-analysis	1,552 (15 trials)	Variable among 15 studies	Radiotherapy + TKI	Radiotherapy or radiotherapy + chemotherapy	Higher RR, DCR, CNS-TTP and OS in radiotherapy + TKI group; increased rate of any grade AEs

AEs, adverse events; CNS-TTP, time to central nervous system progression; DCR, disease control; EGFR, epidermal growth factor receptor, IDCR, intracranial disease control, NA, not available; ORR, overall response rate; OS, overall survival; RR, response rate; SRS, stereotactic radio surgery; TKI, tyrosine kinase inhibitor; TMZ, temozolomide; WBRT, whole brain radio therapy.

as part of multimodality treatment is not clarified yet. Therefore, clinical trials that include patients with BM are warranted to help clarify the optimal timing of TKIs and cranial radiotherapy in NSCLC, with the view to reserve neurocognitive function and improve clinical outcomes.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Metastatic lung cancer in the age of targeted therapy: improving long-term survival

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Provenance: This is a Guest Perspective commissioned by Section Editor Guangliang Qiang, MD (Department of Thoracic Surgery, China-Japan Friendship Hospital, Beijing, China).

Comment on: Lin JJ, Cardarella S, Lydon CA, *et al*. Five-Year Survival in EGFR-Mutant Metastatic Lung Adenocarcinoma Treated with EGFR-TKIs. J Thorac Oncol 2016;11:556-65.

Abstract: *Epidermal growth factor receptor* (*EGFR*) mutations are the most frequent targetable genetic abnormality observed in non-small cell lung cancer (NSCLC). More than a decade after *EGFR* mutations were shown to predict sensitivity to EGFR-tyrosine kinase inhibitors (EGFR-TKI), retrospective cohort studies are now identifying and characterizing 5-year survivors. While these studies indicate subsets of patients achieving long-term survival, there is paucity of data pertaining to the long-term survival benefits of these targeted therapies at a population level. Improving access to molecular testing and treatment are key to maximizing the survival benefits at a population level.

Keywords: Lung cancer; epidermal growth factor receptor (EGFR); genetics; erlotinib; survival

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Lin *et al*. recently estimated and identified factors associated with the 5-year survival rate among patients with *epidermal growth factor receptor* (*EGFR*)-mutant metastatic lung adenocarcinoma treated with an EGFR-tyrosine kinase inhibitor (TKI) (1). In order to appreciate the relevance of the results, we will briefly review the evolving lung cancer treatment landscape and previous survival estimates. We will then provide our perspective on the necessary next steps to maximize the population-wide survival of this historically recalcitrant cancer.

Lung cancer, the majority of which is non-small cell lung cancer (NSCLC), is the leading cause of cancer death for both men and women in the United States (2). According to the latest data, more than half (55%) of the NSCLC diagnosed in the United States presents at an advanced stage, wherein the 5-year survival rate is only 4.9% (3). Until the early-2000s, platinum-based chemotherapy was the standard of care for patients with newly diagnosed advanced NSCLC (4). However, responses to chemotherapy were modest at best with randomized clinical trials indicating response rates between 17% and 22% and median overall survival (OS) between 7 and 8 months (4-6). Starting in the mid-2000s, identification of actionable oncogenic driver mutations and mechanisms of resistance to targeted therapeutics have become increasingly important in the management of NSCLC.

The most extensively studied gene in this context is *EGFR*, which has a high prevalence of mutations (10–28%) among NSCLC patients (7). Tumors harboring *EGFR* mutations tend to be highly sensitive to orally active EGFR-TKIs: erlotinib, gefitinib and afatanib (8-12). In patients with advanced disease, randomized clinical trials have

consistently demonstrated improved response rates (56–83%) and progression free survival (9–14 months) with EGFR-TKIs than with standard chemotherapy (9,12,13). The impact of EGFR-TKIs on long-term outcomes has been less consistent. Although several clinical trials have also shown longer OS among patients with *EGFR*-mutant tumors treated with EGFR-TKIs compared to chemotherapy alone, a significant improvement in median OS has only been reported for afatinib (31–33 *vs*. 18–21 months) (14,15). The lack of an OS advantage has been attributed largely to the crossover design of the clinical trials, indicating that these drugs may be similarly active regardless of line of treatment (12,13,16). Moreover, most of the previous studies have had limited follow-up and/or have not reported long-term survival stratified by EGFR-TKI exposure status. Thus, it has been difficult to determine the true effectiveness of these agents, particularly outside of a clinical trial setting.

With these knowledge gaps in mind, Lin *et al*. sought to estimate and identify factors associated with 5-year survival among patients treated with erlotinib or gefitinib. Briefly, 137 patients from the Dana-Farber Cancer Institute who were diagnosed with *EGFR*-mutant metastatic lung adenocarcinoma between 2002 and 2009, treated with an EGFR-TKI and had completed follow-up for at least 5 years were included in the study. The median OS for these patients was 30.9 months and 20 patients (14.6%) were 5-year survivors. In multivariate analysis, exon 19 deletions, absence of extrathoracic or brain metastasis and non-current smoking status were associated with 5-year survival.

The results from this study are promising and finally indicate that a sizable subset of metastatic NSCLC patients, who can be readily identified, are attaining the previously elusive 5-year survival mark. These results also appear to be in agreement with the reported outcomes from a much larger (n=1,657) multicenter Japanese cohort that included patients with advanced or recurrent *EGFR*-mutant NSCLC who received EGFR-TKI treatment between 2008 and 2012 (17). Briefly, Inoue *et al*. reported a median OS of 30.8 months and an estimated 5-year survival rate of just over 20%. Although there was not complete agreement on which factors were associated with survival, *EGFR* mutation type was again found to be associated with survival.

An important caveat in interpreting the results of these two studies is that the presence of the *EGFR* mutation in itself may be a favorable prognostic marker. Previous studies have shown superior outcomes for patients with *EGFR*-mutant tumors compared to patients without these mutations, irrespective of stage and treatment (18,19). Thus, restricting studies to *EGFR*-mutant positive patients who are treated with an EGFR-TKI makes it impossible to determine if the survival benefit is due to tumor characteristics and/or treatment.

Although the agreement between these two studies is encouraging, we would advise caution be taken before generalizing the 5-year survival estimates to the population level. In a random sample of over 1,300 NSCLC patients from the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) program, we found that only 16.8% patients overall and 22.6% of stage IV adenocarcinoma patients underwent *EGFR* testing (20). In striking contrast to our series which included patients diagnosed in 2010, the frequency of *EGFR* testing in the Lin *et al*. study was 71%, which again included patients diagnosed between 2002 and 2009. Further, roughly 63% of the patients with *EGFR* mutations received an EGFR-TKI in the Lin *et al*. series compared with only 48% of patients from our series. Although we did not have sufficient follow-up time to estimate 5-year survival, the estimated median OS among the *EGFR*-mutant positive lung adenocarcinoma patients who received an EGFR-TKI in our series was only 23 months. Thereby, although the survival estimates from our population level data also indicate improved outcomes among *EGFR*-mutant positive patients who receive EGFR-TKIs compare to NSCLC patients as a whole, the magnitude of the observed improvement at a population level was attenuated. Variations in observed median OS likely reflect differences in patient demographic, tumor and health characteristics and/or the quality of care received at select institutions compared to the national experience.

Ultimately, access to molecular testing and treatment are key to realizing the benefits of precision oncology—the premise that treatment choices tailored to individual patients using personalized cancer genomic data may markedly improve outcomes—at a population level. Given the profusion of potentially targetable molecular alterations and the complexities of obtaining tissue samples and that of testing, it is important to have a national strategy to facilitate widespread and uniform implementation of molecular profiling. Such nationwide efforts have been reported both from the Europe and the United States. The French Cooperative Thoracic Intergroup study involved over 3,500 clinicians and 28 certified molecular genetics centers covering the whole of France and conducted molecular analyses on tumors from over 17,000 NSCLC patients over a 1-year period (21). In the United States, the Lung Cancer Mutation Consortium analyzed samples using multiplex

genotyping from 700 patients with adenocarcinoma at 14 centers, identifying a targetable driver mutation in over 60% (22). These studies underscore the feasibility of large-scale utilization of molecular profiling in lung cancer.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Disclaimer: The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

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Afatinib in lung cancer harboring *EGFR* mutation in the LUX-Lung trials: six plus three is greater than seven?

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Provenance: This is a Guest Editorial commissioned by Section Editor Hongbing Liu, MD, PhD (Department of Respiratory Medicine, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China).

Comment on: Park K, Tan EH, O'Byrne K, *et al*. Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase IIB, open-label, randomised controlled trial. Lancet Oncol 2016;17:577-89.

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Non-small cell lung cancer (NSCLC) harboring activating mutations of the epidermal growth factor receptor (*EGFR*) gene, about 90% of which is either small deletion in exon 19 (Del19) or a leucine to an arginine substitution at codon 858 (L858R), is very sensitive to EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib (1). Phase III trials comparing these agents with platinum doublet chemotherapy showed significant prolongation of progression free survival (PFS) in favor of EGFR-TKIs (*Figure 1A*) (2-5). Nonetheless, those tumors inevitably acquire resistance about half of which are due to secondary *EGFR* mutations resulting in threonine to methionine substitution at codon 790 (T790M) (13). In these clinical trials, patients with acquired resistance to the first-line EGFR-TKI are likely to be treated by platinum doublet as a second-line treatment, while those patients treated initially by platinum doublet therapy are to be treated by EGFR-TKI that works well in this second-line setting. Owing to this "crossover" of treatment, there has been no statistically significant difference in overall survival (OS) of the patients in these trials (2-5) (*Figure 1A*).

Afatinib is one of the so-called 2nd generation (2G) EGFR-TKIs, because it can covalently bind to a cysteine at codon 797 in the presence of T790M whose affinity to 1G EGFR-TKI, i.e., gefitinib or erlotinib, in comparison with ATP is markedly diminished. Hence, IC50 value of afatinib is remarkably lower compared with 1G TKIs (14). However, wild-type *EGFR* is more sensitive to afatinib than *EGFR T790M*, resulting in lack of inhibitory effect of T790M in clinically achievable concentration of afatinib. Indeed, LUX-Lung 1 (afatinib clinical trials are designated as LUX-Lung X, and will be abbreviated as LL hereafter) study did not demonstrate prolongation of OS for patients who acquired resistance to gefitnib or erlotinib, although patients were not tested for T790M mutation but were enriched only by progressive disease after good response to the first-line EGFR-TKIs (15).

LL 3 (9) and LL 6 (10) studies are both phase III trials comparing afatinib with platinum doublet chemotherapy (cisplatin/pemetrexed in LL 3 and cisplatin/gemcitabine in LL 6). Although these studies showed that afatinib prolonged PFS significantly over platinum doublet chemotherapy, apparent difference in OS favoring afatinib did not reach statistical significance. However, when these two studies were combined (LL 3 + LL 6) and *EGFR* mutations were confined to common mutations, i.e., Del19 and L858R, OS of patients in afatinib group was significantly longer than those in chemotherapy group (11). This was the first time that there was a significant OS advantage in the trials comparing EGFR-TKI with platinum doublet chemotherapy although hazard ratio (HR) was 0.81 which was not so impressive (11). This survival advantage is not attributable to low crossover rate to EGFR-TKI in chemotherapy arm. In fact, the higher crossover rate is, the lower the HR is or the more the benefit of afatinib is. For patients in countries where EGFR-TKI is not reimbursed, crossover rate and HR were 52% and 0.84. In contrast, in countries where EGFR-TKI is reimbursed, they were 91% and 0.70 (16).

What is most intriguing in this analysis is the fact that

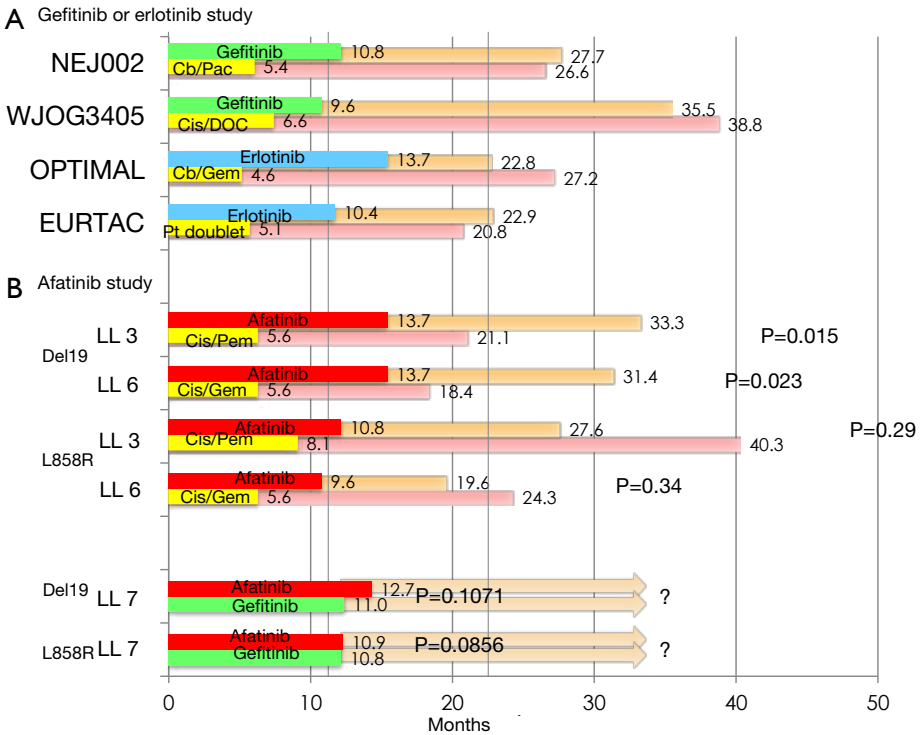

Figure 1 Progression free survival and overall survival in trials comparing chemotherapy with the first-generation EGFR-TKIs (A) (2-8) and LUX-Lung trials (B) (9-12). EGFR-TKIs, epidermal growth factor receptor-tyrosine kinase inhibitors.

survival advantage from afatinib looks different between Del19 and L858R (11). For patients with Del19, the OS difference is greater than overall population with a HR of 0.59 (11). In contrast, for those with L858R, HR is 1.25, although this difference does not reach statistical significance (*Figure 1B*) (11). In both trials, PFS of afatinib group is significantly prolonged compared with chemotherapy in both Del19 and L858R (*Figure 1B*). It is a little curious to note that the superiority of PFS for patients with L858R in afatinib group is reversed in OS, i.e., post-progression survival (PPS) in afatinib group is far shorter compared with that in chemotherapy group resulting in shorter OS. On the contrary, in Del19 patients, PPS in afatinib group is very long compared with chemotherapy group (*Figure 1B*). Although each LL 3 + LL 6 pooled two trials to increase statistical power with elimination of rare mutations, these trends are consistent in each LL 3 and LL 6 (*Figure 1B*).

There is no plausible explanation for this difference. One may be able to speculate that second-line TKI (mostly gefitinib and erlotinib, because afatinib was not commercially available at that time) in chemotherapy group worked very well and responsible for long PPS for L858R patients. There is a possibility that precedent chemotherapy might have affected the sensitivity to the second line TKI or vice versa (17), depending on *EGFR* mutational status.

Patients in the chemotherapy group in LL 3 or LL 6 trial are thought to have received very similar treatments to those in the chemotherapy group of earlier phase III trials of gefitnib or erlotinib such as WJTOG3405 or NEJ002, in which there was no significant OS difference with gefitinib or erlotinib group as mentioned earlier. Taken these together, it appears that afatinib may not be a drug of choice for patients with L858R and that either IG TKI or chemotherapy may be recommended as the first-line treatment for patients with L858R.

LL 7 trial is a randomized phase IIB study that directly compares afatinib with gefitinib for 319 patients with NSCLC harboring common mutations of the *EGFR* gene (12). PFS, the primary endpoint, is significantly longer in afatinib (HR =0.73, P=0.0165). This trend is true for both Del19 (HR =0.76, P=0.1071) and L858R (HR =0.71, P=0.0856) (*Figure 1B*). Median PFS is numerically better in patients with Del19 than those with L858R in both afatinib and

gefitinib group (12.7 *vs.* 10.9 for afatinib and 11.0 *vs.* 10.8 in gefitinib) (12). As expected, toxicity is in general greater in afatinib arm (12).

The authors say "*…our data support the use of afatinib as a treatment option in both patients with L858R and Del19 mutations*" (12). For patients Del19, LL 7 is a confirmation of superiority of afatinib over gefitinib and therefore if the patients are fit enough, afatinib is highly recommend as an initial therapy. Then, how do the LL 7 results compromise with above-mentioned seemingly detrimental OS effect in L858R patients in LL 3 + LL6 trials? Considering that LL 7 is a phase IIB trial without OS results and that LL 3 and LL 6 is phase III studies each of which enrolled more than 300 patients with OS results, until we see very dramatic difference in OS in LL 7 later this year, 1G TKI or chemotherapy still may be recommended even after LL 7 results as discussed earlier.

Last November, osimertinib, 3G EGFR-TKI that is active for T790M secondary mutation, was approved in US and its approval was followed in EU and Japan. Response rates and PFS of patients with acquired resistance due to T790M is ~60% and 10 months, respectively. We do not know exact incidence of T790M after afatinib, although a small study reported the similar incidence of ~50% (18). It is also not very clear whether incidence of T790M is different between Del19 and L858R. Out of 411 patients enrolled in AURA extension cohort and AURA 2 study which are phase II study of osimertinib for patients with T790M, 68% had Del19 while only 29% were L858R (19). Considering that baseline incidence of Del19 is only slightly higher than that of L858R, it appears that Del19 may be more likely to develop T790M. Furthermore, although number of the patients are small, osimertinib as the first-line treatment for patients with EGFR mutations looks promising with a median PFS of ~20 months (20). Therefore, we have to carefully stay tuned for what is evolving in the EGFR world and also we have to keep the enormous value of molecular analysis of patients' specimens in mind.

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Footnote

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Afatinib in the treatment of squamous non-small cell lung cancer: a new frontier or an old mistake?

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Provenance: This is a Guest Editorial commissioned by the Editorial Board Member Ying Liang (Associate Professor, Department of Medical Oncology, Sun Yat-sen University Cancer Center (SYSUCC), Guangzhou, China).

Comment on: Soria JC, Felip E, Cobo M, *et al*. Afatinib versus erlotinib as second-line treatment of patients with advanced squamous cell carcinoma of the lung (LUX-Lung 8): an open-label randomised controlled phase 3 trial. Lancet Oncol 2015;16:897-907.

Abstract: Lung squamous cell carcinoma represents approximately 20% of all non-small cell lung cancer (NSCLC) and is associated with a very poor prognosis. In the randomized phase III LUX-Lung 8 trial afatinib showed a statistical significant efficacy advantage compared to erlotinib as second-line treatment of advanced/metastatic squamous NSCLC. Despite its well-built design and the statistical significant results, in our opinion the study is still far from being clinically relevant for this subset of patients. Moreover, during the last years other drugs have shown encouraging activity with low toxicity in pretreated lung squamous cell carcinomas. In particular, nivolumab in the treatment of platinum-pretreated squamous NSCLC has recently radically changed the treatment paradigms in this histology. Sure, LUX-Lung 8 trial achieved its primary endpoint progression-free survival showing some afatinib activity in one of the most difficult-to treat and genetically complex neoplasm but we haven't found the most active drug in this subset of patients yet. The purpose of this editorial is to discuss some of the most controversial aspects of the LUX-Lung 8 trial focusing especially on its rational and design.

Keywords: Afatinib; squamous histology; non-small cell lung cancer (NSCLC); LUX-Lung 8; erlotinib

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Lung squamous cell carcinoma represents approximately 20% of all non-small cell lung cancer (NSCLC) cases (1). It is associated with a very poor prognosis, with less than 5% of patients alive after 5 years (1). In non-squamous NSCLC the discovery of driver oncogenes, such as epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) translocations, has radically changed the treatment paradigm and patients' clinical outcome (2,3). In 2004, three groups at the same time, discovered the presence of EGFR activating mutations in those patients who dramatically responded to EGFR tyrosine kinase inhibitors (TKIs). Since then, several randomized trials unequivocally established the superiority of TKIs versus chemotherapy in EGFR mutated patients (2,3). EGFR mutations are present in approximately 10–15% of NSCLCs, but they are sporadic in squamous histology. For this reason EGFR molecular testing is not routinely done in the clinical practice for this patient subgroup (4).

Afatinib is a second generation TKI that irreversibly inhibits ErbB family tyrosine kinase receptors.

At present, it is approved by the Food and Drugs Administration (FDA) for the first line treatment of advanced/metastatic EGFR mutated NSCLC (2). Some preclinical data suggest that the lung squamous cell carcinoma pathobiology has a strong dependency from the ErbB family pathway. HER2 and HER3 are overexpressed

in 20–30% of squamous cell carcinomas and they present genetic aberrations in almost 3% and 4% respectively. Furthermore several genetic alterations are present in various signaling molecules depending by the ErbB receptors (NF1 11%, KRAS 3%, HRAS 3%, RASA1 4% and BRAF 4%) (5,6). In these, the rationale relies. The LUX-Lung 8 study (7) authors postulated that afatinib, inactivating multiple ErbB dependent signaling pathways, was a promising candidate to the treatment of squamous NSCLC independently by EGFR mutational status. Nevertheless frequently in the past the evidence of a pre-clinical or phase I clinical drug activity revealed a failure in more advanced study phases. So in our opinion few early positive results, are not sufficient to jump-start a phase III trial.

The LUX-Lung 8 study (7) is a large multi-national, phase III trial, specifically designed in a population where EGFR mutations are almost absent. The study randomized 795 advanced stage squamous NSCLCs who had progressed after a platinum based chemotherapy, to receive either afatinib or erlotinib. Results are positive from the statistical point of view, reporting an advantage both in the primary endpoint progression free survival (PFS) and in the secondary endpoint overall survival (OS), less than 1 month and 1.1 months, respectively. It is also reported a modest improvement in terms of disease control rate (DCR), disease-related symptoms control and patient-reported outcome. A detailed analysis, reveals a well-built design of the study. The large sample size and the centralized analysis are both important quality guarantees. Finally, the programmed bio-molecular analysis, even if still partially published, is certainly another strength of this study. However, the comparison between the toxicity profile of afatinib and erlotinib does not seem so favorable for afatinib. If we consider grade 3–4 adverse events there is a difference ranging from 16% for erlotinib and 25% for afatinib. Looking in more depth into the results, we can also observe that the diarrhea is almost doubled in the afatinib arm (69% *vs.* 33%) and that patients having a grade 3–4 diarrhea are fourfold in the afatinib (10%) than in the erlotinib arm (2,5%). We highlight that a grade 3 diarrhea requires hydration and grade 4 is life threatening. This means that 1 out of 10 patients require at least parenteral support.

At the time LUX-Lung 8 trial (7) was conceived, in squamous lung carcinoma limited therapeutic options existed, especially for patients progressed after first-line platinum based chemotherapy. Historically docetaxel became the gold standard second line therapy (3,8) and in 2005 also erlotinib was approved by FDA for second and third line therapy in all NSCLCs independently by EGFR mutational status (9). In 2012, when the first patient was enrolled into the trials, the two therapeutic options were considered equivalent in this setting, without any significant interaction between treatment and histology (9). The available literature data from three distinct studies (9-11) and the similar route of administration were the reasons given by the investigators to justify the choice of erlotinib as comparator arm. Some comments on these topics are needed.

In the meta-analysis by Li *et al.* (10) EGFR TKIs showed better tolerability and comparable OS in second line therapy compared to chemotherapy both in unselected and EGFR wild-type NSCLC patients. But really, according to the results of the same meta-analysis, chemotherapy compared with EGFR TKIs significantly prolongs PFS in EGFR wild type patients. Moreover, even in EGFR mutated patients, EGFR TKIs reported significant differences only in PFS and not in OS. Failure in detection of differences in OS between the two groups could be justified from cross-over as well as from other confounding factors.

In the discussion of LUX-Lung 8 study, authors affirm that in the subgroup analysis of the phase III BR.21 trial (9) erlotinib improves PFS and OS in patients with squamous NSCLC with results similar to docetaxel. They also underline that in the TAILOR trial (11) there is not a statistically significant difference in terms of OS between docetaxel and erlotinib in patients with squamous histology (HR 0.9, 95% CI: 0.49–1.65). Nevertheless in the BR.21 subgroup analysis erlotinib was compared to placebo, so the relevance of OS and PFS advantage is questionable. Then, the reported equivalence with docetaxel efficacy, derives from an indirect comparison between the BR.21 and Shepherd *et al.* trial data (12). Finally, the TAILOR study (11) clearly suggests that second-line docetaxel is superior to erlotinib in all patients with EGFR wild-type NSCLC, this trend is present also in patients with squamous histology and the lack of statistical significance is probably due to the small patients sample size and to the worse performance of docetaxel in squamous NSCLC than in adenocarcinoma. Finally, no interaction was found assessing a differential effect either for docetaxel or erlotinib for histology.

As regards the same oral route of administration, certainly this could be an additional parameter in terms of results quality and comparability but, in this context, it is evident the lack of a double-blind design. We think that it would have been possible and easily achievable and it would have been

another warranty of impartial judgment and data reliability for the trial. Moreover, a double-blind design would have guaranteed a greater reliability on quality of life data.

The LUX-Lung 8 selected population and the exclusion of docetaxel as comparator arm are other hotspots. Patients with lung squamous cell carcinoma represent about one fourth of all lung cancers, so although this histology is diminishing, placing afatinib in this niche covers an important unmet need. Erlotinib is the only already approved TKI for the second line therapy in squamous cell lung cancer, but this trial could be the springboard for afatinib approval by the FDA and by European Medicines Agency (EMA) in this setting. However today, in clinical practice, TKIs are not the first therapeutic choice after failure of first line therapy in squamous cell lung cancer. Unless an oral therapy is a specific patient request or there are contraindications to chemotherapy, the oncologists commonly use second line chemotherapy in these patients. So it would have been interesting to have a third chemotherapy arm in the study, for example a docetaxel treatment group. Moreover EGFR wild type squamous cell carcinoma patients are not certainly the most helpful population to be selected for such comparison. It is just well known the significant advantage of TKIs compared to chemotherapy in patients with EGFR mutated non squamous NSCLC. Therefore, a direct comparison between the three currently used inhibitors (gefitinib, erlotinib and afatinib) would have been much more helpful in this subset. In our opinion, data deriving from the ongoing phase II LUX-Lung 7 study (13), that compares afatinib versus gefitinib in EGFR mutated advanced adenocarcinoma, will be more interesting and of greater clinical importance.

Despite all these considerations, we have to highlight the relevance of the declared study purpose: to respond to the need of effective treatments for patients with advanced lung squamous cell carcinoma. Unfortunately, although the statistical significant results, we think that LUX-Lung 8 (7) is still far from the identification of a drug able to achieve this aim. The median PFS or OS remain globally, in both treatment groups, unsatisfactory: there is an advantage of just a month or a little over a month, at the cost of significant grade 3 or greater scale world health organization (WHO) toxicities with both TKIs.

Finally, over the last two years other drugs have shown encouraging activity in pretreated lung squamous cell carcinoma. Particularly, two distinct phase III trials, REVEL (14) and CheckMate-017 (15), have led to the ramucirumab and nivolumab FDA approval in platinum-pretreated NSCLC patients, the first both in squamous and non-squamous histology. The angiogenesis is one of the hallmarks of cancer. Formation and proliferation of blood vessels are inhibited by blockade of vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) signaling. Ramucirumab, a fully human IgG1 monoclonal antibody directed against the extracellular domain of VEGFR-2, binding to the receptor, prevents the interaction with all VEGF ligands and inhibits receptor activation (16). The phase III trial REVEL (14) compared the combination of docetaxel plus ramucirumab versus docetaxel alone, in patients with squamous and non-squamous platinum-pretreated NSCLC, showing a statistically significant even if modest improvement in OS (HR 0.86, 95% CI: 0.75–0.98; P=0.02) in the combination group. This improvement was maintained both for squamous and non-squamous histology. However the addition of ramucirumab to docetaxel was associated with a significant increase in toxicity. Much more relevant is the current clinical impact of immunotherapy (17). Newly developed immune checkpoint inhibitors, targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PD1) receptor and programmed cell death 1 ligand (PD- L1) are changing current treatment paradigms in all NSCLCs, especially in squamous histology (15,17). Nivolumab, a human IgG4 anti-PD-1 monoclonal antibody, blocks PD-1 receptor on activated T cells causing an increase in the immune-mediate antitumor response. Recently, in the Check-Mate 017 study (15) nivolumab showed a significant advantage in OS compared to docetaxel in squamous NSCLC second line therapy, being able to reduce the risk of death by 41%, to extend the median OS of 3.2 months and to nearly double the survival rate at 1 year. In this study the early separation of the Kaplan-Meier curves suggests that the advantage given by nivolumab is evident from the first weeks of treatment. The benefit in OS (primary endpoint) is reinforced by the results of all the secondary efficacy endpoints (38% reduction in the risk of progression and an almost doubled response rate, with many long responses in the nivolumab group). As regard the safety profile, nivolumab showed to be significantly less toxic than docetaxel: in nivolumab group only 7% of patients had grade 3 or 4 events and no grade 5 event was recorded; in the docetaxel group, 86% patients had events of any grade, 55% had grade 3 or 4 events, and 2% had an event of grade 5. Typical immunological adverse events, including immune-mediated pneumonia, were generally rare. According with these data in March 2015,

FDA granted the fast track designation for nivolumab in the treatment of platinum-pretreated squamous NSCLC.

By an indirect comparison between Check Mate 017 (15) and LUX-Lung 8 (7), considering the poor prognosis of lung squamous carcinoma after first line therapy, OS (primary end point of Check Mate 017) rather than PFS (primary endpoint of LUX-Lung 8) is the best parameter to assess the treatment value. Moreover docetaxel seems to us a more valid comparator than erlotinib and the lower rate of grade 3 and 4 adverse events reported with nivolumab than afatinib is clinically encouraging. The preliminary data obtained with other immunological agents such as pembrolizumab and atezolizumab are moving in the same direction and other phase II and III trials, that could change the current therapeutic scenario, are ongoing (17). So is the era of targeted therapies in squamous NSCLC ended with immunotherapy? The answer is certainly no. In fact, LUX-Lung 8 (7) study provided *in vivo* the rationale that targeting EGFR in squamous cell carcinoma, although in a still unclear way, could be an useful therapeutic option. The trial achieved its efficacy endpoints showing some afatinib activity in one of the most difficult-to treat and genetically complex neoplasm. Several ErbB dependent signaling pathways are implicated in squamous NSCLC pathobiology (HER2, HER3 etc.). The afatinib role on the inactivation of these pathways and its potential cytotoxic activity are very interesting issues (5,6). Just for this reason we look forward the results of LUX-Lung 8 (7) programmed bio-molecular analysis. Furthermore, a new generation of targeted therapies are coming up, targeting FGFR1, DDR2, PI3K (5,6) and many phase II trials are quickly running.

In conclusion, in our opinion, today only those who present a specific gene alteration, can obtain significant therapeutic advantages from targeted and personalized therapies such as afatinib or other TKIs. In the majority of advanced NSCLC, including squamous cell carcinoma, there is still a long way to go for TKIs category alone. According with the available data, afatinib can not be considered a standard second line treatment in squamous NSCLC. To date, although in the absence of a direct comparison in randomized trials, nivolumab should be preferred to afatinib, in terms both of efficacy and toxicity and it should be considered the new standard second line therapy in this subset. However, the unexpected activity showed by afatinib in this setting deserves more research, not excluding proper trials in combinations with other agents in the future.

Only the identification of prognostic or predictive markers of response could help oncologists in choosing the most effective treatment (TKIs versus chemotherapy versus immunotherapy).

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Footnote

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Afatinib in first-line setting for NSCLC harbouring common *EGFR* mutations: new light after the preliminary results of LUX-Lung 7?

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Abstract: The development of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) changed dramatically the history of non-small cell lung cancer (NSCLC) harboring EGFR sensitive mutations. Several randomized prospective trials confirmed the superiority of these target agents about survival and response rate when comparing with platinum-based chemotherapy. Knowledge about EGFR mutations increased gradually during the development of target agents and different clinical trials. EGFR mutations cannot be considered all equal, but different entities should be considered in our clinical practice: exon 19 deletions, exon 21 mutation (L858R) and uncommon mutation (exon 20, exon 18 and double mutation). Nowadays, we dispose of three different EGFR TKIs (afatinib, erlotinib and gefitinib) approved for the treatment for first-line treatment of patients di NSCLC carrying EGFR, that was compared only by indirect analysis, producing data not always clear and convincing. This research highlight is an overview of data about EGFR TKIs in first-line setting, focusing on differences about exon 19 deletions and L585R mutation in patients treated with different TKIs. In addition, we report the preliminary results of the first head-to-head randomized clinical trial between two different EGFR TKIs, the LUX-Lung 7 (LL7) that compared afatinib and gefitinib showing interesting results.

Keywords: Non-small cell lung cancer (NSCLC); epidermal growth factor receptor (EGFR); afatinib; common mutations; LUX-Lung 7 (LL7)

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The history non-small cell lung cancer (NSCLC) is changing deeply in the last years. In patients with advanced or metastatic NSCLC harboring driving mutation, the survival improved significantly using target agents as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) or ALK inhibitors, prolonging survival when compared with standard chemotherapy (1,2).

In patients harboring EGFR mutations, different randomized trials confirmed the significant superiority of EGFR TKIs versus standard platinum-based chemotherapy in first-line setting about progression-free survival (PFS), quality of life (QoL) and safety profile. No randomized clinical trials evaluating erlotinib, gefitinib, or afatinib, showed a statistical improving in overall survival for patients treated with EGFR TKIs, when considered individually and based on overall population (3-11).

Although these trials seems to be very similar, exploring the same indication and end-points with different EGFR TKIs (afatinib, erlotinib, gefitinib), presents many differences about study design, patients population and statistical analysis.

The Iressa Pan-Asia Study (IPASS) trial was performed to confirm that first-line therapy with an oral EGFR TKI would be at least as effective as chemotherapy with carboplatin-paclitaxel, in a selected Asian population, with lung adenocarcinoma. On a total of 1,038 patients enrolled, 261 were positive for EGFR mutations [53.6% Del19/42.5% L858R/4.2% exon 20 (T790M)/3.8% other

mutations/4.2% multiple mutations]. In a mutation positive subgroup of patients, PFS was significantly longer among patients treated with gefitinib than among those that received chemotherapy (HR =0.48; 95% CI, 0.36–0.64; P<0.0001) (4).

After the IPASS trial, different prospective randomized clinical trial, all undertake in Asian population, showed that gefitinib and erlotinib, improved PFS and response rate, in EGFR-mutant NSCLC.

The European Tarceva vs. chemotherapy (EURTAC) trial was the first randomized phase III trial that evaluated the efficacy of erlotinib in non-Asian population of patients with NSCLC harbouring EGFR mutations. In this trial, 173 patients were randomly assigned to receive erlotinib or standard platinum-based chemotherapy. In this trial, a pre-specified evaluation about type of mutation (exon 19 deletion vs. L858R) was performed. These results confirm the just well-known data that EGFR TKIs are most effective than chemotherapy, improving PFS. In addition, the EURTAC trial reported interesting data about the efficacy of erlotinib about of exon 19 deletion and L858R mutation. In patients with EGFR exon 19 deletion, median PFS was 11.0 months (95% CI, 8.8–16.4), and in patients with L858R mutation was 8.4 months (95% CI, 5.2–10.8) (5).

Based on the results of the IPASS trial and EURTAC trial, gefitinib and erlotinib were approved for the treatment of EGFR mutation positive NSCLC.

Thanks to the results achieved by these first generation EGFR TKIs (erlotinib and gefitinib), the history of patients with NSCLC harbouring EGFR mutation changed dramatically in the last years, doubling survival and improving QoL, also thanks to manageable safety profile. Recently, many evidences confirmed the high activity of afatinib, a second-generation irreversible TKI that inhibits signaling from all dimers of ERBB receptor family members (including EGFR, HER2, ERBB3, and ERBB4) (12).

Afatinib was evaluated in the LUX-Lung3 (LL3) conducted on a mixed population (Caucasian and Asian patients) and LUX-Lung 6 (LL6) conducted exclusively on Asian population. In both trials, mutation-positive patients were stratified by mutation type (exon 19 deletion, L858R, or other), and PFS analysis was prespecified for patients with common EGFR mutation, considering together exon 19 deletions and L858R mutations. For both trials, the primary end point was PFS assessed by independent review. Secondary end points included tumor response, overall survival, adverse events, and patient-reported outcomes (PROs) (9,10).

Considering singularly the LL3 and LL6 trials, the results confirmed the efficacy of afatinib in EGFR mutation positive NSCLC, overlapping the previous trials with reversible EGFR TKIs. Indeed, this trials showed a median PFS in ITT with afatinib of about 11.0 months compared with 6.9 months of chemotherapy arm (HR =0.58; 95% CI, 0.43–0.78; P=0.001). The results reported by the authors of LL3, considered only patients with common mutations (exon 19 deletions and L858R) showed an increased PFS of 13.6 months (HR =0.47; 95% CI, 0.34–0.65; P=0.001). PFS resulted more improved in patients with tumours harbouring exon 19 deletion followed by L858R mutation.

Data regarding overall survival of patients treated with afatinib in LL3 and LL6 was evaluated in a pooled analysis including only patient with common EGFR mutations (exon 19 deletions =355 and L8585R =276). Median OS based on overall population was 27.3 *vs.* 24.3 months, HR =0.81 (95% CI, 0.66–0.99; P=0.037). The median OS of patients with deletion 19 mutations, was 33.3 months (95% CI, 26.8–41.5) in the afatinib group *vs.* 21.1 months (95% CI, 16.3–30.7) in the chemotherapy group (HR =0.54; 95% CI, 0.36–0.79; P=0.0015) in LL3; and was 31·4 months (95% CI, 24.2–35.3) *vs.* 18.4 months (95% CI, 14.6–25.6), respectively (HR =0.64; 95% CI, 0.44–0.94; P=0.023) in LL6. By contrast, there were no significant differences by treatment group for patients with EGFR L858R-positive tumours in either trial: in LL3, median overall survival was 27.6 months (95% CI, 19.8–41.7) in the afatinib group *vs.* 40.3 months (24.3–not estimable) in the chemotherapy group (HR =1.30; 95% CI, 0.80–2.11; P=0.29); in LL6, it was 19.6 months (95% CI, 17.0–22.1) *vs.* 24.3 months (95% CI, 19.0–27.0), respectively (HR =1.22; 95% CI, 0.81–1.83; P=0.34) (13).

Considering individually the overall survival data coming out from all randomized clinical trials with erlotinib, gefitinib and afatinib, it is not possible to found a statistically significant superiority of one drug on the other.

However, the results of pooled analysis showed that a significant improvement in overall survival with afatinib was achieved in patients with tumours harboring the EGFR del19 mutation.

These data confirmed the multiple evidences suggesting that exon 19 deletions and L8585R are two different disease entities. Notably, different retrospective analysis considering both reversible and irreversible TKIs using for NSCLC carrying exon 19 deletions, showed that treatment with EGFR TKI improve OS when compared with standard chemotherapy (14).

In addition to these data about the efficacy of different EGFR TKIs compared with chemotherapy, recently during ESMO-Asia congress was presented the preliminary results of LL7, a phase IIb trial of afatinib versus gefitinib for the treatment of first-line EGFR mutation-positive adenocarcinoma of the lung. In the LL7, the first randomized clinical trial evaluating two different EGFR TKIs, 319 patients with adenocarcinoma of the lung carrying common EGFR mutation (Del19 and L858R), were randomized at a 1:1 ratio to receive afatinib 40 mg/daily or gefitinib 250 mg/daily. Patient population was stratified by mutation type (Del19/L858R) and brain metastases (present/absent). Primary endpoint was independent PFS, time to treatment failure (TTF) and OS; secondary endpoints were overall response rate (ORR), time to response, duration of response (DoR), duration of disease control, tumour shrinkage, QoL and safety profile.

Considering overall randomized population, results about PFS showed no difference between two arms: 11.0 *vs.* 10.9 months (HR =0.73%; 95% CI, 0.57–0.95; P=0.0165). But it is very interesting to underline that 2-year survival rate was 18% *vs.* 8% (P=0.0184) in favour of afatinib treatment. In patients with Del 19 mutations, median PFS was 12.7 *vs.* 11.0 months (HR =0.76%; 95% CI, 0.55–1.06; P=0.1071), while in patients with L858R mutation, median PFS was 10.9 *vs.* 10.8 months (HR =0.71%; 95% CI, 0.47–1.06; P=0.0856). Interesting results coming out from the analysis of TTF that showed a statistical significant clear improvement in favor of patients that received afatinib treatment: 13.7 *vs.* 11.5 months (HR =0.73%; 95% CI, 0.58–0.92; P=0.0073). Afatinib treatment was associated with an improvement of objective response rate (70% *vs.* 56%; P=0.0083) and DoR (10.1 *vs.* 8.4), evaluated by independent review. Safety profile overlaps the results of the previous clinical trial; discontinuation rate was low and equal for both treatment arms (6.3%). Discontinuation rate was more frequent due to diarrhea (3.1%) skin toxicities (1.3%) and fatigue (1.3%) in patients treated with afatinib while due to ALT increase (3.1%), AST increase (1.95%) and interstitial lung disease (ILD) (2.5%) for patients that received gefitinib (15).

These preliminary results regarding PFS, TTF, ORR and DoR, confirm a slight trend in favor of afatinib. Indeed considering the median PFS, only the results about Del19 showed a difference in favour of afatinib, although not statistically significant (P=0.1071). Survival curves about PFS in Del19 and L858R showed a durable response in favor of afatinib after 1 year of treatment, maybe for the activity of afatinib in delaying the development of resistance.

In the era of precision medicine, it will be very interesting to understand the T790M rate in patients treated with afatinib as front-line therapy. Indeed, the only preliminary results of a prospective trial that evaluated the presence of T790M in TKI-naïve patients that progressing to afatinib, showed that the presence of T790M mutation was less common (33%) then is expected with first generation EGFR TKIs, thought these data are based on a small group of patients (16).

Waiting the results of the first randomized phase III trial, comparing two different EGFR TKIs (dacomitinib *vs.* gefitinib) ARCHER-1050 trial, the LL7 (phase IIb) open a new era of clinical trial evaluating two different EGFR target agents, reducing statistical issue developed from indirect comparison analysis (17).

As reported by the discussant Pasi Jänne, probably the choice of first-line EGFR-TKI has no effect on subsequent therapy, considering that the development of EGFR T790M mutations is one the major causes of resistance to first-generation TKIs, also in patients treated with afatinib. The combination of first-generation TKI plus bevacizumab or the treatment new EGFR TKI, could be change our approach to our patients, developing the most effective and tolerable strategy to prevent or delay resistance for as long as possible.

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Footnote

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Afatinib as first-line treatment for patients with advanced non-small-cell lung cancer harboring EGFR mutations: focus on LUX-Lung 3 and LUX-Lung 6 phase III trials

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In patients with advanced or metastatic non-small-cell lung cancer (NSCLC) carrying epidermal growth factor receptor (EGFR) positive mutations, the use of EGFR tyrosine kinase inhibitor (TKI) showed to improve survival and safety profile, when compare with standard chemotherapy. These results were reported in different randomized clinical trials with erlotinib as EURTAC and OPTIMAL (1-3), and with gefitinib IPASS, NEJ002, First-SIGNAL and the West Japan Thoracic Oncology Group Study (3-6). In these studies the median progression-free survival was around 10-12 months. After the results of the IPASS trial, gefitinib was approved for advanced NSCLC with EGFR positive mutation in all setting of treatment in Europa and Asia; while erlotinib that received in 2005 the indication in second- and third-line treatment in patients unselected for EGFR mutations after the Br.21 trial, recently was approved by FDA for the first-line treatment in patients with NSCLC harboring EGFR mutations, based on the results of the EURTAC trial in Europe, Asia and USA.

In addiction to these interesting data, the results of LUX-Lung 3 (LL3) (7) and LUX-Lung 6 (LL6) (8) trial showed and confirm the activity of afatinib, an irreversible EGFR TKI, as front-line therapy in patients with EGFR positive mutations, compared with standard chemotherapy.

In the LL3, patients were randomly assigned, with 2:1 ratio, to receive afatinib 40 mg daily or chemotherapy with cisplatin and pemetrexed every 21 days. Mutation-positive patients were stratified by mutation type (exon 19 deletion, L858R, or other) and race (Asian or non-Asian). The results showed a median PFS of 11.1 months for afatinib and 6.9 months for chemotherapy (HR 0.58; 95% CI: 0.43 to 0.78; P=0.001). A pre-planned analysis of PFS in patients (n=308) with exon 19 and 21 deletions was 13.6 months for afatinib and 6.9 months for chemotherapy (HR 0.47; 95% CI: 0.34 to 0.65; P=0.001). Higher response rates were observed in afatinib groups compared with chemotherapy 69% and 44%, respectively. These efficacy data regarding afatinib in mixed population, was confirmed by LL6 trial (final results are not yet published) that compared afatinib with standard chemotherapy in Asian population were PFS was 11 *vs*. 5.6 months (HR 0.28; 95% CI: 0.20 to 0.39; P<0.0001). Overall, these results confirmed the efficacy of afatinib in selected patients for EGFR mutations, and overlaps the previous trials with reversible EGFR TKIs, as erlotinib and gefitinib in first-line setting.

More attention it is needed to evaluate the toxicity profile of afatinib based on the results of LL3 and LL6 trials. Diarrhea (95.2%) and skin rash (89.1%) were the most common treatment-related AEs with afatinib; discontinuation rate was 8% for patients receiving afatinib and 12% of those receiving chemotherapy. Comparing these results with those from LL6 that enrolled Chinese population, it is very interesting to underline that in this trial the incidence of toxicities was lower than LL3. It is difficult to explain this issue, and it is not simple, at this time, to understand if afatinib is better tolerated in Chinese population. Comparing these results with those of pivotal trial with gefitinib and erlotinib, these results showed a little bit of more toxicities in patients treated with afatinib, when compared with erlotinib or gefitinib. Though this results are not get along with the results of quality of life (QoL) and symptoms improvement (9). Indeed, though afatinib

treatment was associated with high rate of non-hematologic AEs, as skin rash and diarrhea, in this group of patients there were an improvement of global health status and QoL, physical role, and cognitive functioning. In addiction, in patients that received afatinib there was a delayed time to deterioration for cough and dyspnoea compare with chemotherapy arm.

In June 2013, after the results of LL3, FDA approved afatinib as front-line therapy for patients with NSCLC harboring *EGFR* mutations.

Nowadays we have different drugs (afatinib, erlotinib and gefitinib) available for patients with EGFR positive mutations in first-line setting, approved in Europe and USA. The survival rates of these drugs are very similar but afatinib seems to be a more potent TKI. It is need to understand deeply how to interpret the results regarding toxicity profile. Non-hematologic toxicities from EGFR TKIs present a different timing and profile comparing with those toxicities from chemotherapy. Although these three drugs showed different incidence of non-hematologic AEs, at this time there is no direct data that evaluate the response after a close and correct management.

Waiting for the result of LUX-Lung 7 trial, a head-to-head study comparing afatinib with gefitinib, now we have three TKIs available for our patients with EGFR mutation, and further analysis not only of efficacy but particularly for safety profile are needed.

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Footnote

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Afatinib plus chemotherapy versus chemotherapy alone after progression on afatinib: new insights on old question?

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Epidermal growth factor receptor (*EGFR*) mutations occur in about 5–10% of non-small cell lung cancer (NSCLC) in non-Asian population and 40–45% Asian population (1,2). Activating *EGFR* mutations (exon 19 deletion or *L858R* substitution in exon 21) predict high response rate to first line EGFR tyrosine kinase inhibitors (EGFR-TKIs). Approved EGFR-TKI's for the treatment of EGFR mutant lung cancer include first generation TKIs (erlotinib and gefitinib) and second generation TKI (afatinib). Third generation EGFR-TKIs have also been developed to target resistance mutation T790M and spare wild type *EGFR*. Afatinib is an irreversible ERB family blocker that potently inhibits signaling of all homodimers and heterodimers formed by the EGFR, human epidermal growth factor receptor (HER)-2, HER-3, and HER-4 receptors. Afatinib has been evaluated in various settings in LUX-Lung trials summarized in *Table 1* (3-9). A subgroup pooled analysis of LUX-Lung 3 and LUX-Lung 6 showed a survival advantage of afatinib over chemotherapy in patients with exon 19 deletion (10).

After an initial response to EGFR-TKIs, resistance develops invariably through various mechanisms including T790M mutations (50–60% of patients), *MET* amplification, epithelial to mesenchymal transformation, *HER-2* amplification or transformation to small cell lung cancer (11). Development of acquired resistance is heterogeneous process with multiple mechanisms of resistance developing at separate metastatic sites in same patient or at the same site at different time points. Therefore, it is possible that radiographic progression at one site may not imply that all other sites would also be resistant to EGFR-TKI. Supporting this hypothesis, a flare phenomenon has been reported where discontinuation of EGFR-TKI for radiological progression, results in rapid, symptomatic progression at other sites attributed to presence of TKI sensitive clones (12). This phenomenon has given rise to concept of continuing EGFR-TKI beyond progression. Multiple retrospective studies have shown that EGFR-TKI can be continued beyond progression in combination with loco-regional (surgery, radiation) treatment or chemotherapy and may lead to better outcomes (13,14).

In the phase III LUX-Lung 5 trial (15), published in *Annals of Oncology*, Schuler *et al*. evaluated whether continuation of afatinib with paclitaxel is superior to paclitaxel alone after progression on EGFR-TKI in a cohort of lung cancer patients clinically enriched for *EGFR* dependency. The trial was conducted in two parts: part A enrolled patients who had progressed on one or more chemotherapy regimen, had a clinical benefit (complete response, partial response or stable disease) of ≥12 weeks on first generation EGFR-TKI (erlotinib or gefitinib) and must have attained ≥12 weeks of clinical benefit on afatinib monotherapy with subsequent radiologic progression. The patients weren't screened for *EGFR* mutation status as it was not standard clinical practice at the time of study

Table 1 LUX-Lung trials evaluating afatinib in various settings

Study	Study design	Patient population	Treatment arms	Primary endpoint	Results
LUX-Lung 1	Phase 2b/3 randomized	*EGFR*+ progressed on first generation EGFR-TKI (n=595)	Afatinib *vs*. placebo	OS	10.8 *vs*. 12 months (HR =1.08, 95% CI: 0.86–1.35; P=0.74)
LUX-Lung 2	Phase 2 single arm	Second line or higher *EGFR* positive after chemotherapy (TKI naive) (n=129)	Afatinib	ORR	61%
LUX-Lung 3	Phase 3 randomized	First line *EGFR*+ (n=345), adenocarcinoma	Afatinib *vs*. cisplatin plus pemetrexed	PFS	11.1 *vs*. 6.9 months (HR =0.58, 95% CI: 0.34–0.65, P=0.001)
LUX-Lung 4	Phase 2 single arm	Adenocarcinoma progressed on first generation EGFR-TKI (n=61)	Afatinib	ORR	8.2% (95% CI: 2.7–18.1%)
LUX-Lung 6	Phase 3 randomized	First line *EGFR*+ (n=364)	Afatinib *vs*. cisplatin plus gemcitabine	PFS	11 *vs*. 5.6 months (HR =0.28, 95% CI: 0.20–0.39, P<0.0001)
LUX-Lung 7	Phase 2b randomized	First line *EGFR*+ (n=319)	Afatinib *vs*. gefitinib	Coprimary end points (PFS, OS and TTF)	PFS: 11 *vs*. 10.9 months (HR =0.73, 95% CI: 0.57–0.95, P=0.017); TTF: 13.7 *vs*. 11.5 months (HR =0.73, 95% CI: 0.58–0.92, P=0.0073); OS not mature
LUX-Lung 8	Phase 3 randomized	Second line squamous cell (n=795)	Afatinib *vs*. gefitinib	PFS	2.4 *vs*. 1.9 months (HR =0.82, 95% CI: 0.68–1, P=0.0427)

HR, hazard ratio; CI, confidence interval; ORR, objective response rate; OS, overall survival; PFS, progression free survival; TTF, time to treatment failure; *vs*., versus.

planning but were clinically enriched based on disease control with EGFR-TKI for ≥12 weeks. Patients in part A, who derived clinical benefit from afatinib monotherapy were then screened for randomization in 2:1 fashion to afatinib plus paclitaxel (based on pre-clinical evidence of synergism) versus dealer's choice chemotherapy (part B). The primary end point was progression free survival (PFS), secondary endpoints included overall survival (OS) and objective response rate (ORR). The median PFS was significantly longer with afatinib plus chemotherapy versus chemotherapy alone [5.6 *vs*. 2.8 months, hazard ratio (HR) =0.60, 95% confidence interval (CI): 0.43–0.85, P=0.003]. The median PFS in the chemotherapy alone arm was the longest for paclitaxel (3.8 months). There was no difference in median OS (12.2 months) between the two groups. The ORR was 32.1% in the combination arm versus 13.2% in chemotherapy only arm (OR =3.41, 95% CI: 1.41–6.79, P=0.005).

Authors should be commended for successfully conducting a prospective randomized study in fourth line setting for treatment of lung cancer. The study was designed to address an important question of EGFR-TKI continuation beyond progression in combination with chemotherapy and showed that in selected patient population, continued EGFR blockade with chemotherapy may improve PFS compared to single agent chemotherapy after progression on EGFR-TKI monotherapy. Although LUX-Lung 5 provides prospective validation of this concept, its reliability and clinical utility is limited due to several factors. The study was underpowered as the initial number of 351 projected patients to part B was considered unachievable and not recruited. Of the 1,154 patients treated with afatinib monotherapy, 223 patients with clinical benefit of 12 weeks were screened and only 202 patients were randomized. Of the 299 patients with progression after initial benefit on afatinib monotherapy most declined participation due to general health deterioration. This high drop out in enrollment after progression on third line afatinib monotherapy suggests that most patients were not able to have subsequent treatment and patients who continued treatment might be part of a selected population with good performance status, low co-morbidity or even

different disease biology.

Recently multiple reports have demonstrated that treatment of *EGFR* mutant patients after radiologic progression on EGFR-TKI can be complicated by the heterogeneous nature of progression (16,17). Clinical outcomes can be widely variable depending on the subtype of progression, which can be defined as single site, oligo-sites, central nervous system only, systemic or multi-site and asymptomatic or symptomatic. ASPIRATION was a prospective single arm trial in Asian *EGFR* mutant lung cancer patients, designed to evaluate effect of continuing TKI therapy after radiologic progression at the discretion of physician and patient. Results of this study showed that survival post TKI progression can be increased and systemic chemotherapy delayed for a select group of patients without compromising the OS (18). Although this study was limited by the nature of design, it showed that some patients may continue to have indolent course even after radiologic progression on EGFR-TKI. It is possible that most patients who went on to fourth line treatment in LUX-Lung 5 study had less aggressive disease and if patients were not classified based on type of progression, an imbalance in these subgroups between the two post afatinib progression arms could have created the difference in PFS.

Other trials of post progression first generation EGFR-TKI continuation with chemotherapy after initial benefit have not shown any advantage compared to chemotherapy alone. Our group had conducted a small randomized phase II study of chemotherapy (pemetrexed or docetaxel) versus chemotherapy plus erlotinib in patients with progression after initial clinical benefit from erlotinib. There was increased toxicity with addition of erlotinib to chemotherapy without any added benefit in response rate (13% *vs.* 16%) or PFS (5.5 *vs.* 4.4 months) (19). Similarly IMPRESS was a large randomized phase III trial investigating the role of continuing gefitinib in combination with chemotherapy for *EGFR* mutant patients after development of acquired resistance to initial treatment with gefitinib. The primary end point for the trial was PFS, which was same in two treatment arms (5.4 months) indicating lack of benefit with addition of gefitinib to chemotherapy (20). However an exploratory subgroup analysis suggested potential clinical benefit from continued gefitinib treatment after progression, if *EGFR T790M* was not found in circulating plasma DNA (21). One could argue that positive results in LUX-Lung 5 are related to use of second generation EGFR-TKI with irreversible EGFR inhibition and some activity against known mechanism of acquired resistance such as *T790M* mutation or *HER-2* amplification.

The choices of chemotherapy in the chemotherapy only arm (a practical decision as there is no established standard fourth line) between the two arms makes the arms unbalanced. Also 13% of patients in the chemotherapy arm had received the same agent previously. In the absence of placebo arm, it is difficult to ascertain if chemotherapy alone had any significant effect on progression. Since we know from ASIRATION study that EGFR-TKI, when continued beyond progression can delay further tumor growth, it is possible that PFS advantage in the afatinib and paclitaxel arm could be entirely due to afatinib.

Serious treatment related adverse events were more common in the combination arm versus chemotherapy only arm (11% *vs.* 3%). It is interesting to note that 36% of patients in the chemotherapy arm received two additional lines of therapy versus 15% in the combination arm, implying a sizable proportion of patients overall went on to receive six or more lines of treatment, which is uncommon for most lung cancer patients treated outside the clinical trial.

LUX-Lung 5 study was more relevant at the time when it was conceived, since then multiple new agents have been approved or are in clinical trial for *EGFR* mutant patients with acquired resistance. Third generation EGFR-TKIs are the treatment of choice for patients with *EGFR T790M* mutations based on high response rate (61% and PFS of 9.6 months) and low toxicity secondary to sparing of wild type *EGFR* (22). Osimertinib is approved after progression on first or second generation EGFR-TKI in patients with *T790M* mutation. For those patients with non-T790M mediated resistance, combination of afatinib and cetuximab (23) has shown a response rate of about 30%.

Ideal future studies on patients with *EGFR* mutations should focus on preventing or delaying emergence of resistance and identifying targets for new resistance mechanisms. The LUX-Lung 5 study with its limitations of being underpowered amongst others is unlikely to find afatinib a new niche.

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Footnote

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Is afatinib a treatment option for brain metastases in patients with *EGFR* mutation-positive non-small cell lung cancer?

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Introduction

Treatment for advanced non-small cell lung cancer (NSCLC) depends on the molecular characteristics of the tumor. Mutations of the gene for the epidermal growth factor receptor (EGFR) are present in ~32% of Asians and ~7% of individuals of other ethnic groups with NSCLC, with deletions in exon 19 and an L858R point mutation in exon 21 accounting for ~90% of such genetic alterations detected at diagnosis (1). NSCLC tumors that harbor *EGFR* mutations are oncogene addicted and therefore usually sensitive to treatment with EGFR tyrosine kinase inhibitors (TKIs).

Three EGFR-TKIs—gefitinib, erlotinib, and afatinib—are widely available in the clinic. Gefitinib was the first such drug to be approved for patients with NSCLC positive for *EGFR* mutations. The IPASS study assessed gefitinib in comparison with carboplatin-paclitaxel as a first-line treatment for patients with advanced NSCLC in East Asia (2). A subset analysis of this study found that gefitinib significantly improved progression-free survival (PFS) compared with the standard chemotherapy in patients with *EGFR* mutation-positive NSCLC [9.5 *vs.* 6.3 months; hazard ratio (HR) of 0.48 with a 95% confidence interval (CI) of 0.36–0.64; P<0.001]. Overall survival (OS) was not increased by gefitinib, however, in this subset of patients (21.6 *vs.* 21.9 months; HR of 1.00; P=0.990) (3). Another two phase III trials performed in Japan reported similar outcomes (4,5).

Erlotinib was also found to be beneficial in first-line treatment of *EGFR* mutation-positive NSCLC. The EURTAC trial compared erlotinib with platinum-doublet chemotherapy in European patients, finding that the median PFS for erlotinib was 9.7 months compared with only 5.2 months for chemotherapy (HR of 0.37 with a 95% CI of 0.25–0.54; P<0.0001) (6).

In contrast to gefitinib and erlotinib, both of which are reversible inhibitors, afatinib is a highly selective, irreversible EGFR-TKI, often being referred to as a second-generation EGFR-TKI. In a phase III trial (LUX-Lung 3) performed with *EGFR* mutation-positive NSCLC patients, afatinib improved PFS compared with cisplatin-pemetrexed in the first-line setting (11.1 *vs.* 6.9 months; HR of 0.58 with a 95% CI of 0.43–0.78; P=0.001) (7). Similar results were obtained in the LUX-Lung 6 trial, which compared gefitinib with cisplatin-gemcitabine in patients in East Asia (PFS of 11.0 *vs.* 5.6 months; HR of 0.28 with a 95% CI of 0.20–0.39; P<0.0001) (8). The LUX-Lung 7 trial further showed that afatinib was superior to gefitinib in terms of OS in the first-line setting (9).

Brain metastases (BM) in non-small cell lung cancer (NSCLC)

BM are manifest in 16% to 20% of NSCLC patients at diagnosis (10,11). The introduction of magnetic resonance imaging and improvement in OS of such patients likely account for a recent apparent increase in the incidence of

Table 1 Outcome of EGFR-TKI treatment for patients with *EGFR* mutation-positive NSCLC and brain metastases

EGFR-TKI	Study design	n	*EGFR* mutation	Treatment line	History of EGFR-TKI treatment	No. of patients with prior WBRT (%)	Intracranial RR (%)	PFS (months)	Intracranial TTP (months)	OS (months)	Ref.
Gefitinib	Phase II	41	Exon 19 del (n=23); L858R (n=15); other (n=3)	Unknown	EGFR-TKI naïve	0 (0)	87.8	14.5		21.9	(14)
Erlotinib	Retrospective	17	Exon 19 del (n=12); L858R (n=5)	First (n=10); second (n=5); third (n=2)	Unknown	9 (52.9)	82.4		11.7	12.9	(15)
Erlotinib	Retrospective	63	Exon 19 del (n=36); L858R (n=26); other (n=1)	Unknown	EGFR-TKI naïve	0 (0)			16	26	(16)
Afatinib	Phase III	48	Exon 19 del (n=28); L858R (n=20)	First (n=48)	EGFR-TKI naïve	13 (27.1)		8.2		22.4	(17)

PFS, TTP, and OS values are medians. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; WBRT, whole-brain radiation therapy; RR, response rate; PFS, progression-free survival; TTP, time to progression; OS, overall survival; Del, deletion.

BM. BM can cause neurological symptoms and thereby reduce quality of life in NSCLC patients.

A review of 1,127 NSCLC patients found that those with *EGFR* mutations were more likely to develop BM than those without such mutations (12). The frequency of BM was thus 31.4% for the mutation-positive patients but only 19.7% for the negative ones [odds ratio of 1.86, with a 95% CI of 1.39–2.49; P<0.001). Of note, BM were smaller (P=0.031) and the frequency of leptomeningeal dissemination was higher (30.8% *vs.* 12.7%; odds ratio of 3.04 with a 95% CI of 1.64–5.78; P<0.001) in the *EGFR* mutation-positive patients than in those wild type for *EGFR*. Median OS after diagnosis of BM was also significantly longer in patients with *EGFR* mutation-positive tumors (HR of 2.23 with a 95% CI of 1.62–3.10; P<0.001). Another study showed that NSCLC patients with a deletion in exon 19 of *EGFR* had more and smaller metastases with a reduced extent of peritumoral brain edema compared with patients with wild-type *EGFR* alleles, whereas the characteristics of BM in patients with the L858R point mutation of *EGFR* were similar to those of the metastases in wild-type patients (13).

The standard management for BM to date has been irradiation [including whole-brain radiation therapy (WBRT) and stereotactic radiosurgery] and surgical resection. Traditional cytotoxic agents usually do not penetrate the blood-brain barrier. However, the possibility of systemic EGFR-TKI treatment for BM in patients with *EGFR* mutation-positive NSCLC is receiving increasing attention.

EGFR-TKIs for treatment of brain metastases (BM)

A phase II study evaluated gefitinib alone (without irradiation) for the treatment of BM in 41 patients with *EGFR* mutation-positive NSCLC (14). The response rate (RR) for BM, median PFS, and median OS were 87.8%, 14.5 months (95% CI of 10.2–18.3 months), and 21.9 months (95% CI of 18.5–30.3 months), respectively (*Table 1*). This favorable outcome suggested that EGFR-TKIs might delay the need for irradiation and the associated risk of neurocognitive decline in such patients. Erlotinib achieves a higher cerebrospinal fluid concentration than gefitinib (18), but the clinical efficacy of erlotinib alone for BM has not been well assessed in a prospective study. A retrospective study of erlotinib treatment in 17 patients with *EGFR* mutation-positive NSCLC and BM found that the RR for BM, median time to progression (TTP) in the brain, and median OS were 82.4%, 11.7 months (95% CI of 7.9–15.5 months), and 12.9 months (95% CI of 6.2–19.7 months), respectively (15) (*Table 1*). Nine of these 17 patients had a history of WBRT. Another retrospective study compared erlotinib, WBRT, and stereotactic radiosurgery for 110 EGFR-TKI-naïve NSCLC patients with BM (n=63, 32, and 15, respectively) (16) (*Table 1*). Although no significant difference in median OS was apparent between the WBRT and erlotinib groups (35 *vs.* 26 months, respectively; P=0.62), median intracranial TTP was significantly longer in the WBRT group than

in the erlotinib group (24 *vs.* 16 months; P=0.04). Among patients in the WBRT group who received erlotinib within 2 months of completing irradiation (n=21), the median TTP for BM during erlotinib treatment was 25 months, which was significantly longer than that in the erlotinib group by univariate analysis (P=0.01) but not significantly longer by multivariate analysis (P=0.20). Thus, although erlotinib appears to prolong TTP in the brain, its effectiveness for treatment of BM in patients with *EGFR* mutation-positive NSCLC might be enhanced by prior WBRT.

Afatinib has been even less well validated for treatment of BM than has gefitinib or erlotinib. A subset analysis for LUX-Lung 2, a phase II study of afatinib for patients with *EGFR* mutation-positive NSCLC, found that the overall RR did not differ significantly between patients with or without BM (65% *vs.* 60%, respectively; HR of 1.20 with a 95% CI of 0.52–2.78) (19).

A recent study reported a subset analysis for patients with common *EGFR* mutations (exon 19 deletion or L858R), and BM in the LUX-Lung 3 and LUX-Lung 6 trials (17) (*Table 1*). Whereas LUX-Lung 3 compared afatinib with cisplatin-pemetrexed in 345 treatment-naïve patients with *EGFR* mutation-positive NSCLC (7), LUX-Lung 6 compared afatinib with cisplatin-gemcitabine in 364 such patients of Asian ethnicity (8). The two trials included 42 (12.2%) and 49 (13.5%) patients with clinically asymptomatic and controlled BM, respectively, most of whom had common *EGFR* mutations [n=81 (89%)]. Among these patients with BM, there was a trend toward improved PFS on treatment with afatinib compared with standard chemotherapy in both LUX-Lung 3 (11.1 *vs.* 5.4 months; HR of 0.54 with a 95% CI of 0.23–1.25; P=0.1378) and LUX-Lung 6 (8.2 *vs.* 4.7 months; HR of 0.47 with a 95% CI of 0.18–1.21; P=0.1060). Combined analysis of both trials revealed a significant improvement in PFS for the afatinib group compared with the chemotherapy group (8.2 *vs.* 5.4 months; HR of 0.50 with a 95% CI of 0.27–0.95; P=0.0297). Of note, the PFS benefit of afatinib compared with chemotherapy was enhanced by prior WBRT treatment, with median PFS values of 13.8 *vs.* 4.7 months (HR of 0.37 with a 95% CI of 0.12–1.17; P=0.0767) for patients with prior WBRT (n=24) and of 6.9 *vs.* 5.4 months (HR of 0.62 with a 95% CI of 0.28–1.36; P=0.2222) for those without prior WBRT (n=57). One possible explanation for this finding is that WBRT followed by afatinib can confer longer intracranial and extracranial PFS, respectively. Alternatively, WBRT might have disrupted the blood-brain barrier and thereby facilitated the entry of afatinib into the brain (20). Rates of central nervous system (CNS) progression in patients with BM at baseline were similar for afatinib treatment [n=9 (45.0%) in LUX-Lung 3 and n=6 (21.4%) in LUX-Lung 6] and chemotherapy [n=5 (33.3%) in LUX-Lung 3 and n=5 (27.8%) in LUX-Lung 6]. Similar rates of CNS progression were observed in the two trials for all patients without BM at baseline [n=3 (3.7%) in LUX-Lung 3 and n=4 (4.7%) in LUX-Lung 6]. Median OS in patients with BM did not differ significantly between afatinib and chemotherapy for LUX-Lung 3 (19.8 *vs.* 33.2 months, respectively; HR of 1.15 with a 95% CI of 0.49–2.67; P=0.7517), for LUX-Lung 6 (22.4 *vs.* 24.7 months; HR of 1.13 with a 95% CI of 0.56–2.26; P=0.7315), or for the combined data set (22.4 *vs.* 25.0 months; HR of 1.14 with a 95% CI of 0.66–1.94; P=0.6412). An OS benefit for afatinib over chemotherapy was apparent for total patients with a deletion in exon 19 of *EGFR*, whereas no significant difference was observed between afatinib and chemotherapy for patients with an exon 19 deletion and BM (22.4 *vs.* 20.6 months, respectively; HR of 0.78 with a 95% CI of 0.37–1.66; P=0.5229) (21). This difference might be due to an effect of subsequent therapy or to the small number of patients with BM included in the analysis. In conclusion, this study demonstrated superiority of afatinib over chemotherapy in patients with *EGFR* mutation-positive NSCLC and BM.

Reported OS times for the various studies of *EGFR* mutation-positive NSCLC patients with BM treated with EGFR-TKIs are similar (*Table 1*). Given that there have been no head-to-head comparisons among gefitinib, erlotinib, and afatinib for such patients, the best EGFR-TKI for their treatment is not yet known. In addition, prospective data are currently limited, with most of the published studies of EGFR-TKI efficacy in this patient population having been retrospective in nature. The combined subset analysis of the LUX-Lung 3 and LUX-Lung 6 trials is the first such report from a phase III study. Given that the data suggest that afatinib is superior to chemotherapy in terms of PFS for patients with *EGFR* mutation-positive NSCLC and BM, this drug is a potential treatment option for such patients.

Whether WBRT or an EGFR-TKI should be selected for patients with symptomatic BM is unclear. Patients with symptomatic or unstable BM have been excluded from most clinical trials of EGFR-TKIs, with traditional WBRT thus still being preferred for such cases. In patients with asymptomatic and stable BM, however, EGFR-TKIs have the potential to prolong the time to the onset of intracranial

radiation therapy and consequent side effects. EGFR-TKIs without irradiation might be appropriate for patients for whom treatment-related neurocognitive decline is a particular concern. The combined analysis of the LUX-Lung 3 and LUX-Lung 6 trials suggested that prior WBRT prolonged PFS in patients with BM treated with afatinib (17). A retrospective study of erlotinib treatment also suggested that prior WBRT prolongs TTP in the brain (16). Whether an EGFR-TKI alone or together with prior WBRT should be selected for *EGFR* mutation-positive patients with symptomatic BM should thus be addressed carefully on a case-by-case basis, with further studies exploring the effects of EGFR-TKIs in such patients being warranted.

What about treatment for patients with BM and NSCLC positive for a secondary T790M mutation of *EGFR*, which confers resistance to gefitinib, erlotinib, and afatinib? The efficacy of osimertinib, a third-generation EGFR-TKI that is effective against the T790M mutant form of EGFR, for such patients is unclear. Furthermore, a recent study found that the CNS metastases including leptomeningeal metastases of 10 of 12 patients whose extracranial tumor was positive for T790M were negative for this mutation (22). If the CNS metastases of most patients with T790M-positive extracranial tumors are indeed T790M negative, then the metastatic lesions may be susceptible to control by first- or second-generation EGFR-TKIs. AZD3759 is an investigational EGFR-TKI that shows high penetrance into the CNS *in vivo* and is currently under evaluation in a phase I clinical trial (23). This agent may thus hold promise for the treatment of patients with *EGFR* mutation-positive NSCLC and BM.

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Footnote

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Afatinib for patients with epidermal growth factor receptor mutation-positive non-small cell lung cancer: clinical implications of the LUX-Lung 7 study

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We thank Professor Lee for his interest in our recent LUX-Lung 7 publication that assessed afatinib versus gefitinib in patients with epidermal growth factor receptor (*EGFR*) mutation-positive non-small cell lung cancer (NSCLC) (1). We agree that, in an ideal world, afatinib and gefitinib would have been compared in a Phase III trial with a formal hypothesis. However, given the lack of data available at the conception of LUX-Lung 7 (2010–2011), we made the pragmatic decision to undertake an exploratory Phase IIb trial. We felt that it was simply not possible to construct a formal hypothesis based on *a priori* evidence available at the time. Rather, we felt that a flexible trial design that assessed multiple clinically relevant endpoints would be the best way to broadly explore any differences between the agents. Notwithstanding its design, we do not think that the relevance of LUX-Lung 7 should be understated. Firstly, the LUX-Lung 7 population (N=319) was as large as many Phase III trials in this setting. Secondly, it was a global trial that encompassed a multicenter, multiethnic population; recruitment of Asian and non-Asian patients was balanced. Thirdly, signals of improved efficacy with afatinib over gefitinib were observed across multiple, independently assessed, endpoints including progression-free survival (PFS), time to treatment failure (TTF) and objective response rate (ORR). Improvements were generally consistent across key patient subgroups (e.g., Asian *vs.* non-Asian, *EGFR* Del19 *vs.* L858R mutation). We do not believe that the Phase IIb design subverts the clinical relevance of these data, especially when one considers the paucity of head-to-head data in this setting.

Regarding the selection of, and amendments to, the primary endpoints of LUX-Lung 7, we chose endpoints that are most clinically relevant for patients and physicians [overall survival (OS) and TTF], while also acknowledging the relevance of PFS as a critical endpoint in the first-line treatment setting. Thus, OS and TTF were included as co-primary endpoints alongside PFS, and the original co-primary endpoint of disease control was re-defined as a secondary endpoint. These protocol amendments occurred before completion of recruitment or any unblinded efficacy analyses. With regards to PFS, we agree with Professor Lee that the absolute difference in the medians between arms was negligible; however, overall, there was a clear and relevant improvement in PFS (HR: 0.73; P=0.017) that was underpinned by the divergence of curves at later time points (≥10% improvements in 18- and 24-month PFS with afatinib *vs.* gefitinib). We hypothesize that these differences reflect the broader and more durable inhibitory profile of afatinib compared with first-generation tyrosine kinase inhibitors (TKIs), which may delay mechanisms of acquired

resistance commonly observed in *EGFR* mutation-positive NSCLC (2). Clearly, it is impossible to infer whether afatinib has PFS benefit over the other first-generation EGFR TKIs, erlotinib and icotinib, based on LUX-Lung 7. However, we do not believe that Professor Lee is correct to cite the Phase III OPTIMAL trial as evidence that erlotinib confers better PFS than afatinib, as cross-trial comparisons are not possible. Indeed, the recent head-to-head CTONG 0901 Phase III trial did not demonstrate any difference in efficacy and safety between gefitinib and erlotinib (3). Furthermore, the ENSURE trial did not reproduce entirely the outcome of OPTIMAL (4).

TTF was chosen as a co-primary endpoint to reflect 'real-world' clinical practice and guidelines, wherein many NSCLC patients continue treatment with EGFR TKIs beyond radiological progression, in the absence of clinical deterioration. TTF reflects both disease progression and tolerability. Accordingly, the significant improvement of TTF observed with afatinib over gefitinib testifies to the manageability of adverse events (AEs) with afatinib and the willingness of patients and physicians to continue afatinib therapy beyond radiological disease progression despite expected AEs. In our view, it is an oversimplification to cite higher rates of treatment-related grade 3 diarrhea and rash/acne as evidence that afatinib is less tolerable than gefitinib. Although these AEs are clearly more frequent with afatinib, other AE rates, notably elevated liver enzymes and interstitial lung disease, are higher with gefitinib. We would argue that, overall, afatinib and gefitinib do not demonstrate overwhelmingly different tolerability based on the identical rate of treatment-related discontinuations in both arms (6% each). Furthermore, although limited in scope, patient-reported outcomes data indicate no difference in health-related quality-of-life between the two arms. These findings indicate that tolerability-guided dose reductions of afatinib effectively manage AEs and facilitate a favorable tolerability profile close to that of gefitinib.

Updated LUX-Lung 7 data, including primary analysis of OS, were recently presented at the European Society for Medical Oncology (ESMO) 2016 congress (5). In this updated report, afatinib maintained significant improvements versus gefitinib in PFS, TTF and ORR. In addition, a 14% reduction in risk of death was observed with afatinib, corresponding to a numerical difference of 3.4 months in median OS, which did not achieve statistical significance (27.9 *vs.* 24.5 months; HR: 0.86; 95% CI: 0.66–1.12; P=0.2580). It should be noted that, despite being recognized as the preferred first-line treatment for *EGFR* mutation-positive NSCLC, it has proved difficult to demonstrate clear OS advantage versus platinum-based chemotherapy in this setting; only afatinib has shown OS benefit (in patients with Del19). The challenge of demonstrating OS advantage is largely attributable to high rates of post-progression therapy. In this regard, it is interesting to note that ~75% of patients in both arms of LUX-Lung 7 received at least one systemic anticancer therapy, and multiple lines of therapy were common; subsequent use of post-study EGFR TKIs was higher with gefitinib than afatinib (55.6% *vs.* 45.9%). This rate of post-progression therapy is somewhat higher than reported in most previous trials. It is unsurprising, therefore, that significant OS benefit was not achieved, especially given that the trial was not powered for this endpoint.

We acknowledge that these data, obtained from a Phase IIb exploratory trial, are not sufficient to claim superiority of afatinib over gefitinib. However, we believe that the overall findings from LUX-Lung 7 could provide relevant guidance to physicians with respect to clinical decision making in their day-to-day management of patients with *EGFR* mutation-positive NSCLC.

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Footnote

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Afatinib and gefitinib: a direct comparison

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During the last decade, scientific literature had already reported data on frequency and characteristics of EGFR mutations among patients with non-small-cell lung cancer (NSCLC) and their response to tyrosine kinase inhibitors (TKIs) (1). Actually EGFR mutation-positive NSCLC is a well-defined molecular type of lung cancer with specific first-line treatment options.

Gefitinib had been largely studied and developed for treatment in first line settings of patients with advanced EGFR mutation-positive NSCLC compared with chemotherapy (2,3) both in Caucasian and non-Caucasian patients (4-6). Erlotinib had also demonstrated benefits in overall survival (OS), progression free survival (PFS), response rate and quality of life, with a favourable tolerability. These benefits were established in first-line setting versus chemotherapy both in Chinese and European patients with EGFR mutation-positive advanced NSCLC (7,8).

More recently a wide-spectrum preclinical activity against EGFR mutations was demonstrated with afatinib, a second-generation, selective, orally bioavailable TKI that irreversibly blocks signaling from EGFR (EGFR/ErbB1), human epidermal growth factor receptor 2 (HER2/ErbB2) and ErbB4 (9,10). Two phase III trials assessed the efficacy of afatinib in first-line setting in patients with advanced or metastatic EGFR mutation-positive NSCLC compared with a standard chemotherapy regimen. In LUX-Lung 3 trial, afatinib was evaluated against cisplatin plus pemetrexed (11) demonstrating a prolongation of PFS compared with chemotherapy (11.1 *vs.* 6.9 months, respectively; HR =0.58; P=0.001), with a greater benefit in patients with exon 19 deletions and L858R mutations. Similarly, in LUX-Lung 6 afatinib was evaluated compared with cisplatin plus gemcitabine. Afatinib led to an increased PFS of 11 versus 5.6 months compared with cisplatin plus gemcitabine (HR =0.28; P<0.0001) (12).

Thus gefitinib, erlotinib and afatinib are actually a standard therapeutic option in advanced-stage NSCLC with activating mutation of EGFR. However there was no trial comparing two TKIs for the treatment of patients with EGFR mutation-positive NSCLC till now.

LUX-Lung 7 is the first trial comparing an irreversible ErbB family blocker (afatinib) and a reversible EGFR TKI (gefitinib) as first-line treatment for this patients population.

Park and colleagues (13) conducted this multicentre, international, open-label, exploratory trial where patients were randomised to receive as first-line treatment afatinib (40 mg per day) or gefitinib (250 mg per day). Patients had a histologically confirmed diagnosis of NSCLC in advanced-stage with a common EGFR mutation (exon 19 deletion or Leu858Arg). They received treatment until disease progression or beyond radiological progression if deemed beneficial. Originally PFS and disease control at 12 months were primary endpoints. Then trial was update to include PFS, time-to-treatment failure (TTF) and OS as co-primary endpoints, while disease control became one of the secondary endpoint. All patients were included in the primary assessment of efficacy and all patients receiving at least one administration of each drug were considered for safety analysis. Number of patients was well balanced between the two treatment arms: 160 patients in afatinib arm and 159 in gefitinib arm respectively. More than 50% of patients were of Asian origin in both arms. In each treatment

arm patients with Leu858Arg and those with exon 19 deletion were 42% and 58% respectively. Only one patient in gefitinib arm presented both EGFR common mutations.

Median PFS in afatinib arm was significantly higher compared with that in gefitinib arm (11 *vs.* 10.9 months; HR =0.73; P=0.017). Also TTF was longer with afatinib than gefitinib: 13.7 versus 11.5 months, respectively (HR =0.73; P=0.0073). Afatinib benefit was observed for PFS and TTF in most patients subgroups except light ex-smokers and, only for TTF, in patients without brain metastases too.

Data about OS were immature at time of analysis, when median OS was 27.9 months in afatinib arm versus 25.0 months in gefitinib arm.

Responses were obtained during the first 16 weeks and objective response rate (ORR) was significantly higher among patients receiving afatinib (70% of patients in afatinib arm and 56% in gefitinib arm; P=0.0083) who presented a longer median duration of response too (12.7 versus 11.1 months, respectively). However patients reached a similar disease control between the two arms (91% for afatinib group versus 87% for gefitinib group, respectively; P=0.24).

PFS and ORR data for afatinib in LUX-Lung 7 are in line with those reported against chemotherapy in LUX-Lung 3 (11.14 months and 56%, respectively) and LUX-Lung 6 (11.0 months and 66.9% respectively).

The significant better PFS in afatinib group increases with time as demonstrated by the progressive separation of curves with time. This could be due to the broader and more durable inhibitory effect of afatinib, blocking irreversibly all ErbB family members (14) and not only EGFR. Although in preclinical studies afatinib had demonstrated activity also in NSCLC with the acquired mutation Thr790Met (9) and the acquired resistance to anti-EGFR TKIs is due in about 50% of cases to this mutation (15).

Similar efficacy patterns were reported for afatinib compared with gefitinib regardless of EGFR mutation. Patients with Leu858Arg presented a median PFS of 10.9 in afatinib arm versus 10.8 months in gefitinib arm (P=0.086), and an ORR of 66% and 42%, respectively. Patients harbouring exon 19 deletion showed a median PFS of 12.7 months in afatinib arm versus 11.0 months in gefitinib arm (P=0.107), and a ORR of 73% and 66%, respectively.

This finding confirmed the evidence of previous literature supporting a better outcome with first generation TKIs for patients with NSCLC harbouring an exon 19 deletion as EGFR mutation (16,17). It suggests that exon 19 deletion and Leu858Arg define two distinct forms of NSCLC.

Among the adverse events in afatinib group any grade of diarrhoea, acne or skin rash were reported, while in gefitinib group were reported liver enzyme elevation and interstitial lung disease as expected. Grade >3 adverse events were increased with afatinib (31%) compared with gefitinib (18%).

The longer TTF could indicate an acceptable and manageable toxicity profile of afatinib besides a clinical benefit beyond radiological progression. Nevertheless, the open-label design of the trial may have biased TTF in favour of newer afatinib treatment.

The trial presented some other limitations. The authors themselves noted that the trial was designed as an exploratory phase 2B trial without a predefined hypothesis, with three co-primary endpoints and a statistical significance not corrected for multiple comparison. Moreover the immature data on OS precluded robust analysis.

However considering the third generation inhibitors in development, as AZD9291 (18) and rociletinib (19), data from LUX-Lung 7 are very interesting to design future trial about combination approaches and/or sequence strategy to overcome the acquired resistance mutations after a first-line treatment with an EGFR TKI.

Although no benefit in OS was reported in this trial in first-line setting, afatinib might be more effective than gefitinib, with a better PFS and response rate and a good toxicity profile, with a low impact on quality of life. These findings and clinical relevant endpoints such as disease control, survival prolongation, tolerability and quality of life are to be taken into account to choose the most appropriate treatment for every patient. In particular the superiority of afatinib versus gefitinib in terms of response rate could be considered for treatment choice in patients with symptomatic disease or with a large tumour burden.

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Footnote

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Patient reported outcomes from LUX-Lung 3: first-line afatinib is superior to chemotherapy—would patients agree?

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Abstract: The LUX-Lung 3 trial was an important randomized phase 3 trial in patients with *EGFR* mutant advanced non-small cell lung cancer (NSCLC). Here, patients were randomized to either afatinib or cisplatin-pemetrexed and the primary endpoint of progression-free survival (PFS) was easily met (HR=0.58, P=0.001). This was the first large-scale trial of this type using a modern chemotherapy comparator, including Asian and non-Asian patients, central radiology review, and utilizing comprehensive patient-reported outcomes. Whilst efficacy for afatinib was markedly superior to chemotherapy, do the patient-reported outcomes reflect this superiority? The symptom control and quality of life (QoL) data from this trial has now been published. Analysis of these demonstrate clear superiority of afatinib over chemotherapy for delay in cough deterioration, and dyspnoea. Notably, given the toxicity profile of afatinib, these improvements translated into significant improvements in global health status, physical, role, and cognitive functioning. The clinical benefits for afatinib over cisplatin-pemetrexed chemotherapy for *EGFR* mutation-positive advanced non-small cell lung patients seem overwhelming, and are clinically meaningful. These results are also consistent with QoL data from other trials of gefitinib/erlotinib, but much more robust, given the larger patient numbers. Would patients agree that afatinib is superior to chemotherapy? On the basis of data presented, the answer is probably "Yes". However, the key unanswered question remaining is "*Which is the best EGFR-tyrosine kinase inhibitor (TKI) to use up front?*" and we will have to wait until ongoing trial data can help answer this.

Keywords: Afatinib; quality of life (QoL); survival

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Eight large-scale clinical trials have now demonstrated the superiority of first-generation *EGFR*-tyrosine kinase inhibitor (TKI) (gefitinib/erlotinib) over platinum doublet chemotherapy (1-8). Afatinib is a second-generation *EGFR*-TKI designed to irreversibly inhibit *EGFR* kinase, including the T790M gatekeeper mutation that accounts for acquired resistance to gefitinib/erlotinib therapy in around 50% of cases (9). The LUX-Lung 3 trial was the first randomized trial of a second generation *EGFR*-TKI compared to a modern chemotherapy doublet—cisplatin-pemetrexed—in patients with treatment naïve *EGFR* mutant advanced non-small cell lung cancer (NSCLC) (6). The trial recruited both Asian and non-Asian patients, as was the largest trial in this indication thus far, utilizing independent radiology review. Afatinib demonstrated marked clinical efficacy over cisplatin-pemetrexed [progression-free survival (PFS) median 11.1 *vs.* 6.9 months, HR=0.58, 0.43-0.78, P=0.001; improving to PFS median 13.6 *vs.* 6.9 months, HR=0.47, 0.34-0.65, P=0.001 when restricted to the common mutations L858R and exon 19 deletions]. Toxicities for afatinib were as observed in previous trials, with diarrhoea, rash, and paronychia the most prevalent (≥ grade 3 adverse events 14.4%, 16.2%, 11.4%, respectively). Of course, these were the worst grade of toxicity reported per patient, and duration of afatinib therapy was markedly longer than that of cisplatin-pemetrexed.

The patient reported outcomes (PROs) from this trial, subsequently reported by Dr Yang are therefore welcome, to put the toxicity and efficacy balance into patient-related context (10). PROs were comprehensively assessed every

21 days until progression using the established EORTC QLQ-C30 and QLQ-LC13 tools, and compliance was high. Compared to chemotherapy afatinib significantly delayed time to deterioration of cough, and dyspnoea; more so in patients symptomatic at baseline. Whilst chemotherapy was associated with a greater proportion of patients reporting worsening of fatigue and nausea, afatinib was associated with worsening of diarrhoea, sore mouth, and dysphagia, but significant improvements in individual items related to activity. Afatinib-treated patients had significantly better mean scores over time for global health status/quality of life (QoL), physical role, and cognitive functioning. Whilst improvements in emotional and social functioning were not significantly improved compared to chemotherapy, mean treatment differences favoured afatinib.

So how do we interpret these findings? Overall afatinib therapy results in significantly improved symptoms that matter to lung cancer patients (dyspnoea and cough); symptoms that are difficulty to effectively palliate by symptom-control alone. These differences are important for a therapy type that has demonstrated marked clinical efficacy by nearly doubling PFS but not improving overall survival (likely due to cross-over to alternative *EGFR* TKI use in the chemotherapy arm post progression), thereby validating the clinical benefit of this therapy. Whilst the typical afatinib toxicities of diarrhoea, skin rash, and paronychia featured in the PRO symptom analyses, longitudinal analysis of global health status compares favourably for afatinib over chemotherapy. Moreover, rates of afatinib-related adverse events seem to have reduced in more recent trials, perhaps due to increasing pre-emptive management strategies, and increased clinical experience with afatinib, although under-reporting cannot entirely be excluded. Thus, in the LUX-Lung 6 trial of afatinib versus cisplatin-gemcitabine in *EGFR* mutant NSCLC (a trial identical to LUX-Lung 3 other than the use of gemcitabine in place of pemetrexed, and set entirely in East Asia) rates of grade 3-4 toxicities diarrhoea, rash, and paronychia have reduced to 5.4%, 14.6%, and 0%, respectively (7). Clearly the patient-reported outcome data from this trial will be important to review to understand the clinical relevance of this reduced reported toxicity profile.

So, would patients agree that afatinib is superior to chemotherapy? The answer is probably "Yes". However, the key question that remains unanswered, is "*What is the optimal EGFR TKI to use in this setting*?" Other first generation *EGFR* TKIs gefitinib and erlotinib have both demonstrated marked clinical efficacy over platinum-doublet chemotherapy. These studies have also demonstrated similar improvements in PRO metrics, for an improvement in lung-cancer associated symptoms and prolongation of time to deterioration of symptoms for gefitinib/erlotinib, although the instruments used in these trials were different to LUX-Lung 3, thereby prohibiting direct comparisons.

Overall, the field is now replete with randomized trials that have comprehensively identified that *EGFR*-directed therapy with gefitinib, erlotinib, or afatinib is clinically superior to platinum-doublet chemotherapy in treatment-naïve *EGFR* mutant advanced NSCLC, and further trials in this paradigm should not now be performed. However, the key question now unanswered for both patients and oncologists alike is "*Which is the best EGFR-TKI to use up front?*" The suggestion of a median PFS for common *EGFR* mutants of 13.6 months with afatinib from LUX-Lung 3, compared with 9-10 months typically observed for gefitinib/erlotinib might suggest potential superiority, but such cross-trial comparisons are fraught with danger and are perilous at best. However, the LUX-Lung 7 trial (NCT01466660) may potentially answer this question. This randomized trial of afatinib versus gefitinib for *EGFR* mutant NSCLC has now completed accrual and results are awaited. In the interim, treatment-naïve *EGFR* mutant patients have robust, clinically-meaningful data to support the use of afatinib should they and their oncologists chose.

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Footnote

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Known and putative mechanisms of resistance to EGFR targeted therapies in NSCLC patients with EGFR mutations—a review

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Abstract: Lung cancer is the leading cause of cancer related deaths in Canada with non-small cell lung cancer (NSCLC) being the predominant form of the disease. Tumor characterization can identify cancer-driving mutations as treatment targets. One of the most successful examples of cancer targeted therapy is inhibition of mutated epidermal growth factor receptor (EGFR), which occurs in ~10-30% of NSCLC patients. While this treatment has benefited many patients with activating EGFR mutations, almost all who initially benefited will eventually acquire resistance. Approximately 50% of cases of acquired resistance (AR) are due to a secondary T790M mutation in exon 20 of the EGFR gene; however, many of the remaining mechanisms of resistance are still unknown. Much work has been done to elucidate the remaining mechanisms of resistance. This review aims to highlight both the mechanisms of resistance that have already been identified in patients and potential novel mechanisms identified in preclinical models which have yet to be validated in the patient settings.

Keywords: Epidermal growth factor receptor (EGFR); molecular targeted therapy; drug resistance; antineoplastic

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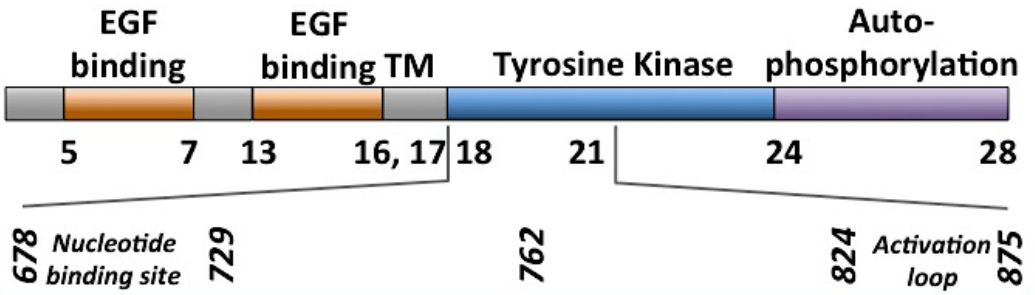
Introduction

Lung cancer is the leading cause of cancer related deaths in Canada (1). In the developed world, non-small cell lung cancer (NSCLC) is the predominant form of the disease, accounting for approximately 85% of cases (2). The advent of molecular profiling has led to the discovery of "driver mutations", targeted therapy, and personalized medicine. Some of the earliest driver mutations discovered and targeted were mutations in the *epidermal growth factor receptor* (*EGFR*) gene (*Figure 1*). EGFR is a receptor tyrosine kinase which, once activated by binding ligand and receptor dimerization, transphosphorylates its cytoplasmic tails, activating cellular signaling pathways such as the phosphoinositide 3-kinase (PI3K)-AKT pathway, the STAT pathway, and the MAPK pathway, ultimately leading to increased cell proliferation, migration, and survival (3-6). Approximately 10-30% of NSCLC patients have activating mutations in *EGFR* (7-9). Targeting EGFR in these patients with activating mutations has shown initial and significant success in the clinic (10,11).

Classical activating mutations, such as the exon 19 deletions and exon 21 L858R substitution, account for approximately 45% and 40% of all *EGFR* mutations, respectively; these two mutations are associated with good responses to EGFR-targeted small molecule inhibitor therapies (11). Initially, these mutations were shown to destabilize the auto-inhibited conformation of the receptor (the normal state of the receptor in the absence of ligand) thus causing constitutive activation of the kinase domain (12-14). More recently, Shan *et al.* (15) reported that the L858R mutation causes a partially disordered state of the EGFR kinase which promotes dimerization and thus aberrant activation. Dixit and Verkhivker (16) recently published the sequence and structure-based computational model which predicted that the L858R mutation synergistically shifts EGFR towards the active state and favours the formation of the asymmetric



EXON 18	EXON 19	EXON 20	EXON 21
G719X (3%)	LREA deletion (45%)	V765A (<1%)	L858R (40%)
	VAIKEL insertion (1%)	T783A (<1%)	L861X (2%)
	L747S (<1%)	V774A (<1%)	T854A (<1%)
	D761Y (<1%)	S784P (<1%)	A871E (<1%)
		T790M *	
		Exon 20 insertion (4%)	
		V769M (<1%)	
		V769M (<1%)	

Figure 1 Missense mutation is represented by the reference amino acid, followed by the residue number, followed by the mutant residue. For summary of somatic mutations found in EGFR. Mutations in green are typically sensitive to EGFR TKIs, those in red are typically resistant. Approximate frequency of occurrence in NSCLC patients of each mutation is shown in parentheses. *T790M is found in ~5% of pre-EGFR TKI treated patient samples and ~60% of post-EGFR TKI treated patient samples. Horizontal numbers represent exons, vertical numbers represent amino acid residues. X indicates when one amino acid has been shown to be replaced by multiple different amino acids, as example, the glycine at position 719 has been shown to be mutated to an alanine, cysteine, or serine. LREA: string of amino-acids leucine, arginine, glutamate, and alanine). VAIKEL: string of amino-acids valine, alanine, isoleucine, lysine, glutamate, and leucine). TM, transmembrane domain; EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors [Modified from Sharma *et al.* (3)].

dimer. The L858R activating mutation has also been shown to decrease ATP binding affinity. Yun *et al.* (17) report that this decreased affinity for ATP essentially creates a "therapeutic window", which renders the oncogenic EGFR mutants more easily inhibited by TKIs, as they now have higher binding affinity than, and thus can outcompete ATP.

Over the years, drugs have been developed which specifically target EGFR. One such class is a group of small molecule inhibitors that inhibit the tyrosine kinase domain of EGFR, and are thus referred to as tyrosine kinase inhibitors (TKIs). The first TKIs shown to have clinical benefit were gefitinib and erlotinib (10,11,18). These two TKIs are considered first-generation; they reversibly bind to the tyrosine kinase domain of EGFR (19). First-generation EGFR TKIs have shown significant success clinically in patients with the most common activating *EGFR* mutations. As first-line treatments, EGFR inhibitors have been shown to produce overall response rates (ORRs) of close to 75% in patients who harbor activating mutations in *EGFR* (3,20,21).

Despite this, the vast majority of patients develop resistance to treatment; the median progression free survival (PFS) after treatment with a first generation EGFR TKI in patients with activating mutations is typically less than one year (20-22). Numerous biological mechanisms of acquired resistance (AR) have been elucidated (*Figure 2*), but in up to 30% of patients, the mechanism of resistance remains unknown (23). To date few patients have been cured by an EGFR TKI alone and almost all patients eventually acquire resistance and relapse (21,24). This review aims to give an overview of the most common mechanisms of primary and AR as well as highlight novel, newly emerging theories.

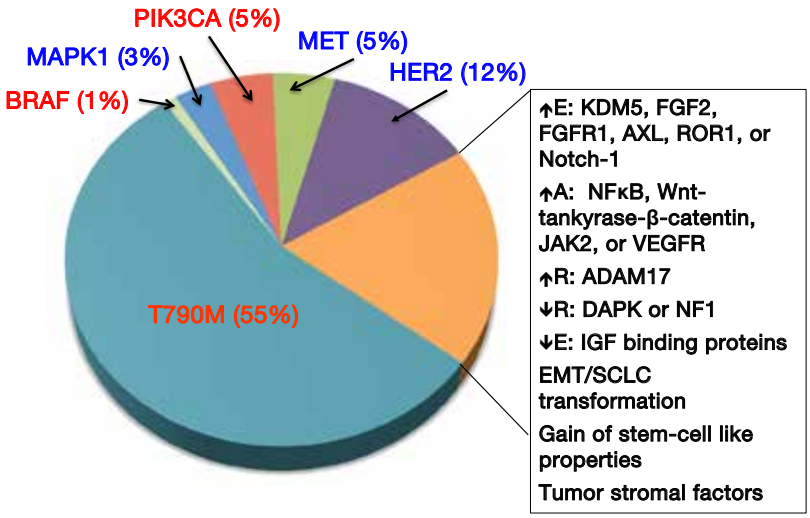


Figure 2 Summary of mechanisms of resistance to first generation EGFR TKIs. Reported occurrence of each mechanism varies somewhat cohort to cohort, thus the shown prevalence rates are approximations. Red text represents mutations, blue text represents amplifications. ↑E, increased expression; ↑A, increased activation; ↑R, up-regulation; ↓R, down-regulation; ↓E, loss of expression.

Primary resistance

EGFR somatic mutations

Depending on the mutation present in *EGFR*, tumors exhibit differential TKI sensitivities. While the most common EGFR-activating mutations, L858R and exon 19 deletion, typically confer sensitivity to EGFR TKIs, other primary EGFR mutations can confer resistance. Exon 20 insertions or duplications, which account for approximately 4-9% of EGFR mutations, appear to be resistant to EGFR inhibitors *in vivo*, despite the fact that these mutations appear to also be activating mutations, at least *in vitro* (25-33). Most of these insertions occur between amino acids 767 to 774 (31). The crystal structure of the exon 20 D770_N771insNPG *EGFR* mutant revealed that the ATP-binding pocket is unaltered, thus EGFR is activated without increasing its affinity for EGFR TKIs (34). Interestingly, loss of these activating *EGFR* mutant genes has been reported *in vitro*, which leads to a decrease in addition to EGFR signaling, gained addiction to both HER2/HER3 and PI3K/AKT signaling, and thus AR to EGFR TKI (35). Other, much less frequent, primary *EGFR* mutations such as G719X and L861X, have been reported (*Figure 1*) (36,37).

Although recognized mainly as a mechanism for AR, another *EGFR* exon 20 mutation, T790M, has also been associated with primary resistance. This mutation is within the gatekeeper residue, and restores the L858R mutant receptors affinity for ATP to wild-type levels, thus decreasing the effect of TKIs (38). Biochemical studies have demonstrated synergistic kinase activity and transformational potential when T790M is concurrently expressed with a TKI-sensitizing, EGFR-activating mutation (39,40).

Minor clones with the T790M mutation have been identified in treatment-naive tumors that contain classic sensitizing mutations. While this mutation has low allelic frequencies in treatment-naive tumors, pressure from TKIs may select for enriched growth of these T790M clones, leading to overall AR. As allelic dilution most likely obscures the detection of *de novo* T790M mutations via conventional Sanger sequencing methods, higher sensitivity assays such as high-performance liquid chromatography, mass spectrometry, locked nucleic acid PCR techniques and next generation sequencing have been suggested as alternate screening methods (41-47). Recent studies using these more sensitive techniques have reported T790M mutations in 35%, 38%, and 79% of *EGFR*-mutant, NSCLC pretreatment specimens (48-50). Interestingly, Rosell *et al.* (48) reported that low levels of BRCA-1 negates the desensitizing effects of the T790M mutations and is associated with longer PFS to erlotinib. Conversely, high levels of BRCA-1 lead to increased DNA damage repair capacity and thus *de novo* resistance.

EGFR germ line polymorphisms associated with primary resistance

T790M

This mutation has also been identified rarely in patients as a germline polymorphism; it has been identified in 0.5% of never smoker-lung cancer patients' blood samples (51). Furthermore, the T790M mutation has also been putatively associated with familial cancer syndromes (52). In short, the proband's mother, maternal grandfather and great uncle all succumbed to bronchioloalveolar carcinoma in their 60's and 70's. Furthermore, three out of the four siblings, including the proband, also developed lung cancer; two of these individuals (including the proband) failed to respond to gefitinib treatment, alone or in combination with chemotherapy. The third sibling was only recently diagnosed at the time of the referenced publication, thus their cancer treatment and subsequent response were not reported. Tumor specimens were available from two of the siblings (five independent primary tumors from the proband and a biopsy from metastatic disease from a brother). *EGFR* sequencing identified the T790M mutation in all tumors in a 1:1 ratio with the wild-type allele. Three of the five tumors from the proband had additional EGFR somatic mutations that typically respond to EGFR TKI therapy (two with L858R, one with delL747_T751); the biopsies from the remaining two primary tumors revealed no additional mutations in *EGFR*. The biopsy from the brother's metastatic lesion also harbored the G719A *EGFR* mutation, which typically confers sensitivity to EGFR TKI therapy. Most intriguingly, the T790M mutation was also present in the germline (measured from peripheral blood mononuclear cells) of both individuals as well as their other two siblings (52). In the report by Girard *et al.* (51), no response information to EGFR TKI was reported.

V843I

In 2008, there was a care report about a woman with a family history of lung cancer (father and a brother) who was diagnosed with multiple adenocarcinomas that exhibited either L858R or L861Q *EGFR* mutations as well as a rare germline *EGFR* mutation, V843I. Three of her four remaining siblings were sequenced, two of whom also harbored the germline mutation, neither of whom had developed lung cancer despite their advanced age (67 and 72 years of age) (53). Another report was published in 2011 on a family with a history of cancer where four of the family members exhibited the germline V843I mutation (54). Three of these family members developed lung cancer, and all of them had the *EGFR* somatic L858R mutation. Only the proband underwent EGFR TKI therapy, however they did not respond to either gefitinib or erlotinib. The most recent report of this germline variation was in 2013, which described the first Caucasian patient with this mutation as well as the first patient without concomitant additional known *EGFR*-activating mutation (55). This patient did not respond to erlotinib and their tumors continued to grow rapidly while on this treatment. Modeling analysis of V843I suggests that ATP and TKI affinities for EGFR are not affected by this mutation; the mechanism of action for a possible germ line predisposition of V843I to develop lung cancer remains unknown. Matsushima *et al.* (56) demonstrated that the V843I mutation increased the phosphorylation of EGFR and downstream signaling proteins compared to wild type EGFR, especially when induced by EGF, suggesting a potentially oncogenic role for this mutation. Furthermore, they demonstrated that the double V843I/L858R mutant did not have increased phosphorylation levels, however the double mutant was resistant to erlotinib, gefitinib, afatinib and dacomitinib. Finally, structural modeling suggests that TKI binding to EGFR would be sterically hindered by Arg841 in the V843I/L858R double mutant (56).

Other genetic polymorphisms

BIM

Despite our furthered understanding of the sensitizing effects that various *EGFR* mutations have to TKIs, patients with identical mutations can demonstrate a spectrum of responses. One explanation for this variability in responses lies within the apoptotic machinery. Recent studies have demonstrated up-regulation of BIM in response to EGFR TKIs in mutant cell lines, which correlated with apoptotic response. *EGFR*-mutant patients with low BIM expression prior to treatment exhibited less tumor shrinkage and shorter PFS after TKI therapy (57-61). Variances in BIM expression levels have been suggested to be due to a genetic polymorphism in BIM, leading to alternative splicing and altered function (58,59,62,63). Clinically, the *BIM* deletion polymorphism has been reported in 12.9% of East Asian individuals. Furthermore, patients with NSCLC who harbor this *BIM* polymorphism exhibit significantly inferior responses to EGFR-TKI treatments compared to wild-type *BIM* counterparts (64). Indeed, Nakagawa *et al.* (64) demonstrated sensitization in EGFR-TKI resistant cell lines that harbor BIM polymorphisms by combination therapy

with HDAC inhibitor vorinostat. Recent results from the randomized phase III EURTAC trial demonstrated that high BIM expression prior to treatment was a marker of longer PFS (HR =0.49; P=0.0122) and overall survival (HR =0.53; P=0.0323) (65). As such, BIM appears to act as both a biomarker and mediator of TKI-induced sensitivities in several oncogene-driven cancers.

Acquired resistance (AR)

Secondary EGFR mutations

The earliest reported mechanism of resistance to TKIs in *EGFR*-mutant NSCLC is the T790M mutation (see previous section on primary resistance), which accounts for approximately 50-60% of cases with AR to EGFR TKI therapy (24,66-69). Despite the multiple avenues of enhanced oncogenicity, tumors harboring T790M mutations often exhibit surprisingly slow growth rates (70). A retrospective study examining T790M status on rebiopsy specimens from 93 patients with *EGFR*-mutant lung cancer and AR to TKIs found that T790M patients had a better prognosis. Furthermore, lack of T790M at time of rebiopsy was associated with a poorer performance status at progression, earlier development of new metastatic disease sites, as well as shorter post-progression survival (24).

Other secondary mutations in *EGFR* linked to AR have also been identified such as D761Y, T854A, and L747S. However, the structural basis for how these mutations confer resistance remains unknown (71-73).

Gene copy alterations of alternative pathways

MET

Amplification of the *MET* gene is considered one of the more common causes of AR in *EGFR*-mutant NSCLC. Heterodimerization of MET and ERBB3 leads to sustained activation of the PI3K/AKT signaling pathway, bypassing the inhibition of EGFR conferred by TKIs (74). Initial reports suggested that *MET* amplification accounted for approximately 22% of AR cases, independent of T790M status. However, two recent studies, each testing 37 patients with AR to EGFR TKIs for MET amplification by FISH, suggest that this prevalence is closer to 5% (44,75). This discrepancy between studies may be in part due to technical difficulties in identifying this genetic alteration in clinical samples. The initial studies with the higher reported percentage of *MET* amplification used several methods of assessment such as array comparative genomic hybridization (aCGH), quantitative real-time PCR, as well as FISH. On its own, FISH is the most widely acceptable technique in clinical laboratories, however technical difficulties arise due to both *MET* and *EGFR* being on chromosome 7. Furthermore, polysomy of chromosome 7 is common in NSCLC, particularly in samples with *EGFR* activating mutations (76). As such, it's been suggested that new clinical protocols to distinguish meaningful *MET* amplification and copy number gain from underlying polysomy in both *EGFR*-mutant and wild-type lung cancers, is required. Aberrant activation of MET and subsequent AR has also been reported via excessive hepatocyte growth factor secretion, the natural ligand for MET (77,78). MET-amplification may not be solely a mechanism of AR but also an inherent event. Low frequencies of MET-amplified subclones have been identified in treatment naive specimens (79). Similar to the development of AR in tumors with low frequencies of T790M, the dominant mechanisms of AR at the time of disease progression in the majority of these cases has been *MET* amplification (80). Recent and on-going attempts to overcome AR due to overriding EGFR inhibition via aberrant MET signaling is to inhibit both receptors simultaneously (80-83). Overall, there is reasonable rationale for clinical trials to evaluate MET inhibitors in patients who developed AR to EGFR TKI therapy via MET amplification mechanism.

HER2 amplification

Recently, amplification of *HER2* has been reported in three of 26 (12%) *EGFR*-mutant NSCLC patients who have AR to TKIs. Similar to *MET*, it is believed that *HER2* is able to signal parallel to inhibited EGFR and thus reactivate common downstream signaling pathways (84).

MAPK amplification

Due to *KRAS* mutations' associations with primary resistance to EGFR inhibitors, recent studies have focused on RAS/MAPK signaling as potential mechanisms of AR (85). *KRAS* mutations themselves are known to be mutually exclusive with *EGFR* mutations in patients. Thus, despite their role in primary resistance, no *KRAS* mutations have been identified in *EGFR* mutant patients with AR (75,85,86). However, Ercan *et al.* (87) identified MAPK1 amplification in an erlotinib-resistant *EGFR*-mutant NSCLC patient. The investigators further demonstrated that a mechanism of resistance to the irreversible EGFR TKI WZ4002 was increased ERK signaling due to amplification of MAPK or down regulation of negative

regulators of ERK signaling. This resistance was overcome by inhibition of MEK or ERK and prevented the development of subsequent resistance.

Mutations in downstream effector molecules of EGFR

PIK3CA mutations

Alternative to parallel pathways being activated, downstream effector molecules of the EGFR signaling pathway have also been reported to be mutated, leading to AR (76). *PIK3CA* mutations have been reported in 5% of *EGFR*-mutant patients who have AR and preclinical studies demonstrate the ability of these mutations to confer resistance via activation of downstream AKT (88). PI3K phosphorylates PIP2 to PIP3; *PTEN (*phosphatase and tensin homolog), reverses this phosphorylation. The loss or decreased expression of PTEN has also been linked to AR (89,90).

BRAF mutations

A recent retrospective study identified point mutations in *BRAF* in two out of 195 (1%) lung cancer patients with AR to EGFR TKIs. The investigators further confirmed *BRAFs* potential role in AR by inducing ectopic expression of mutant BRAF in drug-sensitive *EGFR*-mutant cells, inducing resistance to EGFR TKIs. The addition of a MEK inhibitor was able to overcome induced resistance (86).

Epigenetic and other mechanisms

Epigenetic

Although the genetic basis for acquiring TKI resistance has been well established, a number of recent observations reveal a reversible epigenetic mechanism of drug resistance. Firstly, genetic mechanisms alone cannot account for the high prevalence of TKI-resistant tumors. Secondly, many NSCLC patients who previously developed TKI resistance respond to TKI again after being off the drug for a period of time. Such a phenomenon indicates that acquired TKI resistance might not require a permanent genetic alteration. Thirdly, there is still a significant proportion of TKI resistant tumors that do not harbor any known genetic alterations and activation of alternative signaling pathway. Finally, tumors exhibit not only genetic but also epigenetic heterogeneity within cell populations (91,92).

Epithelial-to-mesenchymal transition (EMT)

EMT, as the name suggests, is a cellular phenotypic change. It can be characterized molecularly by a loss of epithelial markers such as E-cadherin, and a gain of mesenchymal markers, such as vimentin (93). At the cellular level, EMT leads to enhanced motility, invasiveness, and *in vitro* EGFR TKI resistance (94-96). EMT has also been identified in subsets of clinical EGFR TKI-resistant specimens. Despite the growing evidence that EMT may play a role in resistance to treatments, the underlying biology of this change and specific mechanisms of resistance remain unknown (75). Recent work demonstrated the efficacy of blocking ERK1/2 in preventing EMT in lung cancer cells and enhancing their sensitivity to EGFR TKIs. By inhibiting MEK1/2 (MAPKK1/2), an epithelial phenotype was promoted and maintained in NSCLC cells despite exogenous stimulation by TGF-beta. Furthermore, cells that exhibited *de novo* or AR to gefitinib demonstrated decreased cell migration and enhanced sensitivity to the EGFR TKI when MEK was inhibited long enough to trigger changes in EMT marker expression (97).

Histological transformation

Several studies have reported the histological transformation to small cell lung cancer in *EGFR* mutant NSCLC patients with acquired EGFR TKI resistance, accounting for resistance in possibly up to 3% of the patients. Interestingly, the conversion to SCLC was associated with sensitivity to standard SCLC treatment while the original *EGFR* mutation was still maintained in the tumor (75,98). The mechanism underlying this histological transformation still remains unknown.

AXL activation

AXL is a tyrosine kinase receptor which induces cell proliferation, migration and invasion in cancer. Recently, several groups reported that activation of AXL signaling pathway may confer TKI resistance in *EGFR* mutant NSCLC (99,100). Activation of AXL signaling pathway can occur through overexpression of AXL or its ligand GAS6. Small-molecule AXL inhibitors, MP-470 and XL-880 were able to restore the TKI sensitivity in TKI resistant NSCLC cells. Forced overexpression of AXL in TKI sensitive NSCLC cells can confer TKI resistance. These investigators also found an association between the overexpression of AXL and vimentin, a marker of EMT in the TKI resistant NSCLC cells. In their exploratory analysis of patient samples, approximately 20% of EGFR TKI resistant NSCLC patients were found to have tumors with upregulated AXL, GAS6 and vimentin.

NF-κB activation

NF-κB is an important transcription regulator of the genes that controls cell proliferation and cell growth, including tumor growth. Bivona *et al.* (101) reported previously that activation of NF-κB signaling pathway can confer TKI resistance in *EGFR* mutant NSCLC cells. The investigators introduced a shRNA library to target >2,000 cancer relevant genes in the TKI insensitive H1635 NSCLC cell line. This line had an *EGFR* mutation, but no other identifiable mutations or activation of alternative signaling pathways that could confer insensitivity to EGFR TKI. Among the screen hits conferring TKI sensitivity in H1635, 18 target genes were linked to the NF-κB signaling. Inhibition of NF-κB signaling could enhance TKI sensitivity in H1635 and other *EGFR*-mutant NSCLC cells, and they reported that higher NF-κB activation state was correlated with worse PFS and decreased overall survival in *EGFR*-mutant NSCLC patients treated with TKI. However, a recent clinical study of the combination of PF-3512676, an inhibitor for toll-like receptor 9 which activates NF-κB, and erlotinib did not increase PFS as compared to erlotinib alone in patients with advanced recurrent *EGFR*-mutant NSCLC patients (102).

IGF1-R and KDM5A activation

Sharma *et al.* (103) reported that a subpopulation of NSCLC tumors developed reversible TKI resistance by engaging the IGF1-R signaling pathway and an altered chromatin state due to a histone demethylase, KDM5A. These TKI resistant cells had upregulated IGFBP-3, KDM5A and increased phosphorylation of IGF-1R. In this subpopulation, IGF1-R inhibitor, depletion of KDM5A or histone deacetylases (HDACs) could markedly suppress the TKI-resistant outgrowth of NSCLC cells in combination with TKI by restoring the TKI sensitivity of TKI resistant cells. Furthermore, inhibition of IGF1-R could lead to decreased KDM5A expression and restoration of H3K4 methylation, suggesting a direct link between IGF-1R signaling pathway and KDM5A function. Altogether, the authors demonstrated that a transient altered chromatin state could potentially mediate TKI resistance in NSCLC. Unfortunately, a recent randomized Phase II study concluded that the combination of IGF1-R inhibitor (R1507) with erlotinib did not provide any PFS or survival advantage over erlotinib alone in unselected NSCLC patients (104). A clinical study to evaluate the combination of erlotinib and HDAC inhibitor, SNDX-275 *vs.* erlotinib alone in treatment of NSCLC patients has just been completed (NCT000602030), but the results have not been reported.

Other alternative signaling pathway activation

Recently many more signaling pathways have been reported to mediate resistance to EGFR TKI in NSCLC models, but as yet lack evidence for efficacy in patients. These pathways include: activation of Wnt-tankyrase-β-catenin pathway; reduced expression of NF1; downregulation of DAPK through DNA methylation of its CpG island; overexpression of FGF2 and FGFR1 in FGF2-FGFR1 autocrine pathway; upregulation of ADAM17 in heregulin-HER3 autocrine loop; activation of JAK2-related signaling pathway; overexpression of ROR1 caused by NKX2-1; activation of VEGF signaling pathway in stromal cells; overexpression of Notch-1 and its enhancement of EMT; loss of IGF binding proteins; acquisition of stem-cell like properties; and involvement of tumor stroma and cancer-associated fibroblasts derived from EGFR-TKI-resistant tumors (105-118). Many of these pathways have been known to be relevant in cancer development and progression.

Current clinical strategies to overcome AR

When patients relapse secondary to AR, alternative treatment strategies are desired. There is increasing evidence to support patient tumor rebiopsy upon development of resistance to determine the optimal second-line treatments; some cancer centers and clinical trials are already implementing this strategy (119,120). For various cancer sites, rebiopsy is a fairly simple procedure. For lung cancer patients, however, rebiopsy is often a highly invasive procedure, and in many cases, there is a difficult choice of which of multiple metastatic sites should be considered for biopsy. Some patients who develop initial resistance to an EGFR TKI respond again upon a second challenge, after a defined period of a TKI drug holiday (121-124). Song *et al.* reported that, based on multiple studies, over 50% of patients who progressed on a first line EGFR TKI and then stopped the TKI treatment, benefited from a subsequent second course of the same EGFR TKI (125). There is currently a poor understanding of the mechanisms of reversal of resistance conferred by such a drug holiday.

Optimal therapies have not been established for the majority of *EGFR*-mutant lung cancer patients who develop disease progression after merely 10 to 14 months on TKIs (20,24,126). *Table 1* summarizes the results of clinical trials to date using second and third generations TKIs that were supposed to overcome AR. Second-generation EGFR TKIs

Table 1 Response rates to second and third generation EGFR TKIs in clinical trials

Agent	Study	Prior chemo-therapy	Prior EGFR TKI therapy	EGFR mutation required	No. of pts with EGFR mutation	ORR (in all pts, %)	No. pts with T790M	ORR (in T790M+ pts, %)
Second generation TKI								
Neratinib	NCT00266877 (127)	Yes & no	Yes & no	No	91	3	12	0
Afatinib	LUX-Lung 3 (128)	No	No	Yes	345	56	NR	NR
	LUX-Lung 6 (129,130)	No	No	Yes	242	67	NR	NR
	LUX-Lung 2 (131)	Yes & no	No	Yes	129	61	1	NR
	LUX-Lung 1 (132)	Yes	≥12 weeks E/G	No	62	7	4	NR
	LUX-Lung 4 (133)	Yes	≥12 weeks E/G	No	56	8	2	NR
	LUX-Lung 5 (134)	Yes	≥12 weeks A	No	NR	32	NR	NR
Afatinib + cetuximab	NCT01090011 (135)	Yes	Yes	Yes	126	29	71	32
Dacomitinib	ARCHER 1009 (136)	Yes	Yes	No	47	11	NR	NR
	BR26 (137)	Yes	Yes	No	157	7	NR	NR
Third generation TKI								
AZD9291	NCT01802632 (138)	Yes	Yes	Yes	199	55	132	64
HM61713	NCT01588145 (139)	Yes	Yes	Yes	93	17	27	66
CO-1686	NCT01526928 (140)	Yes	Yes	Yes	88	58*	55	58*

pts, patients; EGFR, epidermal growth factor receptor; NR, not reported; ORR, objective response rate; E/G, erlotinib/gefitinib; A, afatinib; TKI, tyrosine kinase inhibitor; *, ORR was calculated from phase 2 which included only T790M+ pts.

have been developed to overcome resistance, however, results from clinical trials have not been as promising as was anticipated.

Second-generation EGFR TKIs form irreversible covalent bonds with the ATP-binding site of EGFR as well as other members of the HER family of receptors (excluding Her3). Neratinib (HK1-272) did not show good response rates (RR) in patients with T790M mutations thus further development was halted (127). Afatinib (BIBW2992) has been investigated as a second- and third-line treatment in patients who have AR to first-generation EGFR TKIs (LUX-Lung 1, 4, and 5 program) and as a first-line treatment in *EGFR*-mutant patients (LUX-Lung 2, 3, 6 and 7). Thus far, afatinib has been shown to improve the disease control rate and prolong PFS in both LUX-Lung 1 and 2 (131,132). The LUX-Lung 4 trial demonstrated a modest benefit of afatinib as a third- or fourth-line treatment for patients who had previously progressed while receiving erlotinib and/or gefitinib (133). The LUX-Lung 5 trial demonstrated the benefit of combining paclitaxel with afatinib after patients with AR to gefitinib and/or erlotinib progress on afatinib monotherpy (134). Dacomitinib (PF-00299804), another second-generation, irreversible pan-HER TKI, has shown activity against NSCLC cell lines that harbor the T790M mutation. Dacomitinib efficacy was studied in two phase II trials. The first was to evaluate benefit (compared to erlotinib) after failure of one or two chemotherapy regimens, the second compared its benefit as a second- or third-line treatment in patients with advanced NSCLC after failure of at least one prior chemotherapy regimen and prior treatment with erlotinib (141,142). While the results of these two studies seemed initially promising, two randomized phase 3 studies, the ARCHER 1009 trial and the NCIC CTG BR.26 trial, failed to meet their objectives (136,137). The ARCHER 1009 trial did not demonstrate any statistically significant PFS in advanced NSCLC patients treated with dacomitinib compared to erlotinib in the second- and third-line therapy of advanced NSCLC (136). The NCIC CTG BR.26 trial, which included patients with advanced NSCLC who failed previous standard therapy with both chemotherapy and an EGFR TKI, failed to demonstrate significant prolongation of overall survival in those treated with dacomitinib versus placebo, though there was significant improvement in response rate, PFS and time to symptom deterioration in patients with *KRAS* WT NSCLC (137). In neither of these

trials were patients selected specifically for the presence of the T790M mutation.

Recent studies have demonstrated the benefit of combining therapies in overcoming resistance that arises through secondary mutations in the driver oncogene. In both cell line-derived and transgenic mouse models harboring T790M mutations, concurrent administration of the irreversible EGFR TKI, afatinib, and EGFR monoclonal antibody, cetuximab, resulted in dramatic tumor shrinkage (143). A phase I/II trial investigating the same drug combination in NSCLC patients with *EGFR* mutations and AR to EGFR TKIs demonstrated responses in 40% of patients (135,143). The mechanisms underlying the synergistic effect of this combination appear to be a dramatic inhibition of both phosphorylated EGFR and total EGFR. In contrast, afatinib appears to affect only phosphorylated EGFR and cetuximab appears to only affect the total EGFR protein expression (143). Meador *et al.* (144) developed resistance to the afatinib/cetuximab combination in PC-9/BRc1- (exon19 deletion/T790M mutant *EGFR* NSCLC cell line) derived xenografts and found that this occurred via the additional amplification of the *EGFR* gene. They further demonstrated sensitivity in this resistant model to the third-generation EGFR TKI AZD9291.

Third-generation EGFR TKIs specifically target both activating mutations and T790M mutations in *EGFR*. These agents seem promising; early results from phase I trials on three 3rd generation EGFR TKIs were presented at the 2014 ASCO Annual Meeting. The first study of HM61713 in advanced NSCLC patients with EGFR mutations who had failed previous EGFR TKIs (NCT01588145) demonstrated disease control rates of 76.5% when treated <4 weeks, and 73.1% when treated ≥4 weeks; 18 of 27 patients carrying T790M mutations showed a decrease in the target lesion sizes (139). The use of AZD9291 in EGFR mutant NSCLC patients (NCT01802632) resulted in (unconfirmed) response rates of 64% in 89 patients with T790M (with disease control in 96%) and only 23% in 43 patients without T790M mutations documented. Importantly, RECIST responses were observed at all dose levels and in brain metastases (138). For the 3rd generation EGFR TKI, CO-1686 (NCT01526928), preliminary results found that, of nine patients carrying T790M mutations, six demonstrated partial responses (PRs), two achieved stable disease, and the final patient achieved PR after transitioning to the HBr form of CO-1686 (140). Despite these promising, early clinical results, resistance to at least one of these third-generation TKIs, CO-1686, has already been demonstrated by an EMT mechanism (145).

Summary

Targeting EGFR in NSCLC patients with activating mutations holds great promise, however AR remains a currently insurmountable hurtle. Mechanisms behind AR have been identified in patients, such as secondary mutations within *EGFR*, activation of alternate proteins that are downstream of EGFR signaling or activation of proteins that feed into the EGFR signaling cascade. Further mechanisms of AR have been identified in cell lines and remain to be observed in patients. Novel treatment regimens of EGFR TKIs in combination with therapies that target EGFR in different ways or that target alternate proteins are being attempted to overcome known mechanisms of resistance. Third generation EGFR TKIs are being developed in the hopes of overcoming the most common mechanisms of resistance, T790M; to date, the results are preliminary but excitingly optimistic.

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Footnote

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Whacking a mole-cule: clinical activity and mechanisms of resistance to third generation EGFR inhibitors in *EGFR* mutated lung cancers with *EGFR*-T790M

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Abstract: Epidermal growth factor receptor (*EGFR*) mutations, especially *EGFR*-exon 19 deletions and *EGFR*-L858R, are the most frequent actionable genomic events in lung adenocarcinomas. Tumors arise due to constitutively activated EGFR signaling and are susceptible to EGFR tyrosine kinase inhibitors (TKIs). First generation EGFR TKIs (gefitinib and erlotinib) and the second generation EGFR TKI afatinib are approved worldwide. Although targeted therapies against EGFR mutants induce dramatic initial responses, acquired resistance (through multiple biological mechanisms) to erlotinib, gefitinib and afatinib emerges within the first 1-2 years of continued monotherapy. *EGFR*-T790M accounts for more than half of acquired resistance to first or second generation EGFR TKIs by modifying ATP affinity and drug binding kinetics. Two new studies have shown that two covalent pyrimidine inhibitors—AZD9291 and rociletinib of EGFR-T790M (i.e., third generation EGFR TKIs) shown remarkable clinical activity in patients with acquired resistance to erlotinib, gefitinib and afatinib when the tumor carries *EGFR*-T790M in conjunction with an activating mutation. However, and regrettably, acquired resistance to these third generation EGFR TKIs has already been reported in preclinical models and clinical specimens; such as a tertiary mutation at *EGFR*-C797S that prevents covalent binding of EGFR TKIs. The experience with sequential EGFR TKI monotherapy highlights tumor heterogeneity and adaptability (i.e., relentless game of whack-a-mole played between TKIs and cancer), and will help shape future clinical development of novel combinatory approaches to manage *EGFR* mutated lung adenocarcinomas.

Keywords: Epidermal growth factor receptor (EGFR); T790M; C797S; AZD9291; rociletinib; CO-1686; acquired resistance

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The mutational landscape of lung adenocarcinomas is complex and defined by heterogeneous subpopulations of tumors that can be addicted to oncogene-driven proliferative and anti-apoptotic signaling (1). Epidermal growth factor receptor (*EGFR*) mutations which were identified in 2004 (2-4)—are the poster children for the concept, as lung adenocarcinomas that harbor activating kinase domain *EGFR* mutations become addicted to deranged EGFR signaling and are susceptible to small-molecule compounds that disrupt EGFR activity (5). The clinically-relevant and most frequent *EGFR* mutations are inframe deletions/insertions (around amino-acid residues 747 to 752) of exon 19 (these account for up to 40-50% of all *EGFR* mutations) and the L858R mutation (this

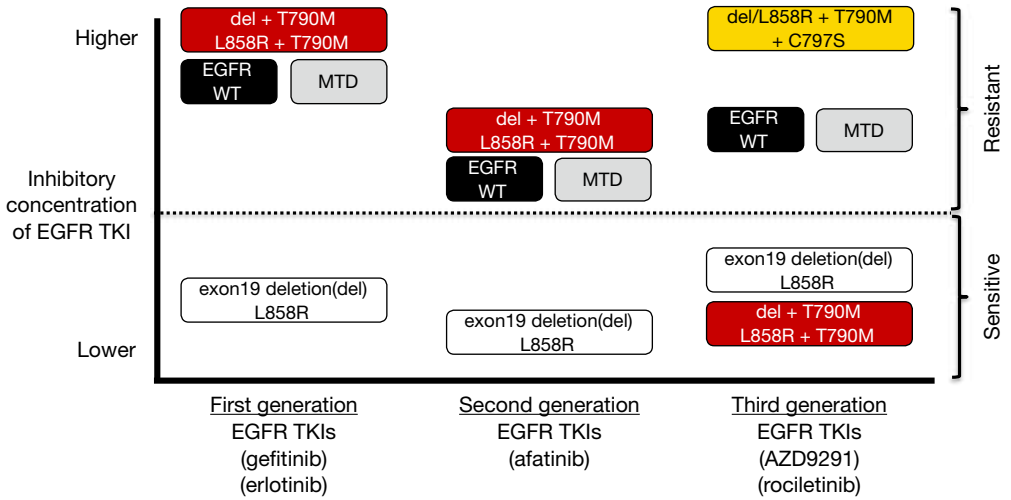

Figure 1 Pictorial graphical display of *in vitro* inhibitory concentrations of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) against various isolated EGFR proteins and the wild-type (WT) kinase. The y axis depicts lower (more sensitive to TKI) and higher (more resistant to TKI) inhibitory concentrations. First, second and third generation TKIs are shown, with maximum tolerated dose (MTD) matching the inhibitory doses for EGFR WT. The therapeutic window of each class of EGFR TKI represents *EGFR* mutated proteins that are inhibited below the MTD.

accounts for up to 30-40% of all *EGFR* mutations) of exon 21 (5-7). The transcribed EGFR mutant proteins favor the active kinase state, induce sustained mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinases (PI3K) cascades, resulting in hyperproliferative and anti-apoptotic cell phenotypes (5). Acute inhibition of EGFR through tyrosine kinase inhibitors (TKIs) in these oncogene addicted lung adenocarcinomas disrupts the intracellular signaling balance, leading to cell cycle arrest and apoptosis (8-10). The concept of "oncogene-addiction" (11) may be the shared basis of pathogenesis for all oncogenic kinase-driven tumors (12-14). These insights into the biology of *EGFR* mutations translated into the clinical real with the development of the first generation EGFR TKIs gefitinib and erlotinib, both of which are reversible ATP mimetic quinazoline derivatives (5,15,16); and also with the development of the second generation EGFR TKI afatinib, an irreversible inhibitor that binds to the C797 amino-acid residue of EGFR (17). First and second generation EGFR TKIs were originally developed to target the wild-type (WT) EGFR but are significantly more potent against common *EGFR* mutations and have a favorable therapeutic window (*Figure 1*) in tumors driven by EGFR-exon 19 deletions or EGFR-L858R (5). Over the last several years, a multitude of randomized clinical trials have compared an EGFR TKI (gefitinib, erlotinib or afatinib) against systemic platinum-based chemotherapies in advanced lung adenocarcinomas. In all of these trials, the response rates (RRs) with the EGFR TKIs exceeded 70% being >2 times higher than platinum-doublets, the median progression-free survival (PFS) times were significantly longer (with a median of approximately 10-12 months) than that with chemotherapy and the median overall survival (OS) times augmented to over 24 months, especially in tumors with *EGFR*-exon 19 deletions (18)—despite a high rate of cross-over from chemotherapy to EGFR TKI (1,15-17). The combined data from these studies now define the clinical management of *EGFR* mutated lung cancers. Erlotinib, gefitinib and afatinib are approved worldwide for the first line treatment of lung adenocarcinomas with *EGFR*-exon 19 deletions or *EGFR*-L858R mutations (5,18).

The advances brought forth by first and second generation EGFR TKIs not only validated EGFR as an important target for lung cancer but also highlighted some of the limitations of these EGFR TKIs. Acquired resistance to erlotinib/gefitinib and afatinib therapy can come about through multiple biological mechanisms that highlight tumor heterogeneity and adaptability [i.e., a game of whack-a-mole analogy (*Figure 2*)]: (I) the gatekeeper kinase *EGFR*-T790M mutation that modifies ATP affinity, drug binding properties and shifts inhibitory curves (5); (II) activation of bypass signaling cascades that reactivate the MAPK and

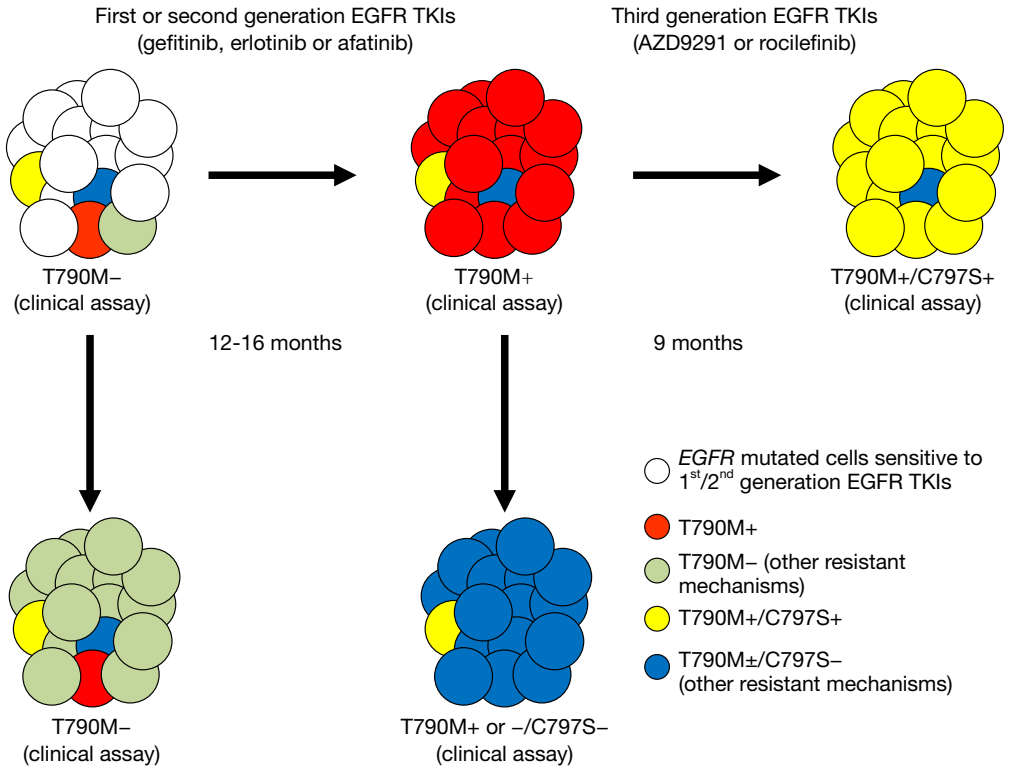

Figure 2 Clonal selection of heterogeneous mass of epidermal growth factor receptor (*EGFR*) mutated lung adenocarcinomas under pressure of EGFR tyrosine kinase inhibitors (TKIs). The graphical display portrays a hypothetical sequence of changes in clonal predominance upon long-term exposure/adaptation of the tumor mass to first/second and subsequently third generation EGFR TKIs. We highlight clones that have the secondary *EGFR*-T790M and the tertiary *EGFR*-C797S mutations. Note that pre-existing mutations may or may not be detected in the clinical setting. This figure attempts to make visualization of the relentless game of whack-a-mole that is constantly being "played" between TKIs and a highly heterogeneous/adaptable cancer.

PI3K downstream pathways (5); and (III) phenotypic and genomic neuroendocrine transformation that silences the expression of or dependence on EGFR protein (19-21). By far, the selection of tumors harboring the original activating *EGFR* mutation with concurrent *EGFR*-T790M is the most common (>50-60%) mechanism of acquired resistance to first/second generation of EGFR TKIs (5,22-26). We and others first identified *EGFR*-T790M in 2005 (22,27), which leads to a threonine (T) to a methionine (M) amino-acid change at the 790 "gatekeeper" regulatory position of the EGFR kinase (22,27). In addition to its effect on ATP affinity and drug binding (5), EGFR-T790M can stimulate other oncogenic signals—such as the β-catenin pathway (23). Originally, *EGFR*-T790M was reported as an acquired mutation after exposure to first generation EGFR TKIs; however, recent progress with sensitive sequencing technologies has revealed that pre-existing *EGFR*-T790M clones can be detected in patients with TKI-naïve tumors (28); as indicated in *Figure 2*. In this context, emergence of *EGFR*-T790M may be due to selection of "*EGFR*-T790M-positive" clones under pressure from a first/second generation EGFR TKI (*Figure 2*).

Since we first reported that an irreversible EGFR TKI can inhibit EGFR-T790M *in vitro* (29), efforts has been made to identify potent irreversible (i.e., C797-binding) EGFR TKIs to overcome resistance caused by EGFR-T790M. However, the initial selected clinical compounds (i.e., second generation EGFR TKIs such as afatinib and dacomitinib) failed to induce responses in the clinical acquired resistance to gefitinib/erlotinib setting (5). These disappointing results can be explained by lack of EGFR mutant selectivity of second generation EGFR TKIs and

their inexistent therapeutic window towards EGFR-T790M when compared to WT EGFR (*Figure 1*). Afatinib and others in the same class are extremely potent WT EGFR inhibitors and achievable serum/plasma levels (limiting toxicities include skin and gastrointestinal adverse events) in patients are unable to inhibit *EGFR*-T790M bearing lung adenocarcinomas (5,6,30).

A major breakthrough in targeting EGFR-T790M occurred in 2009 with the identification of a novel class of covalent EGFR pyrimidine TKIs that are more selective for EGFR-T790M and EGFR TKI-sensitizing mutations than to WT EGFR (31). This class of TKIs against EGFR-T790M heralded the clinical development of third generation EGFR TKIs (*Figure 1*). The two compounds that have advanced the furthest are AZD9291 (AstraZeneca, with a proposed name of mereletinib) and rociletinib (Clovis Oncology, formerly named CO-1686). The impressive results from the expanded phase I first-in-human studies for both drugs were published in April 2015 (32,33). The phase I trial of AZD9291 (AURA) evaluated escalating doses of the drug in patients with advanced *EGFR* mutated lung cancer with resistance to treatment with the first generation EGFR TKIs (erlotinib/gefitinib) (32). A total of 253 patients were included in doses of AZD9291 of 20 mg up to 240 mg daily but a dose of 80 mg daily was considered as optimal to maximize efficacy and minimize skin/gastrointestinal adverse events observed at the higher doses (32). A total of 138 patients had tumors that were confirmed to harbor *EGFR*-T790M and 127 were evaluated for responses; with a RR of 61% (95% CI, 52-70%), disease control rate (DCR) of 95% (95% CI, 90-98%) and a median PFS of 9.6 months (95% CI, 8.3-not reached). As expected, tumors not harboring *EGFR*-T790M (61 evaluable patients) fared worse with a RR of 21% (95% CI, 12-34%), DCR of 61% (95% CI, 47-73%) and a dismal median PFS of 2.8 months (95% CI, 2.1-4.3 months). AZD9291 has been granted breakthrough therapy designation, orphan drug and fast track status by the United States Food and Drug Administration (FDA); and its approval with a companion diagnostic for *EGFR*-T790M is imminent based on results of the aforementioned AURA study and an ongoing phase II trial of AZD9291 80 mg daily for *EGFR*-T790M mutated lung adenocarcinomas (AURA-2 study). This third generation EGFR TKI is also being investigated in randomized trials after progression on gefitinib, erlotinib or afatinib against evidenced-based chemotherapies (AURA-3 study), as a first line therapy for *EGFR* mutated lung adenocarcinoma against gefitinib or erlotinib (FL-AURA study), and in combination with anti-PDL1 immunotherapies (MEDI4736), MEK inhibitors (selumetinib) or MET inhibitors (AZD6094) as part of the TATTON study. The phase I-II trial of rociletinib (TIGER-X) evaluated escalating doses of the drug in patients with advanced *EGFR* mutated lung cancer with acquired resistance to first or second generation EGFR TKIs (33). A total of 130 patients were enrolled and received escalating doses of free-base and subsequently hydrogen bromide salt (HBr) drug formulations, with therapeutic doses considered to encompass 900 mg twice daily of free-base and 625,750 or 1,000 mg twice daily of HBr rociletinib (33). A total of 46 patients had tumors that were confirmed to harbor *EGFR*-T790M and were evaluated for responses; with a RR of 59% (95% CI, 45-73%), DCR of 93% and a median PFS of 13.1 months (95% CI, 5.4-13.1 months). Tumors not harboring *EGFR*-T790M (17 evaluable patients) fared worse with a RR of 29% (95% CI, 8-51%), DCR of 59% and a median PFS of 5.6 months (95% CI, 1.3-not reached). Interestingly, the predominant grade 3 adverse event was hyperglycemia thought to be secondary to a rociletinib metabolite that inhibits the type I insulin-like growth factor receptor (33); and the latter adverse event (often requiring anti-diabetic medications) in addition to concerns related to cardiac QT prolongation may hamper the rapid clinical development of this drug. Rociletinib has been granted breakthrough therapy designation by the FDA with data from the aforementioned TIGER-X and a global registration phase II trial in *EGFR*-T790M positive lung adenocarcinomas (TIGER-2 study) being evaluated for safety plus efficacy. This third generation EGFR TKI is also being investigated in randomized trials after progression on gefitinib, erlotinib or afatinib against evidenced-based chemotherapies (TIGER-3 study) and as a first line therapy for *EGFR* mutated lung adenocarcinoma against gefitinib or erlotinib (TIGER-1 study).

Despite the thrilling responses seen with AZD9291 and rociletinib in lung adenocarcinomas with acquired resistance to gefitinib, erlotinib or afatinib harboring the recalcitrant *EGFR*-T790M mutation (32,33), it is painfully evident that tumor plasticity and selection pressure continue to drive tumor adaptation and resistance to third generation EGFR TKIs (*Figure 2*). The clinical investigators of the AZD9291 clinical trials have convincingly shown that biological mechanisms of resistance to this drug can be readily identified in cell-free plasma DNA from patients (34). The most frequent (40% of 15 *EGFR*-T790M cases treated with AZD9291 in the AURA study) mechanism

identified was the acquisition of the *EGFR*-C797S mutation in exon 20 of *EGFR*. These investigators and other show in preclinical models that EGFR-exon 19 deletion + T790M + C797S and EGFR – L858R + T790M + C797S generate proteins that are resistant to AZD9291, rociletinib and all irreversible EGFR TKIs (including quinazolone- and pyrimidine-based compounds) by impairing covalent binding of these drugs to the C797 amino-acid residue of EGFR (34-36). Plasma samples also showed that another 33% of cases with AZD9291 progression only had *EGFR*-T790M and the original sensitizing mutation detected (*Figure 2*), and in another 27% of cases the *EGFR*-T790M was no longer detected (34). Although the plasma DNA was unable to evaluate for non-*EGFR* mutational mechanisms of acquired resistance, plentiful preclinical reports using third generations EGFR TKIs (including AZD9291) have consistently demonstrated bypass activation of the MAPK-ERK-RAS pathway (through *MAPK1* amplification, downregulation of negative regulators of ERK, *NRAS* mutation/amplification, *KRAS* amplification among others) as a major escape valve to EGFR inhibition (37,38). The clinical investigators of the rociletinib clinical trials have also demonstrated similarly that resistance to rociletinib in the TIGER-X study can be accompanied by putative bypass mechanisms in the presence or absence of *EGFR*-T790M or *EGFR*-T790M amplification (39). In addition, their group also reported neuroendocrine transformation of adenocarcinomas to small cell lung cancer with genotypic/phenotypic silencing of EGFR protein expression as a mechanism of resistance in 16% (2/12 cases) of rociletinib re-biopsies (39). Future reports of tumor and liquid biopsies of lung adenocarcinomas resistant to third generation EGFR TKIs will help define the true frequency of *EGFR*-C797S, MAPK pathway activation and small cell transformation as mechanisms of resistance to this new class of TKI (*Figure 2*).

EGFR mutated NSCLCs (those with exon 19 deletions or L858R) have come a long way in the last decade. Most patients with a new diagnosis of advanced *EGFR* mutated NSCLC in 2015 can expect to receive multiple lines of monotherapy with first, second and third generation EGFR TKIs and can anticipate a median OS that exceeds 2-3 years, which is undoubtedly a tremendous success given that a median OS in pre-EGFR TKI era was less than 1 year. However, the use of monotherapies with EGFR TKIs has also underscored the painful reality that a relentless game of whack-a-mole is constantly being played between TKIs and a highly heterogeneous/adaptable cancer; with the lung adenocarcinoma eventually winning out through mutations (*EGFR*-T790M and/or C797S), bypass mechanisms or histologic/genotypic transformation (*Figure 2*). The next decade of research milestones for *EGFR* mutated lung adenocarcinomas will need to address current unmet clinical needs; which include: the role of first, second and third generation EGFR TKIs in the management of earlier stages (I-III) of NSCLC, the need for improved management of difficult-to-treat sanctuary sites such as the central nervous system (40), and the requisite for treatment strategies (most likely combination therapies with PI3K/MAPK inhibitors, immunotherapies or cytotoxic agents) that can delay or overcome acquired resistance to first, second and third generation EGFR inhibitors. We hope that we will eventually "catch all moles" (*Figure 2*) and "win" the game between TKIs and oncogenic kinase-driven tumors.

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Footnote

Conflicts of Interest: Daniel B. Costa has received consulting fees and honoraria from Pfizer Inc and Boehringer Ingelheim, respectively. Daniel B. Costa also conducts unremunerated clinical trials using AZD9291 (AstraZeneca) and rociletinib (Clovis Oncology). Susumu S. Kobayashi has received honoraria from Bristol-Myers Squibb. No other conflict of interest is stated.

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Treating acquired resistance to EGFR-tyrosine kinase inhibitors: still a work in progress

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Abstract: While most patients with metastatic non-small cell lung cancer (NSCLC) containing sensitizing mutations in the epidermal growth factor receptor (EGFR) gene will achieve an objective response to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib or gefitinib, patients inevitably develop resistance to these agents. One of the strategies being tested to overcome acquired resistance to EGFR TKIs is the use of irreversible EGFR inhibitors such as afatinib. In the randomized phase 2b/3 LUX-Lung 1 trial in advanced NSCLC patients who progressed after at least 12 weeks of benefit from EGFR TKIs, afatinib failed to improve overall survival compared to placebo. Although the liberal entry criteria likely allowed the inclusion of some patients without true acquired resistance, the failure of this study calls into question the viability of irreversible EGFR inhibitors in this patient population.

Keywords: EGFR; acquired resistance; tyrosine kinase inhibitors

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The last decade has seen major progress in the understanding of non-small cell lung cancer (NSCLC), with the growing recognition that NSCLC is not a single disease but rather a collection of many different subgroups with identifiable and potentially targetable genetic lesions. The first targetable driver mutations were sensitizing mutations in the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) gene (1,2), now known to be present in about 10% of NSCLC in Caucasian patients and conferring a high degree of responsiveness to the oral tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib (3). A number of prospective clinical trials have now established that EGFR TKIs induce objective responses in about 70% of patients whose tumors harbor mutations, with a significantly increased median progression free survival (PFS) compared to cytotoxic chemotherapy (4). Nonetheless, most of these patients will eventually progress despite TKI therapy, a phenomenon termed acquired resistance (AR).

Acquired resistance to EGFR TKIs can be achieved through a number of different mechanisms. The most common mechanism (50%) is the development of a secondary T790M mutation in exon 20 of the *EGFR* gene (5). Other less common mechanisms include increased signaling through parallel receptor tyrosine kinases such as the MET (6) and transformation into a small cell phenotype (7). Presumably this heterogeneity of mechanisms would make a single approach unlikely to be successful at overcoming AR, but nonetheless a number of strategies have been proposed and are being tested in randomized trials. One such strategy is the use of second-generation EGFR inhibitors such as XL 647 (Exelixis Inc., San Francisco, CA) and irreversible pan-HER inhibitors such as neratinib (HKI-272; Wyeth/Pfizer, New London, CT), PF00299804 (Pfizer), and afatinib (BIBW 2,992; Boehringer Ingelheim Pharma GmbH, Ingelheim, Germany). Although these agents have shown some ability to inhibit T790M mutant NSCLC in vitro (8,9), evidence of clinical activity of these agents in patients with AR is lacking (10,11).

The LUX-Lung 1 trial was a randomized, double-blind, international phase 2b/3 trial of single agent afatinib versus placebo in 585 patients with advanced lung adenocarcinoma who had not progressed after at least 12 weeks of treatment

with either erlotinib or gefitinib. This study population was intended to represent a clinically defined group with AR to EGFR TKIs, and the primary endpoint was overall survival. Although the response rate (7% versus 0.5%) and PFS (3.3 *vs.* 1.1 months; P<0.0001) were improved in the afatinib group compared to placebo, there was no difference in median overall survival (OS) between the arms (10.8 months for afatinib *vs.* 12 months for placebo; P=0.74) (12). Of note, tissue was not required for entry in the study, and as a result only 141 of the 585 pts (24%) had tissue available for analysis. Of those, 68% were found to have EGFR mutations, evenly split between the treatment and control arms. Only 8 patients (4 in the afatinib arm) had identifiable T790M mutations, and no other known mechanisms of AR were tested.

The intent of the study investigators was to test the efficacy of afatinib in patients with EGFR mutant lung cancer who had developed AR, but the way they went about it was problematic. For one thing, they did not require testing for *EGFR* mutations prior to enrollment, which diluted the study sample with patients with wild-type *EGFR* who would perhaps be less likely to benefit from an irreversible EGFR TKI. Second, efforts have been made to rigorously define clinical acquired resistance to EGFR TKIs, to allow maximum enrichment of patients in trials such as the LUX-Lung study. The most widely accepted definition is the Jackman definition: prior treatment with a single-agent EGFR TKI and either or both of the following: a tumor that harbors an *EGFR* mutation or objective clinical benefit from treatment with an EGFR TKI (PR/CR or stable disease for ≥6 months); systemic progression of disease while on continuous treatment with the TKI within the last 30 days; and no intervening systemic therapy between cessation of the TKI and initiation of new therapy (13). By this strict definition only 34% of patients in the afatinib arm (*vs.* 42% in the placebo arm) would have had true AR, and the magnitude of benefit was indeed numerically higher in this group with a PFS of 4.5 *vs.* 1 month although not statistically significant.

So are we able to draw any conclusions at all from this trial? The liberal definition of AR, the lack of tissue testing to determine mutational status and mechanisms of resistance, and the high degree of subsequent treatment (68% and 79% in the afatinib and placebo arms) combined to muddy the waters. However, if we extrapolate from the minority of patients with available tissue, then we can assume that most patients had tumors with *EGFR* mutations and that most had AR of one mechanism or another. If that is the case then this study, along with the prior failure of neratinib to show benefit in this population (11), casts doubt on the strategy of using irreversible EGFR TKIs as monotherapy in patients with AR.

Interestingly, there is preliminary evidence that afatinib has activity in AR, including T790M, when combined with the anti-EGFR antibody cetuximab (Imclone, owned by Eli Lilly and Company, New York, NY and Bristol-Myers Squibb Company, Princeton, NJ) (14). We know that cetuximab combined with erlotinib has no activity in the AR population (15), raising the intriguing idea that irreversible EGFR inhibitors may have promise in AR when combined with other agents. More mature, peer reviewed results from this trial are anxiously awaited. The indisputable lessons from LUX-Lung 1, however, are that future trials in the EGFR TKI acquired resistance population must be rigorous in defining their target population, and that every patient enrolled must have tissue available for molecular testing so that clear conclusions can be made from the results.

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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

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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

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

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

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

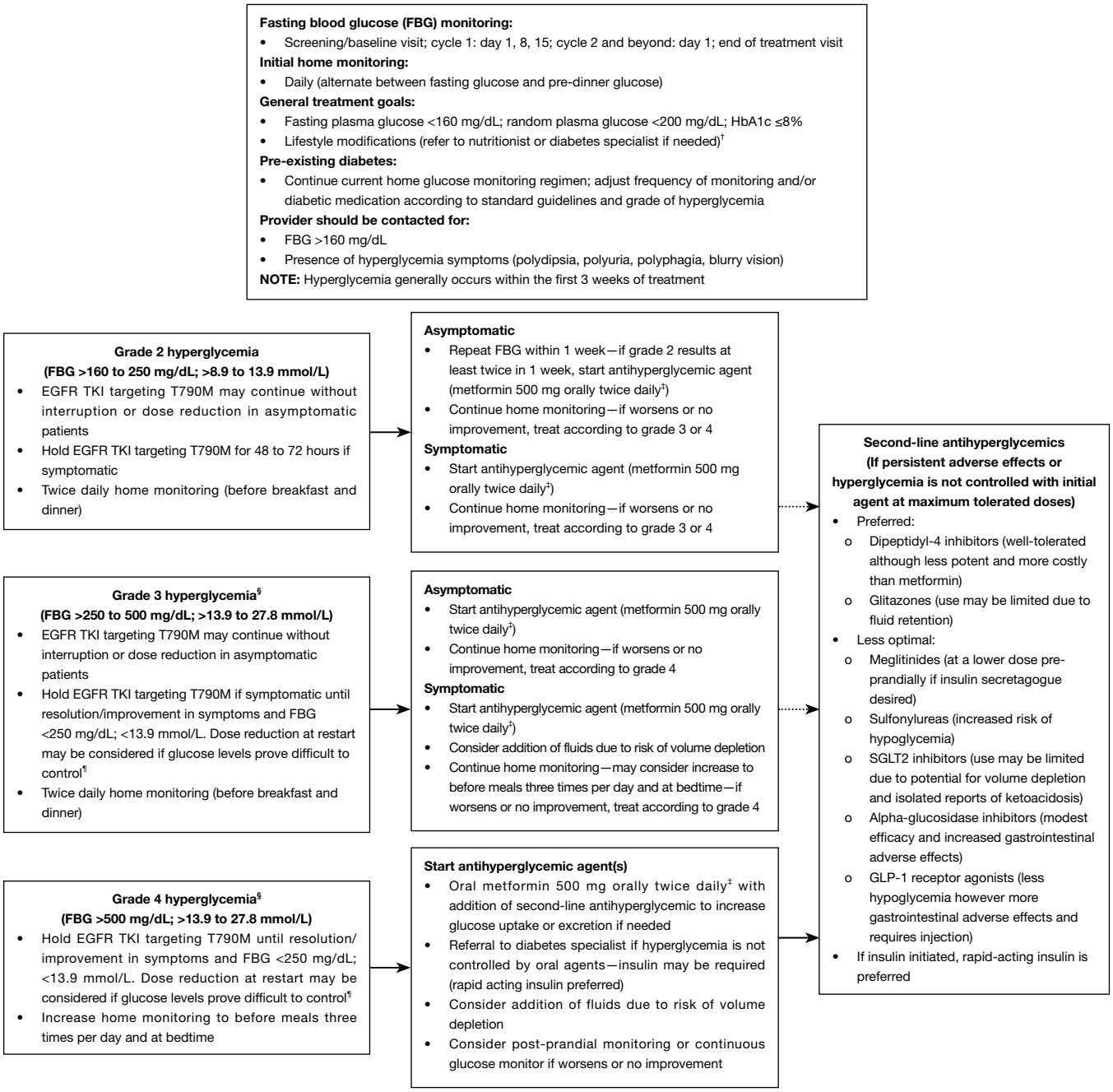

Figure 1 Initial management of hyperglycemia induced by EGFR TKIs targeting T790M. †, Some patients may be able to stop therapy with therapeutic lifestyle changes; ‡, U.S. labeling recommends that metformin should be held for computed tomography scans and should not be used if serum creatinine is >1.3 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.

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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Histopathological transformation to small-cell lung carcinoma in non-small cell lung carcinoma tumors

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Abstract: Lung cancer is the principal cause of cancer-related death worldwide. The use of targeted therapies, especially tyrosine kinase inhibitors (TKIs), in specific groups of patients has dramatically improved the prognosis of this disease, although inevitably some patients will develop resistance to these drugs during active treatment. The most common cancer-associated acquired mutation is the epidermal growth factor receptor (EGFR) Thr790Met (T790M) mutation. During active treatment with targeted therapies, histopathological transformation to small-cell lung carcinoma (SCLC) can occur in 3–15% of patients with non-small-cell lung carcinoma (NSCLC) tumors. By definition, SCLC is a high-grade tumor with specific histological and genetic characteristics. In the majority of cases, a good-quality hematoxylin and eosin (H&E) stain is enough to establish a diagnosis. Immunohistochemistry (IHC) is used to confirm the diagnosis and exclude other neoplasia such as sarcomatoid carcinomas, large-cell carcinoma, basaloid squamous-cell carcinoma, chronic inflammation, malignant melanoma, metastatic carcinoma, sarcoma, and lymphoma. A loss of the tumor-suppressor protein retinoblastoma 1 (RB1) is found in 100% of human SCLC tumors; therefore, it has an essential role in tumorigenesis and tumor development. Other genetic pathways probably involved in the histopathological transformation include neurogenic locus notch homolog (NOTCH) and achaete-scute homolog 1 (ASCL1). Histological transformation to SCLC can be suspected in NSCLC patients who clinically deteriorate during active treatment. Biopsy of any new lesion in this clinical setting is highly recommended to rule out a SCLC transformation. New studies are trying to assess this histological transformation by noninvasive measures such as measuring the concentration of serum neuron-specific enolase.

Keywords: Anaplastic lymphoma kinase (ALK); epidermal growth factor receptor (EGFR); neuroendocrine cells; drug resistance

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Introduction

Lung cancer represents the primary cause of cancer mortality worldwide (1). The World Health Organization (WHO) classifies lung cancer into two subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (2). NSCLC represents 85% of cases of lung cancer, and is divided into adenocarcinoma, squamous-cell, and large-cell carcinoma (3). SCLC represents 14–15% of all lung cancers, and more than 30,000 new cases are diagnosed each year in the United States (4).The oncogenes involved in lung cancer development have been studied extensively and a great variety of tumor promoter and suppressor genes play important roles in the development of lung cancer (5).

Promoter gene alterations: in NSCLC it is common to observe mutations in *KRAS* (6), *HRAS* (7), and *NRAS* (11p15.5; 1p13) (8). Specifically, lung adenocarcinoma can harbor overexpression of the epidermal growth factor receptor (*EGFR*) (9), *ROS* proto-oncogene 1 (10), and rearrangements of the anaplastic lymphoma kinase (*ALK*) (11). All of these alter autocrine and paracrine cell growth (12). Adenocarcinoma and neuroendocrine large-cell carcinoma, can have amplification and overexpression of *c-myc* (13), *l-myc* (14), and *n-myc* (1p32; 2p2.41) (15). These augment proliferation and inhibit cell differentiation (16). Suppressor gene alterations: neuroendocrine carcinoma and NSCLC can have missense mutation in *p53* (17p12-13), which inactivates tumor suppression (17). In SCLC, mutation and deletion in retinoblastoma 1 (*RB1*) (13q14) can be observed, which produces loss of control of the G1 phase of the cell cycle and the arrest of the cell cycle (18).

Alterations in the methylation pattern of DNA have been recognized in many human cancers, and lung cancer is no exception. Aberrant promoter methylation has been shown in various genes, including the retinoid acid receptor β-2, tissue inhibitor of metalloproteinase-3, p16, O6-methylguanine-DNA-methyltransferase, death-associated protein kinase, E-cadherin, p14, glutathione S-transferase P1, the ras effector homologue *RASSF1A*, and the protein tyrosine phosphatase receptor type O. The presence of aberrant methylation in precursor lesions of lung carcinomas identifies it as a reasonable candidate biomarker for early lung cancer diagnosis (5).

Advanced clinical stages of NSCLC that harbor mutations in *EGFR*, *ROS-1*, or *ALK* rearrangements have a distinct clinical course compared with conventional NSCLC. The use of modern therapies for lung cancer such as tyrosine kinase inhibitors (TKIs), some of which inhibit *EGFR* and others *ALK*, has improved survival in patients with specific genetic anomalies of their tumors (19-21). These treatments are preferred over standard intravenous chemotherapy, not only because of their advantages in terms of outcomes, but also because of the better quality of life that patients report. Other advantages include fewer visits to chemotherapy infusion centers and the convenience of administration (22). However, most patients develop resistance to the treatment after 12–15 months of continuous therapy (23-26). This review is focused on standards not only for analysis of the histopathological structure, but also in the molecular mechanisms that drive the histopathological transformation to SCLC in NSCLC tumors.

Histological and genetic characteristics of lung adenocarcinoma and SCLC

Lung adenocarcinoma is the most prevalent subtype of lung cancer among women, nonsmokers, and young men. It commonly presents with *EGFR* mutations or *ALK* translocations, which represent the main objective of current targeted therapies. It is defined as a malignant epithelial neoplasia with glandular differentiation, pneumocyte phenotype, or mucus production. The WHO recognizes many histological subtypes: lepidic, acinar, papillary, micropapillary, and solid (2). In general, the same tumor can have many subtypes and the pathology report must state which one is the most prevalent: this is very important because it can impact the prognosis (27). Immunohistochemistry (IHC) is only recommended in cases in which diagnosis is not made with conventional hematoxylin and eosin (H&E) stain. Typically, the IHC markers used are cytokeratin 7 (CK7) and thyroid transcription factor 1 (TTF-1) (27).

With the development of targeted therapies, molecular testing must be included in the work-up of these tumors. The most common genes targeted by mutations in adenocarcinoma include *EGFR*, *KRAS* and *BRAF*, *ALK*, *ROS1* and *RET* translocations, *MET* and *FGFR1* amplification. *EGFR* mutations are observed in 10–15% of European patients, most commonly in nonsmokers and women, but in up to 40% of Asian patients (3,28,29). Commonly, patients with these mutations respond to targeted treatment and these therapies are approved as first-line treatment in these patients (30,31). *EGFR* activation promotes tumor proliferation and arrests cell apoptosis through stimulation of oncogenic pathways such as *MAPK* and *PIK3/Akt/PTEN/mTOR*. Activating mutations of *EGFR* are localized in exons 18–21, which is the coding region of the intracytoplasmic tyrosine kinase receptor. Ninety percent of these activating mutations are small deletions in exon 19 (deletions of codons 747–750) or point mutations in exon 21 (L858R). Between 5% and 8% are insertions in exon 20 and 2–5% are point mutations of exons 18 and 20. *KRAS* mutations and *MET* amplification are associated with a worse prognosis and *EGFR* mutations with acquired resistance (32,33).

A fusion between echinoderm microtubule-associated protein-like 4 (*EML4*) and *ALK* is present in 2–7% of adenocarcinomas and is more commonly observed in nonsmokers. This group of patients benefits from *ALK* inhibitors (34). The physiological function of *ALK* is not

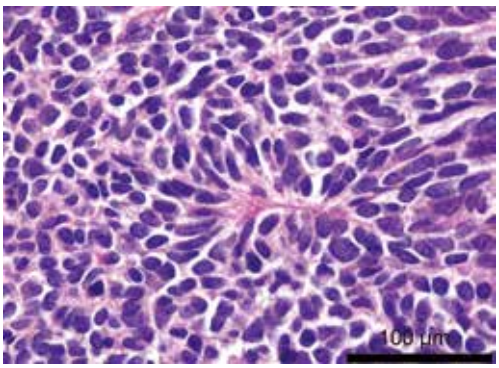

Figure 1 Small cell lung carcinoma. Hematoxylin and eosin (H&E) stain. Tumor composed of nests of small cells with fine granular chromatin nuclei, inconspicuous nucleoli, and scarce cytoplasm.

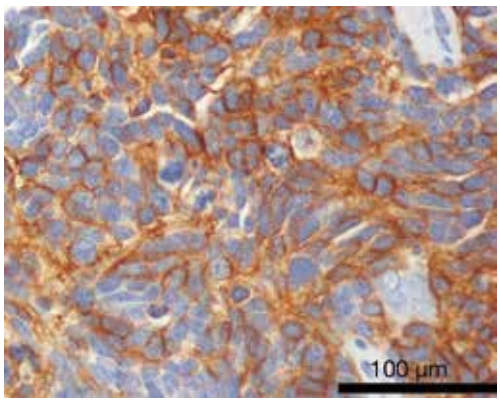

Figure 2 Small cell lung carcinoma. Positive immunohistochemistry (IHC) for CD56, with membranous pattern. This supports the neuroendocrine lineage of the neoplastic cells.

clearly defined; in adult human tissues it is found in low levels in the small intestine, testicles, and nervous system. Histological subtypes of adenocarcinoma that more commonly harbor *ALK* rearrangements include the solid—cribriform, papillary, and micropapillary, and the presence of signet cells with abundant intracellular mucin (34-36).

On the other hand, SCLC usually affects men with a mean age of 60 years and 99% of the patients are smokers (37). By definition, it is a high-grade tumor, so it is very aggressive and very common that patients already have mediastinal lymph node metastases at the time of diagnosis. Histologically, it is a malignant epithelial neoplasia composed of small, oval, rounded, and fusiform cells with scarce cytoplasm, irregular borders, fine granular chromatin, and inconspicuous nucleoli. The cells generally have nuclear molding. Necrosis is extensive and the mitosis count is high (19). It was previously known as oat-cell carcinoma, small-cell anaplastic carcinoma, undifferentiated small-cell carcinoma (SCC), intermediate cell type, and mixed small-cell/large-cell carcinoma; however, these terms are no longer recognized (2). By light microscopy, mitotic rates are high, with an average of 80 mitoses per 2-mm^2 area (2,38-40). The tumor can show different growth patterns, including nests, rosettes, organoid pattern, tubules, ductules with glandular differentiation, and/or peripheral palisading (2). DNA encrustation on vessel walls, which can be observed as basophilic material (also known as the Azzopardi effect), can also be observed in some necrotic zones (19).

The most recent consensus statement of the WHO in 2015 recognizes only two types of SCLC: pure SCLC and combined SCLC (2). When the tumor is composed exclusively of small cells, it is classified as pure SCLC. However, if in addition to the small cells observed in the tumor, it contains at least 10% of large cells, it is classified as a combined SCLC. In surgical samples, neoplastic cells have better formalin fixation and under the microscope the cells can appear larger (19,38). In addition to combined carcinoma composed of small and large cells, one can have combined SCLC with squamous-cell, spindle-cell, or giant-cell carcinoma or adenocarcinoma. Diagnosis of adenocarcinoma or squamous-cell carcinoma can be made if there is any level of frank disease; unlike combined SCLC, no minimum percentage is required. The frequency of diagnosis of mixed carcinoma depends specifically on the size of the biopsy, the type of specimen, and the pathologist's experience (1). In a surgically resected case series, Nicholson *et al*. (38) found combined SCLC in 28% of cases, with 16% combining SCLC with large-cell carcinoma, 9% with adenocarcinoma, and 3% with squamous-cell carcinoma.

Pure SCLC is easily diagnosed in small biopsies (obtained through bronchoscopy) and cytology specimens. The most important technical aspect for accurate diagnosis is a good histological slide and a high-quality H&E stain (*Figure 1*). In most cases, an H&E stain is enough to establish the diagnosis. IHC is used to confirm the diagnosis and in difficult cases. Staining with pancytokeratins such as AE1/AE3 helps to demonstrate that the tumor is a carcinoma rather than a lymphoid lesion (2,19). The most useful neuroendocrine markers include CD56, chromogranin, and synaptophysin, which are best used as a panel (19,38,41). Up to two-thirds of SCLC will be negative for chromogranin and synaptophysin (19). CD56 will stain 90–100% of cases (*Figure 2*) (42-44). Nonetheless, neuroendocrine marker

staining may be focal or weak and only one or two markers may be positive. In <10% of cases, all neuroendocrine markers may be negative and the diagnosis can still be established by morphology (19).

Although a high percentage of SCLC and large-cell neuroendocrine carcinoma (LCNEC) show genetic changes, with some aberrations also seen in carcinoids, some genetic differences between LCNEC and SCLC have been demonstrated (44,45). Therapeutic strategies for SCLC and LCNEC differ substantially. Therefore, because they are two different pathological entities (46), identification of a noninvasive way to detect potential disease transformation before repeated biopsy is crucial.

In addition, an augmented expression of insulin-like growth factor type 1 receptor (IGFR-1) protein and gene copy number has been observed in SCLC, with a significant correlation between protein expression and gene copy number. IGFR-1 inhibitors are beginning to be tested for SCLC in research trials (19,47).

SCLC and LCNEC show a high frequency of loss of heterozygosity (LOH) for 3p, *RB*, 5q21, 9p, and p53 compared with typical carcinoid and atypical carcinoid (19). LOH of 5q21 was found significantly more frequently in SCLC than in LCNEC, and in high-grade carcinoma than in carcinoid (48). The *P16^{INK4}/cyclin D1/RB* pathway that is involved in the regulation of G1 arrest in the cell cycle is frequently affected in neuroendocrine tumors (49,50). *RB* loss is frequent in SCLC and LCNEC, but not in typical carcinoid, although it can be found in 60% of atypical carcinoid. Igarashi *et al*. demonstrated overexpression of cyclin B1 in a high percentage of LCNEC and SCLC (50).

Positive membranous-cytoplasmic expression of the c-kit protein (also known as CD117) is frequently observed in high-grade pulmonary neuroendocrine tumors. Pelosi *et al*. reported expression in 44–77% of LCNEC and 67–80% of SCLC (51), but in only 7% of carcinoid tumors. Araki *et al*. (52) and Casali *et al*. (53) found c-kit staining in 55% and 61% of SCLC and LCNEC, respectively. Casali *et al*. reported a significantly worse prognosis and a higher rate of recurrence for patients with c-kit-positive LCNEC (53). In contrast, neither Pelosi *et al*. (51) nor Araki *et al*. (52) found any prognostic significance of c-kit expression within LCNEC or SCLC tumors.

Mechanisms of acquired resistance to targeted cancer therapies

This section reviews the molecular characteristics that are secondarily acquired during histopathological transformation. Oral TKI-targeted therapies approved for locally advanced or metastatic *EGFR*-mutated NSCLC adenocarcinoma have changed substantially the way this aggressive tumor is treated. They are approved as first-line therapies, based on the observation that 90% of active mutations arise from exon 19 deletion and exon 21 L858R point mutation (54,55). Currently, three drugs are available in most countries as first-line therapies: afatinib, gefitinib, and erlotinib (23,24,56). Unfortunately, some patients develop resistance to the therapy after 1 year or less of response to active treatment (57).

Repeated biopsies in this group of patients have been the vehicle to understand the underlying molecular mechanisms of acquired resistance to *EGFR* TKIs. These include mechanisms that are related to the reactivation of intracellular signal pathways: secondary mutations of *EGFR* Thr790Met (T790M), *MET* receptor tyrosine kinase amplification, and *PIK3CA* mutations (1,58).

These biopsies have also been very useful to observe the phenotypic and histological changes of the so-called histological transformation from NSCLC to SCLC (1,3,59) and epithelial-to-mesenchymal transition (EMT) (60). EMT consists of the loss of the epithelial morphology of the neoplastic cells that develop into a form that resembles that of mesenchymal neoplasms. These phenotypic changes include changes in the IHC-detected expression of vimentin and E-cadherin and also the preservation of the *EGFR* mutations (1).

The most common acquired resistance mechanism is the T790M mutation of *EGFR* (1,61), which is reported in 50–60% of biopsies of patients who develop resistance to current targeted therapies. This acquired mutation augments the ATP receptor and allows signaling from the *EGFR* in the presence of the inhibitor drug (59). Published data from clinical trials focused on this subgroup of patients showed that treatment with a new generation of TKIs resulted in excellent outcomes and drug tolerability (62,63). Other mechanisms that do not involve signaling through the *EGFR*, such as *MET* and *HER2* amplification, make up 15–20% of acquired resistance to *EGFR*-targeted therapies (64-66).

Histopathological transformation to SCLC from NSCLC has been reported as a mechanism of acquired resistance to *EGFR* TKIs in 3–15% of patients (1,3,67). This phenomenon of transformation has been previously reported in case reports and has been confirmed with repeated biopsies in patient cohorts (59,60,68,69). Clinicians

must be aware of this possibility in patients receiving targeted therapies who clinically deteriorate. Little is known about the exact mechanisms that lead to this transformation, but two hypotheses have been proposed to explain it. One states that NSCLC and SCLC have a common cell of origin and that the morphological-phenotypic transformation occurs after treatment with TKIs. The other hypothesis proposes that at the time of the original tissue diagnosis, both types of carcinoma were present, but because of the sampling only the adenocarcinoma was diagnosed (54). The scientific evidence suggests that this latter hypothesis is probably wrong and in many cases it is discredited because some patients originally respond to targeted therapies for months or even years (3).

Synchronous development of adenocarcinoma and SCLC has been observed in *EGFR*-mutated tumors before active targeted therapy (67). This observation suggests that the presence of SCLC in *EGFR*-mutated carcinomas is not exclusively the result of *EGFR* inhibition. In addition, in a series of cases of combined carcinoma, the original biopsy of two adenocarcinomas that transformed to SCLC did not show an *EGFR* mutation. It is improbable that the original *EGFR* report of the tumors was a false-negative result, because both cases were whole resections and one had a *KRAS* mutation (67). This suggests that the transformation can occur independently of the *EGFR* mutational status.

In a 1986 case series, before the discovery of the *EGFR*-activating mutations, when some patients developed conventional chemotherapy or radiotherapy resistance, around 5% of patients with an original diagnosis of NSCLC presented with recurrences in the form of SCLC (70). It is unknown whether the tumors of these patients had any *EGFR*-activating mutations, but they showed SCLC transformation independent of *EGFR* inhibition. Sequist *et al.* (1) did not find any SCLC transformations among 79 patients with stage III NSCLC using surgical samples of tumors with nonmutated *EGFR* that were treated with chemotherapy and radiotherapy (1). This suggests that NSCLC with nonmutated *EGFR* has less tendency to SCLC transformation compared with *EGFR*-mutated tumors. There is a need for studies of larger cohorts of patients to understand better the histological transformation to SCLC from NSCLC with mutated and nonmutated *EGFR*.

In addition, the common clinical presentation differs between these two clinical entities. *EGFR*-mutated adenocarcinomas are more common among nonsmokers and have a more indolent clinical course compared with classical SCLC, which is exclusively a disease of smokers with a rapid growth and early metastases. Clinically, patients with histological transformation to SCLC have an accelerated decline after an initial response to therapy (60).

In many cases that have been studied with repeated biopsies, all the SCLC-transformed tumors retained the initial *EGFR* mutations of the adenocarcinoma (68,69). An autopsy of a patient with histological transformation of NSCLC into extensive metastatic SCLC disease in the lungs, mediastinal and subdiaphragmatic lymph nodes, and liver demonstrated conservation of the *EGFR* L858R mutation of the original lung adenocarcinoma without any additional mutation. However, there are reports of rare cases where tumors not only maintain the original mutations, but also acquire additional changes such as mutations in *PIK3CA* (3,70). These findings suggest that resistance mechanisms involve the phenotypic transformation of the tumor.

Zhang *et al.* (71) reported the case of an 80-year-old man with lung adenocarcinoma (stage IB) who had an *EGFR* mutation (deletion of exon 19). Second-line treatment with *EGFR*-TKI after progression failed, and the progression was accompanied by increased concentrations of the serum tumor marker neuron-specific enolase. The patient's disease progressed during one month of active TKI therapy. Later, repeated biopsies of the metastatic and primary surgical lesions identified a pathological transformation from adenocarcinoma to SCLC, which retained the same *EGFR* mutation. Chen *et al.* (46) suggest that, in the case reported by Zhang *et al.* (71), the transformation occurred before the initial period of TKI treatment. By contrast, in most cases, patients have a long progression-free survival under TKI treatment, which supports the possibility that the transformation might occur during TKI treatment. These conflicting findings suggest the possible existence of factors other than *EGFR* inhibition that might promote the transformation from *EGFR*-mutant adenocarcinoma to SCLC (46). In this case, in addition to the poor response to TKIs, the increased concentration of serum neuron-specific enolase, which rose from 17.9 ng/mL at the early stage of the disease to 211.10 ng/mL at the stage when progression was detected (reference range <15 ng/mL), could be a way to predict potential disease transformation (71).

Genetic analyses of *EGFR*-mutated adenocarcinomas with acquired resistance to TKIs secondary to histological SCLC transformation showed that these tumors can lose *EGFR* expression and have low levels of *EGFR* amplification (60). It is known that SCLC has lower expression of *EGFR*

Table 1 Demonstrated mechanisms of acquired resistance to EGFR TKIs. The most common is the acquired mutation of EGFR Thr790Met, which has been reported in 50–60% of studied biopsies

Secondary mutation of EGFR (T790M)
MET receptor tyrosine kinase amplification
HER2 amplification
PIK3CA mutations
Histopathological transformation from NSCLC to SCLC
Epithelial to mesenchymal transition

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; T790M, Thr790Met; NSCLC, non-small cell lung carcinoma; SCLC, small-cell lung carcinoma.

compared with NSCLC, but the underlying mechanism of this is unknown (68). SCLC with *EGFR* mutations responds less strongly to TKIs compared with *EGFR*-mutated NSCLC, probably secondary to mechanisms that suppress *EGFR* expression in these tumors (3). However, Araki *et al.* (52) reported the case of a patient with SCLC with mutated *EGFR* that responded to conventional TKI treatment. This must be confirmed with studies that include more patients. A summary of mechanisms of acquired resistance to *EGFR* TKIs is listed on *Table 1*.

EGFR-mutated carcinomas that transform to SCLC also have epigenetic changes; miRNA analyses have demonstrated that SCLC-transformed cells express miRNAs that are commonly upregulated in classical SCLC. However, SCLC-transformed cells also express miRNA subtypes that are typically expressed in adenocarcinomas, but not in SCLC. This suggests that transformed SCLCs have some characteristics of the original adenocarcinoma, but that the mRNA expression profile and the clinical course indicate that this neoplasia behaves similarly to classical SCLC (60,62,63,72).

In laboratory studies, the *BCL-2*, *BCL-XL* inhibitor *ABT-263* is one of the few therapies to date to exhibit marked efficacy against SCLC, although recent results from single-agent clinical trials with *ABT-263* demonstrated responses in only a minority of SCLC patients. Transformed SCLC *EGFR*-mutant cells were highly sensitive to single-agent *ABT-263*, and markedly more sensitive than *EGFR*-TKI-resistant NSCLC cell lines harboring the T790M resistance mutation. *ABT-263* treatment induced a robust apoptotic response in *EGFR*-mutant SCLC compared with the resistant *EGFR*-mutant NSCLC. The gene expression and drug sensitivity of the SCLC-transformed cells more closely resemble classical SCLC than *EGFR*-mutant NSCLC (73).

ALK inhibitors provide a better response than cytotoxic chemotherapy in patients with *ALK*-positive NSCLC (34,36). Despite these favorable results, a group of patients will have progression of the disease after 1 or 2 years of active treatment. The resistance mechanisms to TKIs for *ALK*-positive patients include *ALK* domain modification and upregulation of parallel signaling pathways such as those involving *EGFR* and *cKIT* (36,74). To our knowledge, there are only three case reports in the literature describing SCLC transformation in *ALK*-positive patients. The first detected an *EML4-ALK* fusion gene through *ALK* IHC analysis and direct sequencing of cDNA in a surgically resected specimen (75). The second confirmed *ALK* rearrangement by multiplex reverse transcription-polymerase chain reaction (PCR) in a biopsy before treatment (76). The third case described a 67-year-old nonsmoking woman with a diagnosis of *ALK*-positive adenocarcinoma that underwent SCLC transformation during active treatment with the *ALK* inhibitor alectinib (36).

Molecular mechanisms involved in the transformation from NSCLC to SCLC

Two SCLC genome-sequencing projects have been completed, which included analysis of the genome, transcriptome, and the copy number. Both identified a high prevalence of *TP53* and *RB1* mutations (77,78). *MYC* amplification was observed in 16% of the studied cases (77). *MYCL1* knockdown produces diminished proliferation in cells of SCLC (78), which suggests that *MYC* can function as an oncogenic controller in a subgroup of SCLC tumors. Signal activators including *ERK*, *EGFR*, and *KRAS* are more common in adenocarcinomas. By contrast, the loss of *RB1* is more common in SCLC (79).

Because the loss of *RB1* was found in 100% of sequences of SCLC tumors in humans, it was concluded that it plays an important role in tumorigenesis and is essential for its development (3,77,79). Analyses of repeated biopsies of patients with *EGFR*-mutated adenocarcinomas that underwent SCLC transformation have shown that all the tumors had lost *RB1* (60). Evaluation of *RB1* status in 11 samples of *EGFR*-mutated tumors by analysis with IHC, quantitative PCR, next-generation sequencing (NGS), and array comparative genomic hybridization showed that classical SCLC had alterations in *RB1* and did not express *EGFR* (60,77,79-81). However, it is of interest that in *RB1* knockdown experiments in *EGFR*-mutant cell lines, the

loss of *RB1* was insufficient to cause resistance or induce neuroendocrine differentiation. These cell lines do not possess the pluripotent cells that are present in a tumor *in vivo* and that have the capacity to differentiate into many cell types including SCLC. It is suggested that pluripotent cells differentiate to NSCLC when *EGFR* is active, in the same way as *EGFR* activity is associated with alveolar differentiation (60,82). The SCLC transformation could suggest that adenocarcinoma and SCLC originate from a common cell, probably a multipotent stem cell (3). If this could be confirmed, the genetic heterogeneity of neoplasia would again be demonstrated.

Western blotting revealed loss of *RB* expression specifically in resistant *EGFR*-mutant cell lines with SCLC histology also lacking *RB* expression. The universal nature of *RB* loss suggests that this may be a necessary event for the resistant SCLC tumors to emerge. *RB*-deficient adenocarcinomas serve as further evidence that loss of *RB* alone is insufficient to promote transformation to SCLC (83).

Achaete-scute homolog 1 (*ASCL1*) is a basic helix-loop-helix transcription factor pivotal for neuroendocrine differentiation that is expressed in pulmonary neuroendocrine cells and in SCLC. *ASCL1* promotes more aggressive adenocarcinoma growth *in vivo* and may interact with the central retinoblastoma protein-tumor protein 53 (*RB-p53*) axis in the carcinogenesis of neuroendocrine lung cancers. *ASCL1* contributes to enhanced proliferation and migration in lung cancer cells *in vitro* by targeting cyclin-dependent kinase 5 (CDK5). *ASCL1* expression is regulated downstream of neurogenic locus notch homolog (*NOTCH*) signaling, mediated through four different receptors, which causes polyubiquitination-mediated *ASCL1* degradation. Alteration in *NOTCH* receptor signaling is frequently found in malignant neoplasms. The mutated domain determines the functionality, because activating mutations are located in the proline-glutamic acid-serine-threonine-rich (*PEST*) domain and inactivating mutations in the *EGF*-like and ankyrin (*ANK*) repeats. Meder *et al.* investigated signaling via the *NOTCH*- and *ASCL1*-dependent pathway *in vitro* (83). They used amplicon-based NGS to identify mutations on *RB1* and *TP53*. Mutual RB1 and *TP53* mutations were identified only in SCLC cell lines. Thus, *RB1* mutations correlated with the lack of *RB* protein expression. Using different amplicon-based panels, they identified other oncogenic mutations, including *EGFR* mutations in *PC9* and H1975, while *RB* can be inactivated by phosphorylation. They also performed Western blot analysis to determine the total *RB* protein and phosphorylation status. *ASCL1* clones showed higher expression of serine-phosphorylated *RB*. Therefore, *ASCL1* overexpression caused inactivation of *RB* by phosphorylation. Phosphorylation of *RB* is triggered by CDKs. CDK5 was upregulated in *ASCL1* clones compared with the EV control. Because *ASCL1* is targeted by *NOTCH* signaling, Meder *et al.* also performed siRNA-mediated knockdown of *NOTCH1* and *NOTCH2* in PC9 cells, and observed increased *ASCL1* and CD56 expression. Flow cytometry revealed stable *RB* protein expression and significantly increased phosphorylation of *RB* at Ser780, but this was not as strong as in *ASCL1* clones. Meder *et al.* proposed that *ASCL1* overexpression induced CDK5 upregulation and thereby *RB* inactivation by phosphorylation, and that *p53*-mutated cells had a selective advantage when *RB* was inactivated. *ASCL1* assists the central *RB-p53* signaling axis in the establishment of a SCC phenotype. Meder's group examined four mutations in *NOTCH* genes (*NOTCH1-4*), *RB1*, and *p53* by NGS and also assessed representative cases of neuroendocrine pulmonary carcinomas. They suggested that mutual biallelic alterations of both genes were a prerequisite for SCC formation. For secondary SCC, biallelic *TP53* mutations in the non-small-cell precursor, which are more frequent in squamous cell carcinoma than in adenocarcinoma, may be a prerequisite. *ASCL1* expression alone was not sufficient to induce a full SCC phenotype but it was reported that *ASCL1* may cooperate with *RB* and *p53* loss when forming SCC. However, clinical observations also suggest that SCCs may arise as secondary neoplasms from a non-small cell cancer background in the form of relapses after genotoxic chemotherapies or targeted therapies (1,84,85). The complex patterns of inactivating *NOTCH* mutations in the context of mutual *RB1* and *TP53* alteration in tumors with neuroendocrine differentiation indeed suggest that some neuroendocrine neoplasms may represent a NSCLC-dependent secondary tumor overgrowing its non-small cell origin. The results suggested that one inactivating *NOTCH* mutation was sufficient to induce neuroendocrine differentiation from nonneuroendocrine tumor cells or tumor precursors (*Figure 3*). Reactivating *NOTCH* signaling may represent an important therapy option for SCLC patients (86,87).

We lack clinical trials that address the best way to treat SCLC transformed from NSCLC tumors. Case-reports and series of cases in the literature, used standard chemotherapy (platinum-based and etoposide) and reported a response in 75% of the patients. The benefit of radiotherapy to the chest is unknown in this group of patients (1,69,88).

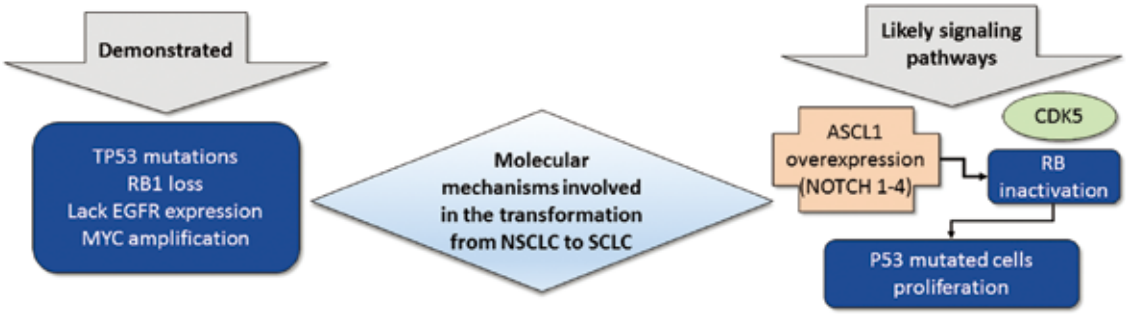

Figure 3 Molecular mechanisms involved in the transformation from NSCLC to SCLC. They include *TP53* mutations, *RB1* loss, lack of *EGFR* expression and *MYC* amplification. The most studied signaling pathway is the *ASCL1* which is regulated by four different *NOTCH* receptors. NOTCH alterations promote *ASCL1* and CD56 overexpression. These changes induce CDK5 activity and inactivation of *RB* by phosphorylation. With inactivated *RB*, *p53* mutated cells have a selective advantage. NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; *RB1*, retinoblastoma 1; *EGFR*, epidermal growth factor receptor; *NOTCH*, neurogenic locus notch homolog; *ASCL1*, achaete-scute homolog 1; CDK5, cyclin-dependent kinase 5.

Conclusions

Clinicians must be aware that transformation to SCLC from NSCLC can occur at any time during active treatment. The specific moment when the transformation occurs has not been elucidated. After *EGFR*-specific TKI treatment, resistant pluripotent cells can accumulate genetic alterations (such as the loss of *RB1* and *TP53*), which give them a distinct epigenetic state and capability of differentiation in a lineage that does not require *EGFR* signaling, such as SCLC. The *EGFR*-specific TKIs silence that signaling pathway, facilitating differentiation to other lineages. This same mechanism could also explain SCLC transformation in patients with *ALK*-positive NSCLC receiving targeted therapy. Other genetic pathways that are probably involved in the histopathological transformation are *NOTCH* and *ASCL1*. A biopsy is recommended for patients with NSCLC and rapid clinical decline to rule out SCLC transformation.

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Footnote

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Recent advances in the development of mutant-selective EGFR inhibitors for non-small cell lung cancer patients with EGFR-TKI resistance

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Over the last decade, first-generation epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKI) (erlotinib and gefitinib) for the treatment of advanced non-small cell lung cancer (NSCLC), especially adenocarcinoma, have demonstrated remarkable advances and leaded to improvement in patients' survival time, either progression-free survival or overall survival. EGFR-TKI therapies also provided a superior quality of life in specific patient populations (1). Erlotinib and gefitinib are orally administered small molecules that reversibly target the EGFR tyrosine kinase domain and interfere with tumor growth. Activating *EGFR* mutations, such as exon 19 deletion and exon 21 L858R point mutation, have been associated with dramatic responses to first-generation EGFR-TKIs. Their side effects like dose-dependent skin rash and diarrhea are usually mild to moderate. However, patients receiving first-generation EGFR-TKIs will eventually experience disease progression because of acquired resistance. *EGFR* T790M mutation was identified in more than half of patients with resistance to gefitinib or erlotinib, and it was the most common mechanism of acquired resistance. At present, there is no standard targeted therapy for patients with EGFR-TKI resistance (1).

The second-generation EGFR-TKIs, including afatinib and dacomitinib, were developed as irreversible pan-HER (human epidermal growth factor receptor) inhibitors which may interfere with the EGFR signal transduction pathway more completely compared with the first-generation EGFR-TKIs (2). They are effective in NSCLC with activating *EGFR* mutation, and also have ability to overcome T790M activity in preclinical models. Nevertheless, the irreversible second-generation EGFR-TKIs as monotherapy failed to overcome T790M activity in NSCLC patients with acquired resistance to gefitinib or erlotinib, because the drug concentrations to inhibit T790M *in vitro* could not be achieved in patients as a result of nonselective wild-type EGFR inhibition-related toxicity. Dual EGFR inhibition with afatinib and cetuximab in NSCLC patients with acquired resistance to EGFR-TKIs has demonstrated a 29% response rate in T790M mutation-positive NSCLC, but this therapy is associated with a significant degree of cutaneous and gastrointestinal toxicities (2). Therefore, in order to pursue better therapies for overcoming T790M-mediated resistance and sparing wild-type EGFR, the third-generation EGFR-TKIs were developed to target T790M and classic *EGFR* mutation while sparing wild-type EGFR.

The third-generation EGFR-TKIs, including AZD9291, CO-1686, and HM61713, are oral, irreversible, mutant-selective EGFR inhibitors that target T790M and have low affinity for wild-type EGFR, while remaining effective against classic *EGFR* mutations. In the recent preliminary reports, the response rates of AZD9291, CO-1686, and HM61713 in patients with T790M mutation were 64%, 58%, and 29%, respectively (3-5). AZD9291 demonstrated promising efficacy against T790M-positive tumors. A multicenter phase I trial of AZD9291 recruited 199 patients, including Asian and Caucasian NSCLC patients with *EGFR* mutation and acquired resistance to EGFR-TKIs (3). This

study revealed an overall response rate of 51% (91/177 patients). In the subgroup of 132 patients with T790M mutation status, the overall response rates were 64% (95% CI: 53-74%) in 89 T790M-positive patients and 23% (95% CI: 12-39%) in 43 T790M-negative patients. Better efficacy was observed in the T790M-positive than -negative tumors. A 96% disease control rate (85/89 patients) was revealed in T790M-positive patients. The longest duration of response was reported to be more than 8 months, but the median duration of response is still pending. The efficacy of AZD9291 to overcome T790M-mediated resistance was demonstrated to be better than that of second-generation EGFR-TKIs.

AZD9291 was designed with reduced affinity for wild-type EGFR. Because of sparing wild-type EGFR in the skin and gut cells, the common side effects, such as skin rash and diarrhea, were milder and fewer than first-generation EGFR-TKIs. No dose-limiting toxicities at 20 to 240 mg/day were discovered in the recent trial (3). The most common drug-related adverse events in the study of AZD9291 were low-grade diarrhea (30%), skin rash (24%), and nausea (17%). The most concerning toxicity was interstitial lung disease (ILD)-like events, and five ILD-like events were reported. All of them responded to treatment, and were resolved without fatalities.

In a recent study, another third-generation EGFR-TKI, CO-1686, also demonstrated considerably lower rates of common EGFR toxcicities, including low-grade diarrhea (22%) and rash (4%), compared with first-generation EGFR-TKIs (4). In addition, the skin toxicity of CO-1686 is also milder and fewer than AZD9291. However, CTCAE grade 3 hyperglycemia in 22% of patients, and prolonged QT corrected (QTc) interval in 7% of patients were observed. Unlike CO-1686, the study of AZD9291 revealed no significant aberration of blood glucose or QTc interval (3). Therefore, AZD9291 treatment in NSCLC patients with diabetes mellitus may be better than CO-1686 when considering the side effect of hyperglycemia.

The third-generation EGFR-TKIs targeting *EGFR*-mutated tumors while sparing wild-type EGFR provide higher efficacy against T790M-positive tumors, and at the same time, they have demonstrated fewer toxicities and good tolerability. However, the efficacy of these third-generation TKIs compared with first-generation TKIs in treatment-naïve *EGFR*-mutated NSCLC is still not clear, nor is the treatment for T790M-negative tumors in patients with acquired EGFR-TKI resistance. Further investigations are ongoing to determine the relevant clinical benefit of these mutant-selective, third-generation EGFR-TKIs, and their role in the first-line setting or treatment for TKI-resistant lung cancer.

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Footnote

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EGFR mutation heterogeneity and mixed response to EGFR tyrosine kinase inhibitors of non small cell lung cancer: a clue to overcoming resistance

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Abstract: The presence of an *EGFR* activating mutation is predictive of benefit from reversible and irreversible EGFR tyrosine kinase inhibitor (EGFR-TKI) allowing personalized medicine in lung cancer. However, intratumoral heterogeneity in *EGFR* mutation status has recently been described and ranged from 13.9% to 27% in some studies. Intratumor heterogeneity may have important consequences for personalized-medicine approaches that commonly rely on a single tumor-biopsy to portray tumor mutational landscape. *EGFR* mutation heterogeneity could also explain the mixed responses phenomenon and act as a mechanism of acquired resistance to EGFR-TKI. In order to a better tailored treatment in advanced non-small cell lung cancer (NSCLC), it is extremely important to elucidate the relevance and degree of heterogeneous distribution of the targeted biomarker regarding the metastasis localisation, previous systemic treatments and interval between primary tumor and metastasis. Additionally, these findings would also help us to design new strategies for patients with lung cancer harboring heterogeneous *EGFR* mutations.

Keywords: *EGFR* mutation; heterogeneity; mixed responses; lung cancer

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Personalised medicine in non-small cell lung cancer (NSCLC) is a reality in our days and tumor genomic landscape is based on a single tumor biopsy results. Several studies have demonstrated that the presence of an *EGFR* activating mutation is predictive of benefit from reversible and irreversible EGFR tyrosine kinase inhibitor (TKI) in non-small cell lung cancers, with significant advantage compared to chemotherapy in progression free survival and response rate (RR) in first line. Among those *EGFR* mutant patients the tumor RR to first-line EGFR TKI is in the range of 58-84%, indicating that there are additional factors mediating the sensitivity of tumors to EGFR TKI (1-8). This phenomenon may be explained by heterogeneity in *EGFR* mutation status within an individual tumor. On the contrary to this theory, because the driver mutation is acquired in an early step of progression, subsequent clonal expansion distributes the mutation through the tumor. However, Gerlinger *et al.* (9) map out the remarkable intratumoral heterogeneity within a single renal cell cancer respect to somatic mutations in driver and passenger genes, which may foster tumor adaption and therapeutic failure via Darwinian selection. Intratumor heterogeneity may have important consequences for personalized-medicine approaches that commonly rely on single tumor-biopsy samples to portray tumor mutational landscape. This heterogeneity has been investigated regarding *EGFR* mutation in NSCLC.

Chen *et al.* (10) studied discordance in *EGFR* mutation status using direct DNA sequencing in paired samples of lung adenocarcinoma and regional lymph nodes or distant metastases in 180 Asian patients. In case of discordance between the primary tumor and the metastasis, results were confirmed using the high-resolution melting method (HRM). The overall discordance rate was 13.9%.

Heterogeneity was significantly higher in patients with multiple pulmonary nodules (24.4%) than in patients with distant metastasis (14.3%), lymph nodes metastases (10.2%) or metachronous primary tumors (9.1%). Additionally, the discordance also was higher between paired samples from metachronous tumors (15.7%) than samples from synchronous tumors (7.5%). These results are in contrast to a study by Yatabe *et al.* (11) who did not find *EGFR* mutation heterogeneity by reverse transcriptase polymerase chain reaction among 77 *EGFR* mutant patients with paired primary and metastatic site samples or among 54 primary and recurrent tumor pairs. The authors also performed a transactional analysis of 50 lung adenocarcinomas carrying *EGFR* mutation. Three parts of each individual tumor were selected and examined for their *EGFR* mutation status and all three parts demonstrated identical mutations. Also, five tumors were dissected into more than 100 pieces and examined for EGFR status and again no *EGFR* mutation heterogeneity was found. The authors concluded that heterogeneous distribution of *EGFR* mutations is extremely rare and that pseudoheterogeneity is observed as a result of the use of less sensitive methods of detection. Other studies using heteroduplex analysis or Scorpion Amplification Refractory Mutation System (ARMS) method have reported *EGFR* mutation heterogeneity in the range of 16.8% to 27%, respectively (12). Tomonaga *et al.* (13) described intratumor heterogeneity of *EGFR* mutation by PCR in nine out of 38 patients with resected mixed-type lung adenocarcinoma and it was significantly associated with smoking history.

Recently, 45 tumors of patients with *EGFR* mutant stage IIIA-IV NSCLC with palliative surgery in which *EGFR* mutations were determined using Denaturing High Performance Liquid Chromotography and ARMS revealed 30% of intratumoral *EGFR* mutational heterogeneity, accompanying with low EGFR copy number. The prognosis of the patients was also related to the *EGFR* mutation heterogeneous status (14). These findings suggest that patients with advanced lung cancer harbor *EGFR* mutational heterogeneity and this heterogeneity might have clinical consequences in the efficacy of EGFR-TKI, and it could be a mechanism of resistance to EGFR TKI. Taniguchi *et al.* (15) demonstrated that those patients harboring heterogeneous tumors had a statistically significant decreased survival compared with those patients harboring mutation-positive tumors cells only after gefitinib treatment.

It is not well understood if systemic therapy may influence the expression of different biomarkers such as *EGFR* mutation in the tumor. In the Chen *et al.* (10) study, those patients that had received systemic therapy had a higher *EGFR* mutation discordance than those without exposure to any systemic therapy, suggesting potential mutagenic effects of chemotherapy. In a cohort of 264 advanced NSCLC patients, chemotherapy significantly decreased frequency of *EGFR* mutations from 34.5% in the prechemotherapy plasma samples to 23.1% in the postchemotherapy plasma samples (P<0.001). It is interesting to underline that the majority of *EGFR* mutation changes after chemotherapy were from mutant state to wild type (16). Notwithstanding these results, Rosell *et al.* (17) demonstrated no statistically significant differences in RR, PFS and OS in *EGFR* mutant patients receiving EGFR TKI in either first- or second-line setting. Also, data from the SATURN trial showed a compelling PFS HR of 0.10 in patients positive for *EGFR* mutation who received erlotinib as a maintenance treatment after standard chemotherapy (18). Chen *et al.* (10) also reported in a multivariable analysis that heterogeneity was significantly higher in patients with EGFR TKI exposure. The *EGFR* mutation heterogeneity accounted 8.9% of TKI-resistant cases. It is difficult to estimate whether discordance biomarker expression between pre and post treatment samples is due to a change in a biomarker status or simply a reflection of the pre-existing tumor genetic heterogeneity that can influence tumor phenotype after EGFR TKI treatment. Taniguchi *et al.* (15) tested EGFR mutation in multiples areas in 21 resected tumors, and six of them had both *EGFR*-mutated and wild type NSCLC cells. This fact could explain why patients with multiple pulmonary nodules had a higher heterogeneity in *EGFR* mutation status in the Chen *et al.* study (10).

EGFR mutant heterogeneity could explain mixed responses to EGFR TKI, suggesting that EGFR TKIs should be continued beyond progression in combination with other therapies such as chemotherapy to act in all cell clones that are part of the tumor in those patients. The IMPRESS study (NCT01544179) is currently evaluating the role of gefitinib combined with chemotherapy in patients with EGFR mutations that have progressed to gefitinib.

Tailored treatment in advanced NSCLC is going to improve in the next years based on new research on druggable biomarkers. Treatment of patients with advanced NSCLC and a positive biomarker requires that all tumor clones are eradicated. The question is if a single biopsy might represent the mutation status of the entire tumor,

and the answer would probably be no. Then, it gets increasingly important to elucidate the relevance and degree of heterogeneous distribution of the targeted biomarker regarding the metastasis localisation, previous systemic antineoplastic treatments and interval between primary tumor and metastasis (synchronous or metachronous). Furthermore, and based on these results, to perform a rebiopsy when the treatment fails to offer an individually tailored treatment would be crucial to determine the status of the druggable biomarker. Since not all patients are suitable for a rebiopsy of all tumor lesions, new techniques such as liquid biopsies might help us to distinguish those patients who could have higher *EGFR* mutation heterogeneity (19,20). These findings would also help us to design new strategies for patients with lung cancer harboring heterogeneous *EGFR* mutations.

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Footnote

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Third-generation epidermal growth factor receptor-tyrosine kinase inhibitors in T790M-positive non-small cell lung cancer: review on emerged mechanisms of resistance

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Abstract: Osimertinib, third-generation epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), has been approved in the US and EU for the treatment of *EGFR* mutant T790M-positive non-small cell lung cancer (NSCLC) patients resistant to first- or second-generation EGFR-TKIs, such as gefitinib, erlotinib and afatinib. Although exciting survival data and response rates have been registered in patients treated with this and other third-generation EGFR-TKIs, unfortunately acquired resistance still occurs after approximately 10 months. Mechanisms determining progression of disease are heterogeneous and not fully understood. *EGFR*-dependent resistance mechanisms (such as new *EGFR* mutations), bypass pathway activation [as erb-b2 receptor tyrosine kinase 2 (*HER2)* or *MET* amplification] and histological transformation [in small cell lung cancer (SCLC)] have been reported, similarly to previous generation TKIs. Here, we review principle mechanisms of innate and acquired resistance described in literature both in clinical and preclinical settings during NSCLC treatment with third-generation EGFR-TKIs.

Keywords: Epidermal growth factor receptor; non-small cell lung cancer (NSCLC); third-generation tyrosine kinase inhibitor; T790M; resistance

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Introduction

EGFR mutated lung cancer represents approximately 10–15% of non-small cell lung cancer (NSCLC) in Caucasian population. Exon 19 deletion (del19) and exon 21 p.L858R mutation account for about 85–90% of all *EGFR* activating mutations and are the most relevant predictive factors of response to EGFR-TKI (1). To date, gefitinib, erlotinib and afatinib are the best therapeutic choice in first-line treatment of patients with advanced *EGFR* mutated NSCLC (2). However, acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) is an unavoidable process and usually appears after 10–12 months of therapy. The occurrence of a second *EGFR* mutation p.T790M in exon 20 represents the most frequent mechanisms of acquired resistance with a prevalence ranging between 49% and 63% (3-5). The secondary T790M point mutation increases receptor affinity for ATP binding with a consequent drastic reduction in drug activity. New EGFR-TKIs with specific capability to bind T790M mutated receptor have been developed and successfully tested in patients with acquired resistance (6-8). Moreover, thanks to the higher ability to spare *EGFR* wild-type counterpart, third-generation TKIs have demonstrated high tolerability. With these evidences, AZD9291 (osimertinib), CO-1686 (rociletinib), HM61713 (olmutinib) and others (EGF816, ASP8273) are object of several clinical trials and osimertinib

has already obtained FDA and EMA approval for the treatment of *EGFR* mutant T790M-positive NSCLC.

Although exciting survival data and response rates have been registered in patients treated with third-generation EGFR-TKIs, unfortunately acquired resistance still occurs after about 10 months (6,7). Mechanisms determining progression of disease are various and not fully understood. Patients who failed treatment with third-generation EGFR-TKIs showed *EGFR* modifications, alternative pathway activation or histologic transformation, suggestive of overlapping mechanisms of resistance occurring under the intensive pressure of EGFR inhibition.

The aim of this review is to elucidate resistance mechanisms to third-generation EGFR-TKIs that have been described both in clinical and preclinical settings, giving perspectives on possible future therapeutic options to overcome them.

EGFR-dependent

To date, the main mechanisms of resistance to third-generation EGFR-TKIs reported involve *EGFR*, with new tertiary mutations (C797S and others), similarly to T790M for first- and second-generation TKIs, with *EGFR* gene amplification and with reduction or disappearance of T790M cell clones (*Table 1* and *Figure 1*).

Tertiary *EGFR* mutations

C797S mutation

The emergence of a new *EGFR* mutation is one of the first mechanisms described in patients with acquired resistance to third-generation EGFR-TKIs. Similarly to p.T790M, p.C797S occurs in *EGFR* exon 20 determining the substitution of a cysteine with a serine in the position 797. The aminoacid cysteine located at the position 797 represents the site used by all third-generation EGFR-TKIs for the covalent binding to the receptor, which is necessary to contrast the increased affinity for ATP determined by p.T790M (19). Therefore, the aminoacidic substitution caused by the point mutation translates in the TKI inability to suppress EGFR activity.

Several authors documented the appearance of p.C797S in preclinical setting (18,20). Ercan and colleagues published a study in which mutagenesis was applied to evaluate *EGFR* mutations conferring resistance to osimertinib, rociletinib or WZ4002 (18). Their results confirm that C797 represents the most common site of acquired mutations conferring resistance to third-generation TKIs. Interestingly, basing on their models, T790M-negative cells with p.C797S could maintain sensitivity to quinazoline-based EGFR inhibitors, such as gefitinib or afatinib. Similarly, Niederst *et al*. present a study conducted on cell lines treated with increasing doses of WZ4002 and found out that resistant cells expressed C797S point mutation, *in cis* with p.T790M in 85% of cases (20). They observed that cells with mutations *in trans* could be sensitive to a combined therapy with first- and third-generation TKI, while those with mutations *in cis* are resistant to any EGFR-TKI both alone and combined. Finally, they described the emergence of p.C797S in the absence of p.T790M, a possible scenario in case of first-line therapy with third-generation EGFR-TKI; in preclinical models, these cells retained sensitivity to afatinib or gefitinib.

The first evidence of p.C797S isolated in NSCLC patients was documented by Thress *et al*. (10). The authors analyzed plasmatic samples from 19 patients with acquired resistance to osimertinib and identified the emergence of p.C797S in 6 of them (31%). Considering only patients with p.T790M detectable in pre-treatment samples, the prevalence of p.C797S raises to 40% (6 out of 15). All patients with post-osimertinib p.C797S retained p.T790M after progression and presented EGFR del19 as activating mutation; p.C797S occurred both *in cis* and *in trans* with p.T790M. Moreover, in two patients undergone to tumor re-biopsy, they described, by using Next Generation Sequencing (NGS), two different plasmatic DNA alterations encoding for p.C797S (T→A and G→C), while the biopsy only revealed one of them (T→A), highlighting the ability of plasmatic analysis to reflect different tumoral clones.

Similar results were reported in other patients series treated with osimertinib (9,13), while some differences were evidenced after rociletinib treatment (11,16). By using cancer personal profiling by deep sequencing (CAPP-seq), Chabon and colleagues analyzed pre- and post-treatment plasma samples collected from 43 patients treated and progressed to rociletinib (11). The results evidenced a high heterogeneity in acquired resistance mechanisms, stressing the importance of plasmatic monitoring to obtain a wider spectrum of developed alterations. In particular, only one patient out of 43 (2%) presented p.C797S *in cis* with p.T790M, a lower frequency if compared to osimertinib series (10). These findings were confirmed by Piotrowska *et al*. who found no p.C797S

Table 1 EGFR-dependent mechanisms of resistance to third-generation EGFR-TKIs

Mechanism	Author	Sample	N° of patients	T790M	Method	Other mechanisms associated	3rd TKI
C797S	Yu *et al*. [2015] (9)	Tissue	1	Present	NGS	—	Osimertinib
	Thress *et al*. [2015] (10)	Plasma/ Tissue	6	Present	NGS, ddPCR	—	Osimertinib
	Chabon *et al*. [2016] (11)	Plasma	1	Present	CAPP-Seq	—	Rociletinib
	Song *et al*. [2016] (12)	Tissue	1	Present	NGS	—	Olmutinib
	Ortiz-Cuaran *et al*. [2016] (13)	Tissue	1	Present	NGS	Intermediate MET amp [1]	Osimertinib
Other mutations							
C797G	Menon *et al*. [2016] (14)	Tissue	1	Present	NGS	EGFR and MYC amp [1]	Osimertinib
L798I	Chabon *et al*. [2016] (11)	Plasma	1	Present	CAPP-Seq	EGFR amp [1]	Rociletinib
E709K		Plasma	1	Present	CAPP-Seq	—	Rociletinib
L692V		Plasma	1	Present	CAPP-Seq	—	Rociletinib
L718Q	Bersanelli *et al*. [2016] (15)	Tissue	1	Present	NGS	—	Osimertinib
T790M reduction or disappearance; T790M reduction, T790M loss	Chabon *et al*. [2016] (11)	Plasma	28	Reduced	CAPP-Seq	Several mechanisms associated	Rociletinib
	Piotrowska *et al*. [2015] (16)	Tissue	6	Absent	NGS	SCLC [2]	Osimertinib
	Thress *et al*. [2015] (10)	Plasma	4	Absent	ddPCR	—	Osimertinib
	Chia *et al*. [2016] (17)	Tissue	2	Absent	ddPCR	MET amp [1]	Osimertinib
EGFR amplification	Menon *et al*. [2016] (14)	Tissue	1	Present	NGS	EGFR C797G and MYC amp [1]	Osimertinib
	Chabon *et al*. [2016] (11)	Plasma	4	Present	CAPP-Seq	EGFR L798I [1], PIK3CA mut [1], CDKN2A mut [1]	Rociletinib
	Piotrowska *et al*. [2015] (16)	Tissue	3	Present	NGS	—	Rociletinib
L844V	Ercan *et al*. [2015] (18)	Ba/F3 cells	Pre-clinical	—	Site direct mutagenesis	—	WZ4002

The number of patients with each specific associated resistance mechanism is indicated in parenthesis. amp, amplification; CAPP-Seq, cancer personal profiling by deep sequencing; ddPCR, droplet digital polymerase chain reaction; mut, mutation; NGS, next generation sequencing; SCLC, small cell lung cancer; 3rd TKI, third-generation tyrosin kinase inhibitor; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; CDKN2A, cyclin dependent kinase inhibitor 2A; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

in a group of 12 patients progressed to rociletinib (16). This raises the hypothesis of different pattern of resistance between rociletinib and osimertinib.

Finally, to our knowledge, only a case report has been published demonstrating the presence of p.C797S, along with p.T790M and *EGFR* del19, in the lymph node re-biopsy of a patient progressed to olmutinib (12).

Interestingly, recently a variant of C797 mutation has been described in a patient progressed to osimertinib with massive pleural effusion (14). Authors isolated a new p.C797G mutation *in cis* with T790M and associated with focal *MYC* and *EGFR* amplifications.

Other EGFR mutations

In their report, Chabon *et al*. pointed out the occurrence of rare tertiary mutations in plasma samples of patients progressed to rociletinib (11). Beyond p.C797S mentioned above, they reported subsequent *EGFR* mutations: p.L798I, p.L692V and p.E709K. Whilst p.E709K and p.L692V have been previously described as activating mutations occurring in EGFR exon 18, this report for the first time describes

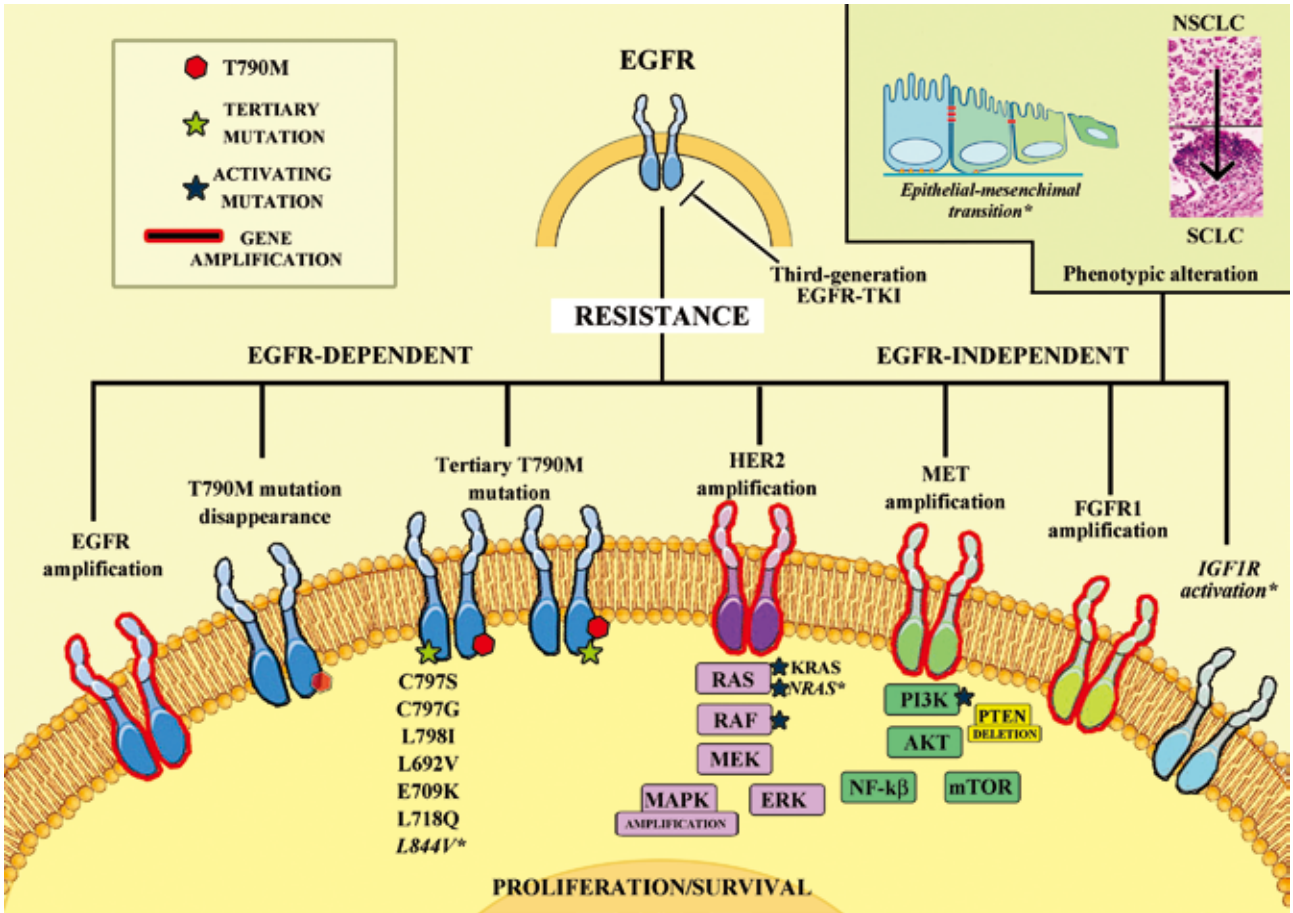

Figure 1 Mechanisms of resistance to third-generation EGFR TKIs. Schematic representation of innate and acquired resistance described both in clinical and preclinical settings during treatment of non-small cell lung cancer with third-generation epidermal growth factor receptor tyrosine-kinase inhibitors. Mechanisms listed in Italic and with * were observed only in pre-clinical setting. Amp, amplification; del, deletion; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; FGFR1, fibroblast growth factor receptor 1; HER2, erb-b2 receptor tyrosine kinase 2; IGF1R, insulin-like growth factor-1 receptor; EMT, epithelial-mesenchymal transition.

the point mutation L798I, never isolated before neither *in vitro* nor *in vivo* (21,22). L798 residue is located nearby C797 and its modification could theoretically interfere with drug binding. In this patient the mutation was associated to *EGFR* CNG (Copy Number Gain) and, accordingly with previous observations, coexisted with p.T790M *in cis*.

Our group published a case report of a patient with activating EGFR L858R initially treated with gefitinib and, after T790M-mediated resistance, with osimertinib (15). When patient progressed to osimertinib, the re-biopsy showed the presence of a new p.L718Q mutation, not detectable in the pre-osimertinib tissue specimen. This mutation has been described before in third-generation TKI-resistant cells and, similarly to p.C797S, cells harboring p.L718Q but p.T790M negative were sensitive to quinazoline-based EGFR-TKIs (18). Another tertiary *EGFR* mutation was described in preclinical models, p.L844V, responsible of resistance due to interference with drug binding (18). In cell models, when associated to p.T790M, p.L718Q and p.L844V determined resistance to all EGFR-TKIs.

T790M reduction/disappearance

The selective pressure determined by third-generation TKI treatment could result in a reduction or disappearance of T790M mutated neoplastic clones, with consequent acquired resistance, as observed by different authors,

including Piotrowska and colleagues (16). Of 64 patients treated with rociletinib in a phase I/II trial, 12 presented sufficient paired pre- and post-therapy biopsy. Six out of 12 patients showed absence of T790M mutation in post-therapy biopsy but 2 of these presented small cell histology transformation. Longitudinal observation, through plasmatic monitoring with BEAMing (beads, emulsion, amplification, and magnetics), allowed to distinguish two different resistance pathways: one with increasing plasmatic levels of p.T790M and activating mutation, reflecting the emergence of a resistant clone still carrying p.T790M and probably with new acquired mechanisms; the other with plasmatic T790M disappearance, suggesting the prevalence of T790M-negative clones no more sensitive to drug inhibition. Plasmatic findings in this study always corresponded to post-progression biopsy results and anticipated evidence of radiological progression, as previously observed with first-generation TKIs (23). An interesting correlation between high baseline plasmatic p.T790M levels and better tumor shrinkage was reported, suggesting that high p.T790M burden, expressed as T790M/activating mutation ratio, could represent a useful tool to predict benefit from rociletinib therapy. Similar results were obtained also by Chabon *et al.* (11).

In addition, also Thress *et al.* reported that 4 of 15 T790M-positive patients lost T790M plasmatic expression after progression to osimertinib, remaining positive for EGFR activating mutation, which levels increased after progression (10). T790M disappearance was reported also by Chia *et al.* in a short communication describing two patients treated with osimertinib (17). At the time of progression to osimertinib, both underwent re-biopsy and p.T790M was not detectable; pre- and post-osimertinib biopsies sites were different for both patients and inter-metastatic heterogeneity may have played a role. In fact, despite T790M-negative biopsy, a patient presented increasing p.T790M plasmatic levels before progression to osimertinib.

EGFR amplification

EGFR amplification was known as a potential mechanism of acquired resistance of first-generation TKI (3,24), but emerging clinical evidences demonstrated that could mediate acquired resistance also after third-generation TKI treatment.

Piotrowska and colleagues observed that three patients developed *EGFR* amplification in the resistance biopsy, not identified in pre-treatment specimens (16). All three patients maintained activating *EGFR* and p.T790M mutations along with *EGFR* amplification. Interestingly one patient presented intrinsic resistance, even if had a significantly lower CNG (6.4) if compared with the other two patients (both reporting CNG >25) progressed after initial response. Moreover, in one of the last two patients, the second post-progression biopsy, in a different anatomic site, showed histological transformation with no *EGFR* amplification. Also Chabon and colleagues identified somatic copy number alteration (SNCA) involving *EGFR* gene in plasmatic samples from 4 out of 43 (9%) patients progressed to rociletinib (11). Three of them presented others detectable genetic alterations: *EGFR* L798I mutation, cyclin dependent kinase inhibitor 2A (*CDKN2A)* mutation and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA)* mutation plus *ERBB2* SCNA. To determine if *EGFR* CNG can mediate drug-resistance, they transfected *EGFR* L858R/T790M double positive cells with lentiviral vectors encoding *EGFR* and observed a significant decreased of rociletinib inhibitory potency. Moreover, these authors demonstrated that patients with CNGs in pre-rociletinib samples presented higher risk to develop primary resistance. These observations suggest that CNGs could represent negative predictive factor for third-generation TKI therapy. *In vitro*, the presence of *EGFR* amplification was reported also by Niederst *et al.* in cell lines derived from a pleural effusion of an erlotinib resistant patient and exposed to increasing dose of WZ4002 (20).

EGFR-independent

Bypass pathway activation

Similarly to first- and second-generation EGFR-TKIs and ALK-inhibitors, also in case of third-generation TKIs, alternative mechanism of resistance can occur involving bypass pathway. Alterations of several pathways have been evidenced in clinical and/or preclinical studies, such as erb-b2 receptor tyrosine kinase 2 (*HER2)* and *MET* amplification, *PIK3CA* activating mutations, *PTEN* deletion, *RAS* mutations and others (*Table 2* and *Figure 1*).

HER2 and MET amplification

HER2 and *MET* amplification may be considered the second most common findings of acquired resistance under first-generation EGFR-TKIs, seen in 10–20% of patients (3-5).

Table 2 EGFR-independent mechanisms of resistance to third-generation EGFR-TKIs

Mechanism	Author	Sample	N° of patients	T790M	Method	Other mechanisms associated	3rd TKI
HER2 amplification	Planchard *et al*. [2015] (25)	Tissue	1	Absent	CGH/FISH	—	Osimerinib
	Oxnard *et al*. [2015] (26)	Plasma/tissue	2	Absent	NGS/CGH	—	Osimertinib
	Chabon *et al*. [2016] (11)	Plasma	4	Present [3]; absent [1]	CAPP-Seq	MET amp [1], CDKN2A mut [1], EGFR amp and PIK3CA mut [1]	Rociletinib
	Ortiz-Cuaran *et al*. [2016] (13)	Tissue	3	Present	FISH	MET amp [1]	Rociletinib/ Osimertinib
MET amplification	Planchard *et al*. [2015] (25)	Tissue	1	Absent	NGS/CGH/IHC	—	Osimertinib
	Ou *et al*. [2016] (27)	Tissue	1	3%	NGS	—	Osimertinib
	Chia *et al*. [2016] (17)	Tissue	1	Absent†	ddPCR	—	Osimertinib
	Ortiz-Cuaran *et al*. [2016] (13)	Tissue	3	Present	FISH	HER2 amp [1]	Osimertinib
	Chabon *et al*. [2016] (11)	Plasma	11	Present [7]; absent [4]	CAPP-Seq	CDKN2A mut [1]; PIK3CA mut [1]; PIK3CA, KRAS and MET mut [1]; HER2 amp [1]	Rociletinib
PIK3CA mutations	Chabon *et al*. [2016] (11)	Plasma	5	Present [4]; absent [1]	CAPP-Seq	MET amp [1]; MET amp, KRAS and MET mut [1]; EGFR and HER2 amp [1]	Rociletinib
	Oxnard *et al*. [2015] (26)	Biopsy	1	Absent	NGS	—	Osimertinib
PTEN loss	Kim *et al*. [2015] (28)	Tissue	1	Present	NGS	—	Osimertinib
RAS-MAPK pathway activation							
KRAS mut	Ortiz-Cuaran *et al*. [2016] (13)	Tissue	1	Absent	NGS	C797S in plasma	Osimertinib
	Chabon *et al*. [2016] (11)	Plasma	3	Present	CAPP-Seq	MET amp, PIK3CA mut and MET mut [1]; KIT mut [1]	Rociletinib
BRAF mut	Oxnard *et al*. [2015] (26)	Tissue	1	Absent	NGS	—	Osimertinib
MAPK1/AKT3 overexpression	Kim *et al*. [2015] (28)	Tissue	1	Absent	NGS	—	Osimertinib
FGF2-FGFR1 autocrine-loop	Kim *et al*. [2015] (28)	Tissue	1	Absent	NGS	—	Osimertinib
SCLC transformation	Piotrowska *et al*. [2015] (16)	Tissue	2	Absent	NGS	—	Rociletinib
	Kim *et al*. [2015] (28)	Tissue	1	Absent	NGS	—	Osimertinib
	Ham *et al*. [2016] (29)	Tissue	2	Absent	NGS	EGFR amp [1]	Osimertinib
EMT	Walter *et al*. [2013] (30)	NCI-H1975 cells	Pre-clinical	Present	RNA-seq	—	Rociletinib
NRAS mutation/CNG	Eberlein *et al*. [2015] (31)	PC9 cell lines	Pre-clinical	—	NGS	—	Osimertinib
IGF1R activation	Park *et al*. [2016] (32)	PC9 cell lines	Pre-clinical	—	Western blot	—	WZ4002

The number of patients with each specific associated resistance mechanism is indicated in parenthesis. †, absent also in plasma sample. amp, amplification; CAPP-Seq, cancer personal profiling by deep sequencing; CGH, comparative genomic hybridization; ddPCR, droplet digital polymerase chain reaction; CNG, copy number gain; mut, mutation; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; NGS, next generation sequencing; SCLC, small cell lung cancer; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; IGF1R, insulin-like growth factor-1 receptor; EMT, epithelial-mesenchymal transition; FGFR1, fibroblast growth factor receptor 1; HER2, erb-b2 receptor tyrosine kinase 2.

Planchard *et al*. reported for the first time *HER2* amplification as a potential mechanism of acquired resistance to third-generation TKI (25). One patient treated with osimertinib for more than 12 months developed acquired resistance due to significant *HER2* amplification found by comparative genomic hybridization (CGH) analysis in the lung sample and confirmed by fluorescent in situ hybridization (FISH) (HER2/CEP17 ratio: 6.65). NGS analysis showed the absence of EGFR T790M mutation in presence of activating del19 mutation. Absence of *HER2* amplification was assessed on pre-treatment samples. The *EGFR* T790M mutation and *HER2* amplification appear to be mutually exclusive as described for first-generation TKIs (33). Similar findings were also presented by Oxnard *et al*. in 2 of 40 patients treated with osimertinib (26).

The same mechanism of resistance was observed also in cohort of patients treated with rociletinib (11). Four patients presented *HER2* amplification in post-treatment specimen: two of these were concurrent with other SCNA and single nucleotide variation (SNV). Despite of the cases treated with osimertinib, the cohort with *HER2* amplification treated with rociletinib seems to retain the T790M mutation; only in one patient was not detectable, but he presented a very low level of T790M also at baseline.

Ortiz-Cuaran *et al*. described in their cohort two cases of *HER2* amplification (13). In a patient treated with rociletinib *HER2* amplification was detectable already after three weeks of treatment, while for the patient treated with osimertinib was detectable in lung sample biopsy collected before treatment. The authors described another patient treated with osimertinib with concurrent amplification of *HER2* and *MET*, but lacking of pre-treatment sample. These findings lead the authors to hypothesize that *HER2* amplification might substitute for *EGFR* signaling and explain the lack of response to third-generation TKIs occurred in these patients.

Regarding *MET* amplification, Planchard *et al*. reported first evidence in a patient treated with osimertinib (25). This case, treated with osimertinib for 10 months until progression of pulmonary disease, showed significant amplification of *MET* (cMET/CEP7: 5.32) confirmed with CGH analysis and by immunochemistry. NGS analysis showed presence of activating mutation L858R but no *EGFR* T790M mutation. Due to unavailability of the pre-osimertinib tissue, the authors were not able to demonstrate if *MET* amplification was absent prior to osimertinib treatment. Instead, Ou *et al*. compare genomic profile of the pre- and post-osimertinib tumor demonstrating *MET* amplification as mechanism of acquired resistance to third-generation EGFR-TKI (27). In fact, they reported one osimertinib treated patient that presents high level of *MET* amplification (30 copies). *EGFR* T790M mutation was detected at 21% reads immediately prior to starting osimertinib, but only present in about 3% of the sequencing reads in the post-osimertinib progression sample. Clinically, the tumor grew rapidly within two months, indicating *MET* amplification as a potential potent driver of rapid tumor growth.

Also Ortiz-Cuaran *et al*. showed high-level amplification of *MET* either in tumor biopsy collected before treatment in a patient that experienced primary resistance to rociletinib and in the post-treatment biopsy of a patient that developed resistance after stable disease to osimertinib (13). Thanks to *in vitro* models they could provide functional evidence that *HER2* and *MET* amplification may induce innate and acquired resistance to this new class of EGFR inhibitors, confirming clinical observations (13). Other pre-clinical studies confirmed the role of *MET* amplification as resistance mechanism to third-generation TKI, suggesting a potential role of MET-inhibitor, alone or in combination, to overcome this resistance (34,35).

In the cohort of patients treated with rociletinib presented by Chabon *et al*. *MET* copy number gain was the most frequent mechanism of acquired resistance (11). Among the 43 patients, 11 (26%) had *MET* amplifications; of these, 7 patients presented only *MET* amplification, 3 had also SNV in other genes (*PIK3CA* and *CDKN2A*) and 1 presented concurrent *HER2* amplification, similarly to Ortiz-Cuaran *et al*. (13). The authors, analyzing an expanded cohort of 16 patients T790M-positive and with *MET* copy number gain in pre-treatment biopsies or plasma, observed that this group displayed significantly less tumor shrinkage and shorter median progression-free survival (PFS) than patients without *MET* alterations. These findings underlying that the presence of different mechanisms at the baseline of third-generation TKIs is associated with an inferior therapeutic response to EGFR-TKI.

PIK3CA activating mutations

Activating mutations of the catalytic subunit alpha (*PIK3CA*) of PI3K lipid kinases family through *PI3K/AKT/mTOR* pathway characterize 2–4% of adenocarcinoma of the lung in a not-mutually-exclusive manner to other oncogenic driver mechanisms (36,37). Shorter median survival has

been described in patients with coexistence of *PIK3CA* and *EGFR* mutations, suggesting synergistic effects likely due to stronger activation of the relevant downstream signals (36,37).

Chabon *et al.* identified two activating mutations, p.E542K and p.E545K, of *PIK3CA* gene as potential mechanism of acquired resistance in 5 patients treated with rociletinib (11). Only two patients present activating mutations in *PIK3CA* alone, while the others presented also SCNA in *MET*, *EGFR* and *HER2* genes. In particular, in a patient that presented concurrence of the p.E542K and *MET* amplification, the SCNA was presenting also prior to start rociletinib. This patient was classified to have an innate resistance to rociletinib, according to a PFS shorter than 3 months. The subclone with *MET* copy-number gain increased over the course of therapy while the abundance of two different activating *PIK3CA* mutations varied over the time. p.E545K was described also in a patient of Oxnard's cohort (26).

PTEN deletion

PTEN loss was previously described as a mechanism of resistance to EGFR first-generation TKI (38). Recently, Kim *et al.* reported a case of a patient with *EGFR* p.T790M mutation and a *PTEN* deletion before osimertinib therapy and with a following increase of the proportion of tumors with *PTEN* deletions and EGF mRNA levels in post-treatment tumors (28). This gradual increase of *PTEN* deletions and EGF overexpression might contribute to focal progression to osimertinib. *EGFR* mutational analysis confirms the retention of activating and resistance mutations. The limited panel of genes studied and therefore the potential genetic alterations underestimated and the presence of *PTEN* deletions before osimertinib treatment in a patient with tumor response should be considered in the interpretation of real potential role of *PTEN* deletion as resistance mechanism.

RAS-MAPK pathway activation

The emergence of *KRAS* activating mutation in patients treated with first-generation EGFR-TKIs was previously described and postulated as a potential mechanism of escape from EGFR-TKI inhibition (39). Ortiz-Cuaran and colleagues described a patient treated with osimertinib that presented p.C797S in a plasma sample with corresponding re-biopsy C797S and T790M-negative but *KRAS* G12S-positive (13). EGFR inhibition through osimertinib may functionally deplete oncogenic EGFR signaling to a level that would allow the emergence of cells harboring *KRAS* mutations. These data are supported by the results of Hata *et al.* and Unni *et al.* (40,41). Also Chabon *et al.* observed the emergence of three *KRAS* activating mutations (p.G12A, p.Q61H and p.A146T) as a potential mechanism of acquired resistance to rociletinib (11). Only the patient with *KRAS* p.G12A mutation presented a single mechanism of acquired resistance, while the other two showed heterogeneous mechanisms: concurrent *KRAS* p.Q61H with *PIK3CA* p.E81K, *MET* p.D1304H point mutations and *MET* amplification and concurrent *KRAS* p.A146T with *KIT* p.L576P mutation.

Another gene involved in pathway of RAS-MAPK and associated to acquired resistance was described by Oxnard *et al.* (26). In a cohort of 40 patients treated with osimertinib NGS analysis performed on tumor biopsy revealed that one patient presented loss of T790M and the presence of p.V600E *BRAF* mutation.

MAPK1 amplification was described as a resistance mechanism to WZ4002 in pre-clinical study performed by Ercan *et al.* (42). Kim *et al.* presented amplification of *MAPK1* gene in a patient treated with osimertinib (28).

Eberlein and colleagues conducted a very meaningful pre-clinical study regarding the involvement of RAS-MAPK pathway in acquired resistance to third-generation TKIs (31). With a comparison across 32 populations of cell lines with acquired resistance to different EGFR-TKIs, the authors detected, as frequent mechanisms of resistance to osimertinib, *NRAS* missense mutations (including a novel E63K mutation) or *NRAS* copy number gain. All these resistant cell lines were sensitive to inhibition by MEK inhibitor selumetinib in combination with EGFR-TKI. Similar results were registered by Ortiz-Cuaran *et al.* that observed *in vitro* that $PC9^{KRAS\text{-}G12S}$ treated with osimertinib and trimetinib showed a full inhibition of MAPK signaling (13). Combined therapy was also tested in study published by Tricker *et al.* where the authors observed a mechanism of WZ4002 acquired resistance mediated by the rapidly reactivation of *ERK1/2* (43). Combination of third-generation TKI with trametinib prevents *ERK1/2* reactivation, increases WZ4002-induced apoptosis and inhibits the emergence of resistance in WZ4002-sensitive models.

These results support use of MEK inhibitors, such as selumetinib and trametinib, in combination with new EGFR-TKIs to overcome acquired resistance mechanisms or to delay/prevent resistance to EGFR-TKI. A phase I trial (NCT02143466) testing the combination of osimertinib and selumetinib is ongoing (*Table 3*).

Table 3 Up-coming combination trials with third generation EGFR-TKIs

Eudract Number	No. of arms	Trial phase	No. of estimated patients	Inclusion of patients pre-treated with 3rd generation TKI	EGFR-TKI	Combined drug	Target of the combined drug
NCT02496663	1	1	30	No	Osimertinib	Necitumumab	EGFR
NCT02503722	1	1	36	Yes (in dose escalation phase)	Osimertinib	INK128	TORC1/2
NCT02520778	1	1	50	Yes (in dose escalation phase)	Osimertinib	Navitoclax	Bcl2 family
NCT02335944	1	1b/2	80	No	EGF816	INC280	MET
NCT02323126	2	2	100	No	EGF816†	Nivolumab†	PD-1
NCT02789345	2	1	74	No	Osimertinib	Ramucirumab	VEGFR2
					Osimertinib	Necitumumab	EGFR
NCT02143466	3	1b	198	Yes (depending on the specific cohort)	Osimertinib	Selumetinib	MEK
					Osimertinib‡	Durvalumab‡	PD-L1
					Osimertinib	AZD6094	MET

†, the other arm will test nivolumab plus INC280; ‡, arm closed due to toxicity. EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; PD-1, programmed cell death 1; VEGFR2, vascular endothelial growth factor receptor 2; PD-L1, programmed cell death 1 ligand 1.

FGF2-fibroblast growth factor receptor 1 (FGFR1)

FGF2-FGFR1 autocrine loop-mediated resistance mechanism was described by Kim *et al*. in one patient treated with osimertinib (28). Osimertinib-resistant tumor harbored focal *FGFR1* amplification and displayed approximately 20-fold higher FGF2 mRNA compared with baseline tumor. NGS analysis showed the loss of *EGFR* T790M mutation in post-osimertinib tumor. This mechanism was supported also by *in vitro* analysis, where a FGF2 supplement conferred resistance to osimertinib in EGFR-mutant NSCLC cells.

Insulin-like growth factor-1 receptor (IGF1R) pathway

Recently, a preclinical study evidenced, in two cell lines resistant to WZ4002, an aberrant activation of IGF1R accompanied by loss of IGF binding protein-3 (IGFBP3) (32). Down-regulation of IGF1R by shRNA, as well as inhibition of IGF1R activity either by a small molecule or a monoclonal antibody restored the sensitivity to WZ4002 both *in vitro* and xenograft. These results suggest that activation of the IGF1R pathway associated with IGFBP3 loss can induce an acquired resistance to EGFR-TKI, as WZ4002. Therefore, a combined therapy of IGF1R inhibitors and EGFR-TKIs might be a viable treatment strategy for overcoming acquired resistance or delay/prevent resistance.

Phenotypic alterations

SCLC transformation

Piotrowska *et al*. reported, for the first time, two patients treated with rociletinib that developed acquired resistance via small cell lung cancer (SCLC) transformation (16). Consistent with previous reports referred to acquired resistance to first-generation of TKI (20), the transformed SCLCs continued to harbor their original EGFR-activating mutations, but not T790M; one patient developed a mutation in *RB1* and the other lost expression of RB1, evaluated by immunohistochemistry.

Kim *et al*. and Ham *et al*. published the same mechanism of acquired resistance for osimertinib separately (28,29). Ham *et al*. reported two cases of acquired resistance mediated by SCLC transformation after osimertinib therapy. The two patients presented disease progression after 14 and 18 months, respectively, and histological analysis of tissue biopsies of both showed SCLC, positive for CD56. NGS analysis showed persistence of *EGFR* activating

mutation (L858R mutation for first patient and Del19 for the second one) but loss of T790M. The authors reported for first patient also *EGFR* gene amplification that is not clear if present before osimertinib treatment. Kim *et al*. described post-osimertinib tumor with neuroendocrine morphology and expression of CD56, chromogranin A and synaptophysin, not present in pre-treatment. Also in this case NGS analysis revealed the depopulation of *EGFR* T790M-mutant clones in post-osimertinib tumor with a loss of *RB1*, similarly to patients described by Piotrowska *et al*.

Epithelial-mesenchymal transition (EMT)

EMT has been previously associated to EGFR-TKIs resistance in NSCLC (44) and it was firstly presented as a potential *in vitro* mechanism of resistance to third-generation TKIs by Walter and colleagues (30). They treated cell lines harboring L858R and T790M for several months with increasing doses of rociletinib until developed of resistance. Comparison results of RNA-seq from cell lines that developed acquired resistance with the parental ones underlying a significant enrichment of genes involved in EMT. This finding was also confirmed with qPCR and Western Blot analysis showing an up-regulation of vimentin, *AXL*, *ZEB1*, *CDH5* and *FN1* expression and a down-regulation of E-Cadherin, *MIR200B*, *CLDN4*, *EPCAM* and *CLDN*7 consistent with a mesenchymal signature in the resistant clones. *EGFR* expression was moderately reduced in the resistant cell clones compared with the parental cell line and no additional *EGFR* mutations were observed.

Discussion

Basing on results discussed in this review, the pattern of acquired resistance to third-generation EGFR-TKIs seems to be extremely various and heterogeneous, probably more complex than that of first- and second-generation EGFR-TKIs. Higher heterogeneity may be the result of wider sequencing approaches employed, of more sensitive molecular analysis techniques used and also of the assessment of plasmatic samples in several studies.

In particular, liquid biopsy appears to be the more promising source to fully understand mechanisms of acquired resistance, bypassing the limit of inter-metastatic heterogeneity. This concept is clearly evidenced by Chabon and colleagues who found out evidence of multiple resistance mechanisms at a very high frequency (46% of T790M-mutant patients) (11). However, liquid biopsy presents a relevant limitation, related to the impossibility to detect histological transformation, described as resistance mechanism of all generations EGFR-TKIs (16,28). Invasive and non-invasive biopsy methods have areas of overlap as well as distinct advantages or disadvantages in the evaluation of patients with disease progression on targeted therapies, being together able to highlight multiple mechanisms, as reported by Ortiz-Cuaran *et al*. (13).

Despite the typology of emerged resistance mechanisms, all studies evidenced the original *EGFR* activating mutation as detectable at the time of resistance, except only one patient in Kim *et al*. cohort (28), suggesting that EGFR remains the principal driver for neoplastic clones even after drug selective pressure. For this reason, new EGFR inhibitors and combined therapies with other target agents are under evaluation (*Table 3*). Jia *et al*. have recently published the results of preclinical tests of a new molecule, EAI045, obtained from the EGFR allosteric inhibitor EAI001 (45). Whilst EAI045 seems to be inactive towards del19 variants, it demonstrated, when combined to cetuximab, to potently inhibit both double mutant L858R/T790M and triple mutant L858R/T790M/C797S cells.

In a preclinical model of acquired resistance to rociletinib via *MET* amplification, Chabon and colleagues raised the hypothesis that combination of target therapy for both *EGFR* an *MET* genes could overcome drug resistance (11). Rociletinib resistant cells were treated with rociletinib and crizotinib, MET inhibitor, with consequent restoration of rociletinib sensitivity. Similar results were obtained also with a new third-generation EGFR-TKI, as EGF816 combined INC280, a cMET inhibitor (46). Moreover, to address resistance via *MET* amplification recently a bispecific EGFR-cMET antibody was developed with very encouraging results *in vitro* and *in vivo* (47). Similarly, as mentioned above, different studies, presenting activation of RAS-MAPK pathway as mechanism of acquired resistance, provide results of a combination of third-generation TKI with a MEK inhibitor (13,31,43). Overall, these data support the use of a combination of EGFR-TKIs with an inhibitor of a different pathway (MET, MEK, IGFR, etc.) to delay or prevent resistance to EGFR-TKI or to treat patients who have progressed with a specific resistance mechanism. Several trials have been developed and are now recruiting patients, offering combined therapies with third-generation EGFR-TKIs (*Table 3*).

Other ongoing studies were initiated evaluating combination EGFR-TKIs with a programmed cell death 1 (PD-1) axis inhibitors, based on a presumption that a highly

active therapy as an EGFR-TKI could induce immune priming and up-regulation of PD-L1 (48).

About C797S point mutation, the most frequent mechanism of acquired resistance to osimertinib, preclinical data suggested that the presence of the mutation *in cis* or *in trans* with p.T790M might have important implications in therapeutic decisions (20). In fact, giving that C797S positive cells seem to retain sensitivity to quinazoline-based EGFR-TKIs, the occurrence *in trans* is the premise for a combined therapy with first and third-generation TKIs, aiming to suppress C797S and T790M positive alleles respectively. Unfortunately, more frequently the two resistance mutations occur *in cis*, a condition that determines resistance to all available EGFR-TKIs, even if combined. In this situation, new generation of irreversible and reversible mutant EGFR inhibitors with strong noncovalent binding properties and with high inhibitory activities against the cysteine-mutated L858R/T790M/C797S are in development (49).

These findings raise questions regarding the best treatment sequence in clinic practice. Trials currently ongoing comparing first- with third-generation EGFR inhibitors in TKI-naive patients will be critical to determine not only the clinical efficacy but also the resistance mechanisms to these drugs when used in this setting. In fact, the sequential treatment of a third-generation followed by first-generation TKI should be considered for those patients developing C797S mutation without T790M. Combinations with other target agents (see above), combination of multiple generations EGFR-TKIs as well as of EGFR-TKIs plus EGFR antibodies (18,20) could be more effective than single agent therapy, but it has not been tested in clinic yet. Clinical trials evaluating these different approaches are awaited to further improve the treatment of EGFR-mutated NSCLC.

The acquisition of C797S is more frequent in patients progressed to osimertinib, approximately one third of treated patients (10), than in patients progressed to rociletinib, raising the hypothesis that acquired resistance could be drug-specific. These differences may be due to different potencies or pharmacokinetics of the two drugs, as well as potential off-target activities. Therefore, in case of resistance to rociletinib, combined or sequential therapeutic approaches with first-third generation TKIs may be not so relevant. Sequist *et al*. published interesting results from a group of patients progressed to rociletinib and successfully treated with osimertinib, opening a possible scenario of sequential strategy with third-generation TKIs (50). This scenario may be analogous to observations in NSCLC ALK positive patients, in whom the next-generation ALK inhibitors (ceritinib, alectinib or brigatinib) can induce responses in patients who developed resistance to the less potent crizotinib (51). Thus, rational sequencing of drugs with different patterns of resistance mechanisms may be a generalizable strategy for maximizing therapeutic benefits. However, recently the clinical development of rociletinib and also of olmutinib was interrupted.

Potential predictive factor of EGFR-TKI resistance were also indicated in this review. The ratio of T790M/activating-mutations (11,16) could predict the patients able to obtain a longer benefit from third-generation TKI, just as the pre-existing copy number gains in some genes like *MET*, *HER2* and *EGFR* (11,13). In particular, amplification of these genes could lead to an innate resistance to third-generation TKIs and justify a combination therapy. Piotrowska *et al*. also observed that EGFR amplification is very common findings especially if drug concentration is not above the level needed to suppress adequately the target (16). They speculate that higher drug concentrations or a more potent TKI-agent could not be as susceptible to this resistance mechanism.

In conclusion, the availability of third-generation EGFR-TKIs targeting T790M-mutant-specific NSCLC represents a significant development in the treatment of EGFR-mutated patients. As indicated in this review, escape mechanisms EGFR-dependent or -independent are likely to emerge, highlighting the importance of repeat tumor biopsies and/or to collect plasma circulating tumor DNA (ctDNA) at the time of disease progression. An understanding of the mechanisms of resistance is key in the future development of the next-generation of EGFR-TKIs and of new agent combinations.

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Footnote

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Molecular testing in lung cancer in the era of precision medicine

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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

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)

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

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

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

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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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

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Precision medicine in lung cancer: the battle continues

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Comment on: Papadimitrakopoulou V, Lee JJ, Wistuba II, *et al.* The BATTLE-2 Study: A Biomarker-Integrated Targeted Therapy Study in Previously Treated Patients With Advanced Non-Small-Cell Lung Cancer. J Clin Oncol 2016. [Epub ahead of print].

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Lung cancer remains the leading cause of cancer-related mortality in the United States, with approximately 160,000 estimated deaths in 2016 (1). Non-small cell lung cancer (NSCLC) accounts for 87% of lung cancers, and 40% of patients have metastatic disease at presentation (2,3). Chemotherapy, the standard treatment of metastatic lung cancer, results in a modest survival benefit compared to best supportive care, and has reached a plateau with no meaningful differences among the many platinum-based regimens used (4).

The approval of the small molecule tyrosine kinase inhibitors (TKIs) of *epidermal growth factor receptor* (*EGFR*) marked the beginning of the era of targeted therapies in lung cancer. Since then, the understanding of markers for response to EGFR TKI has evolved from clinical variables, such as female gender, Asian ethnicity, never-smoker status and adenocarcinoma histology, to genetic markers for response, namely activating mutations in the *EGFR* tyrosine kinase domain, including the most frequent exon 19 deletions, and exon 21 L858R mutations (5). Prospective studies conducted in patients with activating *EGFR* mutations consistently demonstrated improved progression-free survival (PFS) with first line EGFR TKI therapy over platinum-doublet chemotherapy, with erlotinib, gefitinib and afatinib approved by the Federal Drug Administration (FDA), based on the benefit demonstrated in randomized clinical trials (6-9).

The discovery of the *echinoderm microtubule-associated protein-like 4* (*EML4*)-*anaplastic lymphoma kinase* (*ALK*) gene fusions as oncogenic drivers in lung cancer in 2007 marked another therapeutic advance in the treatment of lung cancer (10). The serendipitous finding of activity of the *MET* inhibitor crizotinib in this molecular subset led to an expansion cohort of patients with ALK positive NSCLC treated with crizotinib (11). Subsequent clinical trials demonstrated PFS superiority of crizotinib over both front-line and second-line chemotherapy in patients with *ALK* positive NSCLC, leading to its approval in 2011 (12,13).

Despite the initial therapeutic benefit from molecularly targeted agents in *EGFR*-mutant and *ALK* positive NSCLC, patients eventually develop disease progression. Tissue specimens obtained from re-biopsy in patients with *EGFR*-mutant NSCLC at the time of disease progression have shown histologic changes such as differentiation into small cell lung cancer (14). At the molecular level, the most common mechanism of resistance is the *EGFR* T790M resistance mutation, which is seen in approximately 50% of cases (14). This finding has led to the development of third generation mutant specific EGFR TKI's to target T790M. Osimertinib is the first agent in this class to be granted accelerated approval by the FDA for the treatment of *EGFR* T790M positive NSCLC in 2015 based on the impressive results from the phase 2 trial (15).

Similarly re-biopsies in *ALK*-positive NSCLC have provided information on the mechanisms of crizotinib resistance. *ALK* kinase domain mutations, including L1196M, C1156Y and G1202R among others, have been observed in approximately a third of patients (16). The activity of next generation ALK inhibitors such ceritinib and alectinib may depend on the secondary *ALK* mutations. While both

ceritinib and alectinib are active against L1196M, only alectinib has activity against C1156Y and neither is active against G1202R (17,18). Although the sequencing of these agents is still being investigated in clinical trials, it is possible that resistance mutations identified on repeated biopsies may influence the treatment choice.

The Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) trial evaluated utility of targeted therapies in refractory lung cancer, with a unique trial design of biopsy-mandated prospective adaptively randomized therapy, based on tissue biomarker status (19). A total of 255 pre-treated patients with NSCLC were randomized to agents that were promising at the time of study design in 2005, including erlotinib, vandetanib, erlotinib plus bexarotene, and sorafenib. Patients were assigned based on testing results for *EGFR* mutation or copy number, *KRAS* or *BRAF* mutation, VEGF or VEGFR-2 expression and RXRs, cyclin D1 expression or *CCND1* copy number on study-related core biopsy specimens. The primary endpoint of the study was 8-week disease control rate (DCR), which was noted to be 46% overall, and as high as 79% in patients with *KRAS* or *BRAF* mutations treated with sorafenib. Importantly, this study showed the feasibility of performing re-biopsies on patients in real time and assigning patients to treatment accordingly, as well as the utility of 8-week DCR being used as a surrogate for overall survival (OS). Some of the study limitations included the selection of biomarkers associated with limited predictive value such as RXR and grouping markers such as *EGFR* mutation and copy number by FISH, which have distinct predictive value.

The BATTLE-2 study was developed based on the experience from the previous study, following the umbrella design with adaptive random assignment of therapy and performed in two stages (20). Nevertheless, there was a specific focus on optimizing treatments for *KRAS* mutant NSCLC, one of the most common driver mutations for which there is no specific therapy. Since there are already established treatment options for *EGFR* mutation and *ALK* translocations, patient harboring these alterations were excluded from the study. In the initial stage of the study (stage 1), 200 patients were assigned to study treatment by adaptive random assignment. Based on the discovery markers found in the initial stage, an additional 200 patients were assigned to one of the treatment arms in the stage 2. The four treatment arms were erlotinib alone (arm 1), erlotinib in combination with an AKT inhibitor MK-2206 (arm 2), MK-2206 in combination with a MEK inhibitor AZD6233 (arm 3), and sorafenib (arm 4). Patients were stratified by *KRAS* mutation status. Two hundred patients, including 27% with *KRAS* mutated tumors, were adaptively randomly assigned to the 4 treatment arms. The primary endpoint of DCR at 8 weeks was achieved by 48% of patients. The overall response rate was 3%, with median PFS of 2 months (95% CI: 1.9–2.8 months), which was not statistically different among the four treatment groups. For patients with *KRAS* mutant NSCLC, the DCR was 20%, 25%, 62% and 44% for arms 1, 2, 3 and 4 respectively, while in patients with *KRAS* wild-type tumors, the DCR was 36%, 57%, 49% and 47% for arms 1, 2, 3 and 4 respectively.

Although the BATTLE-2 study did not show a better strategy in patients with *KRAS* mutant NSCLC, it demonstrated the feasibility of re-biopsy and use of an umbrella protocol to assign patients to a particular treatment based on molecular profile. Unlike basket studies, which are based on the hypothesis that the presence of a molecular marker predicts response to therapy independent of tumor histology, and are designed to test a single drug in patients with a single gene alteration regardless of the primary tumor, umbrella studies are designed to test the impact of different drugs on different mutations in a single type of cancer (21). The rationale for the umbrella trials is to facilitate screening and accrual, since a large number of patients can be screened in the same study for multiple and often low prevalence biomarkers for which individual studies would otherwise require a large number of screened patients to achieve the target accrual. In addition to the BATTLE, there are several ongoing umbrella trials in NSCLC including the Lung Cancer Mutation Consortium (LCMC) for adenocarcinoma, the lung Master Protocol (Lung-MAP) for squamous lung cancer, and the Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trials (ALCHEMIST) in the adjuvant setting (22,23). The main objective of these trials is to facilitate the pathway towards rapid test and approval for promising novel therapies in the case of LCMC and Lung-MAP or the testing of approved drugs for metastatic disease in the adjuvant setting in the case of ALCHEMIST.

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None.

Footnote

Conflicts of Interest: D Morgensztern: Advisory Board

Bristol-Myers Squibb. The other author has no conflicts of interest to declare.

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