Key Leaders’ Opinion on Lung Cancer

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KEY LEADERS’ OPINION ON LUNG CANCER (FIRST EDITION)

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Will scholarly journals perish? This is a question that has puzzled me for years.

The introduction of online journals results in the inevitable recession of print journals. The uprise of the open access journals has been changing the structure of scholarly journals ceaselessly. What keeps me thinking is the open access of clinical trials data. What would be the bigger picture if open access to clinical trials data becomes the mainstream?

It is interesting that with the primary bottleneck lying in the availability of open data, the Big-data Clinical Trial (BCT) seems to stay where it was in spite of the increasingly popularity of “Big Data” among scientists. It has to be the fact that without open data, a statistical analysis is restricted to a particular area (or several areas). Even with big enough data, the study can only be termed as “research with big data sets” rather than “big data research”, which are totally different concepts. Big Data is constituted by a plurality of dimensions. On one hand, for an individual (e.g., a patient), the relevant data covering his/her disease course is big enough; on the other hand, for the entire population, as more as individuals (e.g., patients) are expected to be included, to contains all the elements just like the “universe set” in set theory; by doing so, scientists expect to carry out the so-called clinical studies in real-world settings.

Why do the real-world-based clinical trials so appealing? It is understandable that the results and conclusions are likely to be altered in studies targeting the same issue using the same research method with sample size changed. In addition, the probability of such a “likely” is quite high. In many top journals, it is a common phenomenon that some authors tend to validate the results of one study in another population using the same research method. However, if the results are “validated” in one population, it only means that they are “repeatable”. Will the results also be repeatable in the second, third, and more populations? If the attempts are not continuing, which should be, the “validation” is equivalent to “self-deception” in a sense.

When clinical research data is open accessed, we can easily integrate data from multiple centers for statistical analysis and meanwhile “validate” the results in multiple populations. If this is the case, then another question arise: can everyone easily publish his/her results/papers in high-profile journals such as the New England Journal of Medicine? My answer is NO.

When the open access to clinical research data becomes mainstream, we can easily find the constant update of database on the Internet. Simply by clicking on a button, we obtain the statistical results of the most current data. A further button click would display the validation results based on a specific population. The database would be updated at a certain period of time (e.g., 1 month or 1 day), and the statistical results would “likely” also be changed accordingly. At that time, the questions may change to “would any researchers publish their findings in a journal?” Well, even if someone is still keen to write such articles, journals may be reluctant to publish them because of the indefiniteness of the findings with the risk of being overturned at anytime.

Eventually here it comes the serious question: will scholarly journals perish? My answer is still NO. Then in what way the scholarly journals would probably lead to?

During my Business Administration course, my teacher distributed to us an article from the Case Study column of the Harvard Business Review. In this highly respected journal, articles in this column often present one case first, followed by the comments from two experts. These comments could either support or oppose each other. My teacher asked us to study the case, read through the comments and then form our own point of views on the case. He encouraged us to interpret the case from different perspectives independently in what form that I found pretty practical.

The course brought a possible answer to me. When the open access to clinical research data becomes mainstream, the entire publishing industry, especially the publication of “scholarly journals”, would eventually experience revolutionary change. It may no longer focus on the rigid and cold outcomes but it would definitely cares more about the reflection on the problems, update of insights, and integration of science and arts.

AME Medical Review Series is a production of the above thinking. As an attempt, we decided to invite experts internationally to provide their views on a specific topic to share their insights with more clinicians and thus benefit more patients. The first chosen topic for the series is the currently controversial one: conventional surgery versus stereotactic body radiotherapy for
the early stage lung cancer. As the first book to the series, we hope it would give you a glance at the coming changes.

The book series will be written by a group of individual experts who are willing to contribute medical reviews and comments to individuals who are interested in clinical research and medical reviews specifically. The book in your hand may possibly be on a heavy subject but we do hope it is presented in an easier way. It will be more than great if it brings you some thoughts and inspire you in some way.

Stephen D. Wang
Founder and CEO,
AME Publishing Company
Lung cancer (LC) is the leading cause of oncological morbidity and mortality, accounting for approximately 1.8 million new cases and 1.6 million cancer deaths every year worldwide (http://globocan.iarc.fr/old/FactSheets/cancers/lung-new.asp). LC is used to be the disease of smokers and therefore was believed to be a largely preventable malignancy. Recent studies demonstrated an alarming increase of LC incidence among non-smokers (1). Tumours arising in smokers and non-smokers show clearly distinct mutation profiles, indicating that these two categories of LC may require distinct avenues for research and medical intervention (2-4).

Lung cancer is usually classified for non-small cell LC (NSCLC) and small-cell LC (SCLC). SCLC is a highly aggressive category of LC, which is rarely treated by surgery but demonstrates substantial sensitivity to cytotoxic therapy. In contrast to NSCLC, gross amounts of primary SCLC tissue are rarely available for investigation. Recent technological advances provided a handful of methods, which allow comprehensive molecular profiling of minimal amounts of tumour cells. Focus on SCLC revealed a number of intriguing observations, which are discussed in this book. In particular, SCLCs are almost always characterized by inactivation of two most known suppressor genes, p53 and RB1. SCLC may develop directly from lung epithelial cell precursors, or, alternatively, evolve from NSCLC, especially if the latter is treated by tyrosine kinase inhibitors (TKI). SCLCs share some similarities with large-cell neuroendocrine lung carcinomas (LCNECs). Recent studies led to identification of molecular events distinguishing SCLC and LCNEC (5). Furthermore, next-generation sequencing analysis of LCNEC revealed a number of potentially druggable targets in this category of tumours (6). Treatment options for SCLC and LCNEC are currently limited to cytotoxic therapy, which usually produces only short-term effects. Recent clinical trial of rualpituzumab tesiromine, a conjugate of DNA cross-linking agent and antibody recognizing a member of Notch receptor ligand family, DLL3, showed promising results in this difficult-to-treat category of patients (7). Various aspects of SCLC and LCNEC biology and treatment are comprehensively discussed in this book.

Notch signalling pathway is implicated in the regulation of cell-cell communication and plays a role in cellular differentiation. Alterations of Notch cascade are involved in pathogenesis of various human malignancies. Recent studies revealed that Notch pathway may be a promising target for the management of KRAS-mutated tumours. RAS-driven cancers account for approximately 15-30% of LCs. Activating RAS mutations are mutually exclusive with the mutations in druggable kinases (EGFR, ALK, ROS1, BRAF, HER2, MET), with no specific RAS-targeted therapy available at the moment. Ambrogio et al., 2016 investigated early stages of KRAS-driven lung cancer transformation and revealed a critical role of DDR1 kinase activation. Combined inhibition of DDR1 and Notch signalling resulted in the regression of KRAS-mutated tumours in mice (8).

Cancer progression was long believed to be a relatively slow gradual process involving multiple consecutive genetic events and more or less time-consuming transition from curable to incurable disease. Therefore, extraordinary efforts have been invested in the development of tools permitting to identify cancer disease at early, yet curable stages. These activities led to some success, for example, to the reduction of mortality from cervical, prostate and some other cancers (9,10). However, there are some unexpected findings. For example, while lung cancer can be relatively easy visualized through the use of various types of X-ray imaging, positive impact on LC mortality has been obtained only with the use of highly sophisticated diagnostic method, low-dose computed tomography (CT) (11). Furthermore, while the reduction of mortality approaches to only 20%, the frequency of false-positive findings and consequent unnecessary medical interventions is unacceptably high. For above reasons, LC low-dose CT screening is recommended only for current or recent heavy smokers aged between 55 and 80 years, and the optimal interval for examinations is believed to be around 2 years. Further details on LC screening are summarized in this book.

It appears that conventional imaging technologies are unlikely to resolve the issue of reliable and early LC detection in the near future, therefore alternative diagnostic approaches are being intensively studied. Use of molecular diagnostic tools is considered to be the most promising, given that somatic genetic alterations are highly specific for transformed cells and that PCR-based technologies are capable to detect single copies of altered genes. None of well-known mutations (KRAS, EGFR, ALK, p53 etc.) occurs in all LC, therefore the perspectives for mutation-based screening may look limited. It has been established that the majority of LC are characterized by hypermethylation of some regulatory gene regions. Furthermore, the pattern of methylated DNA sequences is more or less conservative, so a relatively limited set of methylation DNA markers may theoretically distinguish between cancerous and non-cancerous tissues (12).

Almost all next-generation laboratory diagnostic tools rely on so-called liquid biopsy. Presence of residual tumour cells and/or their fragments in body fluids of cancer patients was acknowledged a long time ago. There is a number of serum protein markers, e.g., PSA, CA-125, CEA, which demonstrate reasonable tissue specificity and are often elevated in patients with prostate, ovarian, or gastrointestinal cancers, respectively. DNA-based diagnostic tools are likely to have advantages as compared to protein markers. As already mentioned above, presence of cancer-related mutations is relatively specific for transformed cells, therefore some common
pathological processes, such as inflammation or hyperplasia, are unlikely to result in false-positive signals. Liquid biopsy may be applied to various body liquids, including blood, urine, saliva etc. Analytical methods may be targeted to circulating tumour cells (CTCs), cell-free DNA, microRNA etc.

There are three main avenues for the use of liquid biopsy. First, it is projected to become a screening method, aimed to replace or assist various imaging techniques. As example, one may refer to already existing PSA screening for prostate cancer. For lung cancer, the main challenge is to compose sufficiently specific panel of markers, which would reflect molecular alterations in the majority of LC. Secondly, there are studies demonstrating that molecular alterations in primary LC may be reliably detected using blood tests. The need for blood-based molecular profiling for already diagnosed LC is limited: in modern oncology the mere diagnosis of cancer disease is almost always based on tumour biopsy, so the cancer tissue is available anyway; furthermore, treatment-naïve neoplasms usually do not show intratumoural heterogeneity with respect to driver mutations. Thirdly, liquid biopsy may serve as a tool for monitoring and analysis of the tumour burden during the treatment. When tailored to molecular markers detected in primary malignancy, it may estimate an overall cancer volume during the treatment. Furthermore, novel drugs, like osimertinib, are tailored to secondary mutations acquired during earlier lines of therapy, e.g. EGFR T790M. The diagnosis of these mutations in tumour tissue may not be feasible, as it requires multiple re-biopsies and ignores possible heterogeneity of treatment resistance pathways in distinct metastases obtained from the same patients. Accordingly, liquid biopsy is clearly a method of choice in these clinical situations. This book offers a comprehensive discussion on various aspects of liquid biopsy.

The discovery of TKI-sensitizing mutations in lung cancer may be regarded as the most impressive achievement of clinical oncology in the last decade. Patients with EGFR, ALK and ROS1 mutations have drastically improved survival if treated with appropriate targeted drugs (13). Testing for druggable mutations has been incorporated in LC management routine. It is of high interest to see the reports of French Cooperative Thoracic Intergroup (IFCT) initiative, which summarized the first results of LC molecular profiling in a nationwide scale (14,15). There are several other nationwide studies carried out in China, Taiwan, Korea, Russia etc., which allowed to investigate some LC molecular markers with an unprecedented level of comprehension (16-20).

This book provides a valuable update on recent LC clinical trials. In particular, extensive efforts are being invested to further optimize the treatment of EGFR-mutated LC. LUX-7 trial performed the direct comparison of gefitinib and afatinib. While the efficacy of gefitinib towards LC carrying EGFR mutations was discovered by retrospective analysis of the results of lung cancer clinical trials, afatinib was intentionally designed to target EGFR mutation-driven LC. In addition, afatinib exerts activity towards other kinases of HER family. LUX-7 trial involved 319 patients. The investigators acknowledged that afatinib demonstrated statistically longer progression-free survival (11.0 vs. 10.9 months, \( P=0.017 \)) and time to treatment failure (13.7 vs. 10.5 months, \( P=0.007 \)) (21). It is of question whether these small advantages, although being clearly statistically significant, are of high medical relevance (22,23), especially given that no differences in overall survival was observed in this trial (24).

There are also trials attempting the simultaneous use of chemotherapy and gefitinib in patients with EGFR-mutated lung cancer (25,26). Development of osimertinib, a potent EGFR inhibitor specific both for gefitinib-sensitizing mutations (ex19del and L858R) and for gefitinib-resistant substitution (T790M) represents a major breakthrough in the LC treatment. Osimertinib demonstrated remarkable activity in LC patients, who progressed on gefitinib via an acquisition of EGFR T790M mutation (27,28). It also produced unprecedented long progression-free survival when given as an upfront therapy (29).

Crizotinib was the first drug approved for the treatment of ALK and ROS1-rearranged lung cancer. It demonstrates significant advantage over chemotherapy with regard to response rate, progression-free survival and control of brain metastases (30-32). Focus on ALK-rearranged cancers led to development of next-generation ALK inhibitors, e.g., ceritinib, which is more potent than crizotinib, capable to penetrate blood-brain barrier and shows activity towards crizotinib-resistant disease. Ceritinib demonstrated pronounced and durable responses both in crizotinib-treated and TKI-naïve LC patients with ALK translocations, including subjects with intracranial metastases (33,34). Similar results were reported for ROS1-rearranged LC (35). Another novel ALK inhibitor, brigatinib, also showed promising activity towards lung carcinomas carrying ALK fusions (36).

Administration of ALK inhibitors usually relies on FISH analysis, which demonstrates the mere fact of the presence of ALK translocation but is unable to identify the type of ALK rearrangement among the diversity of existing fusion variants. This practice may not be supported by the clinical and laboratory evidences, which suggest that the type of ALK translocation may influence tumour responsiveness to crizotinib (37-39).

Crizotinib was initially developed and clinically assessed as a MET inhibitor, however subsequent discovery of ALK and ROS1 translocations led to the change of its indications for tumours carrying rearrangements in the above genes. However, recent studies
revealed that approximately 3% of lung carcinomas carry activating mutations in MET gene, and these tumours are generally responsive to this drug (40). Thus, if one considers frequencies of all crizotinib-sensitizing mutations (ALK: 4-8%; ROS1: 1-2%; MET: 3%), the total fraction of non-squamous non-small LCs amenable to crizotinib treatment would approach to approximately 10%.

Approximately 2% lung carcinomas carry activating mutations in the codon 600 of BRAF oncogene. Treatment strategies for this category of tumours were initially established in melanoma, as this type of skin tumours is generally not responsive to conventional cytotoxic treatment and BRAF V600E mutations are detected in approximately a half of these neoplasms. Similarly, good responses were detected in lung cancer patients, where pronounced and durable reduction of tumour size was observed in the majority of subjects receiving BRAF mutation-specific inhibitor alone or in combination with MEK inhibitor (41-43).

Failure of immune system was long considered to be an essential component of cancer progression. Extensive efforts have been invested to find the signs of systemic immune suppression in cancer patients, however these studies were largely unsuccessful. Recent investigations resolved this apparent paradox. It was revealed, that immune deficiency indeed contributes to cancer development, however its extent is limited to a peritumoural space, as immune suppressors are produced locally either by tumour cells or tumour-infiltrating immune cells (44,45). This decade may be regarded as a triumph of immune checkpoint inhibitors, which demonstrated substantial clinical activity towards many cancer types. Noteworthy, targeted immune drugs led to a breakthrough in the treatment of lung cancer, especially of LC arising in smokers and lacking druggable mutations. Unfortunately, immune checkpoint inhibitors result in clinical responses only in a subset of patients, therefore there is continuing search for better drugs and their combinations. In addition, studies aimed to identify predictive markers for immune therapy are currently underway (29,46-48).

This book is likely to be of high interest for medical oncologists, translational researchers and cancer biologists.

Acknowledgement
This work has been supported by the Russian Foundation for Basic Research (grant 17-54-12007).

References


Translational medicine is a significant and rapidly evolving aspect of current clinical practice, especially in the field of cancer research. It is an umbrella term encompassing multidisciplinary collaborations to bridge the gap between basic research and clinical practice, with the aim of enhancing patients’ preventive, diagnostic and treatment options for a wide range of diseases through the “bench to bedside” mode.

Lung cancer is the leading cause of cancer-related deaths worldwide. In recent years, the biology of lung cancer has been more clearly recognized through basic and clinical researches. Simultaneously, the prognosis of lung cancer patients has also improved greatly with the development of new technologies and interventions, of which the following two are particularly significant: (I) liquid biopsy: this is an emerging technology that may make up for the limitations (e.g., insufficient quality and quantity of advanced-stage patients; inability to dynamically monitor mutation status etc.) of tissue biopsy which is currently regarded as “gold standard”. Body fluids acquired from patients can be used to measure cancer biomarkers such as cell-free DNA (cfDNA), circulating tumor cells (CTC) and exosomes; (II) targeted therapy: treatment strategies for advanced lung cancer, in particular non-small cell lung cancer (NSCLC) are mainly guided by driver gene mutations. Small molecule tyrosine kinase inhibitors, with promising efficacy and acceptable toxicity, have been developed for treating patients with specific gene mutations, including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1).

Actually, novel biomarkers for screening, diagnosing and treating lung cancer are being developed in parallel and incorporated into clinical practice as a result of translational research. In the era of precision medicine, one should note that management of lung cancer is a comprehensive and systematic work which requires multidisciplinary collaborations (Figure 1).

Multidisciplinary collaborations, especially in the field of cancer research, need the establishment of effective academic exchange platforms. Fortunately, AME Publishing Company has provided an excellent academic communication platform for scholars and clinicians. I am really great honored to have served as the Section Editor (Lung Cancer) of Translational Cancer Research in the past year. During my first stage as a Section Editor, fifty original newly published articles with high representativeness and great clinical significance were recommended and more than 300 international experts were invited to comment on those publications. Commentaries, Editorials, and Perspectives are invited as per our editorial arrangements and the preference of the experts. In addition, we also invited the authors of these original articles to write Correspondences based on the comments we received. Between February 2016 and January 2017, we received 9 Commentaries, 27 Editorials, 9 Perspectives and 6 Correspondences authored by international top-rated experts in the field of lung cancer research. All of these articles are included in this Medical Review Serial book and are classified into the following four sections according to their contents: (I) Cancer Biology, (II) Screening and Prevention, (III) Diagnosis and Monitoring, and (IV) Treatment.

I sincerely hope that this book will play an effective role in promoting academic exchange in the high-quality platform of AME Publishing Company and Translational Cancer Research. I believe that the contents of this book will also be helpful to our readers vis-a-vis scientific writing, professional knowledge, study design and clinical practice in the field of lung cancer since the opinions and experience of the international experts are definitely valuable and worth learning. However, I must respectfully admit to certain unavoidable limitations in the depth and width of the topics covered in this book and hope our readers could come up with better suggestions to improve the quality of this book and the Medical Review Serial books in the future.

Last but not the least, here I would like to express my heartfelt gratitude to the Science Editors, Lucille L. Ye and Celine G. Lin for their kind assistance and patience in sending out and following up on our invitations from February 2016 to January 2017. It is my great pleasure to work with them and their excellent work is highly appreciated.
Figure 1 A concise flowchart describing the process from lung cancer screening and treatment.

References


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In the last few years, almost every aspect of thoracic oncology has experienced research breakthroughs. These range from improvements in how we understand the basic biology of cancer, to how we should best detect it, categorize it, treat it, and monitor it for recurrence or acquired resistance to therapy.

Once each development is presented at an academic meeting the job of truly understanding these new data begins. In this setting, a good discussant – one who follows the presentation, acting as the scientific ‘everyman,’ can make this job so much easier. Indeed, perhaps, in addition to the plethora of ‘best of ASCO’, ‘best of ESMO’, and best of ‘WCLC’ selections which focus solely on the new data, many academic meetings should seriously consider creating ‘best discussant’ awards to highlight the invaluable benefit some of our brightest and best minds contribute through this role.

Once the data are finally published, a good editorial, like a good discussant, can similarly enhance a reader’s appreciation of both the strengths and weaknesses of the science. However, as these commentaries are essentially personal views - opinion pieces that write an essay around a particular paper - more than one point of view may exist. Just as illumination of a building at night and from different angles can change our appreciation of its architecture compared to our view of it in the daytime, the value of several different big thinkers looking at the impact of the same new publication can be very revealing.

In this book, Translational Cancer Research has gathered together some of the best minds in Thoracic Oncology – some established leaders, others the key opinion leaders of tomorrow – and captured their thoughts on some of the newest publications in the field. This collection of commissioned editorials offers a unique opportunity to put a whole range of recent research breakthroughs in thoracic oncology into context. In addition, it should provoke the reader into exploring their own opinions and views on the field, helping everyone to feel like they can contribute to the larger conversation that will continue to drive progress forward.

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In the last decade there has been huge progress in identifying molecular mechanisms of lung cancer that can be targeted with specific treatments. In parallel technology advances in genomics, tissue and liquid biopsy have been revolutionary and point to a future where patients may be diagnosed, treated and monitored using precise molecular information detectable in blood samples.

This book presents a timely and comprehensive insight into emerging knowledge that is highly relevant to clinicians and researchers in the field of lung cancer translational research. The book is divided into themes of cancer biology, screening and prevention, liquid biopsy, targeted therapy, chemotherapy, immunotherapy and radiotherapy. It provides up to date evidence for the progress made in these areas as well as unanswered questions and research that remains to be done in order to improve outcomes for patients.

**Fiona Blackhall**
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Preface

Introductory text to a book, the preface can be short and hold warning – not an advertising, or widely developed, such as prolegomena. So, what about the *Key Leaders' Opinion on Lung Cancer*?

As a warning, this is not a new textbook on Lung Cancer. Rather, it is a non-exhaustive compilation giving a special focus on some major advances made in the field of lung cancer during the past two years. It is a book of over 200 pages, involving a large number of authors from around the world and rich in figures and tables that many of us will use tomorrow in our talks.

The approach is original. This is a collection of editorials or commentaries published in the Translational Cancer Research journal about articles leading from prestigious generalist journals, i.e. Nature, The New England Journal of Medicine, Lancet or JAMA, or oncology journals, i.e. Journal of Clinical Oncology, The Lancet — Oncology, Annals of Oncology, Clinical Cancer Research or Cancer Discovery. Faceciously, the authors of the book find themselves in turn, here, in the position of actor, there, in that of commentator of the research on lung cancer. Also creating the impression of a dialogue between the experts, the same articles have been commented by different groups of authors. This approach allows to qualify what for some could hold of the exploit, where for the others it is about the expected result of the scientific approach.

The book is also characterized by the diversity of topics covered and the desire to move from biology to imaging, targeted therapies to chemotherapy, or from cancer screening to palliative radiotherapy. The great challenges of today and tomorrow are put into perspective. In the first chapter, for example, the importance of basic science is posited as a prerequisite for improving the treatment of neuroendocrine cancers. New signaling pathways — NOTCH pathway, or new pathophysiological mechanisms – epithelial-mesenchymal transition or nutrient microenvironment availability, are explored. In the next two chapters, the importance of precision medicine tools such as, circulating tumor cells and tumor DNA, or the ability of a national health care system to make available to all patients the search for actionable mutations (Biomarqueurs France initiative), are put forward. The longest part of the book is devoted to targeted therapies for EGFR and ALK alterations, but also for BRAF V600 and MET exon 14 skipping mutations in adenocarcinoma, and finally for DLL3 protein target as a hope in the treatment of small cell carcinoma. Several articles are devoted in particular to the heterogeneity of ALK rearrangements and its theoretically therapeutic impact, to the difficulty of the management of brain metastases in ALK disease and to the therapeutic hope provided by some second and third generation ALK-inhibitors (ceritinib, brigatinib). The remaining articles focus on the targeting of the EGFR T790M resistance mutation by osimertinib and the double blocking of BRAF/MEK pathway in the treatment of BRAF mutated non-small cell carcinoma. The articles dealing with chemotherapy are exclusively dedicated to the treatment of small cell carcinoma, as if the chemotherapy had disappeared from the non-small cell carcinoma’s therapeutic armamentarium. Two articles question the place of radiotherapy in the treatment of patients with brain metastases, following the rather negative results of the Quartz trial, but which, in our opinion, included a patient population with a particularly poor prognosis.

Finally, one regret, but which could be formulated as a wish to see a second season to this first series of *Key Leaders' Opinion on Lung Cancer*, is the very small place made for the immunotherapy in lung cancer treatment. Using immune checkpoint inhibitors has revolutionized the treatment of advanced non-small cell carcinoma, but also seems to be a hope for small cell carcinoma and mesothelioma and could also have a major role in the therapeutic strategy of localized non-small cell lung carcinoma, in association with surgery or radiotherapy.

Long life to *Key Leaders' Opinion on Lung Cancer*!

Jacques Cadranel, MD, PhD
Head of Chest Department and Expert Center in Thoracic Oncology, Hôpital Tenon AP-HP and Pierre et Marie Curie Paris 6 University, Paris, France
Lung cancer is the leading cause of cancer mortality worldwide. Over the last decade, there have been tremendous strides towards improving outcomes for lung cancer patients, in particular those afflicted with non-small cell lung cancer (NSCLC). This progress stems from increased understanding of the genetic and molecular mechanisms of tumor initiation and progression. Identification of various oncogenic mutant forms kinases, such as EGFR, ALK, ROS1, BRAF, and others now affords a targeted treatment option for many NSCLC patients. Immunotherapy with checkpoint blockade antibodies (against PD1 or PDL1) benefits other molecular classes of NSCLC patients, such as those with squamous cell carcinoma and high tumor expression of PD1 or PDL1. Survival outcomes are now improving at a pace never before witnessed in lung cancer management. Simultaneously, the quality of life for patients is improving as well, as these biomarker-driven molecular treatments are generally less toxic than conventional cytotoxic chemotherapy.

This volume focuses on this recent progress. Current challenges that remain obstacles to transforming lung cancer into a chronic or curable disease are also discussed. These challenges include the clinical problem of drug resistance that limits long-term patient survival. Additionally, the profound inter- and intra-tumor heterogeneity present within a lung cancer in individual patients is emerging as a key challenge to the use of molecularly targeted agents including current immunotherapy.

Potential solutions to these challenges are discussed in this volume, bringing hope for the future. These include understanding the molecular basis of drug resistance and elucidating the extent and clinical relevance of tumor molecular and cellular heterogeneity. Improved molecular diagnostics, such as the use of circulating biomarkers (for instance, circulating free DNA in blood) offer for the first time the possibility of “liquid biopsies” to monitor and quantitatively measure disease burden and tumor recurrence or progression on therapy.

We hope this volume enhances the understanding of the importance of continued research to catalyze even greater progress towards converting lung cancer from a lethal disease into a long-term chronic disease, or perhaps even a curable condition, in the years to come.

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Introduction

Lung cancer has been reported to be one of the leading causes of cancer death worldwide, and especially among various types of lung cancer, small cell lung cancer (SCLC) is the most aggressive type, showing a rapid growth and metastasis, in spite of a temporary response to chemoradiotherapy (1). Improvements in treatment of SCLC have not been remarkable in the past decades, and the standard chemotherapy regimen of cisplatin or carboplatin plus etopside, used for the first-line treatment of SCLC, has not changed over the past four decades (2). Fundamental studies on molecular mechanisms of small cell carcinogenesis have not been fully established, and significant progresses will be anticipated, in order to explore novel therapeutic development as soon as possible. In the recent years, few studies analyzing a relatively large scale, to search for essential and critical molecules in SCLC, were reported (3-5), and the importance of various pathways, such as cell cycle regulation associated with TP53 and RB1, receptor-kinase signaling, transcriptional network, Notch signaling, and guidance molecule system, were pointed out (3-5). In a recent article...
reported by Meder et al. (6), they proposed that secondary SCLC, could be derived from non-small cell lung cancer (NSCLC), through loss of Notch activity, accompanied with increased achaete-scute homolog 1 (ASCL1) activity, with further additional genetic changes in Tp53 and RB1. They analyzed combined type SCLC from the viewpoint of the Notch-ASCL1-p53-RB axis, and—to our knowledge—it was the first article to discuss comprehensively the molecular mechanisms of small cell carcinogenesis regarding these four molecules. This article is very interesting as it reports (I) SCLC could be generalized basically by changes in the Notch-ASCL1-p53-RB axis; (II) pre-acquisition of potential neuroendocrine differentiation through modulating Notch-ASCL1 balance seems to be important in the development of SCLC; and (III) there could be an alternative pathway in small cell carcinogenesis.

In the current perspective, although many issues remain to be solved for understanding the molecular mechanisms of carcinogenesis of SCLC, and making reference to the article reported by Meder et al. (6), we will discuss some of the recent insights into the mechanisms of neuroendocrine differentiation, and expand the argument on small cell carcinogenesis.

Figure 1 Achaete-scute homolog-1 (ASCL1, termed Mash1 in rodents) is a proneural basic helix-loop-helix transcription factor, and functions in small cell lung cancer (SCLC) as not only induction of neuroendocrine differentiation, but also as a regulator of various biological activities such as cell proliferation, survival, shape, motility and chemoresistance. In the contrary, Notch signaling is involved in small cell carcinogenesis (5,6) and functions in small cell carcinoma not only to suppress neuroendocrine differentiation, but also to control various biological activities such as cell proliferation, survival, shape, motility and chemoresistance.

Transcriptional regulation of neuroendocrine differentiation

Pulmonary neuroendocrine cells are specialized epithelial cells, distributed sparsely throughout the lung epithelia, from the bronchus to the bronchio-alveolar junctional area, and could serve to maintain the homeostasis of airway microenvironments (7,8). Various transcription factors have been reported to determine neuroendocrine differentiation in the normal and neoplastic lung epithelial cells, and ASCL1; a proneural basic helix-loop-helix transcription factor, has been regarded as a neuroendocrine inducer and lineage marker (9-12). In normal epithelial cells, transfection of ASCL1 gene directed epithelial cell toward neuroendocrine differentiation, and in a lung adenocarcinoma cell line, cell morphology and proliferation activity were altered by ASCL1 transfection (6,13,14). ASCL1 appears to be involved in the cell growth, survival, differentiation, cell adhesion, and chemoresistance (Figure 1). According to Osada et al. (14), inhibition of ASCL1 suppressed cell proliferation and induced apoptosis in SCLC cell lines, which could signify that ASCL1 plays pivotal role in carcinogenesis of SCLC. Expression of ASCL1 is suppressed by Notch signaling in normal epithelial cells and cancer cells (15-17), and Hes1; one of the representative target genes of Notch signaling pathway and a repressive basic helix-loop-helix transcription factor, is a strong suppressor of ASCL1 in developing mouse lung and in SCLC cells (10,18). One of the ASCL1 candidate regulators in SCLC cells is Repressor element-1 silencing transcription factor (REST, as it suppresses the expression of ASCL1 through epigenetics mechanisms in neurogenesis (19). Besides, REST is deficient in SCLC cell lines (20).

In addition to ASCL1, Brain 2 (BRN2); a POU domain transcription factor, is a developmentally neural-cell specific factor, and could participate in neural differentiation of SCLC cells (18,21). Recently, a zinc-finger transcription factor; insulinoma-associated protein 1 (INSM1), was reported as a crucial regulator for neuroendocrine differentiation for normal lung epithelial cells (22) and SCLC cells (18), and INSM1 could regulate the expression of both ASCL1 and BRN2 in lung cancer cell lines (18). In addition, INSM1 alone can induce neuroendocrine differentiation in NSCLC cell lines (18). Moreover, the expression of INSM1 and ASCL1 was suppressed by the activation of Notch signaling (18).

Another transcription factor that regulates neuroendocrine differentiation...
differentiation in lung cancer cells, is retinoblastoma (RB) gene product. There is an interesting report, which showed that increased pulmonary neuroendocrine cells are observed in Rb1 gene-deficient mouse lungs (23). Considering that Rb1 is one of the essential genetic abnormalities in SCLC, an attractive molecular research field for studying the relation between neuroendocrine differentiation and RB abnormalities remains to be explored. In addition, pulmonary neuroendocrine cell hyperplasia in Rb1 gene-deficient mice disappears with loss of of E2f3, one of Rb1 targets (24).

**Significance of Notch1 in small cell carcinoma**

Notch signaling is one of the most important cell signaling system, and through interaction with ligands of the Delta and/or Jagged/Serrate families, it regulates several genes such as Hes1, cyclinD1, c-Myc and Akt (25). The importance of Notch signaling in carcinogenesis has been reported in controlling the differentiation, metabolism, cell cycle progression, angiogenesis, stemness, and of cancer cells (26). In lung cancer, Notch exhibits both tumor promoting and suppressive functions. A whole genome sequencing study of SCLC cases revealed mutations of Notch family genes in about 25% of the cases examined, suggesting a tumor suppressive nature of Notch in SCLC cells (5). In SCLC cell lines, gene transfection and knockdown experiments clarified that Notch1 plays significant role in suppression of cell proliferation, enhancement of apoptosis, induction of epithelial morphology (mesenchymal-epithelial transition), suppression of motility, acquisition of drug resistance and suppression of neuroendocrine differentiation (Figure 1) (17,27-29). Regarding cell fate determination, Notch1-Hes1 pathway is a repressor of neuroendocrine differentiation through decreased expression of ASCL1 and INSM1 (10,16,18,30). Using immunohistochemistry, pulmonary neuroendocrine cells are positive for Ascl1, but negative for both Notch receptors and Hes1, while lung non-neuroendocrine cells are negative for Ascl1, but positive for Notch receptors and Hes1 (Figure 2A). This mutually exclusive expression pattern is true in lung cancers; as also confirmed by western blotting analyses, which revealed that SCLC cell lines are positive for ASCL1 and/or INSM1, but negative for Notch1, and NSCLC cell lines are negative for ASCL1 and/or INSM1, but positive for Notch1.

The combined type SCLC has both SCLC and NSCL compartments (18). Immunohistochemically, the SCLC compartment is positive for INSM1, but negative for Notch1, and the NSCLC compartment is negative for INSM1, but positive for Notch1 (Figure 2B), which suggests that Notch signaling pathway is important in determination of the subtypes of SCLC. Some molecular mechanisms of down-regulation of ASCL1 by Notch signaling have been proposed. The human ASCL1 promoter region has broad transcriptional enhancer and tissue-restricted transcriptional repressor motifs (31), and the repressor motif, an N-box sequence, is sensitive to Notch signaling activity via Hes1 binding (32). Moreover, Notch signaling can induce degradation of ASCL1 through proteosome activation (15). A combination of inactivation of Notch signaling, with expression of ASCL1, direct lung epithelial cells to a neuroendocrine phenotype (30), and this molecular relationship seems to be essential in the origin of SCLC (6).

**Molecular mechanisms of small cell carcinogenesis**

Frequent mutations in both TP53 and RB1 have been identified in SCLC cells (3-5), and thus it seems reasonable that genetic events with the bi-allelic TP53 and RB1 mutations must determine the process of small cell carcinogenesis. Actually, the experimental study by Meuwissen et al. (33) showed that mice carrying conditional alleles for both Trp53 and Rb1 developed small cell carcinoma in the lung, which supports the fact that inactivation of both Tp53 and Rb1 is a prerequisite event for the pathogenesis of SCLC. Following this study, several mouse models which developed SCLC, have abnormalities in both p53 and Rb1 genes, and detailed analyses of the pathobiology of these SCLC in these models is useful to understand human SCLC (34). According to Meder et al. (6), SCLC has two oncogenic pathways: primary SCLC, which comes from neuroendocrine precursor cells, with bi-allelic TP53 and RB1 mutations, and secondary SCLC, which comes from Notch-defective NSCLC, which already has TP53 mutations and acquire additional RB inactivation.

Regarding to the origin of primary SCLC, they consider that neuroendocrine precursor cells -which are characterized by inactivation of Notch signaling and ASCL1 expression- are the origin of primary SCLC. This hypothesis could be accepted, as in mouse developing lungs, ASCL1 expressing cells could be a progenitor for various epithelial and mesenchymal cells (11), and could have migrating activity (12). However, using cell lineage-restricted Adeno-Cre virus, Sutherland et al. (35) showed clearly
that loss of Tp53 and Rb1 could efficiently transform neuroendocrine, Clara and type 2 alveolar cells into SCLC cells (35). This study suggests that these epithelial cell lineages could be the origin of SCLC, and that inactivation of Notch signaling and ASCL1 expression are not always necessary to initiate SCLC development. Regarding to the origin of secondary SCLC, transformation from NSCLC to SCLC has been noticed, as a result of acquisition of resistance mechanisms against EGFR tyrosine kinase inhibitors (36). Niederst et al. (37) reported that such transformation from adenocarcinoma to SCLC always accompany the loss of RB1 gene, yet RB1 gene knockdown did not induce neuroendocrine differentiation in the EGFR mutant adenocarcinoma cell line. The combined SCLC cases presented by Meder et al. (6), suggest that NSCLC harboring Notch abnormalities, could become SCLC, with the addition of RB1 gene mutations, although abnormalities were reported in Notch2 but not in Notch1, and that inactivation of Notch2 is not a strong inducer of neuroendocrine differentiation (16). Considering that adenocarcinoma with mutant EGFR could transform to SCLC with the addition of RB1 gene abnormalities, the second and alternative pathway could be important in carcinogenesis of combined type SCLC. However, it is necessary to emphasize that combined type SCLC could originate from pure SCLC, and in this context, NSCL component should have active Notch signaling pathway and decreased ASCL1/INSM1 expression, contrary to Notch signaling inactivation and ASCL1/INSM1 expression seen in SCLC (18). Meder et al. (6) seems to emphasize -in their article- that a combination of inactivation of Notch signaling, with Ascl1 expression, precedes RB1 gene mutation, in small cell carcinogenesis, in both the classical and the alternative pathways. This

Figure 2 Notch and neuroendocrine transcription factors in fetal mouse lung (A) and human combined type of small cell lung cancer (SCLC) (B). (A) Serial sections of fetal mouse lung tissues immunostained for Ascl1, Notch1, Notch3, and Hes1. A cluster of pulmonary neuroendocrine cells (arrow; termed neuroepithelial body, NEB) are positive for Ascl1, but negative for Notch1, Notch3 and Hes1 in the nuclei, but non-neuroendocrine cells are negative for Ascl1, but positive for Notch1, Notch3 and Hes1. Counterstained with DAPI in the sections for Ascl1 and Hes1, and stained with rhodamine-labelled phalloidin in the sections for Notch1 and Notch3. Bar =20 µm; (B) combined type of SCLC immunostained for INSM1 and NOTCH1. The small cell carcinoma component is stained for INSM1, but not for NOTCH1. On the other hand, the adenocarcinoma component forming lumens (asterisks) are negative for INSM1, but positive for NOTCH1. Counterstained with hematoxylin. Bar =20 µm. SCLC, small cell lung cancer.
issue may be similar to the question of which came first; the chicken or the egg. As SCLC could arise from different cell lineages, other than neuroendocrine precursor cells (35), the premise of inactivation of Notch signaling and ASCL1 expression is not always necessary to be considered. Prerequisite of genetic alterations in Tp53 and RB1 should be also crucial in the classical pathway for small cell carcinogenesis, and could be important in the alternative pathway in SCLC transformation from adenocarcinoma with mutant EGFR (Figure 3). It is an attractive research filed to clarify molecular network linking the inactivation of Notch signaling pathway, with ASCL1/INSM1 expression and RB1 gene abnormalities.

Acknowledgements

Funding: This study was supported in part by a Grant-in-Aid for Scientific Research (C; No. 25460439) from Ministry of Education, Culture, Sports, Science and Technology of Japan and in part by a grant from the Smoking Research Foundation.

Footnote

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

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In their recent perspective Ito et al. comprehensively reviewed the carcinogenesis of pure and combined small cell lung carcinoma (SCLC) (1) and we appreciate the explicit discussion of our article Meder et al. “NOTCH, ASCL1, p53 and RB alterations define an alternative pathway driving neuroendocrine and small cell lung carcinomas.” (2).

Ito et al. augmented the regulators of neuroendocrine (NE) differentiation. In addition to achaete-scute homolog 1 (ASCL1), they highlighted Brain-2 (BRN2) and insulinoma-associated protein 1 (INSM1) upstream of ASCL1 which regulate NE marker expression and differentiation in normal pulmonary epithelial cells and cancer cells (1). It remains to be explored, whether BRN2 and INSM1 indeed autonomously drive a morphological switch from non-small cell lung cancer (NSCLC) towards a SCLC phenotype as it has been shown for ASCL1 (2,3). However, all three factors comprise a NE signaling network regulated by NOTCH-Hes1 signaling (1).

Critically, inactivating mutations in NOTCH genes seemed to be not sufficient but advantageous for SCLC formation. In our study, we found evidence for genetic inactivation of NOTCH receptors driving NOTCH-ASCL1 dependent NE differentiation in NE-NSCLC, large cell NE carcinomas (LCNEC) and the so called secondary SCLC transitions from NSCLC (2). However, Ito et al. summarized also the findings of Niederst et al. who reported that bi-allelic loss in RB1 alone was responsible for the transition from EGFR mutated adenocarcinomas (AdC) to SCLC. Furthermore, their results from whole exome sequencing did not reveal genetic alterations in NOTCH genes (4). As the NOTCH loci harbor extremely GC-rich sequences, they are frequently under-covered by next generation sequencing analyses (5) and hence, inactivating mutations in NOTCH genes are frequently unreported.

In addition, the question was raised whether combined SCLC may differentiate from pure SCLC upon deregulation of NOTCH signaling and reduction of ASCL1 expression. Brambilla et al. showed already in 1991 in patients suffering from chemoresistant SCLC that tumor cells acquired a more differentiated phenotype and an increased cell size upon therapy resistance (7). Calbó et al. showed in 2005, that there was tumor heterogeneity within SCLC lesions comprising NE and non-NE tumor
cells. Here, they proposed for the non-NE tumor cells an important role in acquiring chemoresistance (8). Interestingly, in 2011 they were able to link oncogenic Ras protein expression to a switch from NE to a mesenchymal non-NE phenotype (9).

Consequently, comprehensive signaling pathway analysis is required to elucidate how NOTCH, RB and Ras may cooperate in the induction of SCLC and SCLC-NSCLC transitions (Figure 1) and how they can mediate therapy resistance. For this purpose, robust and deep analysis of clinical cases is needed to finally overcome acquired therapy resistance and to improve patient outcome. This is a compulsive issue especially for highly aggressive neoplasms, such as SCLC. Thus, it is essential to routinely isolate biopsies from patients before, during and after therapy.

**Acknowledgements**

None.

**Footnote**

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

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p53 and RB alterations define an alternative pathway


Cite this article as: Meder L, Buettner R. Elucidating alternative pathways triggering small cell lung carcinoma tumor biology. Transl Cancer Res 2016;5(S2):S363-S365. doi: 10.21037/tcr.2016.08.02
To develop cancer therapies based on modulating “ivy league” signaling pathways such as Wnt, BMP/TGFβ, Sonic Hedgehog and Notch signaling is a research avenue currently attracting considerable interest both from academia and industry. In a recent report by Ambrogio et al. in *Nature Medicine* (1), the authors provide evidence that a combination therapy based on simultaneously targeting the Notch signaling pathway and the DDR1 kinase may be an interesting approach towards novel therapy development for lung cancer.

The Notch signaling pathway is a cell-cell communication system controlling cellular differentiation in most, if not all, organs and tissues. The Notch pathway has a simple molecular architecture (summarized in Figure 1), yet it is able to generate quite diverse signaling outputs in a cell context-dependent manner. How the simple architecture can read cell context to produce a large array of different signaling outputs appropriate for each cell state is an area of very active research (2). In keeping with an important role in development, dysregulation of the Notch pathway is increasingly linked to disease and cancer. Mutations in genes in the core Notch pathway (Figure 1) cause a number of diseases and specific forms of cancer. In acute lymphoblastic T-cell leukemia more than 50% of the patients carry activating Notch1 mutations. In other tumor types, such as skin cancer, loss-of-function Notch mutations are causative, indicating that Notch can act as an oncogene or tumor suppressor gene, depending on the tumor type (3). In many tumor types, however, there are no direct mutations in the core pathway, but the Notch signaling output is nevertheless dysregulated and the aberrant level of Notch signaling correlates with patient prognosis. In these cases, it is conceivable that auxiliary proteins to the Notch pathway or signaling pathway interacting with Notch are dysregulated, more indirectly leading to the observed aberrant Notch signaling output. In the light of the emerging links between Notch and cancer, it comes as little surprise that finding ways to therapeutically modulate Notch signaling is a highly prioritized goal. The problem has in principle not been identifying drugs that inhibit Notch signaling: γ-secretase inhibitors for example very effectively block Notch receptor cleavage and thus downstream signaling, but as they were originally designed for systemic use, they have in most clinical trials given rise to unwanted side effects in a variety of different organs, including the gastrointestinal system, the immune system, the skin and the central nervous system, reflecting the importance of Notch in these organs.

There are however several clinical trials ongoing with different dosing regimens, but there are yet no functional therapies routinely used in the clinic (3).

In the search for strategies to successfully modulate Notch signaling, the prospect of using combination therapies where Notch signaling and other proteins are simultaneously targeted has therefore received considerable attention, as it may alleviate some of the problems resulting from high-dose mono-therapies for Notch. In the light of this, the report by Ambrogio et al. (1) is interesting, because it provides data that the combined targeting of the kinase DDR1 and Notch signaling in lung cancer seems promising. Lung cancer is classified into small
cell lung cancer (SCLC) and the more common form called non-small cell lung cancer (NSCLC), which in turn is subdivided into three histologically distinct subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma (4). Driver mutations are frequently found in the ErbB, ALK and KRAS genes, and patients carrying KRAS mutations have the worst prognosis and there is a dire need for therapies that reach beyond the currently used conventional chemotherapy, which is based on cisplatin/paclitaxel treatment. In the Ambrogio et al. paper, the authors searched for new strategies to intervene with adenocarcinomas, using a mouse model in which a
mutated KRAS gene was conditionally activated by CRE-recombinase, leading to hyperplasias followed by more advanced adenocarcinoma. They started out by identifying genes upregulated at early stages of tumor development, i.e., the hyperplasia stage. The rationale for this was that full-blown adenocarcinomas exhibit considerable heterogeneity and the chances of identifying candidate genes that could be relevant for fueling a founder stem cell population would be higher if early tumor stages were analyzed. The DDR1 gene came out as a top candidate gene from the transcriptomic analysis. DDR1 encodes a tyrosine kinase protein, which is a member of a larger family of discoidin domain receptor 1, characterized by that they bind to and are activated by collagen and play roles in the interaction between tumor cells and the extracellular matrix (5). A functional involvement of DDR1 was demonstrated when the activated KRAS mice were crossed with DDR1−/− mice (which are phenotypically largely normal with regard to lung development), and whereas tumor formation was initiated, the medial survival time of the tumor-carrying mice was significantly extended in the absence of DDR1. Similarly, pharmacological inhibition of DDR1 reduced tumor burden, further supporting that blocking of DDR1 could be beneficial.

In addition to ErbB, ALK and KRAS mutations, nearly 10% of NSCLC patients carry activating mutations in Notch1 and 30% of the patients show loss of expression of Numb, a negative Notch regulator (6). This notion, combined with the previous report that Notch1 expression is controlled by DDR1 and that Notch1 ICD and DDR1 interact (7), focused the authors’ attention on Notch as an additional therapeutic target. Indeed, combined treatment with DDR1 and Notch inhibitors (the γ-secretase inhibitor LX-411575) was more effective in inducing apoptosis in the KRAS-driven lung adenocarcinomas than either inhibitor alone. In KRAS-activated mice in which the p53 gene was simultaneously removed, which leads to more aggressive tumor formation, mono-therapy with DDR1 and Notch inhibitors was not effective, and only the combined use of DDR1 and Notch inhibitors led to increased apoptosis in the tumors. Finally, patient-derived adenocarcinoma biopsies were grafted to the lungs of immunodeficient mice, and combined blocking of DDR1 and Notch (in this case by using an anti DLL4-antibody, demecizumab, which is in clinical trials for NSCLC) showed higher efficacy than cisplatin/paclitaxel treatment.

The elegant study by Ambrogio et al. provides an interesting new angle for Notch combination therapies and solid support for a link between DDR1 and Notch. With this said, there are however still important questions that remain unanswered. We are for example still relatively ignorant as to how DDR1 and Notch synergize. A few Notch target genes were analyzed in response to DDR1 inhibition, but whether the Notch response is quantitatively or qualitatively blunted by reduced DDR1 levels remains to be established. The use of a Dll4-blocking antibody in the xenograft experiments also leaves open the possibility that the tumor vasculature, rather than the tumor proper, was affected. The authors propose that MAPK signaling may be a common node between DDR1 and Notch, and this is an interesting concept which should be further pursued.

In addition to the DDR1-Notch combination therapy, are there other interesting combination therapies on the horizon? The fact that Notch signaling intersects with several important signaling mechanisms, such as hypoxia, Wnt and BMP/TGFβ (2) suggests that there is potential for progress on several frontiers. For example, a recent study shows that overexpression of Notch1 ICD in a variety of tumor cell lines makes inhibition of other pathways less efficient (8), arguing that hyperactivated Notch signaling negatively affects inhibitor efficacy and that lowering Notch signaling would be beneficial in combination therapies. In a mouse xenograft model, the simultaneous targeting of ErbB2 and Notch proved effective (9,10).

An important consideration for future Notch-based therapies is at which step in the signaling cascade inhibition would best be executed. As discussed above, γ-secretase inhibitors, which were also used in some of the experiments in the Ambrogio et al. paper, are effective in quenching Notch as they block cleavage of all four Notch receptors, but suffer the drawbacks of being designed for systemic use, which may lead to unwanted Notch blocking in tissues other than the tumor. An attractive idea is therefore to redesign existing γ-secretase inhibitors to decrease their distribution and make their effect more local, for example restricted only to a tumor. An alternative promising strategy to block Notch signaling is the use of antibodies that interfere with Notch ligand-receptor interaction or lock Notch receptors into a non-cleavable state. Antibody-based approaches have the advantage of being more specific, capable of targeting individual ligands or receptors rather than wiping out all Notch signaling, as is the case with γ-secretase inhibitors, and recent reports provide encouraging data from antibodies targeting the Dll4 ligand (11,12) (the Dll4 ab was used in the xenograft experiments in the Ambrogio et al. paper), the Jagged ligands (13)
or Notch receptors (14). Other strategies to modulate Notch includes the use of stapled peptides mimicking Mastermind (15), a protein important for the Notch/CSL transcription complex, and small molecule inhibitors for individual Notch receptors (16). This suggests that inhibition at different levels in the signaling cascade is feasible, but recent data suggest that the CSL level maybe should be avoided, as removal/knockdown of the CSL protein leads to an unexpected tumor-promoting rather than tumor-inhibiting phenotype (17-19). The expanding array of posttranslational modifications of Notch ICDs that modulate Notch signaling output includes hydroxylation, acetylation and methylation (3) as well as phosphorylation (20,21) and these Notch-modifying proteins may serve as potentially interesting candidates for pharmacological modification and inspire new, albeit more indirect, strategies for Notch modulation.

In conclusion, the report by Ambrogio et al. is important, as it addresses a form of lung cancer which has proven difficult to combat with conventional therapies, and their discovery of a link between DDR and Notch signaling, as well as the prospect of combination therapy based on these two proteins, is interesting and inspiring.

Acknowledgements
Work in the author's laboratory is supported by the Swedish Cancer Society, the Swedish Research Council and Knut och Alice Wallenbergs Stiftelse.

Footnote
Conflicts of Interest: The author has no conflicts of interest to declare.

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In recent years our appreciation of the function and design of many signalling pathways implicated in cancer has changed substantially. The original idea of intrinsically independent and linear routes has progressively evolved to the concept of complex networks, involving extensive crosstalk and interplay between various signalling pathways. Yet, we are only beginning to understand how these networks are interconnected within cancer cells. This knowledge will be essential for the design of effective drug combinations as well as for the anticipation of potential mechanisms of resistance.

This burden of complexity was evident in our recent manuscript reporting an efficacious preclinical therapy based on the combined pharmacological inhibition of the receptor tyrosine kinase DDR1 and Notch signalling in K-Ras driven lung adenocarcinoma (1). Yet, as pointed out in the recent commentary by Dr. Lendahl, we are still far from understanding the underlying biology and the hierarchy that governs this K-Ras/DDR1/Notch interplay. Indeed, both positive and negative interactions between Notch and Ras have been identified in various tumour types where Notch can sustain or prevent tumour growth depending on the cellular context (2). We hypothesize that in K-Ras driven lung adenocarcinoma this context dependency may in part rely on the function of DDR1.

We identified DDR1 as the top hit in the transcriptional profiling of a subset of mouse lung hyperplasias when analysed shortly after the activation of a resident K-RasG12V oncogene. The limited consensus on a reliable Notch-dependent transcriptional profile in the published literature prevented us from analysing the presence of a Notch signature in these early lesions. Yet, in the early stages of pancreatic adenocarcinoma, oncogenic K-Ras fails to induce Notch target genes suggesting that these pathways converge subsequently during tumour initiation (3). Although we currently lack supporting experimental evidence, we can speculate on several non-mutually exclusive mechanisms that could explain this functional convergence.

In breast cancer cells, the interaction with DDR1 triggers Notch activation (4). We are still ignorant as to whether the same process operates in K-Ras-driven lung adenocarcinoma. If this is the case, and since DDR1 transcription is partly controlled by MAPK activity (5), K-Ras-induced DDR1 expression would precede Notch activation. Yet, it is currently unclear how DDR1 itself becomes activated. Collagens, the only known DDR1 ligands (5) are among the most abundant proteins in mammalian tissue suggesting that additional layers of regulation must exist to prevent unscheduled activation. RTKs are not uniformly activated across the cell membrane but tend to occur upon receptor clustering in specific microdomains (6). DDR1 is not an exception and receptor aggregation has been suggested to participate in its activation (5). Our hypothesis is that in K-Ras-driven lung adenocarcinoma specific membrane microdomains may result in DDR1/Notch clustering facilitating their reciprocal activation. Furthermore, Ras signal output is qualitatively and quantitatively dictated by its own membrane sub-
localization (7). Whether K-Ras<sup>G12V</sup> might also be present in such microdomains remains to be determined, but in any case DDR1/Notch local enrichment may favour some post-translational modifications required for their full activity. As an illustrative example, Src is required both for full DDR1 activity as well as for the proteolytic activation of Notch in certain cellular contexts.

This putative DDR1/Notch co-regulation may be particularly relevant in specific tumour sub-populations. Notch and DDR1 have been reported to play a pivotal role to maintain cancer stem cell (CSC) traits in K-Ras driven lung adenocarcinoma and breast cancer respectively (8-10). In this context both DDR1 and Notch are subject to common regulatory mechanisms such as those involving PKC function (9,10) that, incidentally, also controls K-Ras membrane localization (7), again reinforcing the hypothesis that local clustering might facilitate co-regulation. Interestingly, the pharmacological co-inhibition of DDR1/Notch in lung adenocarcinoma PDX driven by K-Ras mutations eliminates the most aggressive tumour component and considerably delays disease relapse (1). This observation could be compatible with the combined DDR1/Notch inhibition being particularly effective on CSCs.

The therapeutic efficacy of DDR1/Notch co-inhibition may not only be a consequence of its effect in the tumour proper but in additional components. In agreement with an important Notch function in endothelial cells, co-inhibition of DDR1/Notch in lung adenocarcinoma resulted in diminished tumour vasculature as measured by CD31 staining (Figure 1A). Whether this is exclusively reliant on Notch inhibition or whether DDR1 also plays an important role in tumour endothelial cells remains to be determined. In any case it is likely that the decreased vascularization contributes to the extensive tumour necrosis observed upon DDR1/Notch inhibition (1).

Finally, in his recent commentary Dr. Lendahl speculated on whether the Notch response is quantitatively or qualitatively blunted by DDR1 inhibition. It will be important to identify whether any of the four Notch receptors plays a prominent role in K-Ras-driven lung adenocarcinoma. In our study, Notch2 was over-represented in our limited subset of human PDX samples (Figure 1B). Accordingly, in the TCGA dataset Notch2 is the most frequently deregulated (amplified or up-

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**Figure 1** Effect of DDR1/Notch co-inhibition in the vasculature of K-RAS-driven lung adenocarcinoma & expression of Notch isoforms in this tumour type. (A) Representative immunostaining showing the CD31 endothelial marker in sections obtained from human lung adenocarcinoma (PDX). Tumours were orthotopically implanted in recipient mice, divided in three cohorts and treated in parallel with vehicle, cisplatin/paclitaxel chemotherapy or the combination of dasatinib/demcizumab. Samples were collected upon completion of the treatments. Scale bar: 100 µm; (B) qRT-PCR analysis of Notch1–4 levels in a subset of three human lung adenocarcinoma PDX.
regulated) isoform in human lung adenocarcinoma. In any case, and as discussed above, we have no evidence of a vertical relationship between the DDR1 and Notch pathways. Instead, our experimental evidences rather favour the existence of a bi-directional interaction. Whether this synergy is limited to their activation on the cell surface or, in addition, their respective downstream signals converge to sustain pathways essential for tumour progression is currently unknown. In this regard, the activation of the MAPK pathway, known to be essential for the development of K-Ras\textsuperscript{G12V}-driven lung adenocarcinoma, is only efficiently suppressed when both DDR1 and Notch are simultaneously targeted (1). Whether this synergistic effect can be extended to other signalling and/or pro-survival pathways downstream of oncogenic K-Ras remains to be elucidated.

**Acknowledgements**

None.

**Footnote**

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

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Cancer cells show sustained cellular proliferation, which is necessary for tumor formation and cancer progression. Cellular metabolism is one of the biological processes necessary to enable uncontrolled proliferation of cancer cells. Metabolism converts nutrients provided by the microenvironment through a series of biochemical reactions into metabolic products such as energy, biomass precursors, and antioxidants, which are in turn needed for cellular proliferation. Yet, metabolism can be fueled by different nutrients, and these nutrients are used to produce the needed metabolic products through multiple parallel or converging biochemical reactions. Thus, almost a century ago the idea emerged that cancer cells have a different metabolism compared to non-transformed cells (1) and that these differences can be targeted to inhibit cancer cell proliferation (2).

After the challenging discovery that there is no specific “cancer metabolism” that unifies all transformed cells and separates them from all non-transformed cells (3), researchers undertook the endeavor to mechanistically understand how metabolic alterations are linked to an oncogenic transformation. A milestone in this endeavor was the discovery that oncogenes and tumor suppressors regulate the expression and/or activity of metabolic enzymes (3,4). Consequently, it has been concluded that cancer metabolism is defined by the genetic landscape of the transformed cell. Yet, introducing one and the same oncogenic driver into different non-transformed cells often did not result in a consistent change in metabolism (5-7). This finding was explained by the frequently underappreciated influence of the cell origin (3), i.e., non-transformed cells of different origin have per se a different metabolism, which is defined by their natural function within an organ or the whole body. Thus, the metabolic changes stimulated by the introduction of an oncogenic driver in cells of different origin (and therefore different baseline metabolism), result in a metabolism that is defined by both, the oncogenic drivers and the cell origin (3). Yet, with these findings the question rises whether cancer metabolism is only defined by cell intrinsic factors such as the genetic landscape and cell origin. Multiple lines of evidence, such as metabolic regulation and the importance
of extracellular nutrient concentrations on the activity of signaling pathways (8), suggest otherwise. However, the impact of these cell extrinsic factors has hardly been studied in vivo. Consequently, the relevance and interplay between cell intrinsic and extrinsic factors in shaping in vivo cancer metabolism is poorly defined.

Recently, several laboratories addressed the question whether cell extrinsic factors such as alterations in the microenvironment, play a role in defining cancer metabolism. Hensley and colleagues (9) studied the interplay between metabolism and the microenvironment by combining in vivo $^{13}$C tracer analysis with dynamic contrast-enhanced MRI (DCE-MRI) data in non-small cell lung cancer patients with differential status of the oncogenes KRAS and EGFR. Using DCE-MRI they pre-operatively defined higher and lower perfused areas within the cancer mass and used this information to identify cancer cells exposed to different microenvironments, although harboring the same oncogenic drivers and residing in the same organ. The consequent intraoperative $^{13}$C tracer analysis was used to determine differential nutrient usage and metabolic pathway activities (10) of the cancer cells located in different microenvironments (defined by the perfusion magnitude) within the cancer mass. Based on this data they came to three conclusions (Figure 1): first, regardless of the perfusion state all cancer cells showed sustained or even enhanced tricarboxylic acid (TCA) cycle metabolism [defined by pyruvate carboxylase (PC)-dependent anaplerosis and pyruvate dehydrogenase (PDH) activity] despite the fact that they also showed increased glucose uptake (measured by FDG-PET) compared to adjacent lung tissue. This finding is of profound importance, because a long favored model of cancer metabolism postulates a switch from oxidative to glycolytic metabolism.

**Figure 1** Genetic and microenvironment alterations shape the metabolism of cancer cells. Glycolysis, PC-dependent anaplerosis, and oxidative TCA cycle metabolism is increased in KRAS induced lung cancers compared to adjacent lung tissue (6,9). Intra-tumor heterogeneity in the microenvironment defined by the magnitude of perfusion results in the reliance on glucose metabolism in less perfused tumor areas and in the usage of alternative nutrients in higher perfused tumor areas (9). Inter-organ alterations in the nutrient microenvironment contribute to the use of glutamine anaplerosis in primary breast cancers and the shift to PC-dependent anaplerosis in the resulting lung metastases (11). In vitro cultured lung cancer cells use glutamine anaplerosis, while the same lung cancer cells grown in vivo exploit glucose metabolism (6). OAA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase. Italics indicates metabolic pathways or metabolic enzymes. Only selected biochemical reactions are depicted.
However, the data of Hensley and colleagues (9) support the idea that enhanced oxidative TCA cycle metabolism and increased glycolysis are not opposing metabolic states, but can both be the metabolic basis of an oncogenic transformation; second, cancer cells in lower perfused areas within the cancer mass relied predominantly on glucose to fuel TCA cycle metabolism, while cancer cells in areas of higher perfusion used to a large extent alternative carbon sources; third, neither the KRAS nor the EGFR status changed the influence of perfusion on the nutrient usage in the TCA cycle.

These unexpected results were further confirmed and extended by a study of Davidson and colleagues (6) showing that cellular proliferation of KRAS driven non-small cell lung cancers in mice depends in vivo on PC-dependent anaplerosis and PDH activity. Moreover, they discovered that KRAS-driven lung cancer cells used in vivo glucose as major carbon substrate for TCA cycle fueling, while the same cells cultured in vitro switched to glutamine anaplerosis (Figure 1).

Beyond these works on primary lung cancer, Christen and colleagues (11) investigated the role of the microenvironment in shaping cancer metabolism during breast cancer metastasis to the lungs. They discovered that breast cancer-derived lung metastases activated PC-dependent anaplerosis as a function of the nutrient availability within the lung microenvironment (Figure 1). While primary breast cancers often rely on glutamine anaplerosis and are susceptible to glutaminase inhibitors (3), these data suggest that the resulting and genetically similar lung metastases lose this drug susceptibility, because they activate PC-dependent anaplerosis in response to the lung microenvironment (11).

Considering the data provided by these studies three major milestone conclusions emerge: first, cancer metabolism is a function of the in vivo microenvironment resulting in intra-tumor and inter-organ heterogeneity; second, the nutrient microenvironment can overrule metabolic constraints imposed by the genetic landscape; third, a true switch from oxidative to glycolytic metabolism is not a requirement for uncontrolled proliferation of cancer cells.

At first sight the discovered metabolic heterogeneity and flexibility of cancer cells resulting from the microenvironment seems challenging for the overarching goal to translate cancer metabolism research into innovative and effective drugs against cancer. However, a closer look at these results reveals that although cancers within the lung show the flexibility to exchange glucose with alternative carbon sources, the metabolic processing of all these nutrients requires PC and PDH activity (9). Consequently, targeting PC (or PDH) activity has the potential to inhibit the in vivo proliferation of cancer cells in the lung microenvironment regardless of whether they are located in higher or lesser perfused areas (9), or originate from primary breast cancers (11). However, this in vivo vulnerability of cancer cells within the lung microenvironment is not predictable from in vitro studies, because the in vitro provided environment results in a glutamine dependency of lung cancer cells (6). Thus, these findings provide the basis for a paradigm shift, which builds on a microenvironment defined cancer metabolism. Consequently, not only the genetic landscape and the cell origin of cancer cells needs to be considered when developing anti-cancer drugs and defining their efficacy spectrum, but also the microenvironment as defined by the perfusion state of the tumor, the organ in which the cancer resides, and the whole body physiology of the patient.

Acknowledgements

We thank Dr. Stefan Christen (VIB Leuven) for feedback of the perspective.


Footnote

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

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Cite this article as: Elia I, Fendt SM. In vivo cancer metabolism is defined by the nutrient microenvironment. Transl Cancer Res 2016;5(Suppl 6):S1284-S1287. doi: 10.21037/tcr.2016.11.53
Neuroendocrine tumors are a vastly diverse group of tumors developed by neuroendocrine cells. Neuroendocrine cells are found throughout the whole body, and function in hormone regulation and epithelium repair. In the lung, there are various types of neuroendocrine tumors, one being large cell neuroendocrine carcinoma (LCNEC). LCNEC is a rare and aggressive subtype of non-small cell lung cancer (NSCLC) and is usually treated as a type of NSCLC (1-4). However, in recent years, it has been shown that LCNEC shares similar histological, immunohistochemical (IHC), and molecular characteristics with small cell lung cancer (SCLC), despite the different characterization of the size of the cells (5). Consequently, there have been recent studies testing various combination therapies on both LCNEC and SCLC, but have resulted in somewhat poorer outcome amongst LCNEC patients than patients with SCLC (6). This highlights the ongoing ambiguity and lack of optimal clinical treatment of LCNEC versus SCLC, and motivates further genomic investigation of these two types of neoplasms. There are currently no approved targeted therapies specifically for LCNEC or SCLC; chemotherapy is presently the only therapeutic option (2).

Miyoshi and colleagues examined a Japanese patient cohort consisting of a total of 78 formalin-fixed paraffin-embedded (FFPE) LCNEC samples by using a 244 cancer-related gene targeted exon next-generation sequencing approach to discover genomic alterations. This cohort comprised of 55 surgically resected LCNEC, 13 advanced-stage LCNEC biopsies, and 10 combined LCNEC with NSCLC components. The vast majority of the patients were ever smokers (97% and 98% in LCNEC and SCLC, respectively). They then compared the LCNEC genomic profile to 141 SCLC patients’ genomic alterations: 90 biopsy samples; 50 surgically resected samples and one advanced SCLC sample from Miyoshi et al. previous data (7). Of these SCLC samples, 12 were of a combined type; however, it was not specified which SCLC was of a combined form. Comparison of the LCNEC and SCLC genetic alteration data was used to produce a molecular profile of LCNEC to predict molecular targeted therapies and cancer progression (8).

Miyoshi and colleagues identified a high prevalence of mutations in TP53 and RB1 genes in LCNEC; however, SCLC had a much higher frequency of RB1 mutations when compared to LCNEC (Table 1). In addition, they identified targetable activating mutations in KIT, EGFR, ERBB2, and FGFR1 genes, and found a higher copy number gain in ERBB2 and SETBP1 genes in LCNEC when compared to SCLC. Other mutated genes which were significantly more frequent in LCNEC included LAMA1, PCLO, MEGF8, and RICTOR. Yet, overall, Miyoshi and colleagues concluded that LCNEC and SCLC have similar genomic profiles. The majority of the genetic alterations were related to the PI3K/AKT/mTOR pathway, which could be a potential target pathway in LCNEC tumor formation and progression. In patients
with LCNEC combined with adenocarcinoma, Miyoshi and colleagues found known oncogenic mutations within both cell components in the genes EGFR (E746_A750 del, exon 19) and KRAS (G12V), while a patient with LCNEC combined with squamous cell carcinoma had a PIK3CA gene activating mutation in both cell components (8).

In a recent report, Rekhtman et al. compared the genomic profiles of LCNEC and NSCLC. In agreement with Miyoshi and colleagues' data, Rekhtman et al. also found TP53 and RB1 genes to be the two most commonly mutated genes in LCNEC. In the Rekhtman et al. whole LCNEC cohort, they included additional genes STK11, KEAP1, and KRAS to be among the next most commonly mutated genes in LCNEC; however, in the SCLC-like LCNEC subset, (defined as LCNEC with co-altered inactivation of RB1 and TP53), there was a complete absence of STK11 and KRAS mutations, and an enriched existence of MYCL, SOX2, and FGFR1 amplifications. They also identified the PI3K/AKT/mTOR pathway to be among the most frequently altered gene family-functional group. Furthermore, Rekhtman et al. discovered about 30% of the NSCLC-like LCNEC to have a distinct mutation profile, mostly consisting of loss-of-function mutations in the NOTCH family genes, which are key regulators of neuroendocrine differentiation. The most prominent alteration in SCLC-like LCNEC versus SCLC was an increased frequency of KEAP1-NFE2L2 aberrations, which rarely occur in traditional SCLC patients, but are known to be prevalent in STK11/KRAS wildtype squamous cell carcinomas (9).

A small percentage of neuroendocrine tumors may display combined histologies (i.e., SCLC with NSCLC components, and LCNEC with other NSCLC components). In their study, Miyoshi and colleagues included 10 combined LCNEC and 12 combined SCLC. The combined forms clearly indicate the heterogeneity of these tumors and investigating their genetics may offer clues to their potential different cells of origin. However, often the different components of combined forms are tightly intermixed and it may be challenging separating them; in this case sophisticated technology such as laser capture microdissection may be necessary. Unfortunately Miyoshi and colleagues apparently only used coring of tissue blocks and light microscopy, which may not have adequately separated the two components and therefore yielded inconclusive results of this sub-analysis.

The best recognized combined forms of neuroendocrine tumors with non-neuroendocrine tumors in the mixed SCLC-NSCLC histologies, which have been found in a variable number of cases, range from less than 1% to up to 28%. This variability may depend on the availability of tumor tissue and small biopsies do not allow the opportunity to study the tumor in their entirety (13). According to

### Table 1

<table>
<thead>
<tr>
<th>Study</th>
<th>Next-generation sequencing methodology</th>
<th>Altered genes in SCLC patients</th>
<th>Altered genes in LCNEC patients</th>
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</thead>
<tbody>
<tr>
<td>Miyoshi et al. 2016</td>
<td>Targeted exon sequencing of 244 genes</td>
<td>TP53 (81%)<em>; RB1 (41%)</em>; MLL2 (12%); NOTCH family (1/2/3) (11%)*</td>
<td>TP53 (71%)<em>; RB1 (26%)</em>; MLL3 (11%); LAMA1 (10%); NOTCH1 (10%)*; MLL2 (9%)</td>
</tr>
<tr>
<td>Rekhtman et al. 2016</td>
<td>Targeted exon sequencing of 241 genes; tumor/normal blood sequencing</td>
<td>NA</td>
<td>TP53 (78%)<em>; RB1 (38%)</em>; STK11 (33%)<em>; KEAP1 (31%); KRAS (22%)</em>; PTPRT (22%)</td>
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<tr>
<td>George et al. 2015</td>
<td>Whole genome sequencing</td>
<td>TP53 (98%)<em>; RB1 (98%)</em>; KIAA1211 (18%); COL22A1 (18%); FMN2 (18%); CREBBP (15%); NOTCH1 (15%)*</td>
<td>NA</td>
</tr>
<tr>
<td>Vollbrecht et al. 2015</td>
<td>Targeted sequencing of 48 gene hotspot panel</td>
<td>TP53 (65%)<em>; ERBB2 (24%)</em>; PIK3CA (24%); ATM (24%)</td>
<td>TP53 (63%)<em>; ERBB2 (16%)</em>; KRAS (11%)*</td>
</tr>
<tr>
<td>Karlsson et al. 2015</td>
<td>Targeted sequencing of 26 gene hotspot panel</td>
<td>NA</td>
<td>TP53 (88%)<em>; STK11 (16%)</em>; PTEN (13%)*</td>
</tr>
</tbody>
</table>

*, indicates genes common amongst LCNEC and SCLC; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; NA, not applicable.
different reports from George et al. and Swanton et al., loss of the tumor suppressor genes, TP53 and RB1, is obligatory in SCLC, and inactivating mutations in the NOTCH family genes occur in 25% of human SCLC (10,14). There has been more comprehensive reports examining LCNEC combined NSCLC or SCLC components. In an older report from Wagner et al. in 2009, loss of heterozygosity (LOH) analysis was used, which showed similar genetic abnormalities in the individual components of the combined SCLC cases (15). In addition, Buys et al. investigated a patient with combined small cell carcinoma with two different NSCLC components. They used a whole genome analysis by tiling-path array comparative genomic hybridization to evaluate the clonal relationship, which resulted in divergent clonal evolution (16). The analysis for combined LCNEC genomic alteration in this article indicated, five in ten LCNECs with other NSCLC components harbored the same key driver mutations in both components. For combined LCNEC, the median number of genetic mutations was 3.5 and 4 in LCNEC and NSCLC component respectively. The median concordance rate between LCNEC and associated NSCLC components was 71% (range, 60% to 100%).

Considering these data, LCNEC combined with NSCLC components may behave and respond more like their NSCLC component, suggesting tumor heterogeneity and early evolution of the combined LCNEC. Because it is suggested that they perform like their NSCLC counterpart, examination of the two separate types of LCNEC combined NSCLC components could provide more insight. Further investigation of the KEAP1/NFE2L2, PIK3CA genes in LCNEC combined squamous cell carcinoma, and the EGFR, KRAS, and NOTCH family genes in LCNEC combined adenocarcinoma may offer a more targeted approach to the treatment of LCNEC. Together with Rekhtman et al. data, the combined mutation profiles could be used as markers to differentiate the different subtypes within LCNECs and advance targeted treatment for patients.

A case study by De Pas et al. in 2011 reported a never smoker patient with LCNEC harboring an activating mutation on the EGFR gene (L747_A755>AT, exon 19). This patient was treated with gefitinib, an approved drug for specific EGFR mutated NSCLC. After 2 months of therapy, the patient showed dramatic response to the treatment, and after 5 months, the patient showed complete response of the lung primary lesions (17). This suggests LCNEC carrying activating EGFR gene mutations could be treated with gefitinib with positive effects.

Because the PI3K/AKT/mTOR pathway was emphasized in Miyoshi and colleagues’ discussion, perhaps the dual PIK3CA/mTOR inhibitor, PI-103, could be a potential targeted therapy for LCNEC carrying a PIK3CA mutation. This drug has been tested and was shown to be active in NSCLC cell lines with activating PIK3CA mutations (18). Patients with PIK3CA and EGFR-mutated lung cancers may not respond to EGFR targeted therapies like gefitinib because of the double mutation. All these factors should be taken into consideration when evaluating the complex molecular profile of LCNEC for targeted therapy selection.

Miyoshi and colleagues have demonstrated the power of next-generation sequencing, and this proposal supports the ongoing movement of genomics delivering a “personalized” treatment approach by assessing the patient’s tumor mutations and selecting the appropriate therapy for an improved response, subsequently increasing overall survival (19-21). Although the study was not a complete, comprehensive investigation of LCNEC, Miyoshi and colleagues provided valuable data in the advancement of LCNEC analysis, diagnosis, and treatment. Because of the distinct molecular characteristics compared to other lung cancer types, LCNEC should be treated as a separate group and not lumped into traditional NSCLC or SCLC treatments. Moreover, smoking patients need to be taken into consideration; it is known that NSCLC patients who have smoked have a 10-fold higher mutational load than neversmokers, and C>A/G>T transversions predominantly occur in NSCLC tobacco users, whereas C>T/G>A transitions most frequently occur in NSCLC never-smokers (22). It seems that LCNEC do not have distinct, targetable genes or mutations (Table 1), similar to other cancers such as pancreatic (23). Nonetheless, possible future studies with more refined methodologies or technologies, such as laser capture microdissection or tissue macrodissection instead of tissue block coring, tumor/normal (blood) pair sequencing, or using single cell or droplet digital PCR, could minimize tumor component/normal cell contamination and dissect tumor heterogeneity. In addition, the authors did not specify the sequencing mean read depth of coverage; a higher read depth of coverage will provide higher accuracy and detection of true somatic mutations. We believe further investigation within a larger and more diverse cohort comparing LCNEC to SCLC and NSCLC would be beneficial. The Cancer Genome Atlas (TCGA) project has only sequenced lung adenocarcinoma and squamous cell carcinoma cases (585 and 504, respectively), but no
SCLC cases, mainly because of difficult collection of large, resected material (24). Since LCNEC are treated more like NSCLC based on morphology, perhaps there is potential to obtain more LCNEC surgical samples more easily for genomic analysis, based on this presumption. Furthermore, using RNA sequencing, whole exome or whole genome sequencing would provide a stronger interpretation of the LCNEC genomic landscape.

Acknowledgements
None.

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

References
23. Bryant KL, Mancias JD, Kimmelman AC, et al. KRAS:

Cite this article as: McCutcheon JN, Zhao X, Giaccone G. A genomic analysis of large cell neuroendocrine carcinoma versus small cell lung cancer: which is which? Transl Cancer Res 2016;5(Suppl 6):S1088-S1092. doi: 10.21037/tcr.2016.11.19

We thank J. N. McCutcheon, X. Zhao, and G. Giaccone for their insightful commentary on our article regarding genomic studies for large-cell neuroendocrine carcinoma (LCNEC) of the lung (1). We would like to take this opportunity to comment on some of the points that they raised and to discuss our data reported in the original manuscript further (2).

Although LCNEC is distinguished from small cell lung carcinoma (SCLC) based on histological criteria such as a larger cell size and abundant cytoplasm, LCNEC shares many similarities with SCLC in terms of immunohistochemical (IHC) staining results and molecular biology (3,4). However, a multi-center prospective phase II study examining combination chemotherapy with irinotecan and cisplatin resulted in a somewhat poorer outcome among LCNEC patients than among those with SCLC (5), suggesting a possibility of biological distinction between LCNEC and SCLC. Due to its rarity, information about biologically relevant genetic alteration in LCNEC is insufficient. Thus, we examined LCNECs for biologically relevant genomic alterations using next-generation sequencing (NGS) and compared the genomic profiles of LCNECs with those of SCLCs.

McCutcheon et al. pointed out that we have proposed the ongoing movement in genomics to deliver “personalized” treatment approaches to patients with LCNEC (1). We reported that a group of LCNEC patients harbored targetable activating alterations in receptor tyrosine kinase signaling pathways, such as the PI3K/AKT/mTOR pathway and EGFR, ERBB2 and FGFR1 (2). Our results showed that sequencing-based molecular profiling is warranted, since it was capable of identifying a population of LCNEC patients who were likely to benefit from novel targeted therapies even if it was a small population.

Our results showed that LCNEC and SCLC had similar genomic profiles (2). LCNEC is a rare disease, so NGS-based analyses might be helpful for developing novel targeted therapies along with other types of lung cancer, such as SCLC. Rekhtman et al. also reported that the TP53 and RB1 genes were the most commonly mutated genes in LCNEC, in agreement with our data; however, they showed that LCNEC represented a biologically heterogeneous group of tumors with distinct subsets exhibiting the genomic signatures of SCLC, NSCLC, and, in rare cases, highly proliferative carcinoids (6). Although the more than 200 genes that are included in the target-sequencing panel encompass most known, functionally important cancer-related genes, studies utilizing whole-genome/exome sequencing technologies will be desirable to obtain a detailed understanding of the similarities between LCNEC and SCLC. In addition, analysis of a larger cohort of cases will be needed to capture a full spectrum of genomic profiles in LCNEC.

As McCutcheon et al. pointed out, the genomic analysis...
of combined LCNEC is challenging (1). We found that 5 of the 10 cases of LCNECs combined with NSCLCs harbored candidate driver gene alterations that have been previously reported for NSCLC. We diagnosed combined LCNEC as follows: LCNEC with an additional component of some other NSCLC histology that was clearly separated from the LCNEC component. In most cases, the size of the NSCLC component in the combined LCNEC was relatively small. Therefore, we used the core of the specimen for DNA extraction to obtain as much DNA sample as possible. In this study, the median and mean read coverages for all the LCNEC samples (including the NSCLC components) were 360 and 359, respectively. To avoid contamination, pathologists reviewed all the tumor samples before and after tissue punching and evaluated the tumor cell contents of the punched-out sites: a minimum of 50% tumor cells were included in all the samples, and no additional micro-dissection was needed. The variant frequency of the mutations in the NSCLC component tended to be lower than that shared with the LCNEC component. We suppose that the tumor contents in the NSCLC component were generally less than those in LCNEC component. The relatively high concordance rate might be due to the common origin of different components and not due to contamination of the samples during the DNA extraction (2).

LCNEC is a rare and lethal disease with no approved disease-specific targeted therapies (7). Ongoing efforts to collect and analyze samples using more advanced research tools are likely to enable the development of effective and novel targeted therapies. Integrated omics analyses, including RNA sequencing and metabolomics as well as whole exome or whole genome analyses, might provide a stronger interpretation of the LCNEC biology. In addition, co-clinical studies using patient tumor-derived and/or circulating-tumor cell derived xenografts could be used to guide therapeutic strategies for individual patients in the same way as those for SCLC (8,9).

Acknowledgements

None.

Footnote

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

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Cite this article as: Umemura S, Miyoshi T, Ishii G, Tsuchihara K. Treatment approach for large-cell neuroendocrine carcinoma of the lung using next-generation sequencing. Transl Cancer Res 2017;6(Suppl 2):S448-S449. doi: 10.21037/tcr.2017.03.51
Molecular resistance mechanisms of ALK inhibitors and implications for therapeutic management of ALK-rearranged lung cancer patients

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Provenance: This is an invited Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Feb 01, 2017. Accepted for publication Feb 24, 2017.
doi: 10.21037/tcr.2017.03.22
View this article at: http://dx.doi.org/10.21037/tcr.2017.03.22

Dysregulated anaplastic lymphoma kinase (ALK) protein expression has been previously reported in non-Hodgkin’s lymphoma (NHL) (1). However, it was not until 2007 when Soda et al. and others revealed that ALK is constitutively activated in some patients with non-small cell lung cancer (NSCLC), due to ALK gene rearrangement (2,3). In NSCLC, ALK rearrangement results in expression of ALK fusion proteins with aberrant ALK signalling and oncogenic transformation (2) and occurs in about 3–5% of the total NSCLCs (4). Currently, the treatment strategy of so-called ALK rearranged NSCLC relies on selection of an ALK tyrosine kinase inhibitor (TKI). The first in class ALK inhibitor crizotinib was developed and approved through accelerated drug approval by the US Food and Drug Administration (FDA) in 2011 on the basis of high response rates in early phase evaluation (5,6) and was granted regular approval by US FDA in 2013 based on demonstration of superior progression-free survival (PFS) and overall response rate (RR) for crizotinib-treated patients compared to chemotherapy (7). However, despite the significant improvements observed in ALK-rearranged NSCLC patients with crizotinib compared to conventional cytotoxic chemotherapy, resistance to crizotinib occurs with patients often relapsing within 1–2 years (8-10). Several second-generation ALK inhibitors have been developed and tested in patients who have progressed on treatment with crizotinib (11,12). Among these, ceritinib and alectinib were recently approved by the FDA for treatment of crizotinib-resistant ALK-rearranged NSCLC patients (13-15) and have also demonstrated clinical efficacy in crizotinib naïve patients (16,17) but resistance also occurs to these drugs (18,19). The question then is how best to select and schedule ALK inhibitors to optimise treatment for ALK rearranged NSCLC? To address this requires knowledge of ALK TKI resistance mechanisms and how this knowledge can be applied in the clinic. A recent study conducted by Gainor and colleagues from the group of Dr. Alice T Shaw provides new insights into ALK TKI resistance mechanisms that has implications for ALK treatment selection in the clinic (20).

Generally, resistance mechanisms to ALK TKIs can be classified into two main categories, including on-target genetic modifications such as ALK resistance mutations or ALK gene amplification and off-target changes including
Interestingly, no resistant mutations were concomitant with (8.3%) specimens demonstrated ALK gene amplification. Examined specimens were found to have on-target genetic FISH testing as rearranged tumours, 31% of the ALK 36 crizotinib-resistant specimens pre-confirmed by ALK I1171T (2%), S1206Y (2%), and E1210K (2%). Within (frequency) detected were C1156Y (2%), G1202R (2%), the total mutations detected, respectively. Other mutations most common mutations with a frequency of 7% and 4% of patients. L1196M and G1269A mutations represented the fluid (31%), liver (22%), and nodal tissue (18%) from 10 specimens from biopsy sites that included pleural crizotinib resistance mutations were confirmed in 11 (20%, exposure to second generation ALK inhibitors. In this study ALK resistance mutations for different ALK inhibitors and that mutations are more frequent following exposure to second generation ALK inhibitors. In this study crizotinib resistance mutations were confirmed in 11 (20%, N=51) specimens from biopsy sites that included pleural fluid (31%), liver (22%), and nodal tissue (18%) from 10 patients. L1196M and G1269A mutations represented the most common mutations with a frequency of 7% and 4% of the total mutations detected, respectively. Other mutations (frequency) detected were C1156Y (2%), G1202R (2%), 11171T (2%), S1206Y (2%), and E1210K (2%). Within 36 crizotinib-resistant specimens pre-confirmed by ALK FISH testing as ALK rearranged tumours, 31% of the examined specimens were found to have on-target genetic alterations contributing to the crizotinib resistance and 3 (8.3%) specimens demonstrated ALK gene amplification. Interestingly, no resistant mutations were concomitant with the ALK gene amplification. Changes in ALK resistance mutational profile following treatment with the second-generation ALK inhibitors ceritinib (N=23), alectinib (N=17), or brigatinib (N=6) were also examined. The results obtained indicate that within the available specimens of 9 cases of pre-ceritinib/post-crizotinib only 2 (22%) exhibited on-target resistance mechanisms, including ALK resistance mutation S1206Y and ALK fusion gene amplification. Of 24 separate post-ceritinib biopsies (obtained from 23 ceritinib treated patients), 54% harbored ALK mutations, with 17% of the total ALK mutations exhibiting two different mutations concomitantly. The G1202R (21%) and F1174C/L (16.7%) mutations were most common. Of note, a novel ALK G1202del mutation was also identified in 8% of specimens. The authors conducted preclinical studies to determine the functional consequences of the various mutations identified in the clinical cases. Ectopic expression of the EML4–ALK G1202del mutant in Ba/F3 cells suggested that the G1202del ALK mutant confers moderate resistance to ceritinib, alectinib, and brigatinib with crizotinib potency being less affected. For 17 patients treated with alectinib (who had previously received crizotinib), 17 alectinib-resistant biopsies were analysed and ALK resistance mutations were detected in 9 (53%) specimens. Interestingly, the ALK G1202R mutation was present in 29% of cases. Detection of the ALK V1180L mutation (6%) in response to alectinib was confirmed for the first time in an alectinib-resistant patient. Finally, for the 7 patients treated with brigatinib, ALK resistance mutations were observed in 5 of 7 (71%), where the ALK G1202R mutant was detected in three specimens (60%). Overall, these data indicate that patients treated with second generation ALK inhibitors compared to crizotinib as a first generation inhibitor have a higher frequency of ALK mutations with the higher resistance conferred by the ALK G1202R mutation also representing the most common detected mutation. The findings demonstrate that part of the mechanism by which dysregulated ALK tumours adapt to resist treatment with ALK TKIs relies on the potency of ALK inhibition. On exposure to a less potent ALK inhibitor (crizotinib) the ALK mutations that emerge exhibit moderate resistance capacity. However, the more potent second-generation ALK inhibitors are associated with a higher frequency of mutations with higher resistance capacity such as G1202R. However, it is unclear whether the mutation profile that emerges on treatment with a second generation ALK TKI is contributed to by prior exposure to crizotinib. Thus, the question that remains to be answered.

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is whether patients with \( \text{ALK} \) rearranged NSCLC should be treated with second or third-generation \( \text{ALK} \) inhibitors as first line treatment or whether sequencing treatment as \( 1^{\text{st}} \), \( 2^{\text{nd}} \) and \( 3^{\text{rd}} \) generation inhibitors would be more beneficial. A recent pre-planned interim analysis from the J-ALEx clinical trial presented at the 2016 American Society of Clinical Oncology (ASCO) Annual meeting demonstrated prolonged PFS for treatment with alectinib compared to crizotinib in untreated \( \text{ALK} \) rearranged NSCLC patients (median PFS not reached versus 10.2 months; HR: 0.34; \( P<0.0001 \)) (16), and the final data evaluating overall survival (OS) might provide more information about which is the better sequencing treatment strategy when the patients in the “crizotinib treatment arm” are crossed over to alectinib treatment when disease progresses. Furthermore, lorlatinib a third-generation \( \text{ALK} \) inhibitor has been found to be more effective in a number of patient-derived ceritinib-resistant cell lines harbouring \( \text{ALK} \) mutations (20).

In another study by Shaw et al., a patient with metastatic \( \text{ALK} \) rearranged lung cancer developed crizotinib resistance due to a C1156Y \( \text{ALK} \) mutation. The patient did not respond to a second-generation \( \text{ALK} \) inhibitor, but responded to the third generation \( \text{ALK} \) inhibitor, lorlatinib before tumour relapse (23). Sequencing analysis revealed a further lorlatinib resistant \( \text{ALK} \) L1198F mutation in addition to C1156Y. The L1198F mutation was found to enhance the \( \text{ALK} \) crizotinib binding affinity within \( \text{ALK} \) and re-sensitise the tumour to crizotinib (23). Therefore, the usage of second or the third-generation inhibitors as first line treatment in \( \text{ALK} \) rearranged NSCLC patients could be an option. A further consideration for \( \text{ALK} \) inhibitor selection is the type of \( \text{ALK} \) rearrangement and fusion gene. Recent data published by Yoshida et al., that compared the differential crizotinib response duration among \( \text{ALK} \) fusion variants established using RT-PCR demonstrated \( \text{ALK} \) fusion variant 1 to be the most frequently detected variant (54%) (24). The objective response rate (ORR) was 74% and 63% in the variant 1 and non-variant 1 groups, respectively and 69% overall. The median PFS was significantly longer in patients with variant 1, 11.0 (95% CI, 6.5–43.0) months than in those with non-variant 1, 4.2 (95% CI, 1.6–10.2) months, respectively (\( P<0.05 \)) (24). Further investigations evaluating survival endpoints for the different specific types of \( \text{ALK} \) fusions, and for the most common mutations, such as L1196M, G1269A, C1156Y, G1202R, I1171T, S1206Y, and E1210K would be of value to develop optimal clinical algorithms for \( \text{ALK} \) inhibitor selection.

From the aforementioned, it is clear that detection of \( \text{ALK} \) TKI resistance mutations are a critical point in determining treatment strategy, although not yet fully integrated into routine practice. Repeat biopsy and sequencing analysis to assess for the presence and type of resistance mutation following treatment with an \( \text{ALK} \) TKI seems set to become the norm. However, tissue biopsy specimens are always limited to certain locations and hardly reflect the comprehensive molecular signatures of metastatic lung cancer, and intratumour heterogeneity. In contrast liquid biopsies can be obtained from almost all body fluids, thus representing a new source of cancer-derived materials to better reflect the nature of tumour at both primary and metastatic sites. Detection of circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), circulating tumour RNA (ctRNA), exosomes, and tumour-educated platelets (TEPs) in body fluids have potential to provide much more information regarding \( \text{ALK} \) resistance mechanisms compared with tissue biopsies. However, due to the lower frequency of these markers in body fluids, achieving higher detection sensitivity and specificity remains technically challenging (25). Table 1 summaries the current liquid biopsy strategies for \( \text{ALK} \) resistance evaluation with the observed limitations. Evaluation of intrinsic or acquired TKI resistance alterations in patients with \( \text{ALK} \) rearranged NSCLC using liquid biopsy can direct treatment selection based on a time specific mutational profile and anticipate treatment resistance to second generation \( \text{ALK} \) inhibitors. Additionally, liquid biopsy might overcome the tumour heterogeneity limitations of tissue biopsies allowing for the detection of acquired compound mutations associated to specific TKI resistance (Figure 1).

In summary, despite clinical studies that demonstrate better RR and benefits of \( \text{ALK} \) TKIs in patients with \( \text{ALK} \) rearranged NSCLC, resistance to \( \text{ALK} \) TKIs remains challenging. Better knowledge of the genomic profile of the \( \text{ALK} \) rearranged tumours at first diagnosis and monitoring for acquired resistance mechanisms using liquid biopsy approaches have potential to optimise \( \text{ALK} \) TKI and sequencing for improved outcomes.
Table 1 Current liquid biopsy strategies for ALK resistance evaluation and the associated limitations

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<td>Isolation method by size of epithelial tumour cells (ISET) + fluorescence in situ hybridization (FISH)</td>
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</table>
Acknowledgements

Funding: This work is supported by International Association for the Study of Lung Cancer (IASLC) International Mentorship Award.

Footnote

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

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The role of tumor-derived exosomes in epithelial mesenchymal transition (EMT)

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Provenance: This is an invited Commentary commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Jan 10, 2017. Accepted for publication Jan 12, 2017.
doi: 10.21037/tcr.2017.02.13

View this article at: http://dx.doi.org/10.21037/tcr.2017.02.13

Exosomes are membrane-bound small vesicles (30–150 nm) produced by all cell types and present in all body fluids (1). They are a part of the intercellular communication system that is evolutionarily conserved and operates in bacteria as well as all multicellular organisms (2). Tumor cells produce and release masses of exosomes into the extracellular space. These exosomes carry information in the form of molecular signals and/or genetic materials (mRNA, miRNA, DNA) from the parent tumor cell to locally- or distantly-located recipient cells. Exosome-mediated transfer of information results in re-programming of the recipient cell genome and proteome and ultimately leads to the acquisition of new cellular functions (3). In the tumor microenvironment (TME), where tumor orchestrates cellular interactions, tumor-derived exosomes (called TEX) carry messages from the tumor to host cells, to other tumor cells or via autocrine signaling back to the parent tumor cell (4). For this reason, and also because their content in part resembles that of the parent cell, TEX have been of special interest as potential “tumor surrogates” or as biomarkers of the tumor behavior, including its growth, differentiation, progression or the potential for metastasis formation. Today, the mechanisms responsible for TEX-mediated re-programming of recipient cells are under intense investigation, and as our knowledge of TEX expands so does the spectrum of cellular activities that TEX can apparently regulate and alter in a variety of recipient cells.

In a recent paper published in Oncotarget (5), Rahman and colleagues report that exosomes derived from supernatants of highly metastatic lung cancer cells or from sera of patients with lung cancer drive the epithelial to mesenchymal transition (EMT). The EMT is a cell process that drives differentiation of epithelial cells into mesenchymal cells. Epithelial cells undergoing EMT dramatically alter their shape, phenotype (lose E-cadherin, down-regulate EPCAM; acquire vimentin, Zeb1, Twist, Snail) and behavior (e.g., increase motility). Importantly, carcinoma cells that have undergone an EMT not only acquire a distinct molecular signature but become resistant to chemotherapy and immunotherapy (6). TEX have been previously reported to carry a pro-EMT program that includes EMT inducers such as TGF-β, HIF1α, β-catenin, caveolin-1 or vimentin, which increase invasive capabilities of recipient cells and promote the pre-metastatic niche formation [reviewed in (7)]. While the morphological, phenotypic and functional changes accompanying the EMT are well characterized, molecular and genetic mechanisms responsible for driving the process remain unclear. More recent data suggest that TEX carry factors necessary for activation, initiation and support of the EMT (7).

The Oncotarget report (5) provides in vitro evidence that lung-cancer-derived exosomes activate the metastatic process in human bronchial epithelial cells (HBECs) by increasing their metastatic properties such as migration, invasion and vimentin expression. In this report, TEX were isolated from supernatants of non-metastatic and metastatic
lung cancer cell lines by ultracentrifugation and shown to carry the epithelial (E-cadherin, ZO-1) and mesenchymal (N-cadherin, vimentin) markers, respectively. HBECs were co-incubated with TEX and tested for migration in wound healing “scratch” assays; for invasion in matrigel assays; and for expression of mRNA for vimentin as well other EMT markers by RT-PCR. Only TEX produced by the metastatic lung cancer cell line induced activation of the EMT program in recipient HBECs. Importantly, exosomes isolated from sera of patients with the late-stage lung cancer (but not those isolated from sera of normal donors) similarly increased vimentin expression as well as migration and invasion capabilities of recipient HBECs. Finally, exosomes isolated from lung cancer patients sera and labeled with the PKH67 dye (but not exosomes from normal donors’ sera) were shown to be taken up by HBECs and to up-regulate vimentin expression. Further, a successful knockdown of vimentin in serum-derived exosomes reduced migration of the recipient HBECs. These data suggested that vimentin carried by TEX and delivered to recipient HBECs may be one of the key proteins necessary for induction of the EMT. However, the precise mechanism of how vimentin transferred by TEX contributes to the initiation of the metastatic program in recipient HBECs remains unsolved.

The EMT is a complex multistage process that involves progressive changes of the molecular pathways in the tumor and neighboring cells. It has been suggested that TEX play a critical role in all stages of the EMT—from initial activation of the invasive phenotype to metastasis (7). To initiate and sustain the process, TEX have to deliver autocrine or paracrine signals to neoplastic epithelial cells and other cells in the TME. The targeting of TEX to specific recipient cells is probably dependent on the content of TEX cargo, e.g., membrane-associated integrins. It is known that TEX can interact with recipient cells via the receptor/ligand type signaling or integrin-mediated adhesion or they can be internalized by endocytosis or phagocytosis (8). The type of recipient cell probably determines which of these mechanisms are engaged in TEX cross-talk with a recipient cell. Disrobing of the internalized TEX in recipient cells and delivery of nucleic acids, including miRNAs, leads to genetic re-programming and to changes in the proteome and/or transcriptome of the cell. Evidence that TEX may serve as a conduit for EMT-initiating signals is based on observations that: (I) TEX carry and deliver known EMT inducers such as TGF-β, IL-6, β-catenin and others to recipient cells (9); and (II) epithelial neoplastic cells exhibit morphologic, phenotypic and functional alterations that are consistent with the EMT after co-incubation with TEX (10). The pre-metastatic niche formation is then followed by progressive shift toward metastasis, which is also facilitated by TEX delivering signals and cues that culminate in the formation of metastasis at secondary sites (7).

To fully understand how TEX drive the EMT, it will be necessary to better characterize their unique molecular and genetic cargo and to in vivo model cellular changes mediated by TEX delivery in a suitable animal model of the EMT. To do so, TEX isolation from body fluids rather than supernatants of tumor cell lines will have to be accomplished. As body fluids of patients with cancer, while variably enriched in TEX, contain a mix of exosomes derived from different normal cells, capture of TEX for molecular, genetic and in vivo modeling in animal models of the EMT will be essential for comprehending of TEX mechanisms of action and of their relative contributions to the initiation and progression of metastasis. It is expected that rapidly emerging technological advances enabling TEX capture from body fluids of cancer patients and TEX characterization will soon be at hand to help in a mechanistic definition of the role TEX play in the EMT.

Acknowledgements

Funding: The author is supported in part by NIH grants R01 CA168628 and R21 CA205644.

Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Cite this article as: Whiteside TL. The role of tumor-derived exosomes in epithelial mesenchymal transition (EMT). Transl Cancer Res 2017;6(Suppl 1):S90-S92. doi: 10.21037/tcr.2017.02.13
Screening and Prevention

The French initiative paves the way: routine molecular profiling of advanced non-small-cell lung cancer fights inequalities in access to molecular targeted therapy and improves patient outcome

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Provenance: This is a Guest Editorial commissioned by Section Editor Shao-Hua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


This month’s issue of the Lancet showcases the results of the French Cooperative Thoracic Intergroup (IFCT) 1-year nationwide program of routine molecular profiling of patients with advanced non-small-cell lung cancer (NSCLC). In its sheer size and logistic complexity, the endeavor is unprecedented, and its results highlight an impact of targeted therapy on outcome that extends well beyond what can be attributed to baseline prognostic characteristics. Moreover, it represents a striking example of health-policy implementation mobilizing pre-existing but previously scattered resources.

Advances in multiplex genotyping and high-throughput genomic profiling by next-generation sequencing (NGS) allow physicians to routinely gather therapy-relevant molecular information in a timely fashion. As a result of wide genetic mapping of several cancer types, lung adenocarcinoma as a subtype nowadays encompasses a cluster of discreet subtypes characterized by a single driver alteration, potentially actionable through a matching drug. Since 2004, several targeted therapies for molecularly-defined subsets of NSCLC have successfully found their place in the therapeutic armamentarium. Identification of mutations within the EGFR gene resulting in ligand-independent activation (1,2) rapidly led to widespread development and use of EGFR tyrosine kinase inhibitors (TKI), doubling median survival time to more than two years when compared to a similar population not exposed to targeted therapy (3). Following closely with a more efficient development path, the successful targeting of ALK in patients with lung adenocarcinoma bearing rearrangements in the ALK gene yielded similar survival benefits in terms of survival, not explained by baseline prognostic factors, but solely attributable to exposition to the specific targeted therapy (4). In recent reports, median overall survival (OS) of advanced ALK positive NSCLC using optimized sequencing of treatment options has been shown to extend beyond four years (5). Beyond these two oncogenic drivers, for which TKIs are now established as the present standard of care from first-line onwards, other smaller oncogene-addicted NSCLC subsets have been reported with similar sensitivity to targeted approaches (6-8).

Recognizing that lung cancer remains by far the leading cause of death in countries with very high or high human development index (HDI), the translation of these development into nationwide everyday practice is expected to yield tremendous benefits (9). Yet from a public-health point of view, there are further conditions for true personalized medicine in the face of an ever-growing list of molecularly targeted drugs: broad availability of testing, high quality of testing, timeliness of test results compatible with patient care.
as well as satisfactory cost-effectiveness. Importantly these parameters may harbor some very distinct definitions across countries. In this regard, the French initiative is remarkable: acting on the Cancer Plan 2009–2013 calling for equal access to innovative and existing therapy, the French National Cancer Institute and the Ministry of Health have set up a nation-wide network of regional hubs for molecular testing that perform tests free of charge for patients and institutions. Between April 2012 and April 2013, 17,664 NSCLC patients were routinely screened for EGFR mutations, ALK rearrangements, as well as HER2, KRAS, BRAF, and PIK3CA mutations. Considering an estimated annual incidence of close to 39,000 new NSCLC cases with an estimated 85% of which either initially present with advanced stage or with subsequent metastatic relapse, and considering that only 5% of the samples analyzed were of squamous histology; the number of samples tested demonstrates a very high nation-wide testing rate, with very little selection bias. The study thus provides a very comprehensive cohort that carries general applicability.

This high coverage dataset suffers from obvious limitations regarding missing clinical annotations, in particular demographic information, tumor staging details as well as outcome indicators. Capture of such parameters would have brought invaluable knowledge to the field, Nonetheless, the study clearly succeeds in demonstrating the feasibility of the implementation of large-scale nationwide decentralized testing, fulfilling one crucial public health requirement, namely broad unrestricted access to testing.

Quality and reproducibility of molecular analyses represent basic conditions for success, impacting the expected magnitude of benefit of the testing strategy. The authors do not dwell on quality considerations, which are beyond the scope of the article. Nonetheless, the program involved central coordination of the regional centers by the National Cancer Institute, which included the setup of external quality evaluations, the implementation of new molecular assessments, fostering standardization and ensuring high-quality molecular testing in all 28 sites. Turnaround time, a well-known bottleneck in patient management, directly affects physicians’ compliance and their willingness to use a particular test provider and might encourage them to initiate therapy before molecular test results are available. In this particular initiative, overall turnaround time from sample collection to report of the analysis was 19 days for EGFR, 28 days for ALK, 26 days for HER2, and 23 days for BRAF. Most clinicians will consider this long and borderline acceptable for optimal patient care, and this was indeed the case for 23% of patients in the study, whose therapy was started before the molecular information became available, disregarding this information for initial treatment decision-making. Maximal reduction of turnaround time is obviously limited by diverse in-laboratory factors. Some sample require multiple attempts at library preparation, as sample quality and quantity are often an issue in the lung cancer setting, where most tests must be performed on small biopsies or endobronchial ultrasound guided cytological samples. Formalin-fixed and paraffin-embedded samples do often yield poor-quality DNA and contamination with non-tumor cells hampers the detection of tumor-specific mutations. While the French program used sequential Sanger sequencing, or a more sensitive validated allele-specific technique with confirmation by Sanger-sequencing (similarly to the Lung Cancer Mutation Consortium), many laboratories have now implemented NGS methods (10). These usually allow for more rapid sequencing of a large panel of genes in parallel, with time requirements nonetheless ranging from more than ten days for earlier platforms to less than 24 hours for the newest platforms in use (11). As sequencing time falls, the overall turnaround time will then be dominated by human factors such as variant interpretation and report sign-out, as the time required to interpret a large panel of gene sequences and a fortiori whole-gene sequence data is undoubtedly slower than hotspot genotyping because of the wider range of variations detected. In summary, the long turnaround time reported by the French initiative will rapidly shorten as the technology evolves.

From a public health perspective, cost effectiveness remains a key issue when implementing large-scale molecular profile guided therapy. While the cost effectiveness of first-line crizotinib therapy has been called into question, this seems to be mainly a consequence of drug pricing, and not of the magnitude of benefit (12). The cost effectiveness of EGFR mutation testing has already been demonstrated by several studies (13). In France’s health-care system, that relies mainly on public centralized State funding by the Sécurité Sociale, the extrapolation of these savings to the nationwide population may lead to a significant relief of the financial burden.

With regards to patient outcome, the study highlights major differences in progression free survival (PFS) both in first and second line, and OS. OS was 4.7 months longer when a genetic alteration was detected, including alterations not actionable currently, suggesting both a prognostic advantage in some molecular subsets, mixed with the impact of targeted therapy. This is especially striking when considering the median PFS of first-line treatment of
patients with $EGFR$-mutated NSCLC of 15.4 vs. 8.3 months in the overall population, and the median PFS of second-line treatment of patients with $ALK$-rearranged NSCLC of 9.3 vs. 3.1 months in the overall population. These differences dramatically exceed what is to be expected from baseline prognostic differences and pinpoint the immediate effect of EGFR and ALK TKIs, respectively. Interestingly, the inclusion rate into clinical trials was not improved by the initiative. This finding may be related to the specific panel of alterations being tested, that were either of uncertain predictive value for targeted therapies, disappointingly altered to date by available compounds (KRAS, PIK3CA, HER2) or for whom established and registered drugs were already available (EGFR and ALK). Another likely explanation may be insufficient coordination between molecular pathologists and clinical trials investigators; and possibly a lack of collaborative efforts across centers in building shared and distributed clinical trials or in systematically referring patients for research protocols. National registers listing recruiting clinical trials might support maximizing patient enrollment into clinical trials—these were probably not part of this French program.

The recent initiatives aiming at addressing the complex molecular landscape of lung tumors through the design of widely distributed umbrella trials (Battle trials, SAPHYR, Lung-MAP, SPECTAlung,...) is probably the way to move forward, reinforcing an academic and transversal research of quality across regions and countries.

The French program should further encourage worldwide initiatives to provide NSCLC patients with access to personalized therapy; and we anticipate they will demonstrate that molecular stratification of NSCLC for therapeutic purposes is a cost-effective strategy that can be successfully implemented in a centralized health-care system.

Acknowledgements

None.

Footnote

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

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The reality of precision or personalized cancer care is here. The discovery of oncogenic drivers (such as BCR-ABL translocations in chronic myelogenous leukemia, kinase domain mutations of the epidermal growth factor receptor (EGFR) gene or EML4-ALK fusion genes in lung adenocarcinoma, and BRAF V600E mutation in melanoma) has revolutionized the field of cancer biology. These drivers have led to new paradigms in cancer treatment (Table 1). Tumours that harbor these genomic aberrations, now commonly referred to as “actionable mutations”, are highly dependent for their growth and survival on the function of the protein products of these mutated driver genes (1). Patients with driver-addicted tumours can benefit from drugs that specifically inhibit the function of these driver genes, and a high percentage experiences significant treatment response and prolongation of survival.

However, the success of this precision cancer treatment strategy hinges on the availability of routine clinical testing programs to identify these actionable mutations. At present, routine testing for driver mutations is the standard in the prescription of targeted therapies with bona fide predictive biomarkers (2). Nevertheless, the number of approved targeted therapies in specific cancer type remains few (3).

During the last five years, we have witnessed rapid advances. Several cancer genomic profiling projects have identified increasing numbers of potentially actionable mutations across various tumour types, including lung cancer (4–7). Most of these actionable mutations occur at low frequency in each tumour type, and they are mostly mutually exclusive in each patient tumour. These discoveries have led to the acceleration of novel targeted therapy development with associated clinical trials to evaluate their efficacy. There is thus greater incentive for increasing the throughput of driver mutation profiling in patient tumour samples, and tissue availability has become more of a limiting factor. In parallel, there have been rapid advances in DNA/RNA sequencing technologies not only increase the throughput but also lead to rapid reduction in the cost of molecular profiling (8,9). Against this background, many single or multi-institutional studies have been initiated to demonstrate the efficiency and value of broad and higher throughput molecular testing programs. The BATTLE trial (10) demonstrated the feasibility of prospective biomarker dependent clinical trials. In the United States, the Lung Cancer Mutation Consortium (LCMC), a coalition of 14 cancer centers, assessed the feasibility of conducting multiplex genotyping of 10 driver oncogenes in tumour samples of ~1,000 lung adenocarcinoma patients, in six academic but Clinical Laboratory Improvement Amendment (CLIA)-certified molecular testing laboratories (11). While this pioneering pilot project was successful in demonstrating the clinical benefit of obtaining multiplex genotyping information from patients with lung adenocarcinoma, the scale was limited. In contrast, the French Cooperative Thoracic Intergroup (IFCT), supported by the French National Cancer Institute...
(INCa) and in collaboration with the French Ministry of Health, launched a bold initiative that aimed to make molecular profiling available to all cancer patients in all regions of France, free of charge to patients, with tests being conducted in 28 regional molecular genetic centres (12,13). In a recent paper by Barlesi et al. (14), published in *The Lancet*, the IFCT investigators reported the results of this program during its first year of operation, on routine molecular profiling of 18,679 patients with advanced non-small cell lung carcinoma (NSCLC).

The network of 28 certified regional genetic centres was established by INCa in 2006, nationwide across France, approximately one centre per administrative region (12). Each centre was a partnership between several university hospitals and cancer centre laboratories that provided free molecular testing across many tumour types to the hospitals and cancer centre laboratories that provided each centre was a partnership between several university regions of France, free of charge to patients, with tests established by INCa in 2006, nationwide across France, approximately one centre per administrative region (12). Each centre was a partnership between several university hospitals and cancer centre laboratories that provided free molecular testing across many tumour types to the hospitals and cancer centre laboratories that provided each centre was a partnership between several university

<table>
<thead>
<tr>
<th>Actionable mutation</th>
<th>Cancer type</th>
<th>FDA/EMA* approved drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR-ABL translocation</td>
<td>Chronic myeloid or acute lymphoblastic leukaemia</td>
<td>Imatinib, dasatinib, nilotinib, bosutinib, ponatinib</td>
</tr>
<tr>
<td>KIT &amp; PDGFR mutations</td>
<td>Gastrointestinal stromal tumours</td>
<td>Imatinib</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>Breast cancer</td>
<td>Trastuzumab, lapatinib, pertuzumab</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>Gastric cancer</td>
<td>Trastuzumab</td>
</tr>
<tr>
<td>EGFR mutations</td>
<td>Non-small-cell lung cancer</td>
<td>Gefitinib, erlotinib, afatinib</td>
</tr>
<tr>
<td>ALK rearrangement</td>
<td>Non-small-cell lung cancer</td>
<td>Crizotinib, ceritinib, alemtinib</td>
</tr>
<tr>
<td>ROS1 rearrangement</td>
<td>Non-small cell lung cancer</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>BRAF V600 mutation</td>
<td>Melanoma</td>
<td>Vemurafenib, dabrafenib, trametinib</td>
</tr>
</tbody>
</table>

*, FDA, Federal Drug Agency; EMA, European Medicines Agency.

The IFCT reported results for the molecular profiling of six routinely screened genes selected in 2009 for NSCLC, including *EGFR* mutations and *ALK* rearrangements, as well as mutations in *HER2* (ERBB2), *KRAS*, *BRAF*, *PIK3CA*, using Sanger sequencing and/or more sensitive validated sequence-specific techniques. The authors demonstrated that a genetic alteration was present in about half of the tumours analyzed, with a median turnaround time of 11 days between the initiation of analysis and reporting. Importantly, the presence of a genetic alteration affected first-line treatment decisions in 51% (4,176/8,147) of the patients with alterations. The investigators demonstrated that routine molecular profiling of patients with advanced NSCLC is not only feasible, but also provided a significant clinical benefit: the presence of a genetic alteration was associated with a significant improvement in the proportions of patients achieving an overall response to both first-line (37% vs. 33%, *P*=0.03) and second-line treatments (17% vs. 9%, *P*<0.0001), compared with the absence of a genetic alteration; the presence of a genetic alteration was significantly associated with improved first-line progression free survival (10 vs. 7.1 months, *P*=0.0001) and overall survival (16.5 vs. 11.8 months, *P*<0.0001) compared with the absence of a genetic alteration. However, similar to the LCMC study, whether the survival benefit was due to the presence of the alteration (prognostic effect) or the effectiveness of the targeted agent (predictive effect), or both, remained in question and could not be teased apart in this study.

In this large population-based molecular profiling study, the reported prevalence of the six genetic alterations can be compared with the results reported in other studies of more limited scope. *EGFR* mutations were detected in 11% of analyzed tumours, which is significantly lower than the 22% reported in the LCMC (11) or 20.6% in the Canadian province of Ontario (15). The prevalence of *ALK* gene rearrangements was found to be 5% in the IFCT analyses, compared to 7.9% in the LCMC. LCMC detected *HER2* (ERBB2) mutations at a 2.7% frequency, while the IFCT screening reported 0.8%; these are in contrast to the single-institution analyses at Memorial Sloan Kettering Cancer Center (MSKCC) with a 6% mutation rate (16). *BRAF* was detected at 1.9% frequency in the IFCT, comparable to 2.6% in LCMC. The *PIK3CA* mutation rate was 2.3% in the IFCT study, 2% at MSKCC (17), and 0.8% in the LCMC. Institutional referral bias or differences in population characteristics (such as ethnicity) could potentially account for these differences. Given the high incidence of lung cancer, these findings warrant routine
testing of the rarer mutations (HER2, PIK3CA, BRAF) as they represent a substantial number of patients in the population that might benefit from targeted therapy.

The network structure of the 28 molecular laboratories spread across France benefited from rapid, uniform, high quality molecular testing and unhindered flow of information across testing centers. As a push for standardization, France mandated all medical laboratories to obtain an accreditation to ISO 15189 standard by 2016. Moreover, the network allowed for the creation of a large centralized national database that provides a major advantage to precision cancer treatment strategies, as it becomes a large resource for epidemiological analyses on the utility of approved targeted treatments, as well as a mechanism to direct patients harboring specific mutations without approved targeted agents into clinical trials. Prior to this, no program or initiative had been able to set up as extensively as this cancer molecular profiling program, which gathered epidemiological, clinical, histological, and therapeutic data, along with follow-up information. To facilitate rapid recruitment of patients to early-phase clinical trials, the French also utilized a network of 16 INCa-certified early phase centers (CLIP²) distributed across the country with the goal of helping clinicians match patients to early-phase clinical trials (18). With collaborative efforts between academia and pharmaceutical industry, CLIP² allowed selection of potential therapeutic targets to be rapidly investigated in clinical trial. Disappointingly, the promise of increasing clinical trial recruitment by molecular profiling programs has yet to bear fruit. No clear improvement in clinical trial recruitment resulted during the IFCT 1-year period of molecular profiling for NSCLC; only 3% of patients with a molecular alteration were enrolled into clinical trials. This failure is a concern as similar molecular

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**Figure 1** Frequency of genetic alterations in the French Cooperative Thoracic Intergroup (IFCT) study. (A) Overall population; (B) adenocarcinoma only; (C) women only; and (D) never smokers only. [Adapted and reproduced with permission from reference (14)].

<table>
<thead>
<tr>
<th>Category</th>
<th>Overall</th>
<th>Adenocarcinoma</th>
<th>Women</th>
<th>Never smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full WT</td>
<td>15%</td>
<td>15%</td>
<td>12%</td>
<td>9%</td>
</tr>
<tr>
<td>EGFR</td>
<td>11%</td>
<td>12%</td>
<td>21%</td>
<td>12%</td>
</tr>
<tr>
<td>KRAS</td>
<td>29%</td>
<td>32%</td>
<td>27%</td>
<td>14%</td>
</tr>
<tr>
<td>BRAF</td>
<td>2%</td>
<td>2%</td>
<td>6%</td>
<td>3%</td>
</tr>
<tr>
<td>HER2</td>
<td>1%</td>
<td>1%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>2%</td>
<td>2%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>ALK</td>
<td>5%</td>
<td>5%</td>
<td>6%</td>
<td>14%</td>
</tr>
<tr>
<td>Unknown</td>
<td>35%</td>
<td>32%</td>
<td>28%</td>
<td>13%</td>
</tr>
</tbody>
</table>

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profiling studies performed in other centers across the world have also failed to show significant trial participation after testing. The MD Anderson genomic testing protocol matched 83/2,000 (4%) of patients to clinical trials (19). The SAFIR-01 breast cancer trial matched 28/295 (9%) (20). The British Columbia Cancer Agency Personalized Oncogenomics trial only matched 1/78 (1%) patient (21).

As molecular profiling evolved from single gene assays into multiplex genotyping platforms such as next-generation sequencing, common actionable mutations shared across many tumour types are being recognized and provide a strong rationale for using similar targeted agents as treatment. For example, while the ALK-inhibitor crizotinib is only approved for use in EML4-ALK and ROS-1 rearranged NSCLC, the presence of ALK and/or ROS-1 alterations in other tumour types, including breast, colorectal, melanoma, and thyroid cancer, as well as a variety of other blood and solid tumours have led investigators and clinicians to off-label use (22). The problem arises in that most approved targeted therapies have been rigorously tested in a clinical trial for only a specific subset of tumours. Without proper clinical trial investigation, off-label use in non-approved tumour types runs the risk of toxicity with only anecdotal evidence of a treatment benefit. To this end and as part of the French National Cancer plan, France has set up the AcSe (Secured Access to Innovative Therapies) program, to bridge the results of molecular testing with investigative clinical trials of targeted drugs for patients harboring actionable mutations outside a drug's market authorization. The AcSe program will help generate safety and efficacy data on these targeted agents outside their approved indications, and has already shown proof of concept with crizotinib and verumafinib trials in various tumours not currently approved for use (22). Even if the drug's market authorization holder does not submit for a new indication, the safety and efficacy data generated from these trials will be useful for future off-label prescriptions.

The French initiative's synergistic approach has given the world a great example of how to implement a precision or personalized cancer care strategy that benefits all citizens of a country. Moreover, the central database that was included in the establishment of this program provides an invaluable and real time resource for crucial molecular epidemiological studies in personalized cancer care. By offering free molecular testing nationwide, they have provided universal access to predictive biomarkers that may be implemented in clinical treatment decisions. Even if no clinically approved drug is currently available, these patients will not be left out because the AcSe programme allows for a quick way to investigate innovative targeted agents based on their molecular profile. This approach also seems to be cost-effective compared to prior strategies, with the overall cost of molecular testing balanced by the savings on prescription drugs for patients without the intended biomarker or actionable mutation. A similar initiative in the United Kingdom is now ongoing (23), which could be extended to other European countries.

In the United States, the plethora of private health insurance systems might require significant modifications when building such an infrastructure. However, a renewed commitment to precision medicine has been undertaken, and the NCI has launched multiple initiatives with the goal of matching patients with an actionable mutation to an agent that targets that specific molecular alteration or pathway, ensuring clinical trial participation. The NCI Molecular Analysis for Therapy Choice (MATCH) serves as a prescreening histology-agnostic basket trial to designate patients with particular mutations to targeted treatment arms (24). The Lung-Master Protocol (LUNG-MAP) aims to overcome the difficulty in recruiting patients with lung squamous cell carcinoma into specific clinical trials by utilizing an umbrella model, where comprehensive molecular profiling is performed and the results of these tests will determine enrollment in four substudies (25). These substudies are based on targeted treatment of patients with mutations in PIK3CA, CCND1/2/3 or CDK4 amplifications, and FGFR alterations. Those without any defined alterations are placed into a randomized PD-L1 immunotherapy arm or chemotherapy. These new trial designs hope to overcome the many challenges of genotype-matched trials. Leading by example, France, along with other countries, has paved the way for precision cancer care by promoting the revolution of taking action on actionable mutations.

Acknowledgements

Dr. Michael Cabanero is a Research Fellow of the Terry Fox Foundation STIHR on Clinician Scientists in Molecular Oncologic Pathology at Canadian Institutes of Health Research (www.molecularpathology.ca). Dr. Tsao is the M. Qasim Choksi Chair in Lung Cancer Translational Research.

Footnote

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

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Cite this article as: Cabanero M, Tsao MS. Taking action on actionable mutations: a French initiative on universality in precision cancer care. Transl Cancer Res 2016;5(S1):S35-S39. doi: 10.21037/tcr.2016.05.16
In non-small cell lung cancer (NSCLC), the purpose of precision medicine is to use the latest genomic knowledge to adapt treatments to patients. It is essential that drugs are designed to hit a molecular abnormality, mutation or translocation, inducing NSCLC. Compared with other cancers, genetic alterations in NSCLC are notably high (1). In NSCLC, FDA and EMEA have already approved epidermal growth factor receptor (EGFR) inhibitors, gefitinib, erlotinib or afatinib, in the front line setting of EGFR mutated NSCLC and anaplastic lymphoma kinase (ALK) inhibitor, crizotinib, in ALK or ROS1 translocated NSCLC (2,3). Several novel cancer therapies targeting oncogenic mutations as BRAF or MET mutations may be approved in NSCLC in the next years.

The two major issues of precision medicine are the complex biology and the economic costs (4). Thus, targeted drugs need to be accompanied by valid diagnostic tests to identify patients who will benefit of these therapies. EGFR or ALK testing are cost saving as expensive drugs will be exclusively prescribed to patients who will gain benefit (5). However many health-care systems have no funding to pay for these tests.

Proceeding efforts are necessary in molecular dismantling of NSCLC to provide a tailored therapy to a maximum of patients. In France, prescription of EGFR or ALK targeting therapies are conditioned by molecular alterations and these testings are done routinely. In 2006, the French National Cancer Institute (INCa) has set up a national program to support molecular testing with the establishment of 28 regional molecular genetics centres. Screened molecular alterations were selected in 2009, including EGFR mutations, ALK gene rearrangements, but also emerging biomarkers such as KRAS, BRAF, HER2 or PI3KCA mutations. Furthermore, INCa developed a quality assurance program for molecular testing (ISO 15189).

The BIOMARKER France study assessed the characteristics, molecular profiles and clinical outcomes of patients who were screened by this programme from 04/2012 to 04/2013. Data reported in Lancet on more than 17,000 patients show the presence of at least one genetic alteration in about 50% of analysed samples (6). Thus, EGFR mutations were detected in 11% of samples, HER2 mutations un 1%, KRAS mutations in 29%, BRAF mutations in 2% and PI3K mutations in 2% of patients; ALK rearrangements were detected in 5% of the analysed samples (Figure 1). The presence of a genetic alteration affected first line treatment for 51% of patients with a significant improvement in the proportion of patients achieving an overall response in the first line or second line treatment and an improved overall survival [16.5 months (15.0–18.3 months) versus without a genetic alteration 11.8 months (10.1–13.5 months); P<0.0001]. However improved prognosis in NSCLC harbouring EGFR mutations or ALK rearrangements compared to wild-type NSCLC is reported. Thus whether this effect on overall survival is related to specific medications such as EGFR and ALK inhibitors (predictive) or to the prognosis of NSCLC is hypothetical. This systematic biomarker analysis was...
This French project, as well as other initiatives as the German Network Genomic Medicine (NGM), the nationwide Japanese Lung Cancer Screening Network (LC-SCRUM) and the American Lung Cancer Mutational Consortium (LCMC), participate to a better understanding of NSCLC.

In the BIOMARKER France study, no improvement in the inclusion rate of clinical trials was noticed; thus only 3% of patients with a molecular alteration were included in a clinical trial. Molecular alterations were selected in 2009 and emerging biomarkers such as KRAS, HER2, BRAF and PIK3CA mutations were routinely analyzed, also for these molecular abnormalities, no targeted therapies were available. Data on targeting HER2 or BRAF mutations are now robust (8,9). It is not certain that KRAS or PIK3CA are effective targets for tailored therapy and whether these mutations should be routinely detected is speculative. ROS1 testing and MET amplification/mutations are now part of the routine molecular testing on the molecular platforms. Since 2014, INCa supports ACSé program to assess the effectiveness of crizotinib in MET amplified/mutated or ROS rearranged and vemurafinib in BRAF mutated NSCLC (9-11). Further large scale molecular screening studies should collaborate with pharmaceutical companies to target emerging biomarkers. Thus, the Japanese LC-SCRUM study includes a genomic analysis by next generation sequencing multiplexing diagnostics and a collaboration with 13 pharmaceutical companies to deliver drugs on the basis of the patients genomic alteration (12).

Acknowledgements

None.

Footnote

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

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Cite this article as: Ruppert AM, Cadranel J, Wislez M. Screening for mutations in lung cancer in France: purpose of precision medicine. Transl Cancer Res 2016;5(S1):S47-S49. doi: 10.21037/tcr.2016.05.09
Biomarkers France: a first and distinctive step in assessing the impact of non-small cell lung cancer (NSCLC) patients routine molecular profiling

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Provenance: This is a Guest Correspondence commissioned by Section Editor Shao-Hua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Jul 21, 2016. Accepted for publication Aug 02, 2016.
doi: 10.21037/tcr.2016.09.24

View this article at: http://dx.doi.org/10.21037/tcr.2016.09.24

The French Cooperative Thoracic Intergroup (IFCT) recently published the results of the Biomarkers France study, the largest nationwide program of molecular profiling for non-small cell lung cancer (NSCLC) patients (1). As highlighted in the three editorials published by Translational Cancer Research regarding these results, the Biomarkers France study not only shows that a routine molecular profiling is already feasible for all of our advanced NSCLC patients, but also that identification of a molecular alteration changes their outcomes by decreasing their risk of death by 22%.

This program, launched by the French NCI, was initially designed on the basis of available actionable molecular alterations in 2006 and then improved by the addition of emerging biomarkers in 2010. The results reported in the Lancet are based on the molecular profiling done during 2012/2013. Obviously, several changes occurred since 2010 and multiplex testing by NGS is becoming more and more frequent, but mainly in selected centers. Indeed, the number of NSCLC patients really accessing to a molecular profiling by next-generation sequencing (NGS), across US or EU, in daily practice, has still to be assessed, and only very few examples are available to date outside clinical trials (2).

One of the main drawbacks of global health initiatives is often the lack of a comprehensive assessment on the changes provided regarding patients’ outcomes. This is the justification of nationwide studies such the Biomarkers France study. In the same time, collecting data on more than 17,600 patients treated routinely by more than 3,800 physicians was not an easy task. In order to succeed, the choice was made to collect selected data only in order to maximize the chance to get the case reported forms completed by the treating physician(s). Despite some gaps in the data collected, the Biomarkers France study succeeded. This study was able to provide data regarding epidemiological characteristics, turnover time, response rates or survival for prespecified molecular alterations. Furthermore the Biomarkers France study also provided the scientific community and the health authorities with...
unexpected results (less than the half of EGFR mutated patients receiving an EGFR-TKI in first line, 3% and 2% of patients enrolled in clinical trials in 1st and 2nd lines, respectively, etc.). All these data will now be used to adapt the French NCI guided national initiatives but also give the background to set up comparable molecular profiling programs in other countries. Additional improvements will come from the expanded use of NGS to identify additional molecular alterations, from the use of cfDNA to better identify and/or potentially monitor molecular alterations (resistance), from the increasing access to drugs in development across the early phases trials cancer units network (CLIP²) (3), etc. All these improvements will be benchmarked against the data collected via the Biomarkers France initiative.

The IFCT Biomarkers France #2 project, hopefully starting in 2017, will collect all these newly routinely available data in order to assess the impact of these technical and medical changes for NSCLC patients, including the advent of immune-oncology options. A second step toward precision medicine for NSCLC patients!

**Acknowledgements**

None.

**Footnote**

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

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**Cite this article as:** Barlesi F, Soria JC, Maraninchi D, Ifrah N, Moro-Sibilot D. Biomarkers France: a first and distinctive step in assessing the impact of non-small cell lung cancer (NSCLC) patients routine molecular profiling. *Transl Cancer Res* 2016;5(S3):S608-S609. doi: 10.21037/tcr.2016.09.24
Introduction

In view of the prospective results of the largest randomized controlled lung cancer screening trial worldwide, the National Lung Screening Trial (NLST), and baseline results of other trials, interest in low-dose chest CT for lung cancer screening in high-risk individuals is increasing.

In 2011, the U.S. NLST demonstrated that screening using annual low-dose chest CT reduces lung cancer mortality by 15–20% compared to screening by chest radiography (1). This result was translated by several U.S. medical associations, including the U.S. Preventive Services Task Force, into a recommendation to screen subjects at high-risk for developing lung cancer by annual low-dose chest CT (2-5). According to the recommendation of the U.S. Preventive Services Task Force, all individuals between 55 and 80 years old who smoked at least 30 pack-years and quit not longer than 15 years ago are eligible for lung cancer screening. Screening should be discontinued once a person has not smoked for 15 years or develops a health problem that substantially limits life expectancy or the ability or willingness to have curative lung surgery (5).

A drawback of CT screening is the high prevalence of small to intermediate-sized (<500 mm$^3$ or <10 mm) lung nodules, most of which are benign. Up to 66% of participants enrolled in CT screening trials has at least one pulmonary nodule (6). Additionally, about 5–7% of lung cancer screening participants develop a new nodule each year (7). Accurate nodule management is required to differentiate between benign and malignant lung nodules, as over 99% of all screen-detected lung nodules are benign.

Determination of the optimal screen interval plays an important role in the balance between harms for the patients, costs, and benefits of CT lung cancer screening. It is not said that a screening protocol should be uniform for all screening participants over the whole 25-year period of screening. If participants with higher and lower risk of developing lung cancer can be identified during screening, the screening protocol might need to be adjusted for those screeners. Currently, lung cancer screening is being implemented in routine clinical care in the United States, via annual low-dose CTs based on the screening regime as used in the previously mentioned NLST. In the Dutch-Belgian lung cancer screening trial (NELSON trial, a Dutch acronym for Nederlands-Leuvens Longkanker Screening Onderzoek), the largest randomized lung cancer screening trial in which lung cancer screening by low-dose chest CT is compared to no screening, screenees were invited for four screens by low-dose chest CT: at baseline, one year later (round 2), two years later (round 3), and another two-and-a-half years later (fourth round). The mortality results of this trial are awaited. The NELSON strategy with prolonged screen intervals provides a unique...
opportunity for evaluation of the influence of the screen interval length on screening characteristics like sensitivity and specificity (8). A second European study that looked into the influence of prolonged screen interval is the Multi-centre Italian Lung Detection Trial (MILD). Participants were randomized to no screening, or annual or biannual screening. Overall, the study showed no mortality benefit for the CT screen group compared to the non-screen group after five years of follow-up, possibly due to the limited sample size (9).

Optimal screen interval

The NLST used three annual screening rounds. Recently, a retrospective cohort analysis was published in which the necessity of annual screening for all eligible screening individuals was evaluated (10). Patz et al. looked into all NLST participants, N=26,231, who received a baseline (T0) screen. The T0 screen was negative (no nodules with diameter over 4 mm or other suspicious findings) in 73% of participants. Special interest was directed to this group of screenees, and the authors found that a prolonged screen interval after a negative T0 screen might be a reasonable option. Both lung cancer incidence and lung cancer mortality were significantly lower for participants with a negative T0 compared to all T0-screened participants. Furthermore, the yield of screen-detected lung cancer at the T1 screen (first annual screen after baseline) in the negative T0 group (0.34%), was far less than the yield in all T0-screened participants (1.0%). If the negative T0 group would not have received an annual screen, 62 screen-detected lung cancers (3.2 per 1,000 screenees with negative T0) would have been diagnosed by delay. However, even in case all these persons would have died because of lung cancer, lung cancer mortality in the negative T0 group would be lower compared to lung cancer mortality in all T0 participants, suggesting that annual CT might not be needed in case of a negative baseline screen.

Two European studies actually used different screen intervals in their screening protocol, and could thereby directly compare screen characteristics when using an annual, biannual or even 2.5-years screen interval. In contrary to the NLST, this comparison did not include lung cancer mortality data. The MILD trial concluded that biannual screening may save about one third of LDCT scans compared with annual screens, with similar lung cancer detection rate, specificity, sensitivity, positive predictive value, and negative predictive value (11). In the NELSON study, nodule management was based on semi-automatically measured nodule volume instead of manually measured nodule diameter (12). In 2014, Horeweg et al. published the results of an in-depth analysis on lung cancer probability based on the presence and size of lung nodules. In more than half of participants, no baseline nodules were found. Furthermore, the 2-years lung cancer probability of screenees with largest lung nodule with volume of less than 100 mm$^3$ (proposed as new cut-off value for a negative baseline screen) was equally low as compared to screenees with no baseline nodules at all [0.6% vs. 0.4%, respectively (P=0.17)]. These results suggest that a screen interval of at least two years might be safe to apply after a negative baseline screen (13). However, in depth analysis of the fourth screening round, 2.5-years after the third round, showed that the interval cancer rate in the last screening round was significantly higher compared with the annual and biannual screen (8). Moreover, the proportion of advanced staged disease in this round was higher compared with the previous rounds. Therefore, a 2.5-year screen interval seems to be too long, at least when not considering the final screen result (positive or negative) of previous screens.

Conclusions

What are we to conclude from these studies? For participants with a negative baseline screen result, which comprises the majority of screen participants, annual screening might not be necessary. Question remains which screen interval will be the best. The study of Patz et al. suggests that the optimal screen interval differs for participants with different baseline screen results: A negative result may lead to safe extension of the screen interval beyond 1 year (10). Yousaf-Khan et al. showed that a screen-interval of 2.5 years is too long (8). Probably, the optimal screen interval for participants with a negative screen lies somewhere between 1 and 2 years. Further (modeling) studies need to be performed to confirm these results.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.
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Cite this article as: Heuvelmans MA, Oudkerk M. Determination of the optimal screen interval in low-dose CT lung cancer screening: are we there yet? Transl Cancer Res 2016;5(Suppl 6):S1070-S1072. doi: 10.21037/tcr.2016.11.55
Circulating tumor cells (CTCs)

CTCs are shed tumor cells that have entered the bloodstream and are able to survive in the blood environment often by endothelial mesenchymal transition (EMT) (1-4). It is thought that CTCs mirror tumor heterogeneity of both the primary tumor and metastases, making them an excellent candidate that reflects the behavior of cancer. CTCs may replace invasive biopsies of the original tumor as a diagnostic tool, lowering the diagnostic burden placed on patients. Follow-up of number of CTCs will give physicians the opportunity to monitor therapy efficacy and observe relapses in time. A major prerequisite is that enough CTCs are being detected; in small cell lung cancer (SCLC) a variable amount of about 20 to 20,000 CTCs are found in 7.5 mL blood.

CTCs numbers in SCLC

Hou et al. showed that CTCs are prognostic for survival in SCLC patients, and observed decreasing CTCs after therapy in responding patients (5). These findings were confirmed in 2012 (6). Hiltermann et al. showed that a decrease in CTC counts after the first course of therapy already predicted tumor response (7). Similar findings were found in other studies (8-10). CTC enumeration is therefore a very promising biomarker for chemotherapy efficacy. Molecular characterization of CTCs may help us to understand mechanisms of metastasis and resistance, hopefully leading to better treatments in this disease where chemotherapy and radiation are still the only known effective treatments (11).

CTCs and copy number variation (CNV) or aberration status

SCLC has a very high mutation rate, as shown by Peifer et al. when by sequencing 29 SCLC exomes, 2 genomes and 15 transcriptomes of SCLC tumors, they observed 7.4±1 protein-changing mutations per million base pairs (12).

In 2013 Ni et al. described CNV patterns in CTCs of lung cancer patients that are highly reproducible for individual patients (13). Tumor subtyping in adenocarcinoma and SCLC could be made on basis of CTC CNV. These patterns were not affected by drug treatment as described in one SCLC patient. For the 23 genes with significantly increased mutation frequencies in response to chemotherapy, six genes (ALPK2, KIF16B, TP53, MYH7, TTL2, PAK2) were enriched and possibly involved in resistance. In 2017 Carter et al. were the first to demonstrate the predictive value of copy number aberrations (CNAs) in CTCs in 31 patients with SCLC (14). SCLC patients receiving chemotherapy were classified as either chemorefractory [progressive disease (PD) within 90 days after completion of chemotherapy] or chemosensitive (PD after 90 days). First, 88 baseline CTC samples from 13 patients were used to create a CNA-based classifier by combining CNA status with clinical response to...
chemistry. They studied the known 13 gene signatures with frequently altered genes in SCLC (8 amplified and 5 deleted genes). No segregation between chemosensitive and chemoresistant status was observed, which is in line with the findings of Rudin et al. and Peifer et al. (12,15). Afterwards, the CNA classifier was expanded, and using this new 16 CNA profile classifier the concordance between predicted and clinical outcome (chemosensitive vs. refractory) was 83%. CNV patterns in CTCs were also not influenced by chemotherapy. Initial chemosensitive patients who became chemoresistant had similar CNV patterns in their CTCs. One of the issues is that the CNV test is not sensitive enough and not the right test to detect the many known resistance mechanisms for platinum and etoposide at DNA or protein level.

The 16 CNA profile classifier was subsequently validated in a new set of 18 patients with 112 CTC samples. The classifier correctly assigned 15 out of 18 patients (83.3%) to either the chemosensitive or chemoresistant group. However, the prediction became worse when 1–4 individual CTC calls were in disagreement with the majority of CTCs. This CTC heterogeneity remains an important issue for biomarker studies and therefore a substantial number of single cells are needed to perform robust treatment predictions.

**Hurdles in CTC detection**

Isolation and detection of CTCs is based on the different physical and biological properties of CTCs compared to normal cells. Different methods to identify and isolate the CTCs (including different definitions of a tumor cell) make comparisons of studies difficult. At this time, the Cell Search system—based on the expression of the epithelial cell adhesion molecule (EpCAM)—is still the only FDA approved system. Other techniques such as the ISET platform (RareCells Inc.) which is based on size and the Clear Cell system (Clearbridge, BioMedics, Singapore) that separates cells by sorted weights are becoming more commonplace, competing with the cell search platform (16,17). It is not yet clear how CTCs obtained by different techniques mirror the characteristics of the original tumor and/or its metastases best. Different CTC characteristics should be compared to identify the most important cell traits that determine patient outcome. In the Cell Search system however, identified CTCs are fixed and can’t be used for further cell culturing and molecular characterization of CTCs enclosed in a cartridge after enumeration is a challenge. Another way to isolate CTCs is microsieve filtration where single cells are deposited into microwells (18), from which living tumor cells can be isolated for immunocytochemistry or culturing, which is developed by VyCap (VyCap, Deventer). Another filtration technique from the same company was described by de Wit et al. who used a silicon microsieve on cell waste obtained after Cell Search to detect EpCAM-CTCs on which they carried out FISH (19). The ISET system and the Clear Cell system both offer, after the different methods for filtration or isolation, further characterization of CTCs by molecular analysis, FISH, immunofluorescence or culturing (6,17,20-25). These additional CTC applications will offer more detailed information.

**Alternatives to CTCs as liquid biopsy**

Currently, circulating tumor DNA (ctDNA), and tumor RNA derived from platelets are other biomarkers to evaluate tumor response and that may be useful for follow-up. Cell free DNA (cfDNA) are nucleic acids detected in body fluids. In cancer patients, at least a part is ctDNA, thought to originate from dying tumor cells. These approaches may be complimentary to CTCs, where high ctDNA and CTC numbers of untreated patients could indicate a high burden of disease.

Fernandez-Cuesta et al. studied ctDNA in SCLC patients, identifying TP53 mutations in ctDNA in the plasma of 53 SCLC patients (49%) and 123 controls (11.4%) (26). This study illustrates that a substantial number of otherwise healthy people showed TP53 mutations without having cancer. This is an important hurdle in the implementation to ctDNA as a screening test. It showed however that it is possible to identify specific mutations in ctDNA, which could be useful in daily practice.

RNA from the tumor is also found in the blood. In 2011 Nilsson et al. showed that tumor RNA was transferred into blood platelets, so called tumor-educated platelets (TEPs) (27). Using TEPs, Best et al. distinguished 228 patients with localized and metastasized tumors from six different origins from 55 healthy individuals with 96% accuracy. They correctly identified the source of the primary tumor with 71% accuracy and they distinguished MET or HER2-positive, and mutant KRAS, EGFR, or PIK3CA tumors (28).

Although both methods have hardly been studied in SCLC, mRNA and ctDNA can be detected in plasma and
can be used to detect specific mutations or translocations. CTCs may deliver more information than mere plasma ctDNA or mRNA in platelets. Different cellular surface markers can be stained such as PD-L1, a target for checkpoint inhibitors in lung cancer or delta-like 3 (DLL3), a ligand in the NOTCH signalling pathway that shows increased expression on SCLC tumor cells in biopsies and perhaps also on CTCs. The meaning of CTC expression of these markers has not yet been clarified.

In conclusion, in patients with SCLC, liquid biopsies like CTCs may play a major role to determine tumor biology in a non-invasive way. Standardization and validation of CTCs and cfDNA assays are important issues realized by the current EU/IMI consortium CANCER-ID (www.cancer-id.eu).

Acknowledgements
None.

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

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Development of predictive liquid biomarkers for response to treatment in small cell lung cancer

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Provenance: This is an invited Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Abstract: Small cell lung cancer (SCLC), despite being initially chemosensitive, behaves aggressively and tends to progress rapidly after or during first-line chemotherapy. Predictive indicators of response to specific treatment for SCLC have not yet been established. Carter et al. had reported that they established a genetic classifier to predict whether SCLCs were “chemosensitive (sensitive relapse)” or “chemorefractory (refractory relapse)”. They used whole genome amplification products of native circulating tumor cell (CTC) from patients to develop this classifier. These CTC classifiers could accurately identify patients with SCLC as “sensitive relapse” or “refractory relapse” to first-line chemotherapy. Although this study represented a remarkable step forward in biomarker research in SCLC, classifiers obtained in the same fashion at disease progression could not predict the response to further treatment. This may imply that the inherent genetic background for the initial response to first-line chemotherapy differs from that for newly acquired resistance to treatment. In order to improve our understanding of the biological backgrounds of SCLC, extensive research into concepts such as cancer stem cells, epithelial mesenchymal transition/mesenchymal epithelial transition, and circulating tumor microemboli might be necessary.

Keywords: Circulating tumor cell; liquid biopsy; small cell lung cancer; tumor biomarker

Submitted Mar 03, 2017. Accepted for publication Mar 07, 2017.
doi: 10.21037/tcr.2017.03.56
View this article at: http://dx.doi.org/10.21037/tcr.2017.03.56

Small cell lung cancer (SCLC) is different from non-small cell lung cancer (NSCLC) in that it is more malignant and aggressive; it progresses rapidly and metastasizes by the time of diagnosis. Even though SCLCs are initially highly responsive to first-line chemotherapy, most patients relapse within a few months to a year after the initial therapy (1). The response to first-line chemotherapy and the length of the interval after the last dose of the first-line chemotherapy can predict the subsequent clinical outcome of the second line chemotherapy (2-5). Based on these factors, relapsed SCLCs are classified into “sensitive relapse” and “refractory relapse”. Patients who respond to first-line chemotherapy and then relapse after 2–3 months are considered “sensitive relapse,” whereas patients whose disease progressed during the first-line chemotherapy or whose tumors recurred within 2–3 months after the first-line chemotherapy are considered “refractory relapse.”

Because of the disseminated nature of SCLC, surgical resection or serial biopsies are seldom indicated. The genomic profile and background of SCLC are not as well established as that of many other cancers due to lack of surgical or biopsy specimens. Nevertheless, SCLC was reported recently to show high levels of genetic alterations, including in RB transcriptional co-repressor 1 (RB1) and tumor protein p53 (TP53); indeed, lung cancer is the second most frequent type of cancer associated with genetic
mutations (6-9). Despite recent progress, very few genetic molecular biomarkers are available to predict the clinical outcome of SCLC (10).

Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are the most well-established blood-borne biomarkers for tumors. They are considered tumor-derived cells and DNA which are shed into the bloodstream; they are collected through a process called “liquid biopsy” and studied as reliable alternatives for conventional biopsies. They provide detailed molecular data useful for clinical management of patients with lung cancer, and analysis of these biomarkers may be useful for selecting treatment methods, for tumor monitoring, and for studying resistance mechanisms. In particular, the companion diagnostic test to detect a mutation (T790M) that provides resistance against serum epidermal growth factor receptor (EGFR) was approved by the U.S. Food and Drug Administration; it provides the clinical indication for the use of third-generation EGFR-tyrosine kinase inhibitor to treat patients with advanced NSCLC. On the other hand, many devices have been developed recently to detect and capture CTCs. They are broadly classified as label-dependent and label-independent, according to the methods used for detecting CTCs. In label-dependent assays, CTCs are separated from other blood cells using cell surface markers such as epithelial cell adhesion molecule (EpCAM) and CD45 (11). The label-independent assays rely on biophysical differences between CTCs and blood cells (11). The CellSearch® assay is a well-validated assay to detect EpCAM expressing CTCs. The presence of CTCs detected using this assay is associated with poor prognosis in patients with some cancers, and might be useful for monitoring patients with metastatic breast, colorectal, or prostate cancers (12-14).

Regarding SCLCs, the detection rate of CTCs by the CellSearch® assay has been reported to be relatively high; the majority of patients (67-86%) had 2 or more CTCs per 7.5 mL of blood (15-20). However, the prognostic impact of CTCs and their association with metastases in patients with SCLC remains unknown. Changes in the number of CTCs before and after chemotherapy might indicate the patients’ survival and treatment response (15-20).

As mentioned above, despite recent progress in research on genetic molecular alterations, sufficient data to predict the clinical outcome of SCLC is not available. As an excellent alternative for primary tumor materials, liquid biopsy can be used. It is a feasible, repeatable, and less invasive procedure, and the specimens obtained have been used for molecular analysis to understand the biology of SCLCs and the mechanisms behind the treatment response. However, there are two major limitations for the application of CTCs in genetic molecular analysis. One is the challenge behind separating and extracting single CTCs from a mixture with other contaminating blood cells without losing small amounts of CTCs. The other is the comprehensive investigation of molecular traits of individual CTCs. A recent technological advance, the DEPArray™ technology (Silicon Biosystems S.p.A.) can automatically prepare a suspension of isolated single CTCs, already sorted and enriched by the CellSearch® assay in order to perform the whole genome amplification (WGA) (21).

A recent study by Carter et al. reported a copy-number aberration (CNA)-based SCLC CTC classifier, comprising 16 different CNA profiles, to identify genetic features that could distinguish “sensitive relapse” from “refractory relapse” (22). In this study, blood samples were collected and enumeration of CTCs was performed using the CellSearch® assay, and stored CTCs that were obtained in a previous study were used. Individual SCLC CTCs from the CTC-enriched suspension were extracted using the DEPArray™ system, and the WGA products obtained from them were used for the CNA analysis to develop the SCLC CTC classifiers. They found that 83.3% of cases were correctly classified as “refractory relapse” or “sensitive relapse,” based on the patient's own CTC-based CNA classifier. In addition, the progression-free survival (PFS) of patients was calculated with the baseline CNA classifier. The PFS of patients with “sensitive relapse” was significantly longer than that of patients with “refractory relapse.” Such analyses are called retrospective-prospective analyses, and require further prospective investigation to confirm the results. However, this result suggested that their baseline CNA classifier might be capable of accurately predicting the response for first-line treatment as well as the clinical outcome of SCLCs.

Furthermore, the authors used the same CNA classifiers on the serially collected CTC samples of the corresponding patients to analyze the acquired genetic alterations. Notably, the CTC CNA classifier of patients with initially “sensitive relapse” did not become a “refractory relapse” CNA profile at disease progression, indicating that the CNA profile classified the disease as chemosensitive at the disease progression. No chromosomal changes were detected. This result suggested that the genetic background for the initial response to chemotherapy differs from that for acquired chemoresistance.
These contradictions offer some scope for speculation. As mentioned previously, most epithelium-derived CTCs in the bloodstream are thought to express EpCAM on their cell surface; however, CTCs have also been detected in cancers that do not express markers of epithelial origin (23). In addition, the expression levels of this cell surface marker vary in tumor cells during epithelial-mesenchymal transition (EMT) (23). During EMT, the epithelial tumor cells lose their epithelial features and are subsequently converted into mesenchymal cells; they then migrate into surrounding connective tissues and blood vessels. In contrast, tumor cells in metastatic lesions generally exhibit an epithelial appearance; this suggests that a reversed version of EMT may occur in these metastatic sites. Once tumor cells reach their destination organs, they lose their mesenchymal aspects and regain their epithelial features, and this process is called mesenchymal-to-epithelial transition (MET). During these processes, cell surface markers such as EpCAM are thought to be lost to some extent and their expression levels may vary and become heterogeneous (23). CTCs in the bloodstream may have different characteristics after EMT, and some CTCs may lose their epithelial markers. Carter L. et al. employed EpCAM-dependent CellSearch® assay to detect SCLC CTCs, and this might be why they did not detect epithelial marker-negative CTCs (22,24).

Similar to single CTCs, tumor cell clusters in the peripheral blood, named circulating tumor microemboli, have been reported recently (25). These tumor cell clusters had a higher metastatic potential than single tumor cells (26,27). Apoptotic and proliferating cells were seldom seen in these CTC clusters, indicating that these cells have a survival advantage against cytotoxic chemotherapy and against anoikis, which is a form of programmed cell death that occurs in cells that lose contact with the surrounding extracellular matrix (20,28). These cell clusters had also lost the epithelial cell markers, and expressed mesenchymal markers. Therefore, epithelial-marker dependent CTC detection assays, including the CellSearch® assay, might have missed these cell clusters (16,20).

Furthermore, SCLCs are known to be malignant and aggressive, and the occurrence of rapid relapses after highly effective chemotherapy suggests that SCLCs may contain cancer stem cell (CSC) components (29). As part of CTCs, CSCs may play a crucial role in tumor biology, including tumor heterogeneity, resistance to chemotherapy and radiotherapy, recurrence, and metastasis. Importantly, EMT may induce stem cell characteristics; in fact, some CTCs possess CSC characteristics, and are called, “circulating cancer stem cells” (28-32). These cells may share their origin with CSCs. Therefore, a thorough understanding of CSCs, including circulating cancer stem cells and EMT/MET, would be necessary for understanding the biological features of SCLCs.

As mentioned previously, recent rapid advances in liquid biopsy has made it possible to use ctDNA in the clinical setting to facilitate clinical decision-making. This is undoubtedly an extremely sensitive method to detect the cancer burden, even if they are small. However, ctDNA is not suitable for analyzing proteins or for functional and morphological analysis of cancer cells. CTCs allow structural evaluation of the cancer phenotype, permit in vivo and in vitro assays, make molecular characterization of the disease possible, and enable immunocytochemical labeling techniques, even though their inherent heterogeneity makes it difficult to detect their presence. They can therefore be used as complementary tools with ctDNA (11). Observing a single aspect of tumor biology might deny us full comprehension, and so, deep and extensive research on liquid biopsies, using multiple modalities, is required.

Acknowledgements
None.

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

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sensitivity to first-line chemotherapy are significant prognostic factors in patients with recurrent small cell lung cancer receiving second-line chemotherapy. Cancer 2008;113:2518-2523.


31. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of


Cite this article as: Tokudome N, Yamamoto N. Development of predictive liquid biomarkers for response to treatment in small cell lung cancer. Transl Cancer Res 2017;6(Suppl 2):S348-S352. doi: 10.21037/tcr.2017.03.56
With understanding of lung cancer biology and advances in technology, treatment for advanced non-small cell lung cancer (NSCLC) is currently guided according to the genetic abnormalities, including epidermal growth factor receptor (EGFR) mutation, translocation in ALK, ROS1 or RET, B-raf mutation, etc. In the treatment of EGFR-mutated NSCLC, EGFR tyrosine kinase inhibitors (TKIs) are recommended as first-line therapy based on high response rates and longer progression free survival (PFS) compared to platinum-doublet chemotherapy (1-3). Unfortunately, most patients eventually develop acquired resistance to EGFR TKIs after 10–12 months of PFS. Extensive studies of repeated biopsy demonstrated various different resistant mechanisms. Among them, EGFR T790M mutation confers most common resistance mechanism, accounting for 50–60% of patients with EGFR TKI resistance. Other mechanisms include activation of the alternative pathway such as c-met amplification or HER2 amplification and phenotypic change like small cell lung cancer transformation or epithelial-mesenchymal transition (EMT) (4). Recently, several novel targeted agents to overcome T790M mutation have been developed and being actively investigated. These 3rd generation EGFR TKIs such as osimertinib, rociletinib, or olmutinib are associated with robust efficacy in patients harboring T790M mutation (5,6). Furthermore, combination of EGFR TKI with c-met inhibitor also showed promising activity in patients with high c-met amplification. Therefore, to understand the underlying resistance mechanism and to guide optimal treatment, repeated biopsy is essential and considered the gold standard at the time of progression. However, the invasive nature of repeated biopsies makes it difficult to obtain samples from patients especially those with poor performance or inaccessibility due to tumor location, tumor containing blood vessel or air bronchogram, existence of tumor necrosis, or previous radiation site. Tumor heterogeneity is another limitation and a single snapshot study cannot represent the dynamic changes of genetic abnormalities due to evolving nature of tumor progression.

As minimally invasive methods, the circulating cell free DNA (cfDNA) in plasma and circulating tumor cells (CTCs) have been used as surrogates for tumor tissues in detecting genetic alterations (7). In the article that accompanies this editorial, Yanagita et al. reported prospective longitudinal monitoring of both CTCs and cfDNA in EGFR mutant non-small cell lung cancer patients treated with erlotinib on a phase II trial. Clin Cancer Res 2016. [Epub ahead of print].

Submitted Aug 26, 2016. Accepted for publication Sep 05, 2016.
doi: 10.21037/tcr.2016.10.40
View this article at: http://dx.doi.org/10.21037/tcr.2016.10.40
complementary as non-invasive methods but cfDNA may offer more clinical utility than CTCs for serial monitoring.

The majority of cfDNA is derived from apoptotic or necrotic tumor cells that release their fragmented DNA into the circulation. The isolation of cfDNA remains challenge due to high degree of fragmentation and its low concentration in the blood. However, highly sensitive and specific molecular methods such as ddPCR or BEAMing have been developed to detect genetic alterations including single gene mutation or even whole genome sequencing in cfDNA which can guide personalized treatment decisions (9). A phase IV single arm study of gefitinib as first-line therapy in Caucasian EGFR mutation positive NSCLC patients along with preplanned exploratory analysis of EGFR mutations in paired tumor and plasma samples demonstrated that cfDNA analysis can reliably detect EGFR mutation status of the tumor, suggesting that cfDNA analysis can be considered for mutation analysis if repeated biopsy is unavailable or inaccessible (10). Based on these data, cfDNA test for sensitizing EGFR mutation in NSCLC has been approved in Europe. Recently, a pooled analysis of 20 eligible studies reported 67% of sensitivity and 93% of specificity for detection of sensitizing EGFR mutation in cfDNA (11). cfDNA also offers the potential for longitudinal monitoring for the development of resistance mutations such as T790M. Several studies demonstrated EGFR T790M can be non-invasively monitored in cfDNA during the course of EGFR TKIs treatment and this resistance mutation can be detected even earlier than radiological progression (12). Also, the efficacy of osimertinib or rociletinib has been observed in plasma genotyped T790M positive patients (13,14). Recently, FDA approved Cobas EGFR mutation test using plasma specimens as a companion diagnostic test. This is the first liquid biopsy test approved for use by FDA.

As another source of circulating biomarkers, CTCs have been introduced 10 years before. Given the rarity and lack of consensus on expression markers to identify CTCs, significant challenges still remain in CTC detection even though various methods have been developed for isolation and characterization of CTCs (15). Epithelial cell adhesion molecule (EpCAM) based enrichment is most commonly used. CTC-chip is another method using microfluidic-based platform which can separate CTCs from peripheral blood samples without processing samples. Compared to cfDNA, CTCs have several advantages. First of all, enumeration, isolation and visualization are possible. Several small studies reported that serial CTC counts might be useful in predicting the response to treatment. Also isolated CTCs confer further molecular characterization such as mutation, mRNA, or whole exome sequencing in single cell level (16). However, it still remains challenge that there is no standardized method for detecting CTCs. Moreover, the number of CTCs in NSCLC patients is much lower than those of small cell lung cancer patients.

This single center prospective study analyzed cfDNA and CTCs quite extensively and the results are quite intriguing, but several issues should be considered. First of all, at baseline, 37% of patients did not show either CTCs or cfDNA and only 41% (17/41) of patients had detectable cfDNA. At the time of progression, 42% of patients had no detectable CTCs or cfDNA and only 25% (9/36) of patients had detectable cfDNA. Given that advanced stage of NSCLC patients were enrolled in this study, cfDNA detection rate is quite low compared to the results of previous studies. One explanation discussed by the author was attributed to the use of heparinized tubes which might be associated with potential interference with ddPCR. Furthermore, T790M mutation was noted in 66% (23/35) of patients in repeated biopsy. But T790M mutation was identified only in 18% (7/39) of samples suggesting lower sensitivity than previous reports. The reason for the low sensitivity has not been determined yet. About 60% of patients had no detectable CTCs at progression, suggesting potential possibility of loss of epithelial marker due to epithelial to mesenchymal transition. It does not seem to be correlated between CTCs and cfDNA detection in this study. It appears that cfDNA is more associated with prediction of PFS than CTCs. The author pointed out the limitation of non-invasive method to detect small cell lung cancer transformation either by cfDNA or CTCs, where further improvement of technology should be warranted.

It is clear that the analysis of CTCs and cfDNA has paved new way as liquid biopsy diagnostics and the growing body of evidences suggests that these two methods are complementary. In particular, the analysis of cfDNA and CTCs for detection of genetic abnormalities to guide treatment and to monitor resistance to targeted therapies in NSCLC should be incorporated in daily clinical practice. More standardization of clinical assays and clinical validation should be needed.

Acknowledgements

None.
Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

References


The “liquid biopsy” in non-small cell lung cancer—not quite ready for prime time use

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Provenance: This is a Guest Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Aug 29, 2016. Accepted for publication Sep 05, 2016.
doi: 10.21037/tcr.2016.10.29
View this article at: http://dx.doi.org/10.21037/tcr.2016.10.29

Biopsy of a tumor site has long been the gold standard for the diagnosis of malignancy. Advanced sequencing technology has enabled us to study the molecular changes driving a particular cancer. As we move towards the molecular era of medicine, repeat tumor biopsies are often obtained to assess for development of resistance, which has both prognostic and therapeutic implications. However, the logistics of obtaining repeat tumor biopsies are complicated—many patients with advanced malignancies are unable or unwilling to undergo another invasive procedure and the skillset and personnel required for these procedures are often time consuming, expensive, and leads to delay in care. Furthermore, it is known that tumors develop heterogeneity over time and disease sites and therefore one biopsy may not provide a complete picture of the tumoral landscape.

Advancements in bioinformatics and nanotechnology have brought the “liquid biopsy”—i.e., assessing the genetic material of tumor cells in the blood and urine—to the forefront. There are three major sources for the plasma liquid biopsy: (I) circulating tumor cells (CTCs); (II) cell-free nucleic acids (cfNA); and (III) extracellular vehicles (EVs)—with the bulk of clinical studies thus far focusing on CTCs and cfNA. CTCs are those cells that have been able to detach from the primary tumor, infiltrate the vasculature, and enter into circulation. They have been recognized since 1896 (1), but until recently, the inability to detect and characterize them has limited their use. cfNA (most commonly cell-free DNA, cfDNA) are either passively secreted during tumor apoptosis or actively released due to reasons that are yet unclear. Extracellular vesicles are the least well characterized, but are composed of nucleic acids enveloped within a lipid membrane and are released by both normal and tumor cells. Table 1 describes the liquid biopsies in further depth, while Table 2 compares them to the conventional tumor biopsy.

Non-small cell lung cancer (NSCLC) is a cancer for which there is great potential in the complementary use of the liquid biopsy. Advances over the past 2 decades have revealed that NSCLC, in particular adenocarcinoma, is comprised of distinct entities, driven by specific molecular changes (2). FDA-approved tyrosine kinase inhibitors (TKIs) are currently available for three driver mutations—EGFR, ALK, and ROS-1. Numerous phase I and II studies are ongoing using TKIs for other driver mutations. However, development of resistance mutations to TKIs is common, with the median duration of response commonly cited as about 12 months (3). TKIs have also been developed to target specific resistance mutations. However, assessment for these mutations requires a repeat tumor biopsy, which as described earlier, poses numerous challenges. Studies have shown that a liquid biopsy can be used to detect the presence of these mutations with varying sensitivity and specificity. Most of them, however, have been retrospective in nature.

Yanagita et al. (4) have recently published a prospective
Table 1 Comparison of various “liquid biopsy” tests in NSCLC

<table>
<thead>
<tr>
<th>Type of “liquid biopsy”</th>
<th>Source</th>
<th>Methods of isolation</th>
<th>FDA-approved assays</th>
<th>Sensitivity of available assays (%)</th>
<th>Specificity of available assays (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCs</td>
<td>Shed from primary tumors</td>
<td>EpCAM-independent or EpCAM-dependent assays</td>
<td>None yet approved</td>
<td>23–90 (detection rates)</td>
<td>N/A</td>
</tr>
<tr>
<td>cfNA</td>
<td>Passive release from apoptosis/necrosis or active secretion; from both tumor and healthy cells</td>
<td>Many extraction kits with further enrichment using ddPCR or NGS</td>
<td>cobas EGFR Mutation Test v2* (assesses for EGFR sensitizing mutations)</td>
<td>15–100</td>
<td>89–100</td>
</tr>
<tr>
<td>EVs</td>
<td>Membranous lipid structures secreted by both tumor and healthy cells</td>
<td>Differential ultra-centrifugation (gold standard); assays not needing special equipment to isolate EVs based on their physical properties are also available</td>
<td>None yet approved</td>
<td>N/A (technology still being developed)</td>
<td>N/A (technology still being developed)</td>
</tr>
<tr>
<td>Urine cfNA</td>
<td>cfNA that is either filtered by the kidney or from cells that came into direct contact with urine</td>
<td>Extraction kits with further enrichment through ddPCR or NGS as in plasma cfNA</td>
<td>None yet approved</td>
<td>81–100 (concordance rates)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*‡, Roche. NSCLC, non-small cell lung cancer; CTCs, circulating tumor cells; cfNA, cell-free nucleic acids; EVs, extracellular vehicles; ddPCR, digital droplet PCR; NGS, next generation sequencing; EpCAM, epithelial cell adhesion molecule.

Table 2 Advantages and disadvantages of the different biopsy methods, both traditional and plasma-based

<table>
<thead>
<tr>
<th>Source of biopsy</th>
<th>Advantages</th>
<th>Pitfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating tumor cells</td>
<td>Has potential for the widest application including evaluating for recurrence, monitoring response to therapy, and assisting in prognosis</td>
<td>Current methods are not always accurate in distinguishing between tumor cells and epithelial cells</td>
</tr>
<tr>
<td>cfNA</td>
<td>Directly able to assess for specific mutations</td>
<td>Released by both healthy and tumor cells</td>
</tr>
<tr>
<td>Extracellular vesicles</td>
<td>Nucleic acids are protected by degradation given encapsulation</td>
<td>Released by both healthy and tumor cells</td>
</tr>
<tr>
<td>Urine cfNA</td>
<td>Easiest liquid to obtain; same advantages as plasma cfNA</td>
<td>Filtration of urine by nephrons limits the size of NA fragments that can enter the urine; kidney disease will also affect nephron filtration.</td>
</tr>
<tr>
<td>Tissue</td>
<td>Gold standard; direct assessment of the tumor</td>
<td>Does not account for tumor heterogeneity; invasive procedure; may not always be feasible</td>
</tr>
</tbody>
</table>

cfNA, cell-free nucleic acids.

The authors studied the phase II study of first-line use of erlotinib in patients with EGFR activating mutations who consented to repeat biopsy at tumor progression along with serial blood draws to evaluate for both CTCs and cfDNA. They enrolled 60 patients, of which 44 had progressive disease. Of these 44 patients, 41 had paired CTC and cfDNA blood draws at baseline, 36 had them at progression, and 35 had repeat tumor biopsies at disease progression. The results of their study highlight both the advantages and pitfalls of using the liquid biopsy as a tool to identify mutations that confer resistance to the original TKI. The authors should be commended for their well-designed study, especially for its prospective design.

There was 100% concordance for the EGFR L858R substitution and exon 19 deletion as determined through digital droplet PCR (ddPCR) from blood cfDNA compared to tissue biopsy analysis. Unfortunately, as there were no true negative controls (in tissue or blood), it is not possible...
to calculate the specificity. However, the high concordance rate suggests that cfDNA may serve as a suitable alternative method for initial diagnosis in the right clinical setting and particularly in resource poor environments. For example, the incidence of EGFR mutant lung cancer is as high as 47% in lung adenocarcinomas seen in Asia/the Pacific islands (5). As a first in NSCLC, the FDA recently approved the cobas EGFR Mutation Test v2, a cfDNA assay assessing for the presence of the EGFR sensitizing mutations which will determine patient eligibility for EGFR inhibitors including gefitinib, erlotinib, and afatinib.

At progression on erlotinib, 35/44 patients had repeat tissue biopsies compared to 36/44 that had blood drawn for cfDNA and CTCs. Repeat tissue biopsy from the 35 patients at progression identified the presence of the T790M resistance mutation in 23 (66%) of patients. T790M mutation is the most common gatekeeper resistance mutation seen in 50% of patients treated with first generation TKIs. In contrast, only 9 of 39 blood samples were found to contain cfDNA at progression, of which 7 (18%) were found to have the T790M mutation. Due to an unequal number of tissue and plasma biopsies, it is not possible to calculate the true sensitivity, but would estimate it to be ~30%.

These findings are in contrast to a retrospective study to detect the presence of T790M mutations in cfDNA in patients treated with osimertinib, where the sensitivity was 70% (6). More recently, in a large study of 548 patients with EGFR mutant lung cancer, Wakelee et al. described a sensitivity of 81% for detecting the T790M mutation (7). Despite the lack of sensitivity in the study by Yanagita et al., all seven T790M-positive cfDNA samples corresponded with their respective tissue biopsy. Interestingly, in two patients where repeat tissue biopsy was not possible, plasma cfDNA revealed the T790M mutation. Lower sensitivity to detect resistance mutations may be due to fewer copies of circulating cfDNA, related to heterogeneous resistance patterns and differential shedding coupled with different sensitivities of sequencing technologies.

The other findings of this study are also worthy of further discussion as they highlight potential applications of the liquid biopsy as well as aspects warranting further research and refinement. The authors assessed the prognostic value of baseline CTC/cfDNA burden for progression free survival (PFS) (4). There was no significant relationship between CTC level and PFS (P=0.88), although the association was almost significant for lower baseline cfDNA with improved PFS (P=0.08). In contrast, a prospective study in patients with advanced NSCLC treated with systemic chemotherapy showed that higher baseline CTC (≥5) was significantly associated with poor PFS (P=0.034) (8) and a meta-analysis found a significant relationship between high baseline cfDNA and shorter PFS (9). It is important to note that there is currently not an accepted measure of “high” CTC count, with some studies using the cutoff of ≥3, whereas others use ≥5. Similarly the definition of high levels of cfDNA has also not been set. This is of especial concern when measuring copies of specific mutations by ddPCR, i.e., T790M as the authors did (4). Due to the lack of consistent findings and the absence of agreed upon measurements, cfDNA and CTC burden cannot be used currently as reliable prognostic markers for NSCLC.

Serial evaluation of CTCs and cfDNA failed to reveal a consistent pattern of change throughout therapy. The authors described four major patterns, one of which was no correlation between cfDNA or CTCs at baseline, on treatment, or at progression. In fact, 7 of the 36 samples did not have detectable cfDNA or CTCs at any point throughout the study and 15 of 41 patients did not have detectable CTCs or cfDNA at baseline. At time of disease progression, only 6 of 36 samples had both detectable CTCs and cfDNA. The lack of detection of cfDNA and CTC at baseline and at progression severely impacted the significance of the findings of Yanagita et al., limiting meaningful interpretation. The current study also did not address identification of various mutations using the CTCs. Studies have shown that changes in the tumor genotype during treatment response can be representatively monitored in CTCs (10,11). As for cfDNA, Sacher et al. recently published a prospective validation of cfDNA to detect EGFR mutations in advanced NSCLC treated with a EGFR TKI, also describing multiple patterns of change during serial cfDNA monitoring (12). Preliminary analysis suggests that continued presence of cfDNA may portend increased risk for progression, but the data is still immature. Refinements in blood collection and sequencing technologies will no doubt improve sensitivities of detection.

There has also been growing interesting in the use of the other liquid, i.e., urine, as it has been shown that cfDNA is also found in the urine. The aforementioned study by Wakelee et al. also tested for the T790M mutation in the urine using a quantitative next generation sequencing (NGS) assay and found a concordance rate of 81%. More importantly, the three methods (tissue biopsy, blood, and urine) provided complementary evidence for detection of
T790M. In patients where tissue sample was negative or inadequate for testing were considered, urine identified 169 patients as T790M-positive (7). Given the complementary nature of results obtained from blood and urine, it may be that future endeavors focus on more frequent testing of blood and urine to detect earlier development of resistance mutations. If the patient is found to harbor a resistance mutation through the liquid biopsy, then it may be possible to omit the tissue biopsy and initiate alternative treatment sooner.

Although our ability to obtain genomic information has expanded with the advent of NGS, our capacity to synthesize and apply this information is still quite limited. An excellent example is in the evaluation for resistance mechanisms through cfDNA as ddPCR can only be performed looking for known resistance mutations. As we are already seeing, tumor cells can also develop alternative resistance mechanisms that we have not yet characterized or activate alternative pathways for which we may not have an evaluable biomarker. The lack of sensitivity of currently available assays does not yet preclude the need for a tissue biopsy (13). Therefore, although the results of this and other similar studies have unveiled the enormous potential of the “liquid biopsy” in the diagnosis and prognosis of NSCLC, barring very specific clinical scenarios, it is not quite ready for prime time use.

Acknowledgements
None.

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

References

Introduction

Despite the advances in lung cancer therapeutics perspectives involving personalized therapies and investigation of cancer biomarkers the available epidemiological data presents a disquieting scenario of the lung cancer mortality and morbidity ratio in the near future. Alarming epidemiological reports concerning lung tumors incidence are probably associated with a still low social awareness of adverse effects of cigarette consumption on a human body and low level of interest in tobacco prevention programs. The major problem is also lack of screening tests which could improve detection of tumor developing in an early stage, what seems to be associated with difficulties in selection of cancer high risk individuals. Other emerging issues noted especially in developing countries are both too high cost of screening programs and still restricted accessibility to advanced imaging diagnostics including computed tomography (CT) or low-dose spiral computed tomography (LDSCT) (1-3).

The mentioned issues influence a late detection of most patients when disease is diagnosed in a locally advanced or advanced stage with presence of distant metastases. Unfortunately tumor detected in a late stage of the disease disqualifies patients from radical surgery and in consequence prevents from complete recovery. In the late-tumor stage patients the treatment options include characterized by restricted efficacy: chemotherapy, radiotherapy or radiochemotherapy (4). However, standard chemotherapy regimen is constantly used routinely in most advanced cases, because the number of patients who could achieve from personalized therapy is still limited (5).

Although in some developed countries the CT/LDSCT-based diagnostics was applied for early lung cancer detection, there is still lack of indications or recommendations to introduce such methods to cancer screening in a general population, though tumor development is often asymptomatic, insidious and also not in each case strongly associated with cigarette consumption. It is worth noting...
that CT/LDSCT scans may visualize small pulmonary nodules which are usually confirmed as non-cancerous lesions, what may leading to generation of false positive results. The above presumptions may be probably clarified by expanding of diagnostic perspectives with introduction of molecular markers which could provide early detection of lung cancer, reduce false results rate obtained by imaging diagnostics or even be applied as independent diagnostics tools (6,7).

Among potential lung cancer epigenetic biomarkers, hypermethylation of tumor suppressor genes promoters is meticulously investigated in the last decade. Involvement of DNA methylation phenomena in regulation of key processes of the cell cycle allows considering it as potential diagnostic markers of neoplasms including lung cancer. Moreover, the possibility of investigation of hypermethylation in circulating cell-free DNA (cfDNA) using liquid biopsy make it a promising biomarker of lung cancer (8,9).

The role of DNA methylation in cancer development

DNA methylation of gene promoter is a dominant and the most known epigenetic alteration of human genome. The sequences in genome that undergo methylation are not accidental and especially concern cytosines located within CpG islands [repetitive dinucleotide sequences (5’-CG-3’)]. It is estimated that approximately half of genome sequences consist of CpGs out of which about 70–80% are methylated. In physiology, activity of DNA methyltransferases (DNMTs) provides a stable methylation pattern in cell genome and thereby controls gene expression post-replication. Consequently the methylation of CpG islands is mostly observed in genes encoding tissue-specific proteins, except from the cells which a gene product is typical to. The genetic information concerning gene expression after cell division (DNA methylation pattern) is inherited by progeny cells. It seems to be a main warranty of gene expression pattern in different tissues—DNA methylation pattern is typical for the defined types of cells. However, the 5’ regions of tumor suppressor genes promoters which are rich in CpGs sequences do not undergo methylation due to their key function in healthy cells cycle. Unmethylation of gene promoter regions is a leading requirement for an active and controlled gene transcription (10-12).

The methylation of promoter region causes gene silencing, what leads to changes in structure of chromatin and its conversion into a condensed and inactive form (heterochromatin). It causes blockade of the promoter transcription start site (TSS), so its recognition by transcription factors and transcription of genetic information from DNA to mRNA cannot be processed. Alterations of a methylation pattern which are finding in tumor cells may result in genetic repression of information encoded in the DNA. However, the methylation pattern of CpGs of tumor cells is variable compared to healthy cells throughout the hypomethylation or hypermethylation which in result determine new DNA methylation pattern. The hypermethylation of tumor suppressor genes promoters seems to be a significant epigenetic alteration noted in tumor cells. As regards to methylation of gene CpGs promoter regions in physiology, the normal cells do not undergo such epigenetic modification because of their crucial function as control mechanisms of cell cycle. Furthermore, protective mechanisms against promoter hypermethylation involving regulation of replication, chromatin modification, demethylation of DNA are demonstrated by non-tumor cells. Mentioned mechanisms efficiently prevent the access of DNMTs to DNA. On the other hand, protective mechanisms against methylation are disabled in tumor cells, what causes circumvent of protective systems by DNMTs. Moreover, the overexpression of DNMTs which are responsible for de novo methylation of promoter CpGs is commonly noted in tumor cells (10-13). Due to a large interest in DNA methylation concerning its involvement in cell cycle and observed disorders in methylation processes in tumor cells, this pre-transcriptional gene modification is currently carefully considered as a potential marker for early lung cancer diagnosis.

Development of DNA hypermethylation as a lung cancer biomarker

Currently imaging diagnostics and patients’ clinical factors are often insufficient for early diagnosis of lung cancer. Unfortunately most of cancer cases are diagnosed in a late stage of the disease and require invasive diagnostics tools (e.g., bronchoscopy with EBUS-TBNA or transthoracic biopsy) to obtain tumor sample (cells or tissue) for assessment of lung cancer histology and conduction of molecular examination. Similarly, invasive diagnostic procedures refer to assessment of histology of undefined small pulmonary nodules detected in a LDSCT/CT screening. Moreover, mentioned invasive diagnostics methods may expose lung cancer patients on periprocedural
complications and even revision of minor-surgery in view of the risk of obtaining a non-diagnostic material (lack of tumor cells in the examined sample or degradation of clinical material). It is worth nothing that collected clinical samples are subsequently fixed before examination and then embedded in paraffin blocks or cell-blocks, what may lead to degradation of tumor cells and consequently falsify the results of molecular testing (resulting in manifestation of false positive or false negative results). Based on the above facts clinical material scheduled to molecular testing is preferred to collection with non-invasive manner and should be easy to obtain (14,15).

Although sputum is the easiest diagnostic material to obtain especially in patients with tumor located centrally in mediastinum, its sensitivity in lung cancer diagnosis ranges 22–98% and depends on tumor size, patient’s ability to expectorate sputum and an experience of a pathomorphologist examining specimens (16). Moreover, the sputum is frequently scant in tumor cells what prevents from reliable molecular testing and high specific examination of genes methylation status. In consequence molecular examination of sputum ceased to be a diagnostic standard in early lung cancer detection. Recent studies analyzed hypermethylation status of selected tumor suppressor genes are consistent with above findings. The following genes examined in sputum samples demonstrated the sensitivity and specificity: APC (23.1%/96%), CDH13 (27.6%/75%), CDKN2A (p16) (39.8%/72.8%), DAPK (47.2%/82.2%), MGMT (35.8%/85.6%), RASSF1A (12.2%/93.5%), TCF21 (53.8%/100%), respectively (17-19). However, some studies presented acceptable diagnostic accuracy for assessment of risk of lung cancer development, when methylation was examined simultaneously in loci of a few genes. Belinsky et al. achieved sensitivity and specificity of 64% for lung cancer prediction analyzing methylation status of six following genes p16, MGMT, DAPK, RASSF1A, PAX5β and GATA5. Hypermethylation of mentions genes was associated with a >50% increased lung cancer risk (18). In another study, designed methylation panel analyzed methylation profile of four genes: 3-OST-2, RASSF1A, p16 and APC in sputum samples allowed to distinguish lung cancer patients from healthy individuals with sensitivity of 62% and specificity of 100% (20).

Currently the most promising seems to be investigation of tumor suppressor genes methylation status using liquid biopsy technique (examination of blood sample) which could provide non-invasive diagnosis of cancer. In contrast to physiology, tumor cells demonstrate an increased cellular metabolism, likewise cells uncontrolled proliferation and partial destruction by immunological mechanisms lead to their disintegration in necrosis and apoptosis mechanism. Therefore significantly higher cfDNA concentration is observed in circulation of cancer patients compared with healthy individuals. In cancer patients the cfDNA concentration positively correlates with tumor stage, size, aggressiveness and presence of distant metastases. Moreover, molecular status of cfDNA with estimated presence of epigenetic alterations reflects molecular status in tumor tissue. High vascularization of the lung tissue and tumor potency to angiogenesis stimulates formation of blood vessels network, which promotes a release of high yield tumor cfDNA into the circulation. The investigation of tumor suppressor genes hypermethylation using liquid biopsy technique is enabled by a high stability of cytosines within cfDNA sequence, and lack of cfDNA hypermethylation in the blood of healthy people (21-23). The recent large studies evaluating methylation status of selected genes as early and non-invasive lung cancer markers are presented in Table 1.

### Hypermethylation of tumor suppressor genes promoter in lung cancer screening

In recent years a few randomized clinical trials were conducted to evaluate utility of imaging diagnostics (RTG) or imaging diagnostics supported by sputum examination in high risk individuals of lung cancer development. However, that screening improved the detection rate of lung tumors in the stage I of the disease and increased the 5-year survival rate, the decrease in mortality rate of screened individuals was not achieved (16,30). Recent studies proved that more accurate diagnosis of lung cancer can ensure screening by LDSCT. In a randomized trial conducted by the National Lung Cancer Screening Trial (NLST), the mortality reduction of 20% was achieved in a group of patients screened by LDSCT compared to standard chest RTG. According to the NLST, the chances of developing lung cancer with a positive CT result are below 5%, because lung cancer with CT screening in the NLST study achieved 71% sensitivity and 63% specificity with a 96.4% false positive rate (31,32). In up to 50% of individuals screened by the LDSCT small pulmonary lesions (which diameter do not exceed of 10 mm) with a benign etiology are detected. Unfortunately about 20% of these nodules which are scheduled to thoracotomy do not confirm their malignancy, thus LDSCT may lead to
false positive results and unnecessary surgery (31-33). In such cases the molecular examination analyzing epigenetic markers could support LDSCT and reduce rate of false results.

To date there is a lack of established recommendations concerning the application of gene hypermethylation in lung cancer screening, but the available literature data seem to confirm their high applicability in a daily clinical practice. Current studies draw the particular attention to the application of epigenetic biomarkers as complementary tests for early imaging diagnostics of lung cancer. The main purpose of the simultaneous application of both methods constitutes an improvement of the sensitivity and specificity of screening and the reduction of false

<table>
<thead>
<tr>
<th>Study</th>
<th>Studied gene</th>
<th>Analysed material</th>
<th>Study group (disease stage)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Begum et al.</td>
<td>APC</td>
<td>Serum</td>
<td>76: (58 stage I-II); 30: healthy control</td>
<td>15.8</td>
<td>90</td>
<td>(24)</td>
</tr>
<tr>
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<tr>
<td></td>
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<td>70</td>
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<tr>
<td></td>
<td>MGMT</td>
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<td>17.1</td>
<td>96.1</td>
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<td></td>
<td>RASSF1A</td>
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<td>7.9</td>
<td>96.7</td>
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<td>84.2</td>
<td>56.7</td>
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<tr>
<td>Hsu et al.</td>
<td>ZMYND10</td>
<td>Plasma</td>
<td>63: (41 stage I-II); 36: healthy control</td>
<td>36</td>
<td>87</td>
<td>(25)</td>
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<td>48</td>
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<td>Methylation of any 2 above loci</td>
<td></td>
<td></td>
<td>73</td>
<td>82</td>
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<tr>
<td>Hulbert et al.</td>
<td>SOX17</td>
<td>Plasma</td>
<td>125 stage I-II, 50: healthy control</td>
<td>73</td>
<td>84</td>
<td>(26)</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>CDO1, TAC1, SOX17</td>
<td></td>
<td></td>
<td>93</td>
<td>62</td>
<td></td>
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<tr>
<td>Wielscher et al.</td>
<td>HOXD10, PAX9, PTPRN2, STAG3</td>
<td>Serum/Plasma</td>
<td>23: (8 stage I-II); 23: healthy control</td>
<td>97</td>
<td>73</td>
<td>(27)</td>
</tr>
<tr>
<td>Weiss et al.</td>
<td>SHOX2, PTGER4</td>
<td>Plasma</td>
<td>50: (23 stage I-II); 72: healthy control</td>
<td>90</td>
<td>73</td>
<td>(28)</td>
</tr>
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<td>Powrózek et al.</td>
<td>PCDHB6, RTEL1</td>
<td>Plasma</td>
<td>55: (20 stage I-II); 80: healthy control</td>
<td>67.3</td>
<td>90</td>
<td>(29)</td>
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**Table 1** Recent studies analyzing utility of cfDNA hypermethylation for early lung cancer detection

cfDNA, cell-free DNA.
positive/negative results rate. Then, a positive result obtained in high risk individuals based on the analysis of blood cfDNA methylation may be a first indication to schedule such individuals to the imaging diagnostics. The leading advantage of epigenetic screening over the other examinations is a possibility of their non-invasive detection using a liquid biopsy technique (26-28).

Majority of available papers focused especially on the designation of hypermethylated gene signature which could distinguish cancer patients from healthy individuals or patients with benign lung diseases. Nowadays, only two large studies detailed analyze utility of DNA hypermethylation in lung cancer screening. The NELSON LDSCT trial screened methylation status of sputum DNA of asymptomatic high-risk individuals to detect lung cancer at preclinical stage in a screening interval of 2 years. The selected diagnostic panel of three genes including: RASSF1A, 3OST2 and PRDM14 detected 28% of lung cancer cases within 2 years with specificity of 90%. Sputum cytology examination in contrast to epigenetic screening did not detect any lung cancer cases. As a previously mentioned DNA hypermethylation analysis in sputum may play a potential role in the detection of preclinical disease, but complementary diagnostic markers are needed to improve the low sensitivity (34). The latest study of Hulbert et al. evaluated the utility of plasma and sputum DNA hypermethylation panel as an adjunct test to lung cancer CT screening. Interestingly, the sputum diagnostic panel of three following genes: TAC1, HOXA7 and SOX17 demonstrated a high diagnostic accuracy for early lung cancer detection (stage I–II of the disease) with sensitivity of 98% and specificity of 71% (AUC =0.890) with high negative predictive value (NPV) and positive predictive value (PPV) of 89% and 93%, respectively. Additionally, the plasma cfDNA methylation panel including genes: TAC1, CDO1 and SOX17 presented following diagnostic accuracy for early lung cancer: 93% sensitivity, 62% specificity (AUC =0.770), NPV and PPV 78% and 86%, respectively. Moreover, independent blinded random prediction model combining gene methylation of above genes with clinical factors correctly predicted lung cancer in 91% of individuals using sputum detection and 85% of individuals using plasma detection. Cited study confirmed that, designed methylation panels could be used as a complementary to CT screening, identifying individuals at high risk for lung cancer, reducing false positive results, unnecessary toracotomies and improving the diagnosis of tumor at an earlier stage (26).

The above mentioned data seem to confirm the importance of epigenetic tests in early lung cancer detection and their applicability in screening programs. Careful designation of diagnostic tests may significantly contribute to an improvement of lung cancer detection statistics and lead to a reduction of false results obtained by LDSCT.

Current status and future perspectives

To date hypermethylation of various tumor suppressor genes was investigated as potential lung cancer biomarkers. High methylation frequency of e.g., CDKN2A (p16), MGMT, DCLK1, CDH13, RASSF1A, KARB2 and many others was noted (35-40). Despite the potential utility of above genes in lung tumors detection, these are also widely hypermethylated in other cancers. Therefore the leading challenge in application of methylated genes into routine diagnostics is a determination of tumor specific genes, which undergo hypermethylation only in selected tissue-specific tumors. In consequence, it is difficult to assign hypermethylation of particular genes to defined disease, what is a main limitation of use these in daily clinical practice. An ideal example for that issue seems to be hypermethylation of SEPT9 which is an established epigenetic marker of colorectal cancer and used in its diagnostics. However, recently hypermethylation of this gene was found in lung cancer patients, what undermines its colorectal cancer specificity and put a red flag for diagnostic tests assessing methylation status of SEPT9 (41). The next issue is a total number of CpG islands which could be methylated and their location within promoter sequences. Firstly methylation pattern may depend on cell type which is from cancer development. Secondly methylation process may be restricted to selected CpGs in different cancers. Moreover, the methylation signature may differ between tumor tissue and blood cfDNA (42). The tool which could advance application of cfDNA methylation analysis in daily practice is a wide genome methylation sequencing of DNA and its comparison between tissue and blood samples of cancer patients and healthy individuals. Perhaps, such procedure could provide novel findings which will lead to designation of diagnostic epigenetic based tests.

The next challenge is a validation of previously selected biomarkers into clinics. As noted above process of biomarkers selection for lung cancer detection will required long-drawn and laborious validation process conducted in a large group of lung cancer patients and healthy individuals and even patients suffered from other cancers. Moreover, biomarkers validation should
be conducted by a large independent diagnostic center with application of complicated diagnostic methods such as DNA microarrays and next-generation sequencing. Such considerations seem to be confirmed by a fact that generally only two epigenetic diagnostics tests were registered for early diagnosis or confirmation of tumor presence based on molecular alterations analysis. Mentioned presence of SEPT9 methylation in cfDNA was established as a diagnostic marker of colorectal cancer, whereas the analysis of short stature homeobox 2 gene (SHOX2) methylation in bronchoalveolar lavage samples may be a confirmatory test of ambiguous result of cytology examination for lung cancer detection. Although both tests were certified to in vitro diagnostics (CE-IVD certificate), their diagnostic accuracy is limited. Unfortunately positive results of both tests require invasive diagnostic methods such as colonoscopy or bronchoscopy to confirm cancer occurrence. Nevertheless, the epigenetic based diagnostic tests have not yet been applied in routine diagnostics also due to the availability of very few results of prospective clinical trials, which could confirm the utility of such markers in a daily practice. Moreover, lack of elaborated recommendations or guidelines to carry out diagnostics with their usage limits the estimation of their presence in cancer patients. Consequently this also raises doubts regarding the technique which should be preferred for material collection and finally what material is the most valuable for methylation screening? Despite biomarker validation, each step of a diagnostic procedure (from sample collection to molecular analysis) also needs to be validated. The potential algorithm of markers selection and validation into diagnostics is presented in Figure 1.

It seems that in nearly future the subsequent genes will be examined as potential lung cancer biomarker. Based on currently achieved findings the most promising is selection of tumor-specific hypermethylated genes and combination these into diagnostic panel. Such proceeding could significantly improve accuracy of designed molecular tests. Strongly recommended also seems to be combining of methylation analysis with analysis of other epigenetic alterations such as microRNA expression which potential utility in lung cancer screening was proven in COSMOS and MILD trials. All recent findings indicate that analysis of methylation status of tumor suppressor genes promoters will not be a single diagnostic tool that allows early diagnosis of lung cancer but rather will be applied as adjunct test for imaging diagnostics. Such application of epigenetic tests significantly reduce false results in LDCST examination and prevent patients form unnecessary surgery or invasive biopsy what is always disturbing and stressful. Finally, the sputum examination should not be underestimated in cancer detection and may be used as complementary examination for serum/plasma analysis when the results are inconclusive. Thanks to possibility to examination of methylation status with non-invasive manner (liquid biopsy, sputum collection), analysis of such epigenetic alteration will be one of a leading priority in lung cancer early detection.

**Acknowledgements**

None.
Footnote

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

References


The purpose of cancer screening is to identify those patients for whom survival might be improved by early intervention. The strategy of waiting for symptoms results in a greater proportion of patients with advanced disease, fewer treated with curative intent and even fewer surviving long term. In some patients, such as the very elderly or infirmed, lung cancer may not shorten survival, and screening in them is less likely beneficial.

The National Lung Screening Trial (NLST) performed three annual computed tomogram (CT) screens and had established trial guidelines for managing discovered lesions (1). By design, all the participating institutions had high quality systems to manage discovered abnormalities. The low risk results are unlikely to be matched in the real world; the psychological toll, cost, morbidity, and mortality are more likely to be a burden on society. So, developing an additional test, such as from the blood or sputum, would be of benefit. Many have attempted to improve the diagnostic capabilities of imaging using blood, plasma, serum, sputum, buccal smears, and breath analysis. The best results have had marginal improvement and were impractical to perform.

Combining additional clinical information may improve the sensitivity and specificity of a testing system. For example, important parameters may include age, smoking pack-years, COPD status, forced vital capacity, lesion diameter, density, location in the upper lobe versus other lobes, border characteristics, presence of corona radiata, pleural retraction, presence and character of calcifications, possible contrast enhancement, presence of emphysema, emphysematous changes, to name a few. It remains unclear how this data provides additional help in ruling in our ruling out cancer.

The next concern is typical of screening studies: who are the patients at risk? Yes, smokers with greater than 15 pack-years and older patients are at risk for lung cancer, but there are other patients that may have significant risk factors other than smoking. Those not included in the NLST and other similar trials include patients with inflammatory lung disease, generalized inflammatory diseases such as rheumatoid arthritis, genetic predisposition, and exposures to toxins, such as asbestos. By choosing a test group, the authors may not be studying the patients most in need of an additional screening test.

DNA methylation abnormalities are found in most human tumors including lung cancer. DNA methyltransferase enzymes methylate between 70% and 80% of the CpG dinucleotide sequences in mammalian DNA at position 5 of cytosine. Cytosine methylation is an epigenetic modification that is generally incompatible with gene expression when present at gene promoters. However, there are genomic landmarks that are particularly rich in CpG sequences, yet they are kept in an unmethylated state during organismal development, in most somatic
These methylation biomarkers could be useful for early detection, classification of cancer subtypes, clinical outcome predictions, or even disease management and treatment choices. Just to give one example for tumor classification, the presence of the “CpG island methylator phenotype” (CIMP), which is based on the presence of an unusually large number of CpG island methylation events in a subset of patients (21), is a common occurrence in subgroups of colorectal cancers and brain cancers. In some instances, CIMP has been associated with specific genetic changes in the same tumor specimens. For CIMP in colorectal tumors, the presence of a BRAF mutation has been noted (22), although the exact mechanism how these two events within the genetic and epigenetic landscapes of tumors are linked has remained unclear. IDH1 mutations in lower grade gliomas are also associated with CIMP (23). In this case, the mutant IDH1 protein (commonly IDH1 R132H) produces high levels of the metabolite 2-hydroxylutarate which is a competitive inhibitor of 2-ketoglutarate-dependent dioxygenase enzymes. One popular theory is that 2-hydroxyglutarate inhibits 5-methylcytosine oxidases, the TET enzymes, thus leading to an imbalance of DNA methylation patterns in IDH1 mutant brain tumors.

However, even in the absence of CIMP, most tumors carry several hundred to a few thousand CpG island hypermethylation events within individual tumor specimens. Thus it is generally not too difficult to screen for, identify and then characterize CpG islands that can serve as potential DNA methylation markers. Such a marker needs to fulfill a number of criteria: (I) it should be very commonly methylated in a series of patients with the same malignancy (ideally in all patients); (II) the methylation level, i.e., the frequency of methylated alleles at the target locus, should be as high as possible in the tumor; (III) background methylation levels in normal tissues, both in the target organ and in other healthy tissues that might be present in the analytical specimens should be close to zero. For example, if buccal cells were to be used for methylation analysis, methylation at the biomarker CpG island should be close to zero in normal buccal epithelial cells but also in lymphocytes and other immune cells that may be present in the buccal mucosa.

Having identified a methylation biomarker that fulfills these criteria, the next challenge is to develop a highly sensitive assay for its detection. One advantage of a DNA hypermethylation marker is that it provides a positive signal that might stand out from a large background of having no signal. Ideally the diagnostic test would be a noninvasive one that does not require tissue biopsy or other unpleasant or
risky procedure such as bronchoscopy. Although the latter procedures are likely to sample a substantial fraction of tumor cells for lung cancer diagnosis, they may be difficult to implement in the clinical practice for screening a larger population. Therefore, testing for methylation biomarkers in serum, plasma or sputum have been considered as viable alternatives for lung cancer screening. The main challenge with this approach is the low amounts of tumor-derived DNA found in body fluids such as serum (24). The level of serum DNA is often increased in tumor patients relative to normal healthy individuals. However, the amounts and the integrity of DNA fragments seem to vary considerably between patients. This could depend on a number of parameters, most notably on whether the tumor is present in an early or late stage of malignant progression. Tumor-derived DNA may also be present in sputum and in this case it is likely that tumors near the central airway system would shed more tumor cells into sputum than more peripherally located tumors. With sputum, it may also be difficult to separate tumor cells from normal epithelial cells. Furthermore, sputum samples are much more readily obtained from current smokers than from nonsmokers or from those who have quit smoking a long time ago.

The first studies on lung cancer diagnosis using DNA methylation markers were reported almost two decades ago (25,26). The number of patients was small but in one study it was possible to detect methylated DNA sequences in sputum 3 years prior to clinical diagnosis of a lung tumor (27). Lack of sufficient specificity and problems with the sensitivity of the assays has made it difficult to advance these studies into the clinical practice.

A new report by Hulbert et al, published in Clinical Cancer Research now describes important progress in diagnosing lung cancer using DNA methylation markers (28). The authors designed a case-control study of individuals with suspicious nodules detected by CT imaging. Plasma and sputum were analyzed before surgery. The study included 150 cases of non-small cell lung cancer confirmed by pathology. They were all node negative (stage I and II). The 60 controls had no cancer diagnosis upon pathological examination.

Hulbert et al. used a technique with increased sensitivity “methylation on beads” that was designed to minimize sample loss. A method with extremely high sensitivity is paramount for implementing methylation diagnostics. One of the most sensitive methods is methylation-specific PCR, which uses PCR primers that distinguish between methylated and unmethylated alleles after bisulfite conversion (29). This conversion deaminates cytosine to uracil, which later amplifies as T, but bisulfite cannot deaminate 5-methylcytosine, which amplifies as C. Used in the format of quantitative real-time PCR, this method is very sensitive and specific. To ensure a maximum level of specific amplification of the locus in tumor DNA but not in normal cells or tissue, the methylation state at the queried CpG sites should show the greatest differences possible (see Figure 1). The opposing methylation state in tumor versus normal tissue DNA could be confirmed by high throughput bisulfite sequencing of the targeted region.

The authors started from publically available information to identify six DNA methylation markers that are methylated in a large fraction of patient cohorts, for example as published by The Cancer Genome Atlas (TCGA). These six genes included SOX17, TAC1, HOXA7, HOXA9, CDO1, and ZFP42. The gene set included the homeobox genes HOXA7 and HOXA9, which are very frequently methylated in non-small cell lung cancers as previously reported (7). There is a large set of useful DNA methylation markers to choose from inasmuch as other groups have used different sets of very specific markers to detect lung cancer in sputum (30). Using criteria for highest sensitivity and specificity in patients versus controls,
Hulbert et al. narrowed the gene list down to three, TAC1, HOXA7 and SOX17. With sputum testing, the positive and negative predictive values for these genes were 93% and 58% for TAC1, 97% and 40% for HOXA7 and 96% and 60% for SOX17, respectively (28). For plasma, the gene CDO1 provided better data than TAC1 and was therefore used. The positive and negative predictive values were 86% and 46% for CDO1, 90% and 57% for TAC1, and 92% and 55% for SOX17. Based on these two gene sets, it appears that overall comparable predictions were possible with sputum and plasma as testing material. When the authors included only smokers in their analysis, similar results were obtained. Employment of blinded random forest prediction models also showed that methylation values were more important variables than demographic and clinical variables alone. Using sputum analysis, for example, the authors found that the random forest model with methylation markers correctly predicted lung cancer in 91% of the cases (28). Unfortunately, the authors did not conduct a direct comparison between sputum and plasma methylation data in their patient cohort. It is therefore not clear at the moment if the diagnostic efficacy could be further improved by analyzing plasma and sputum in parallel for the same patients.

In the described trial, the study population are those that had a suspicious lesion on screen CT and who underwent surgical resection, certainly a very select group and not necessarily generalizable to the population at risk. The control patients were from the same analysis, but not found to have cancer in the surgical specimen. Some of the control patients may have had lung cancer not yet discovered and the cancer patients may not necessarily represent a spectrum of biological disease that we currently see; lepidic-predominant disease, then called bronchioloalveolar, to invasive micropapillary with an aggressive histology. Lacking long-term follow-up information also makes it challenging to interpret the data. Finally, when combining an additional test with the CT, a clinician seeks a high negative predictive value, not necessarily a high sensitivity. Providing that information to the clinician will reduce unnecessary testing and reduce the burden of lung cancer screening.

Although Hulbert et al. did not notice a difference in methylation detection according to the size of the tumors and tumors of less than 2 cm diameter were readily scored, it is still likely that the location of the tumor within the bronchial tree may affect the analysis. Also, some patients may not be able to produce sputum, which necessitates analysis of plasma only. In summary, the new study reports promising data on methylation biomarkers that may aid in diagnosing patients with suspicious lesions found on CT scans. This type of analysis should now be extended to larger prospective studies in multi-center clinical trials.

Acknowledgements

None.

Footnote

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

References

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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.

Acknowledgements

None.

Footnote

Conflicts of Interest: C Gridelli has received honoraria as advisory board and speaker bureau member for Roche, Boehringer and Astra Zeneca.
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Cite this article as: Gridelli C, Losanno T. Afatinib and gefitinib: a direct comparison. Transl Cancer Res 2016;5(S3):S425-S427. doi: 10.21037/tcr.2016.08.37
Adding to the targeted therapy toolbox: BRAF and MEK inhibition in the treatment of BRAF V600E metastatic non-small cell lung cancer

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Provenance: This is an invited Perspective commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Abstract: Mutations in the BRAF oncogene are found in approximately 2–4% of non-small cell lung cancer (NSCLC). The most common mutation is associated with substitution of glutamic acid for valine at position 600 (V600E) within the BRAF kinase. Targeted therapy against the BRAF V600E mutant kinase has shown efficacy in other solid tumors including melanoma. In this setting, dual inhibition of both BRAF and the downstream mitogen-activated protein kinase kinase (MEK) improves survival compared to BRAF inhibition alone. Planchard et al. published two recent phase 2 trials evaluating the clinical activity and safety profile of second-line BRAF monotherapy (dabrafenib) and BRAF-MEK combination therapy (dabrafenib plus trametinib), respectively, in patients with stage IV BRAF V600E mutant NSCLC. Here, we review the pertinent findings from each of these studies, discuss their significance in context of the current literature, and consider their potential impact on the management of patients with NSCLC in the clinical setting.

Keywords: Non-small cell lung cancer (NSCLC); BRAF V600E; dabrafenib; trametinib

Submitted Sep 30, 2016. Accepted for publication Oct 18, 2016.
doi: 10.21037/tcr.2016.11.50

View this article at: http://dx.doi.org/10.21037/tcr.2016.11.50

Knowledge of the role of oncogenic driver mutations in tumor initiation and maintenance has transformed the treatment of non-small cell lung cancer (NSCLC). Given the availability of targeted therapies that are approved for first-line use, guidelines now recommend that all patients with non-squamous lung cancer undergo routine testing for mutations in the epidermal growth factor receptor (EGFR) gene and rearrangements in the anaplastic lymphoma kinase (ALK) gene (1–4). The success of targeted therapies for EGFR- and ALK-mutated NSCLC as well as the historically poor outcomes of patients with advanced disease has led to increased interest in identifying additional driver mutations in lung cancer that may similarly be targets for novel therapies. One such potential target is the BRAF oncogene, which encodes a serine-threonine protein kinase within the mitogen-activated protein kinase (MAPK) signaling pathway that regulates cell growth (5). Mutations in BRAF occur in 2–4% of NSCLC with predominance in adenocarcinoma (6–9). The clinical characteristics of patients with BRAF mutant NSCLC tend to be similar to those of patients with BRAF wildtype NSCLC. BRAF mutations occur in both males and females but favor older patients (age >60) and current or former smokers (8,10). At least half of BRAF mutations in NSCLC are characterized by the substitution of glutamic acid for valine at position 600 (V600E) within the BRAF protein, leading to constitutive activation of
the kinase and subsequent tumorigenesis (7,9). Although the remaining non-V600E BRAF mutations are similarly thought to drive tumorigenesis in NSCLC, the efficacy of targeted therapies against these mutations is questionable, and clinical trials in other solid tumors have focused on patients with BRAF V600E mutations in particular (11-13).

Inhibitors of the V600E mutant BRAF kinase, including dabrafenib and vemurafenib, were initially approved for melanoma, which harbors BRAF mutations in >40% of cases (14,15). Based on the efficacy of BRAF inhibitors in this clinical setting and the success of other targeted therapies in NSCLC, there has been interest in pivoting towards the use of BRAF inhibitors for BRAF V600E mutant lung cancer. In Lancet Oncology, Planchard et al. recently published the two largest phase 2 studies to date evaluating the clinical activity and safety profile of BRAF monotherapy and combination BRAK-MEK inhibition, respectively, in previously treated NSCLC (16,17). A third cohort of patients receiving BRAF-MEK combination therapy in the first-line setting has yet to be reported. In the first of the two published studies, 78 patients with stage IV NSCLC who had progressed after one or more systemic therapies were enrolled from August 2011 to February 2014. Notable inclusion criteria included the presence of a BRAF V600E mutation as identified locally by Clinical Laboratory Improvement Amendments (CLIA) approved methods and an ECOG performance status 0-2. Patients with brain metastases that were <1 cm in size, untreated, and asymptomatic were allowed to enroll. All patients received dabrafenib 150 mg twice daily as monotherapy unless adverse events merited a dose reduction. By investigator assessment, the primary endpoint of overall response was achieved in 26 of 78 patients (33%; 95% CI: 23–45%, all partial responses). The majority of these responses (73%) were detectable by the time of the first patient assessment at 6 weeks from baseline. Disease control, defined as the number of patients achieving a response or stable disease for \( \geq 12 \) weeks after the initiation of therapy, was reported in 45 patients (58%; 95% CI: 46–67%). Median progression-free survival (PFS) was 5.5 months, and median overall survival (OS) was 12.7 months.

In the second study, 59 patients with stage IV NSCLC who had progressed after one or more platinum-based systemic chemotherapy regimens were enrolled from December 2013 to January 2015. Inclusion and exclusion criteria were similar to the cohort described above. All patients were treated with dabrafenib 150 mg twice daily plus trametinib 2 mg daily unless dose reduction was warranted due to adverse events. Trametinib inhibits the mitogen-activated protein kinase kinase (MEK), a downstream effector of RAF within the MAPK pathway. An investigator-assessed overall response was documented in 36 of 57 eligible patients (63.2%; 95% CI: 49.3–75.6%), including two complete responses. Disease control was documented in 45 patients (78.9%; 95% CI: 66.1–88.6%), and PFS was 9.7 months. Although median duration of response was 9.0 months at the time of data cutoff, 18 of 36 responses were still ongoing, and the majority of these patients (approximately 16 of 18) had already been on therapy for at least 6 months. Survival data for this cohort is incomplete.

Prior to these results, studies of BRAF inhibition in NSCLC had been limited (Table 1). Early support for

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*a, within this cohort, 34 patients were included in the survival analysis, of which 29 had NSCLC harboring V600E mutations; b, although outcomes were assessed in patients with any BRAF mutation including non-V600E mutations, only one patient with a non-V600E mutation achieved a partial response to targeted therapy; c, the single enrolled patient with NSCLC achieved a partial response to therapy; d, within this cohort, 19 patients were included in the survival analysis, of which 18 had NSCLC harboring V600E mutations; e, within this cohort, 57 patients were included in the survival analysis. ORR, objective or overall response rate; DCR, disease control rate; PFS, progression free survival; OS, overall survival.
BRAF inhibition in NSCLC came from case reports of patients treated off-label with dabrafenib or vemurafenib (18-21). Subsequently, a retrospective analysis of the European BRAF cohort (EURAF) by Gautschi et al. reported outcomes in patients with BRAF-mutant NSCLC who had received BRAF monotherapy as first- or second-line treatment (13). Among 34 patients, 29 with V600E mutations, overall response rate (ORR) was 53% and disease control rate (DCR) was 85%. Although these results were striking, validation by prospective studies has been necessary. In a phase 1 study of dabrafenib monotherapy in various solid tumors, Falchook et al. accrued a single patient with NSCLC who achieved a partial response to dabrafenib with an 83% reduction in tumor size (22). In a larger phase 2 “basket trial” of vemurafenib in non-melanoma tumors, the ORR in a cohort of 20 patients with BRAF-mutant NSCLC (18 with V600E mutations) was 42%, and median PFS was 7.3 months (23). Data supporting MEK inhibition in NSCLC is even more limited by comparison. In a trial of patients with NSCLC, small cell lung cancer, and thymic malignancies treated with selumetinib monotherapy, the ORR was 11% in nine patients with NSCLC (24). However, the study included patients with mutations in any one of multiple RAS/RAF proteins including KRAS, HRAS, NRAS, or BRAF.

These few prospective trials have been limited by small patient numbers, which reflects the low incidence of BRAF mutations in NSCLC. Additionally, many of these studies were conducted as “basket trials” that included patients with multiple tumor types, which limits the inferences that can be drawn about the efficacy of therapy in lung cancer in particular. The studies conducted by Planchard et al. likely benefitted from multiple centers of enrollment as well as a more widespread understanding of the role of multiplex genotyping in improving patient outcomes in lung cancer (25). As a result, Planchard et al. were able to enroll relatively larger numbers of patients with BRAF-mutant NSCLC in each of their two cohorts reported thus far. With respect to the clinical characteristics of the patients enrolled, there was also fairly good correspondence with previous descriptions of individuals with BRAF V600E mutant NSCLC in the literature. In prior studies, median age at diagnosis has ranged from 63–67 years with BRAF mutations occurring predominantly in adenocarcinoma, which matches the cohorts enrolled in each of the studies from Planchard et al. (8-10). The percentage of never-smokers in each of the two cohorts (28% and 37%, respectively) was also similar to what has been reported previously (8,9,26).

With a sizeable cohort and fairly representative sample of patients enrolled, the results from Planchard et al. should set the current standard upon which the efficacy of BRAF monotherapy and BRAF-MEK combination therapy is judged. However, in considering whether dabrafenib or dabrafenib plus trametinib should be used routinely in the second-line treatment of BRAF-mutant NSCLC, it is important to understand what is known about the efficacy of currently approved second-line therapies since the studies from Planchard et al. were not randomized or controlled. When comparing results across trials, one must keep in mind that earlier studies of second-line therapy included patients with NSCLC regardless of tumor genotype whereas the studies from Planchard et al. were designed to evaluate only the subset of patients with NSCLC harboring BRAF V600E mutations. This caveat is especially important given that long-term survival of BRAF V600E mutant NSCLC has been described in select cases (27,28). In addition, at least one study has demonstrated a trend toward better outcomes among patients with NSCLC whose tumors harbor any BRAF mutation compared to those harboring other driver mutations or no mutations at all (10). On the other hand, in a nationwide French study of patients with NSCLC whose tumors were profiled for oncogenic mutations, outcomes among patients with BRAF mutant NSCLC receiving second-line therapy were poor (ORR 9%), with the majority receiving best supportive care only (26).

Per current guidelines, approved second-line therapies following disease progression include single-agent or combination chemotherapy (pemetrexed, docetaxel, gemcitabine, or ramucirumab plus docetaxel), targeted therapy (erlotinib) and newer immunotherapies (nivolumab, pembrolizumab) (1). Second-line chemotherapy agents in NSCLC have generally yielded poor results. Accounting for methodological differences, studies of single-agent gemcitabine reported ORRs ranging from 13–19% with median OS 26–34 weeks (29,30). Single-agent docetaxel by comparison was demonstrated in separate trials to be superior to best supportive care and single-agent vinorelbine or ifosfamide, respectively, but the highest ORR was only 10.8% and the longest median OS was 7.0 months in either of the two studies (31,32). Better outcomes were noted in a trial of docetaxel alone vs. docetaxel plus ramucirumab in which an ORR of 14% was reported for single-agent docetaxel (33). However, the authors of that study attributed such findings to the enrollment of patients with better
The objective or overall response rates (%; complete or partial response) for currently approved second-line therapies in NSCLC are shown along with the response rates recorded in the two recent studies by Planchard et al. For each therapy for which multiple clinical trials have been performed, one representative trial is shown. The response rates for two different doses of docetaxel that were studied in the same trial from Fosella et al. are both shown. Molecular testing in NSCLC is key to identifying appropriate patients with BRAF mutant NSCLC who would benefit from second-line treatment with targeted therapy over other approved agents.

As a second-line treatment option, erlotinib compared to placebo results in a greater ORR (8.9% vs. <0.1%) and median OS (6.7 vs. 4.7 months) (35). Compared to single-agent chemotherapy, however, the benefit of targeted therapy in this setting is less clear. A comparison of pemetrexed vs. erlotinib, for example, demonstrated similar outcomes with chemotherapy and targeted therapy (36). In the TAILOR study, ORR (15.5% vs. 3%) and DCR (44.3% vs. 22%) were higher in patients with wildtype EGFR NSCLC who were treated with docetaxel compared to erlotinib, and thus the benefit of targeted therapy in patients with wildtype tumors is questionable (37). With respect to newer anti-PD-1 immunotherapies, nivolumab compared to docetaxel has been associated with longer OS (12.2 vs. 9.4 months) and higher ORR (19% vs. 12%) (38). Herbst et al. reported similar benefits with pembrolizumab with median OS 10.4 months (2 mg/kg dose of pembrolizumab) and 12.7 months (10 mg/kg dose) and an ORR of 18% at both dosages (39). However, the study excluded patients with negative PD-1 expression <1% and found that the best outcomes were experienced by patients with PD-1 expression >50%.

In the context of these studies, dabrafenib monotherapy and dabrafenib plus trametinib both compare favorably to currently approved second-line therapies. The response rates reported for both dabrafenib alone and dabrafenib plus trametinib are higher than that which has been traditionally reported with either single-agent chemotherapy or erlotinib in EGFR wild-type patients in the second-line setting. Additionally, the median OS of 12.7 months in patients with BRAF mutant NSCLC receiving dabrafenib monotherapy is longer than the survival typically reported with second-line chemotherapy. While newer anti-PD-1 immunotherapies are promising, their efficacy is dependent on PD-1 expression in tumor cells, and it is unclear if they will represent a treatment option for all patients with BRAF-mutant NSCLC. Although some variability in results may be explained by differences in patient populations, enrollment sizes, and methods between studies, targeted therapy nonetheless seems to represent a significant treatment addition for the subset of patients with BRAF V600E mutant NSCLC. This furthermore highlights the importance of molecular testing in patients with NSCLC. To optimize the benefits of BRAF targeted therapy, clinicians must be able to accurately identify patients with NSCLC harboring targetable BRAF V600E mutations who would be candidates to receive dabrafenib or dabrafenib plus trametinib over other standard second-line therapy options for which responses are less robust (Figure 1).

For oncologists tasked with making treatment decisions for patients with BRAF V600E mutant NSCLC, the next dilemma is selecting between BRAF monotherapy vs. BRAF-MEK combination therapy. In melanoma, acquired resistance to BRAF monotherapy leads to eventual drug failure and disease progression (12). Preclinical studies in melanoma cell lines have demonstrated multiple mechanisms of acquired resistance including new mutations in NRAS or MEK and increased expression of COT, CRAF, or PDGF-α (40-45). The rationale for the combined use of BRAF and MEK inhibition is to delay acquired resistance by blocking two sites along the MAPK pathway.
studies in melanoma have demonstrated better outcomes with BRAF-MEK combination therapy compared to BRAF monotherapy (46). Planchard et al. caution against directly comparing the results of their two cohorts since each was studied independently. However, each study employed a similar methodological design and had a similar median duration of follow-up. The comparable baseline characteristics of each cohort with respect to age, sex, performance status, percentage of non-smokers, and histology also makes direct comparisons more palatable. It is worth noting that with respect to ethnicity, the two cohorts were not as well balanced with a greater percentage of patients of Asian ethnicity enrolled in the cohort receiving dabrafenib monotherapy (22% vs. 7%). The potential effect of this discrepancy on outcomes is not clear.

Across nearly all metrics, dabrafenib plus trametinib was superior with a higher ORR, higher DCR, and longer PFS than dabrafenib monotherapy. While the duration of response in each therapy group was similar (9.0 months for dabrafenib plus trametinib vs. 9.6 months for dabrafenib), 18 of the 36 patients receiving dabrafenib plus trametinib who achieved a response remained on therapy at the time of data cutoff. In addition, among all patients receiving dabrafenib plus trametinib, 17 out of 57 (30%) remained on therapy for >12 months. As pointed out by Planchard et al., the response rate of dabrafenib plus trametinib compared to that of dabrafenib monotherapy is closer to the response rates typically reported with other targeted therapies such as erlotinib and crizotinib, although some of these latter studies were conducted using targeted therapy as first-line treatment (2-4,47-50). With this in mind, combined dabrafenib plus trametinib should likely be the preferred option wherever possible but until a head-to-head trial of BRAF monotherapy and BRAF-MEK combination therapy is conducted in NSCLC, clinician experience, patient preference, and the safety profile of each therapy should always be considered. The poor outcomes of patients receiving second-line treatment for NSCLC in general should make even dabrafenib monotherapy an attractive option in cases where combination therapy is contraindicated.

The documented adverse events occurring in patients receiving dabrafenib monotherapy were similar to those reported in melanoma. Planchard et al. reported adverse events of grade 2 or worse in 45 of 84 (54%) patients. By comparison, in a phase 3 trial of dabrafenib monotherapy in melanoma patients, adverse events grade 2 or greater occurred in 53% of patients, the most common of which were skin-related, pyrexia, fatigue, headache, and arthralgia (12). The rate of grade 3 squamous cell carcinoma of the skin was less common in this study of patients with melanoma compared to the Planchard et al. cohort (12% vs. 4%). In the two studies from Planchard et al., patients receiving combination dabrafenib plus trametinib compared to those receiving dabrafenib monotherapy had higher rates of adverse events leading to drug discontinuation (12% vs. 6%), drug interruption (61% vs. 43%), and dose reduction (35% vs. 18%), which has been similarly reported in comparisons of BRAF monotherapy and BRAF-MEK combination therapy in melanoma (46). Serious adverse events were also more common in the cohort receiving combination therapy (56% vs. 42%). However, squamous cell carcinoma was much less common, occurring in only 4% of patients. Regardless of these differences, Planchard et al. reported that both therapies were tolerated well overall. With respect to serious adverse events, it is worth noting that one patient receiving dabrafenib monotherapy who was also on a factor Xa inhibitor died from an intracranial hemorrhage while one patient with a history of a cranial artery aneurysm receiving dabrafenib plus trametinib experienced a subarachnoid hemorrhage. Only the intracranial hemorrhage was attributed to the study drug. Although rare, three patients with cerebral hemorrhage were reported in a trial of dabrafenib plus trametinib in melanoma, and at least one case report of intracranial hemorrhage occurring in a patient receiving dabrafenib plus trametinib therapy has been described previously (46,51). While causality has not been established, the potential for such serious adverse events should be noted as use of dabrafenib and trametinib increases.

Conclusions

In conclusion, the recent studies from Planchard et al. shed new light onto the efficacy of targeted therapy as second-line treatment in patients with stage IV BRAF V600E mutant NSCLC. As existing second-line therapy options in NSCLC have traditionally been associated with poor outcomes, dabrafenib monotherapy and combination dabrafenib plus trametinib should be considered in the management of patients with NSCLC harboring BRAF V600E mutations. Areas for future research remain and include direct head-to-head comparisons of BRAF monotherapy and combination BRAF-MEK inhibition, long-term follow-up of the safety profile of these targeted therapies, evaluation of the efficacy of dabrafenib and trametinib in the first-line treatment setting, and
explorations of treatment options for patients with tumors harboring less common \textit{BRAF} mutations.

**Acknowledgements**

None.

**Footnote**

\textit{Conflicts of Interest:} The authors have no conflicts of interest to declare.

**References**


47. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or


The importance of biological target-based studies is currently highlighted by impressive objective response rates (ORRs) and longer progression-free survivals (PFSs) provided by epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) compared with cytotoxic chemotherapies in the treatment of EGFR mutated non-small cell lung cancer (NSCLC) patients and, more recently, anaplastic lymphoma kinase inhibitors (ALK-Is) in ALK-rearranged NSCLC ones (1-4). As a consequence, recent therapeutic research strategies in NSCLC, particularly in lung adenocarcinoma, focused on innovative potential molecular targets such as KRAS, BRAF, HER2, PIK3CA, and others in frequencies exceeding 1% (5).

Reports of lung cancers bearing BRAF gene mutations generated extensive interest since these alterations could potentially be associated with an increased sensitivity to those agents directly targeting BRAF or BRAF-mediated downstream signaling pathways (6).

BRAF (v-RAF murine sarcoma viral oncogene homolog B1) is a member of the RAF family of serine/threonine kinase which have roles in mediating proliferation and survival and lies downstream of RAS in the RAS-RAF-MEK-ERK signaling pathway: upon activation by RAS, BRAF phosphorylates a dual-specificity mitogen-activated protein kinase (MEK), this leads to the activation of an extracellular signal-regulated kinase (ERK) and finally ERK signaling pathway (7).

Mutations in BRAF are most commonly seen in melanoma (40% to 50% of cases) but they were also detected in 2–3% of lung adenocarcinomas (8). The most frequently observed mutation in BRAF is BRAF valine-to-glutamate amino acid substitution at codon 600 (V600E) which results in a constitutively active protein, since it no longer requires dimerization for its activity, it is transforming in vitro and stands for a driver mutation effectivly targeted with selective BRAF and/or MEK inhibitors (9). BRAF-mutated melanomas harbor a V600E amino acid substitution in exon 15 in more than 80% of cases, but the actual prevalence, distribution, prognostic and predictive role of BRAF mutations and particularly BRAF^V600E ones in patients with NSCLC is currently under investigation. According to Paik et al. the incidence of BRAF mutations in their series of lung adenocarcinomas was 3% [95% confidence interval (CI): 2% to 4%], similar to literature data, with a relative frequency of non-V600E mutations distributed in exons 11 and 15 in 39% and 11% of cases, respectively, while BRAF^V600E mutations were reported in 50% of those patients (10). These data suggest that awareness about the exact type of BRAF mutation and the pathogenesis of such mutations could be critical in defining effective strategies for the targeted treatment of NSCLC with mutated BRAF. In fact, according to Cardarella et al., V600E, G469A, T599_V600insT, and V600_K601delinsE mutations showed increased BRAF kinase activity compared with wild-type BRAF; in contrast, the G496del mutation resulted in a reduced in vitro kinase
activity (11).

In order to increase knowledge about this molecular subgroup of patients highlighting advances in therapeutic approach, the present editorial will discuss about BRAF mutations, particularly BRAFV600E-positive patients, and treatment with dabrafenib alone or in combination in the contest of the latest single-arm, multicentre, non-randomized, open-label, phase II trials conducted by Planchard et al. (12,13).

In the phase II study with dabrafenib alone, 84 previously treated (n=78) and untreated (n=6) patients with stage IV metastatic BRAFV600E-positive NSCLC were enrolled, with the aim to investigate clinical activity and safety of dabrafenib in this specific setting. Of those patients 50% were females, 37% were never-smokers and 96% had adenocarcinoma histology (12).

BRAF mutations in lung cancer, as evidenced also by Planchard et al., are usually detected in adenocarcinoma histology and, according to previous reports, they were often recognized in smokers, although both V600E and non-V600E mutations were also identified in patients who had never smoked (8,11,12). The proportion of never and/or light smokers (≤10 pack-years) did not differ significantly according to BRAF mutation type (V600E or V600-like vs. other BRAF mutations) but Marchetti et al., in 37 of 1,046 screened lung cancers with a BRAF mutation, evidenced that all non-V600E mutations were detected in smokers, whereas BRAFV600E mutation was significantly more frequent in never-smokers and in female patients (11,14). These data where described also by Planchard et al., suggesting that even if BRAF mutations were more frequently observed in smokers they could also be identified in patients irrespective of their smoking history, as opposed to EGFR mutations and ALK rearrangements, which are usually evidenced in patients with no-smoking history (12).

At present, considering the low amount of data, the prognostic significance of BRAF mutations in lung cancer is still uncertain even if the type of BRAF mutation seems to be a prognostic factor. About this issue, Marchetti et al. found that V600E mutation was a negative prognostic factor, significantly associated with shorter OS on multivariate analyses (hazard ratio (HR): 2.18; P=0.014); particularly, patients with BRAFV600E mutations had shorter median disease free survival (DFS) and OS than patients without V600E mutations (15.2 vs. 52.1 months; P=0.001 and 29.3 vs. 72.4 months; P=0.001, respectively) (14).

Considering its predictive role, a strong correlation was observed between tumor initiation and expression/activation of MAPK pathway proteins, providing evidence that both tumor initiation and promotion were dependent on MAPK activation; conversely, suppression of BRAFV600E expression led to tumor shrinkage, accompanied by dephosphorylation of ERK 1 and 2. These findings pointed the interest on the role of BRAF in cancer induction and promotion, also as a driver mutation and consequently as a potential therapeutic target (15).

Dabrafenib is a potent adenosine triphosphate (ATP)-competitive, reversible inhibitor of mutant BRAF kinase. It decreases phosphorylated ERK and causes cell cycle arrest (16). In preclinical studies it was almost 20 times more selective at inhibiting BRAFV600E mutants than wild-type BRAF in multiple cancer cell lines and demonstrated activity in patients with NSCLC harboring BRAFV600E mutation (16). However, despite the success of BRAF-directed treatment in cutaneous melanoma, only small numbers of NSCLC patients received a BRAF-directed therapy in prospective studies so far (16).

Planchard et al., in their study with dabrafenib alone, reported an overall response (OR) in 21 (33%; 95% CI: 22–46) with a disease control rate (DCR) in 34 patients (53%; 95% CI: 40–66), according to the independent review committee, and a median PFS of 5.5 months (95% CI: 3.4–7.3). Particularly, a post-hoc analysis of response based on detailed smoking history (Planchard et al. supplemental files) evidenced that 15 (52%) of 29 patients with no smoking history had a response rate, compared with 6 (24%) of 25 patients with a history of less than 30 pack-years and 5 (21%) of 24 patients with a history of 30 pack-years or more. However considering available literature data, it is still unclear if smoking habits have a predictive value or not in this particular population of patients (12).

Results from Planchard et al. study are encouraging steps towards validating the targeting of BRAF pathway in patients with lung adenocarcinoma harboring a BRAFV600E mutation but, even considering response rates ranging around 60% in patients with melanoma treated with BRAF inhibitors, disease progression inevitably occurs (12,17,18). Several mechanisms of resistance to BRAF inhibitors were described in melanoma, such as activation of PIK3CA, new BRAF mutations, A-RAF and C-RAF increased expression (which can ultimately activate MAPK pathway downstream) and finally the activation of MAPK pathway at a downstream level (19,20).

A possible way to overcome resistance is blocking MAPK pathway downstream to BRAF, MEK inhibitors, such as trametinib which is an oral, reversible, highly selective
allosteric inhibitor of MEK 1/2 activation, exert their inhibitory effect by targeting a different kinase located downstream at the same pathway (6,15). Blocking MAPK pathway at two different levels (BRAF in conjunction with MEK) has the advantage of overcoming some of the resistance mechanisms observed with BRAF inhibitors alone (15).

BRAF and MEK inhibitors dabrafenib and trametinib, as a second line treatment, were tested by Planchard et al. in a prospective, single-arm, open-label, phase 2 study involving 57 NSCLC patients with BRAF\textsuperscript{V600E} mutation, in order to improve efficacy over BRAF inhibitor monotherapy through dual MAPK pathway inhibition (13). As already evidenced in melanoma patients harboring BRAF mutations, also in those with NSCLC, a double blockade increased response and DCR rates suggesting a delay in the development of tumor-resistance when compared to BRAF inhibitors alone. Particularly in Planchard et al. study on dabrafenib and trametinib combination, 36 of 57 patients achieved an OR of 63.2\% (95\% CI: 49.3–75.6); the independent review committee confirmed the investigator-assessed OR with a DCR in 43 patients (75.4\%; 95\% CI: 62.2–85.9) and a median PFS of 8.6 months (95\% CI: 5.2–19.1) (13). Results of this study suggested that a combined approach could be preferable in BRAF-mutated NSCLC, just as it is in BRAF-mutated melanoma (21). Moreover MEK inhibition counterbalances the effect of BRAF inhibitors on keratinocytes, which is responsible for the secondary cutaneous tumors observed with these drugs. In fact in Planchard et al. study on dabrafenib alone, the development of cutaneous squamous-cell carcinomas (cuSCC) grade 3 was evidenced in 10 patients (12\%), four cases had basal-cell carcinomas (5\%) while one (1\%) presented with lip squamous-cell carcinoma; the median time to development of cutaneous squamous-cell carcinoma was 13.1 weeks [interquartile range (IQR): 5.1–21.7], but none of these patients needed for a dose modification or interruption and any other squamous-cell carcinomas were evidenced in other organs. On the contrary, in Planchard et al. publication about dabrafenib and trametinib combination, a better cutaneous toxicity profile was evidenced considering that only two patients (4\%) presented with basal cell carcinoma. This data confirmed that combination of MEK and BRAF inhibitors block a paradoxical activation of MAPK signaling in BRAF wild-type cells reducing the incidence of cuSCC compared with BRAF inhibitor monotherapy (1–3\% vs. 9–18\%) (12,13,22).

In addition to cutaneous toxicity, it is important to underline that both single agent treatment as well as combination therapy do have important, but manageable, toxicities in what remains a palliative situation. In BRAF inhibitor monotherapy study by Planchard et al., more than half of patients (45 of 84, 54\%) had adverse events of grade 2 or worse. One patient died during the study from an intracranial hemorrhage judged to be related to the study drug. With dabrafenib and trametinib combination, Planchard et al. reported that nearly half of patients (28 of 57, 49\%) had at least one grade 3–4 event. Dose reductions were needed for 33 patients (58\%) who received at least 80\% of the planned dose of dabrafenib, and 43 patients (75\%) who received at least 80\% of the planned dose of trametinib (12,13). Of potential concern were the cases of fatal hemorrhage (although anticoagulation was a risk factor in these cases) or haemoptysis which despite there being no strong signal of increased hemorrhagic risk in melanoma, is of particular importance in lung cancer and should be monitored closely in future trials (12,13,21).

If targeting multiple kinases at the same time is confirming to delay disease progression in this subgroup of patients, also alternative strategies are raising up to overcome BRAF inhibitors resistance, primarily in melanoma patients. LGX818 is a selective BRAF inhibitor which potently decreases ERK phosphorylation and inhibits proliferation in BRAF\textsuperscript{V600E} mutant melanoma cell lines; it is currently under investigation in early phase trials, mostly in BRAF mutant melanoma patients (8,23). ARQ736 is a pan-RAF inhibitor, which targets A-RAF, B-RAF and C-RAF. It has been studied in a phase I trial with the strategy of inhibiting all RAF kinases with a single drug to delay disease progression (8,24). Another compound, RAF265, a potent inhibitor of BRAF\textsuperscript{V600E}, wild-type-B-RAF, and C-RAF, is also under investigation on a phase II trial, after promising results demonstrated on the phase I trial (8,25). Finally another area of growing interest is immunotherapy: treatment targeting immune system check-points, such as CTLA-4 and PD-1, were designed to enhance host immune system, oppose tumor immune evasion and generate an effective immune response against tumor cells (15). As already evidenced in melanoma, the pro-apoptotic and cytotoxic effect evidenced after chemotherapy or targeted therapies, such as BRAF inhibitors, may expose intracellular antigens that were previously “hidden” by tumor immune evasion mechanisms (15). This leads to the exciting hypothesis of a synergistic effect: BRAF inhibitors, probably together with the events of immune response at different levels, may expose tumor antigens enhancing the efficacy of
In conclusion, even if advances achieved in the comprehension of \textit{BRAF} mutations and MAPK pathway, mostly in melanoma patients, are leading to an increased knowledge in lung cancer research, it is still not clear if the results observed in melanoma can be undoubtedly translated into a therapeutic benefit for NSCLC patients. Previously described approaches have a role in lung cancer biology; although a better understanding of those mechanisms needs to be further investigated. For now, caution should be exercised in extrapolating definitive results from early-phase, single-arm studies without a comparator arm, but only with these trials intriguing hypothesis about new targeted agents or dual pathways blockade will emerge; the final aim is to optimize new sequencing strategies and stimulate research towards personalized therapy in NSCLC even warranting for additional investigation in future clinical trials.

\textbf{Acknowledgements}

The author thanks Silvia Novello for interesting discussions on the subject matter.

\textbf{Footnote}

\textit{Conflicts of Interest:} The author has no conflicts of interest to declare.

\textbf{References}


Patients with central nervous system (CNS) metastases in general experience have a deterioration in performance status and a limited survival time (1). CNS metastasis of non-small cell lung cancer (NSCLC) has been the subject of renewed interest of late given that small-molecule tyrosine kinase inhibitors (TKIs), such as those that target activated forms of the epidermal growth factor receptor (EGFR), have the potential to improve local tumor control in molecularly selected individuals. Given that the concentration of EGFR-TKIs is much lower in cerebrospinal fluid (CSF) than in plasma, however, frequent isolated CNS metastasis, without other systemic progression, has been detected in patients with advanced NSCLC who show a response to these drugs (2). Similar concerns have also been raised for patients with NSCLC positive for the \textit{EML4-ALK} fusion gene treated with the anaplastic lymphoma kinase (ALK)-targeted TKI crizotinib.

While the need for randomized phase III trials comparing targeted therapy to standard cytotoxic chemotherapy in patients with low-frequency driver mutations such as \textit{ALK} rearrangements has been under discussion, a randomized phase III study (PROFILE 1014) recently demonstrated a superior progression-free survival (PFS), objective response rate, and patient-reported outcomes for crizotinib versus pemetrexed-platinum combination chemotherapy in \textit{ALK} rearrangement-positive NSCLC patients (3). Focusing on the intracranial efficacy of crizotinib in such patients enrolled in the PROFILE 1014 study, Solomon et al. have now reported that PFS was significantly longer with crizotinib versus chemotherapy for individuals with stable treated brain metastases (4). Intracranial time to tumor progression also tended to be longer on crizotinib compared with chemotherapy, although this difference did not achieve statistical significance. Intracranial progression—worsening of existing or development of new intracranial lesions—is often the first manifestation of disease progression in patients treated with crizotinib, and intracranial disease progression as the sole site of progression during crizotinib treatment was more frequent in patients with stable treated brain metastases (38%) than in those without known brain metastases (19%). The management of patients who show recurrent isolated CNS failure during crizotinib therapy is thus an emerging clinical problem.

A case study found that the concentration of crizotinib is much lower in CSF than in plasma (5), suggesting that the likelihood of isolated CNS metastasis is greater than that of disease progression elsewhere in patients with \textit{ALK} rearrangement-positive advanced NSCLC who are treated with crizotinib. Systemic disease progression (also known as acquired resistance) in patients receiving crizotinib occurs through several molecular mechanisms including the acquisition of a mutation at the so-called gatekeeper site in the tyrosine kinase domain of ALK, and activation of bypass pathways (6,7). In contrast, extracranial tumors in patients who experience isolated CNS metastasis as a result of poor drug penetration through the blood-brain barrier (pharmacokinetic resistance) are likely to remain
sensitive to the corresponding molecularly targeted therapy. We have previously shown that the resumption of daily administration of crizotinib after whole-brain radiotherapy or stereotaxic radiotherapy for isolated CNS failure in NSCLC patients was found to be effective for control of extracranial disease (8). Although the molecular mechanisms of resistance to crizotinib operative in the new study of Solomon et al. (4) were not determined by analysis of tissue or CSF samples, most patients who developed isolated intracranial progressive disease during crizotinib treatment received crizotinib for >3 weeks beyond disease progression at the discretion of the treating physician, suggesting that most physicians may consider crizotinib beyond intracranial disease progression to be beneficial.

Novel strategies to enhance exposure of the CNS to ALK inhibitors, including the development of new drugs with a greater ability to cross the blood-brain barrier, are thus warranted. Alectinib is a second-generation, ALK-selective TKI with more potent inhibitory activity toward ALK (9). In animal models, alectinib generates relatively high brain/plasma concentration ratios, ranging from 0.63 to 0.94 (10). Clinically, an objective response was achieved in 48% of ALK rearrangement-positive, crizotinib-resistant NSCLC patients treated with alectinib at 600 mg twice daily in a phase II trial (11). Importantly, 75% of patients with measurable CNS lesions at baseline achieved an intracranial response. The J-ALEX study, a randomized phase III trial comparing the efficacy of alectinib (300 mg twice daily) with that of crizotinib (250 mg twice daily) in Japanese patients with ALK rearrangement-positive, NSCLC, found that alectinib reduced the risk of disease worsening or death (PFS) by 66% compared with crizotinib (hazard ratio of 0.34, with a 99% confidence interval of 0.17 to 0.70; P<0.0001) (12). Of note, the allowed dose of alectinib in Japan is lower than that in the United States, which allows alectinib to be administered at 600 mg twice daily. The global randomized phase III study ALEX (NCT02075840) comparing alectinib (600 mg twice daily) with crizotinib (250 mg twice daily) in treatment-naive patients with ALK rearrangement-positive advanced NSCLC is ongoing. Whether alectinib therapy reduces the risk of CNS progression compared with crizotinib remains unknown, but current evidence suggests that alectinib may prevent or delay the emergence of CNS metastases.

Acknowledgements
None.

Footnote
Conflicts of Interest: K Nakagawa has received honoraria from Chugai Pharmaceutical and Pfizer Japan Inc., M Takeda has no conflicts of interest to declare.

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Cite this article as: Takeda M, Nakagawa K. Crizotinib for *ALK* rearrangement-positive non-small cell lung cancer patients with central nervous system metastasis. Transl Cancer Res 2016;5(S3):S554-S556. doi: 10.21037/tcr.2016.09.34
Intracranial efficacy of crizotinib versus chemotherapy in PROFILE 1014: shining a light on central nervous system endpoints in clinical trials

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Provenance: This is a Guest Perspective commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Abstract: PROFILE 1014 is the first phase III, randomized controlled trial that has prospectively studied the CNS efficacy of crizotinib compared to platinum-pemetrexed chemotherapy in ALK positive NSCLC, including among those with stable, treated CNS disease. Overall, PROFILE 1014 has given us valuable information to inform our optimal first line treatment decision for those with CNS disease at baseline, reassuring us about the efficacy of local treatment and use of crizotinib in these patients. It also highlights some of the design aspects that still need to be addressed for future clinical trials if we are to most informatively assess the activity of drugs in the CNS. Many next generation ALK inhibitors have been associated with significantly increased CNS activity compared to crizotinib and are now entering the clinic. Their CNS activity is so significant that their initial use could potentially allow the use of local CNS therapies, such as radiotherapy, to be deferred for those with CNS disease at baseline. Capturing robust CNS endpoints and learning the lessons from PROFILE 1014 will be vital if we are to determine the optimal use of these new drugs, among those both with and without CNS disease at baseline, in the future.

Keywords: PROFILE 1014; crizotinib; central nervous system

Submitted Aug 24, 2016. Accepted for publication Aug 30, 2016.
doi: 10.21037/tcr.2016.09.38

View this article at: http://dx.doi.org/10.21037/tcr.2016.09.38

PROFILE 1014

The appropriate management of non-small cell lung cancer (NSCLC) that has spread to the central nervous system (CNS) is becoming an increasingly important clinical issue and nowhere is this more obvious than in the battleground of developing tyrosine kinase inhibitors (TKI) for the treatment of anaplastic lymphoma kinase (ALK)-positive disease. In the 1\textsuperscript{st} line PROFILE 1014 study, which compared crizotinib with platinum-pemetrexed chemotherapy in patients with ALK positive disease, 23\% of patients had baseline CNS disease, with estimates of the lifetime incidence of CNS disease in ALK positive NSCLC approaching 50\% (1,2). In contrast, baseline estimates of CNS disease in potentially operable NSCLC (not otherwise specified) have been quoted at 7\%, and in 1\textsuperscript{st} line trials for advanced epidermal growth factor receptor (EGFR) mutant NSCLC at 12–14\% (3,4). Consequently, although small studies looking at the pattern of metastatic spread at diagnosis of stage IV disease have not identified the CNS as a site of spread that is significantly different between dominant oncogene-addicted subtypes of NSCLC, including ALK, there may still be some inherent tropism
of ALK positive disease for the CNS (5). In addition, the successful development of therapies to control the extra-CNS disease in ALK positive NSCLC for months and sometimes years is likely to exaggerate the impact of even a small propensity for CNS spread through its cumulative manifestation over time.

Recognizing the distinct risks of CNS metastases in ALK positive NSCLC, PROFILE 1014 represents the first completed phase III clinical trial to prospectively measure the efficacy of an ALK inhibitor in the CNS as one of its defined endpoints (1). Patients with baseline CNS metastases were permitted to enter the trial provided that their CNS disease was treated and neurologically stable for ≥2 weeks with no ongoing corticosteroid requirement. The main CNS efficacy endpoint in PROFILE 1014 was intracranial time-to-progression (IC-TTP) which was defined as the time from randomization to objective worsening of existing intracranial lesions or the development of new intracranial lesions. Patients with a baseline history of CNS disease had repeat CNS imaging every 6 weeks and if they had no history of CNS disease, every 12 weeks.

In the intention-to-treat (ITT) population (343 patients), crizotinib demonstrated a non-significant trend towards an improved IC-TTP compared to up to 6 cycles of platinum-pemetrexed chemotherapy (with no pemetrexed continuation maintenance option): median not reached vs. 17.8 months, HR = 0.60; 95% confidence intervals (CI): 0.34 to 1.05; P=0.068. In the subgroup with baseline treated and stable brain metastases (tBM; 79 patients), a similar non-significant trend was noted with median IC-TTP 15.7 vs. 12.5 months for crizotinib and chemotherapy, respectively (HR = 0.45; 95% CI: 0.19 to 1.07; P=0.063). In the subgroup without baseline CNS disease (263 patients), again a non-significant trend was noted with the median IC-TTP not reached in either treatment group (HR = 0.69; 95% CI: 0.33 to 1.45; P=0.323).

Therefore, with regard to the protocol defined IC-TTP endpoint, no significant difference between the interventions can be claimed, although there were consistent non-significant trends in favor of the crizotinib in all groups analyzed. Importantly, much of the dataset remains immature and therefore whether any of these trending differences will become significant later remains to be seen.

To generate CNS efficacy data which would mature sooner than IC-TTP, the study investigators performed a post-hoc analysis of the intracranial disease control rate (IC-DCR) at 12 and 24 weeks, which is the major new data contained in this publication, separate from the previously published main trial report (6). The IC-DCR was defined as the percentage of patients with confirmed complete response, partial response, or stable disease in the tBM subgroup at the defined time points. At 12 weeks, the IC-DCR was 85% (95% CI: 70% to 94%) and 45% (95% CI: 29% to 62%) for crizotinib and chemotherapy, respectively (P<0.001). At 24 weeks, the IC-DCR was 56% (95% CI: 40% to 72%) and 25% (95% CI: 13% to 41%) for crizotinib and chemotherapy, respectively (P=0.006).

Overall antitumor activity demonstrated similar statistically significant improvements in progression free survival (PFS; the primary endpoint of the study) with crizotinib over chemotherapy, as in the main IIT analysis, regardless of the presence or absence of baseline CNS disease. In the tBM group, median PFS was 9 vs. 4 months for crizotinib and chemotherapy, respectively (HR = 0.40; 95% CI: 0.23–0.69, P<0.001). In the BM absent group, median PFS was 11.1 vs. 7.2 months for crizotinib and chemotherapy, respectively (HR = 0.51; 95% CI: 0.38–0.69, P<0.001). Similarly, the objective response rate (ORR) was significantly higher with crizotinib than with chemotherapy, as in the main IIT analysis, regardless of the presence or absence of baseline CNS disease.

Discussion on the CNS activity of crizotinib in PROFILE 1014

Previously it has been reported that 46–72% of ALK positive NSCLC patients receiving treatment with crizotinib first progress within the brain and that this is the only site of progression in over 80% of these cases (7,8). From a single case, in which matched blood and cerebrospinal fluid (CSF) crizotinib levels were assessed <0.3% of the levels present in the blood were seen in the CSF, suggesting a plausible pharmacokinetic explanation for the disconnect in activity seen between the body and the brain with this drug (9). Consistent with this, when CNS activity was assessed retrospectively within the Pfizer trials database, among those with untreated measurable disease in the brain at the start of crizotinib therapy, the CNS objective response rate was only 18% (compared to 53% systemically); the median duration of these CNS responses was nearly half that of the systemic response data (26.4 vs. 47.9 weeks, respectively) and the median time to progression was 7 months intracranially, compared to 12.7 months systemically (8).
With multiple prior retrospective reports commenting on the limited activity of crizotinib in the CNS, does the prospective data from PROFILE 1014 now make the case stronger for using crizotinib as first line therapy in patients with CNS disease at baseline?

Well, the answer is both ‘yes’ and ‘no.’ In addition, understanding why there isn’t a simple answer to this question starts to shine a revealing light on just how we are learning to better design and interpret efficacy endpoints relating to CNS metastases within modern cancer clinical trials.

At the most superficial level, it is simple enough to argue that the protocol defined endpoint of a statistically significant improvement in IC-TTP was not met and the IC-DCRs at 12 and 24 weeks represent post-hoc assessments based on relatively few events and are therefore of more questionable validity. For example, among the tBM group, only 21 of 79 patients (27%), across both arms had experienced a CNS progression event at the time of analysis. Among the BM absent group, only 30 of 263 patients (11%) across both arms had experienced a CNS progression event. In addition, as CNS lesions were previously treated and did not have to be of a given size, these were not assessed as RECIST target lesions. Instead, intracranial progression as it related to both IC-TTP and IC-DCR was only defined as either the development of new lesions or ‘worsening’ of disease. In the absence of specific size or percentile change criteria, the term ‘worsening’ was therefore open to subjective variations in interpretation. The consistent general anti-cancer benefit of crizotinib over chemotherapy in terms of both PFS and ORR in the tBM and absent BM subgroups (just as in the overall ITT population) also cannot be interpreted as clearly showing CNS benefit, as the events driving these endpoints (progression/non-progression and non-response/response) will have been overwhelmingly dominated by extra-CNS events. When progression did occur, the CNS was still the sole site of progression in a higher proportion of crizotinib than chemotherapy treated cases, in both the tBM (38% vs. 23%, respectively) and absent BM (19% vs. 6%, respectively) subgroups suggesting that the CNS remains a prominent Achilles heel for crizotinib. That said, it should be recognized that progression occurred on average at a significantly later date with crizotinib than with chemotherapy. So while PROFILE 1014 may not have conclusively proven that crizotinib is better than chemotherapy within the CNS, it also hasn’t shown that it is any worse and it is clearly better when considering efficacy within the patient as a whole, reliably solidifying crizotinib’s case as the initial treatment choice in advanced ALK positive NSCLC compared to chemotherapy.

However, before we come to the conclusion that we don’t have to pay any particular attention to the CNS when we start patients on crizotinib, we should recall the specific details of this study. Patients with CNS disease were only permitted to be enrolled when that disease was treated and stable. Consequently, any conclusions from this study regarding the ‘efficacy’ of using crizotinib for those with established CNS metastases can only be applied to those with CNS disease that has been treated before the drug is commenced. More importantly though, we also have to consider whether this requirement for a priori CNS treatment could, in fact, have influenced the endpoints being assessed within the study.

Among the tBM subgroup, while the significant improvement in IC-DCR at both 12 weeks and 24 weeks for crizotinib over chemotherapy could reflect a true benefit from ALK inhibition in the brain, it could also have been confounded by other differences between the two arms. One of the major variables not presented (besides the number of CNS deposits present in the patients) was the exact nature of the CNS treatments used. While stereotactic surgery or radiosurgery (SRS) should reduce the potential of an individual CNS locus to later progress, the risk of progression at other sites within the brain remains unchanged. In contrast, whole brain radiotherapy (WBRT) may have more of a general protective effect across the entire brain parenchyma and/or cerebral leptomeninges, in addition to any impact on the overall permeability of the blood-brain barrier to subsequent systemic drug exposure (9). In the absence of detailed information on the type of prior treatment, in order to ascribe the IC-DCR benefit to the differences in drug intervention alone, we are left to assume that the rate of WBRT (and its potential broader CNS benefit) was equally distributed between the crizotinib and chemotherapy arms. Yet, given that there were only 39 and 40 cases in the crizotinib and chemotherapy arms of the tBM subgroup, respectively, significant imbalances in the rate of WBRT, when it was not a planned stratification factor, could certainly have occurred.

Admittedly, the consistent numerical improvements in IC-TTP present in both the tBM and the absent BM subgroups suggest that differences in CNS efficacy are unlikely to solely be due to imbalances in prior radiation (given that the absent BM subgroup would not have received any prior therapy). However, as all of these IC-TTP improvements remain non-significant to date, any use...
of these data to support such an argument would have to be qualified by noting that any of these IC-TTP trends could also have occurred by chance.

So where does this leave us from the trials perspective?

A recent survey of 413 open trials assessing systemic drug therapy for adult patients with advanced NSCLC within the clinicaltrials.gov database revealed that 14 and 19% of trials excluded patients with any history of CNS parenchymal or leptomeningeal metastatic disease, respectively (10). Furthermore, 19% of trials contained no explicit mention of CNS disease in their available inclusion/exclusion criteria. Consequently, given the increasing clinical concern about the CNS as a relevant battleground in the treatment of advanced NSCLC, PROFILE 1014 should be applauded for specifically permitting patients with CNS disease entry into the trial in the first place. In addition, it should be applauded for making prospectively defined CNS efficacy (IC-TTP) a prominent secondary endpoint, heralding a move away from some of the problems commonly associated with retrospective analyses of CNS data (9). To further address the issues associated with optimizing clinical trial designs for assessing CNS activity in metastatic disease, the Response Assessment in Neuro-Oncology (RANO) group, an independent, international, collaborative effort, has now begun to publish a series of guidelines on this topic (11,12). Clearly, one area in need of greater attention is the issue of accurately documenting and assessing the potential impact of prior CNS therapy on CNS related endpoints in subsequent drug trials. As in PROFILE 1014, in the clinicaltrials.gov analysis, 41% of trials permitted CNS disease only after prior CNS-directed treatment, which, at best, may limit the interpretation of CNS drug activity due to an overall stabilizing/protecting effect on the CNS and, at worst, if the specific modality (i.e., WBRT) is not balanced between the arms could confound the attribution of any apparent drug benefit in randomized trials (9,10).

And from the first-line ALK positive patient perspective?

Together with the existing data on the limited activity of crizotinib in untreated brain metastases, the new data from PROFILE 1014 in the setting of treated CNS disease, helps us to sketch out a practical decision tree with regard to appropriate action plans for a treatment naïve ALK positive NSCLC patient with parenchymal CNS metastases at diagnosis (Figure 1). In the setting of asymptomatic CNS disease it may be reasonable to commence crizotinib treatment and watch the CNS closely, given that the activity of crizotinib in the CNS is modest, but not zero. On the other hand, if the patient were symptomatic from...
parenchymal CNS disease, local CNS treatment should probably be utilized up front rather than relying on the crizotinib to do the job, when it will not be sufficient in most cases.

An ongoing debate relates to the number of CNS lesions that should prompt a decision for WBRT rather than SRS, which may be influenced by many different factors including access to specific equipment, health economic analyses, regional or national guidelines and general medical philosophies relating to the need to treat more than a certain number of deposits as if a field effect were present versus the utility (or futility) of treating each site individually. However, in the setting of ALK positive disease, the WBRT versus SRS decision also has to consider the emerging data on the marked longevity of these patients. Among 90 patients with CNS disease from ALK positive NSCLC, the median overall survival was 49.5 months, more than enough time to manifest significant cognitive side-effects from WBRT (13). Consequently, a proposal for WBRT might give us pause for thought and, beyond pushing the upper limit of the number of lesions considered appropriate for SRS, prompt us to look for other options (Figure 1). Fortunately, such options are becoming increasingly available. A number of next generation ALK inhibitors with significant activity against disease in the CNS are now either FDA approved in the USA post-crizotinib, approved in the first line setting in other countries, or are being explored in clinical trials across several different lines of therapy, including in the first line setting. For example, both alectinib and brigatinib have shown CNS response rates over 50% in the post-crizotinib setting and are being explored in the first line setting compared to crizotinib (14,15). In addition, in the J-ALEX study conducted in Japan, alectinib has already shown a significantly longer progression free survival compared to crizotinib in the treatment naïve or post-chemotherapy (but ALK inhibitor naïve) setting, solidifying its existing first line license in that country [HR =0.34 (95% CI: 0.17–0.71)] (16). Among those with CNS disease at baseline, the magnitude of benefit from alectinib was even more marked [HR =0.08 (95% CI: 0.01–0.61)]. Whether the absolute difference in PFS will justify transitioning next generation drugs into the front-line for all ALK positive patients, rather than keeping them for sequential use post-crizotinib remains to be determined. However, when they are available, either because they are licensed, or through off-label or trial use, the presence of CNS disease at baseline is likely to be a key factor driving their first-line use over crizotinib, allowing any CNS radiotherapy, but especially WBRT, to be avoided, at least for a while.

**Summary**

PROFILE 1014 is the first phase III, randomized controlled trial that has prospectively studied the CNS efficacy of crizotinib compared to platinum-pemetrexed chemotherapy in ALK positive NSCLC, including among those with stable, treated CNS disease. Overall, PROFILE 1014 has given us valuable information to inform our optimal first line treatment decision for those with CNS disease at baseline, reassuring us about the efficacy of local treatment and use of crizotinib in these patients (Figure 1). It also highlights some of the design aspects that still need to be addressed for future clinical trials if we are to most informatively assess the activity of drugs in the CNS. Many next generation ALK inhibitors have been associated with significantly increased CNS activity compared to crizotinib and are now entering the clinic. Their CNS activity is so significant that their initial use could potentially allow the use of local CNS therapies, such as radiotherapy, to be deferred for those with CNS disease at baseline. Capturing robust CNS endpoints and learning the lessons from PROFILE 1014 will be vital if we are to determine the optimal use of these new drugs, among those both with and without CNS disease at baseline, in the future.

**Acknowledgements**

None.

**Footnote**

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

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Cite this article as: Ng TL, Camidge DR. Intracranial efficacy of crizotinib versus chemotherapy in PROFILE 1014: Shining a light on central nervous system endpoints in clinical trials. Transl Cancer Res 2016;5(S3):S520-S525. doi: 10.21037/tcr.2016.09.38
Central nervous system (CNS) metastases remain a significant problem in the management of patients with \textit{ALK}-rearranged non-small cell lung cancer (NSCLC). The frequency of CNS involvement in \textit{ALK}-positive tumors is extremely high; it approaches 25\% in treatment-naive patients (1) and rises to 50\% in patients treated with crizotinib (2,3). Crizotinib was previously reported to have only minor intracranial activity (4), with poor CNS penetration suggested as the underlying mechanism (5).

The study published this year by Solomon and colleagues in \textit{Journal of Clinical Oncology} addressed the question of intracranial efficacy of crizotinib in PROFILE 1014 trial (1). The study confirmed that crizotinib, as compared with platinum-based chemotherapy, is associated with better progression-free survival (PFS) irrespectively of presence or absence of brain metastases at the time of initial diagnosis [HR 0.4 (0.23–0.29); \textit{P}<0.001] and 0.51 (0.38–0.69; \textit{P}<0.001) for patients with and without CNS metastases, respectively. Most importantly, the results hinted towards better control of intracranial disease with crizotinib as compared to platinum-based chemotherapy. The effect was even more pronounced in patients with brain metastases treated with radiotherapy (RT) before enrollment. For instance, crizotinib treatment was associated with numerically better intracranial time to tumor progression (IC-TTP) both in the intent-to-treat (ITT) population and the subgroup of patients with treated CNS metastases; however, the results were not statistically significant. Crizotinib treatment was also associated with significantly better intracranial disease-control rate (IC-DCR) in patients with previously treated CNS metastases confirming the results of a combined analysis of PROFILE 1005 and PROFILE 1007 studies (4).

Can the study published by Solomon and colleagues provide us with a “yes or no” answer with regards to intracranial efficacy of crizotinib? The answer to the question is no. It is important to emphasize that although IC-TTP was a protocol-specified end-point, the study was underpowered to demonstrate a statistically significant difference in intracranial effects between crizotinib and chemotherapy. In fact, only 15\% of the ITT population had their disease progressed in the CNS. On the other hand, if the existing difference is too small to be picked-up in a large-size cohort study—whether that amount of effect we are looking for in clinics?

Interestingly enough, the intracranial effect of crizotinib was more pronounced in patients with brain metastases at study entry. Is it a pure statistical phenomenon? Imbalances in the baseline patient characteristics, such as male predominance in the CNS metastases subgroup, cannot be responsible for the differences observed. Differences in the schedule assessment between the subgroups may have confounded the results. However, the most possible explanation for larger effect observed with crizotinib in patients with brain metastases treated with RT before the study entry as opposed to patients without CNS metastases is better drug penetration into the CNS resulting from the disruption of the blood-brain barrier by the brain irradiation.
Of note, the results observed in that subgroup are in line with the results of the combined analysis of PROFILE 1005 and PROFILE 1007 studies, confirming higher CNS control rate achieved with brain irradiation (4). Solomon and colleagues were first to demonstrate that better CNS control is not a pure radiation effect (since both arms received brain irradiation), but the effect of the combination, supporting the hypothesis of drug penetration improvement following brain RT. Indeed, higher peak (Cmax) crizotinib concentrations in the cerebro-spinal fluid (CSF) may provide a prolonged CNS control in ALK-rearranged tumors (6-8).

Importantly, another scenario of combining RT with crizotinib in order to achieve intracranial control is administration of cranial irradiation after isolated intracranial progression which allows controlling the disease for another 5-7 months (1,4,9).

Overall, crizotinib used as a sole modality has modest intracranial activity and is only marginally superior to chemotherapy in terms of intracranial disease control. Furthermore, its intracranial effects require brain irradiation to be given at some point in the majority of cases in order to control the disease in the CNS. Whole brain radiation therapy (WBRT), on the other hand, delivered early in the disease course frequently results in long-term cognitive decline and substantial neurological morbidity (10).

New-generation ALK-inhibitors not only have a broader spectrum of activity in terms of resistant mutations in the ALK gene but also possess better CNS penetration. In particular, alectinib administered at a standard dose produces therapeutic concentrations in the CSF (11). Although the data with regards to intracranial activity of newer compounds is limited, it is very promising (Table 1). Thus, new generation ALK-inhibitors might represent a better alternative to RT in case of intracranial progression during crizotinib treatment (8). Furthermore, it is very possible that new-generation ALK-inhibitors are superior to crizotinib in treatment-naïve patients. According to the results of J-ALEX, a Japanese phase III randomized trial evaluating alectinib versus crizotinib in advanced ALK-positive NSCLC patients naïve to ALK-tyrosine kinase inhibitors (TKI), alectinib is superior to crizotinib in terms of PFS (19). The results of ALEX trial having the same design and conducted in the Caucasian population are highly awaited. Noteworthy, permitting patients with asymptomatic CNS metastases and having time-to-CNS progression as a key secondary end-point, ALEX trial is expected to provide important prospective data on the comparative intracranial efficacy of the two agents.

**Acknowledgements**

None.
Footnote

Conflicts of Interest: Elizabeth Dudnik—honoraria for lectures: Pfizer and Roche; Nir Peled—paid membership of advisory boards: Pfizer.

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Cite this article as: Dudnik E, Peled N. Intracranial activity of crizotinib: something to rely on? Transl Cancer Res 2016;5(Suppl 4):S836-S838. doi: 10.21037/tcr.2016.10.77
Clinical data and role of ceritinib a second-generation ALK tyrosine kinase inhibitor for the treatment of ALK positive non-small cell lung cancer

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Provenance: This is an invited Commentary commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


doi: 10.21037/tcr.2016.11.66

View this article at: http://dx.doi.org/10.21037/tcr.2016.11.66

Rearrangements in anaplastic lymphoma kinase (ALK) gene and echinoderm microtubule-associated protein-like 4 (EML4) gene were first described in 2007. This genomic aberration is found in about 2–8% of non-small cell lung cancer (NSCLC) patients. In patients with adenocarcinoma lacking EGFR and KRAS mutations, the prevalence of EML4-ALK translocation could be as high as 42.8% (1). In these patients, ALK rearrangements serve as a key and strong oncogenic driver for NSCLC and represent a critical therapeutic target susceptible to targeted ALK kinase inhibition (2,3).

Ceritinib was the first ALK tyrosine kinase inhibitor (TKI) licensed for treatment of metastatic ALK-positive NSCLC based on the randomized phase 3 trial PROFILE 1014 (4). Despite the initial treatment response of ceritinib, disease progression inevitable develops after about 10 months of therapy. Different resistance mechanisms have recently been described. One relevant mechanism of resistance is the development of mutations in ALK. Novel ALK TKIs have been developed to overcome these mutations. Ceritinib is an oral second-generation ALK inhibitor showing clinical activity in ceritinib-resistant ALK-positive NSCLC but also in treatment-naive ALK-positive disease. Ceritinib has first been investigated in the multicenter, open-label, phase 1 trial ASCEND-1. The initial publication of the trial included 130 patients with advanced cancers harboring genetic alterations in ALK (5).

In a first step, 59 patients were included in the dose-escalation phase and received ceritinib in doses of 50 to 750 mg. The maximum tolerated dose of ceritinib was 750 mg once daily; dose-limiting toxic events included diarrhea, vomiting, dehydration, elevated aminotransferase levels, and hypophosphatemia. This phase was followed by an expansion phase, in which an additional 71 patients. Among 114 patients with NSCLC who received at least 400 mg of ceritinib per day, the overall response rate (ORR) was 58% [95% confidence interval (CI), 48–67]. Among 80 patients who had received crizotinib previously, the ORR was 56% (95% CI, 45–67). Responses were observed in patients with various resistance mutations in ALK and in patients without detectable mutations. Among patients with NSCLC who received at least 400 mg of ceritinib per day, the median progression-free survival (PFS) was 7.0 months (95% CI, 5.6–9.5). The survival rate after 1 year was 65%.

The expansion cohort of this cohort did further include more patients and updated results have recently been published in Lancet Oncology (6). Between January 2011 and July 2013, 255 patients were enrolled and received at least 1 dose of ceritinib 750 mg/d, of whom 246 patients had an ALK positive NSCLC. The data cut-off for this updated analysis was April 14, 2014. At a median follow-up of 11.1 months, 60% of patients who had discontinued ceritinib therapy. Of 147 patients having discontinued ceritinib, 98 patients (67%) stopped therapy due to disease progression.
This update analysis is of high interest as it includes a large number of ALK positive patients with and without previous therapy with other ALK TKIs. Moreover, this analysis also includes data on intracranial activity of ceritinib in patients with treated and untreated neurologically stable brain metastases.

The updated analysis of ASCEND-1 includes 246 patients with ALK positive NSCLC. Of these patients, 163 (66%) were pretreated with another ALK TKI. Of these pretreated patients all had received crizotinib, and five patients had also received alectinib. Other ALK TKIs have not been used before. Most of the pretreated patients (91%) had progressive disease on or within 2 weeks of the last dose of the previous ALK TKI. Baseline characteristics were consistent with those reported in other ALK TKI studies (7,8), and were irrespective of previous ALK TKI therapy. Briefly, most patients were heavily pretreated. In the ALK TKI naive cohort 81% of patients have previously received one or more lines of chemotherapy. In the ALK TKI pretreated population 84% of patients have additionally received one or more lines of chemotherapy. At study entry, half of the patients had asymptomatic or controlled brain metastases and 67% of these patients have previously received brain irradiation. On the basis of investigator assessment the proportion of ALK TKI naive patients who achieved an overall response was 72% (95% CI, 61–82). The proportion for ALK TKI pretreated patients was 56% (95% CI, 49–64). Median time to response was 6.1 weeks for both cohorts and therefore corresponds to the first radiographic evaluation. In addition to responses according to RECIST criteria, most of the patients in both groups reached a certain degree of tumor reduction when comparing measurable disease at baseline and one post-baseline assessment. For ALK TKI naive patients, median duration of response (DoR) was 17.0 months [95% CI, 11.3–non-estimable (NE)] and median PFS was 18.4 months (95% CI, 11.1–NE). Patients previously treated with another ALK TKI exhibited a median DoR of 8.3 months (95% CI, 6.8–9.7) and a median PFS of 6.9 months (95% CI, 5.6–8.7). In a prespecified exploratory analysis of the overall survival (OS), the median has not yet been reached (95% CI, 19.6–NE) in the ALK TKI naive patients and was 16.7 months (95% CI, 14.8–NE) in the ALK TKI pretreated population. It is important to mention that the results of the blinded independent review committee confirmed the investigator-assessed data.

With regard to patients with brain metastases outcome in these 124 patients was similar to those of the overall patient population. A retrospective analysis of intracranial response to ceritinib included 94 patients with independently confirmed brain metastases. Based on RECIST 1.1, 36 patients (38%) of these patients had measurable intracranial lesions at baseline (8 patients ALK TKI naive and 28 pretreated patients). The majority of these patients (69%) have received previous radiotherapy to the brain. Intracranial disease control was documented in 79% (95% CI, 54–94) of ALK TKI naive patients and 65% (95% CI, 54–76) of ALK TKI pretreated patients. Intracranial response rates in patients who had previously received radiotherapy to the brain were similar to those of patients not treated with brain irradiation.

Median duration of exposure to ceritinib 750 mg daily for all 246 patients was 38.6 weeks with a median average daily dose of 664.2 mg and a median relative dose intensity of 82.8%. Overall, 181 patients (74%) had at least 1 dose interruption, and 152 patients (62%) had at least 1 dose reduction. One fifth of patients were in need of 2 dose reductions. At least one adverse event (AE) was reported for all patients in this study with 97% of patients being reported as having a study drug related AE. At least one grade 3–4 AE was reported in 81% of patients and at least one serious adverse event (SAE) was reported in 48% of patients. Treatment-related grade 3–4 AEs were reported in 51% of patients and treatment-related SAEs were reported in 12% of patients. The most common grade 3–4 AEs were gastrointestinal toxicities (diarrhea, nausea, vomiting), increased liver enzymes, increased lipase serum levels and hyperglycemia. Twenty-six patients (11%) discontinued treatment due to AEs, of which 35% were suspected to be related to ceritinib. Two on-treatment deaths were deemed to be related to study drug, one due to interstitial lung disease and the other due to multiorgan failure that occurred in the context of infection and ischemic hepatitis. In summary, the updated report of ASCEND-1 is important as it confirms clinical activity of ceritinib in patients with ALK rearranged NSCLC patients that have progressed in previous therapy with crizotinib and therefore confirm the role of ceritinib as an effective second-line treatment option. Moreover, the reported activity of ceritinib in patients with brain metastases both in the brain and extracerebral is important in a population that has a high rate of intracerebral metastases.

Further trials confirmed the role of ceritinib in the setting of ALK positive NSCLC. The ASCEND-3 study was a phase II study of ceritinib in previously treated ALK TKI naive patients. Updated results have recently been

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presented during the ESMO congress in Copenhagen (9). This single-arm phase II study included 124 patients with ALK positive NSCLC that were ALK TKI naive and have had up to three lines of previous chemotherapy, asymptomatic or neurologically stable brain metastases and a WHO performance status of 0 to 2. Patients were treated with ceritinib 750 mg daily until disease progression or unacceptable toxicity. After a median follow-up of 25.9 months 48.4% of patients were still on study drug. The ORR by independent review was 63.7% (95% CI, 54.6–72.2) and the median PFS was 18.4 months (95% CI, 10.9–26.3). The median OS as not reached at the current data cut-off. The survival rate at 24 months was 67.5% (95% CI, 58.0–75.2). A decrease in tumor burden from baseline was shown in 108/114 patients (94.7%). ORR for patients with brain metastases at baseline (n=49) was 57.1% (95% CI, 42.2–71.2) compared to 74.7% (95% CI, 63.3–84.0) for patients without brain metastases. The respective PFS rates were 10.8 months (95% CI, 7.3–16.6) and 19.6 months (95% CI, 14.5–not reached), respectively. The documented intracranial disease control rate was 76.9% (95% CI, 46.2–95.0).

The ASCEND-5 trial was a randomized open-label trial for patients with metastatic ALK positive NSCLC previously treated with crizotinib and one or two prior lines of chemotherapy regimens including a platinum-based doublet chemotherapy (10). Patients were randomized to either chemotherapy (pemetrexed or docetaxel) or ceritinib 750 mg once daily. Cross-over from chemotherapy to ceritinib was allowed following confirmed progressive disease according to blinded, independent review committee. The current report is based on a median duration of follow-up of 16.5 months. Sixty-four point seven percent of patients on chemotherapy crossed over to ceritinib. Median PFS was 1.6 vs. 5.4 months with a hazard ratio (HR) of 0.49 (95% CI, 0.36–0.67). With a P value of <0.001 ceritinib significantly prolonged PFS compared to chemotherapy. At the data cut-off, median OS was similar for both arms. Ceritinib also significantly improved the ORR (6.9% vs. 39.1%).

In conclusion, current evidence supports the use of ceritinib in crizotinib-pretreated ALK positive NSCLC patients. There is a well-documented activity of drug in patients with brain metastases that is a frequent localization of the disease in this patient population (11). Ceritinib is currently approved for ALK-positive adenocarcinoma patients progressing on crizotinib. As discussed in this overview, ceritinib also showed intriguing activity in ALK positive NSCLC patients that were ALK TKI naive. Several other ALK TKIs (e.g., alectinib, brigatinib, ensartinib, entrectinib, lorlatinib) are currently investigated in clinical trials. Alectinib is one of the most advanced new generation ALK TKI and was recently approved by the FDA for the treatment of patients with ALK positive metastatic NSCLC who have progressed on or are intolerant to crizotinib (12). This approval was based on two single-arm trials including 225 patients treated with alectinib 600 mg orally twice daily (8,13). The ORRs by independent review committee in these studies were 38% (95% CI, 36–52) and 44% (95% CI, 36–53); the median DoR were 7.5 and 11.2 months. In a pooled analysis of 51 patients with measurable disease in the central nervous system at baseline, the CNS ORR was 61% (95% CI, 46–74); the CNS DOR was 9.1 months. The results of a randomized trial conducted in Japan, J-ALEX, assessing alectinib 300 mg BID versus crizotinib in 207 ALK inhibitor-naive patients with ALK positive NSCLC, were recently reported to show a PFS advantage for alectinib over crizotinib (14). The ALEX study will determine whether similar findings are observed in a global population treated with alectinib 600 mg BID.

Studies are still needed to address optimal sequencing of ALK inhibitors in the treatment of patients with metastatic ALK-positive NSCLC.

Acknowledgements
None.

Footnote
Conflicts of Interest: SI Rothschild received honoraria from Novartis, Pfizer and Roche for advisory boards and invited talks.

References


Cite this article as: Rothschild SI. Clinical data and role of ceritinib a second-generation ALK tyrosine kinase inhibitor for the treatment of ALK positive non-small cell lung cancer. Transl Cancer Res 2016;5(Suppl 6):S1173-S1176. doi:10.21037/tcr.2016.11.66
Lung cancer accounts for a leading cause of cancer mortality worldwide. Patients with non-small cell lung cancer (NSCLC) harboring activating mutations in the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) fusion genes benefit from treatment with EGFR tyrosine kinase inhibitors (TKIs) and ALK-TKIs, respectively. Recently, NSCLC harboring ROS1 or RET fusion genes were also found to be sensitive to respective TKIs. In addition, mesenchymal-to-epithelial transition (MET) protein overexpression and MET amplification have been shown in NSCLC irrespective of EGFR mutation status (1,2).
MET inhibitors including antibodies and TKIs for NSCLC have been investigated in clinical trials. Although MetMAb (onartuzumab) was most hopeful antibody, a phase III study comparing erlotinib plus onartuzumab with erlotinib alone in MET positive NSCLC by an immunohistochemistry (IHC) assay did not show its efficacy (J Clin Oncol 2014;32:abstr 8000). Furthermore, onartuzumab did not confer any clinical benefit in the MET IHC-positive squamous cell NSCLC when combined pacitaxel plus platinum (3). In case of MET-TKI, the result of a phase III study comparing tivantinib (ARQ 197) plus erlotinib (n=526) with erlotinib alone (n=522) was already published (4). Forty-seven point four percent (231/476) of tumor samples had high MET expression, which was defined if intensity on IHC was >2+ in >50% of tumor cells. Eleven point four percent (54/476) had MET copy number >4 and only four patients had MET amplification with MET to chromosome 7 centromere (MET:CEP7) ratio >2. Although tivantinib plus erlotinib increased progression-free survival (PFS) (median PFS, 3.6 vs. 1.9 months; P<0.001), overall survival (OS) was similar (median OS, 8.5 vs. 7.8 months; P=0.81). OS might be improved in patients with MET IHC high expression (hazard ratio: 0.70; 95% CI: 0.49–1.01) (4). Thus, MET-TKI did not seem to be so beneficial for MET IHC-positive NSCLC as was EGFR-TKIs for EGFR-mutant NSCLC.

Under the concept that aberrant MET signaling can cause cancer, activating point mutations of MET were proved to occur in human renal, hepatocellular, and gastric carcinomas (1,2). MET mutations were also clonally selected for during the metastasis of human head and neck cancers, as their frequency increased from 2% in the primary tumors to 50% (5). In NSCLC specimen, there was an alternative splice variant with the 47-amino acid exon 14 (juxtamembrane domain) missing in-frame from the MET (6). The skipped transcript produces a constitutively active MET that lacks an E3 ubiquitin protein ligase (Cbl) promoting MET degradation (7,8). Exon 14 skipping in MET was found in 4.3%, which was more than a total (3.9%) of ALK (1.3%), ROS1 (1.7%), and RET (0.9%) fusions in 230 lung adenocarcinoma (9). Frequencies of driver oncogene aberrations in 319 Japanese lung adenocarcinoma was 53.0% in EGFR, 3.8% in ALK, 1.9% in RET, 0.9% in ROS1, and 2.8% in skipping of MET exon 14, which was less than ALK fusion alone (3.8% vs. 2.8%) (10). Thus, we need to know clinical and genomic backgrounds with NSCLC harboring MET exon 14 mutation.

Awad et al. described unique clinical, molecular, and pathologic features in 28 (3.0%) patients with MET exon 14 mutations among 933 non-squamous NSCLC patients (7). Genomic deletions occurred in 17 (61%) of the 28 patients with MET exon 14 mutations, ranging in size from a 2-base pair deletion to a 193-base pair deletion, and point mutations occurred in 11 (39%). The median age at diagnosis was 72.5 years, 19 (68%) were women, 10 (36%) were never smokers, their stages I/II/III/IV were 13 (46%)/2 (2%)/4 (14%)/18 (64%), and their histologic subtypes were adenocarcinoma (64%), pleomorphic or sarcomatoid carcinoma with an adenocarcinoma component (14%), poorly differentiated NSCLC not otherwise specified (18%), and adenosquamous carcinoma (4%). Four patients with pleomorphic or sarcomatoid histology and MET exon 14 mutations represented 26.7% of 15 total patients with pulmonary sarcomatoid carcinoma. Liu et al. also reported that MET mutations exon 14 were identified in 8 (22%) of 36 pulmonary sarcomatoid carcinoma (11). The patients with MET exon 14 mutations were older than patients with EGFR- and KRAS-mutant NSCLC, were more likely than those with KRAS mutations to be never-smokers and more likely than those with EGFR mutations to have a history of tobacco use (7). A higher percentage of patients with MET exon 14 mutations had stage I disease compared with those with EGFR or KRAS mutations.

All 28 NSCLC harboring the MET exon 14 in the Awad' cohort were white, non-Hispanic (7). According to a report from China, MET exon 14 skipping occurred in only 0.9% of lung adenocarcinomas, which was less than half the frequency previously observed in white patients (3%) (12). MET exon 14 mutations occurred at a young median age, 59 years in Chinese patients with stage IV adenocarcinoma, which was similar to the median age of patients with ALK and ROS1 rearrangements. Liu et al. suspected that ethnic difference between Western and Chinese patients could explain the variation. Another report showed that MET exon 14 skipping was detected in 1.3% (23/1,770) of the NSCLC and in 1.6% (21/1,305) of adenocarcinoma in Chinese patients (13). Because MET exon 14 mutation was reported in occurred in 2.8% of Japanese lung adenocarcinomas (10), the difference might be caused by the detection methods. Identifying the intrinsic mutations responsible for MET exon skipping using genomic DNA is difficult because of their highly diverse locations and the occurrence of passenger mutations (8). In 271 Asian NSCLC (stage I mainly) resected at Korean hospital, 1.8% had exon 14 mutation in MET (14). Although the ethnicity was not described, 19% (10/54) in never-smoking NSCLC patients without EGFR, KRAS, ROS1,
Table 1 Characteristics of MET exon 14 mutated lung cancer patients treated with MET-tyrosine kinase inhibitors

<table>
<thead>
<tr>
<th>Patient</th>
<th>MET amplification</th>
<th>MET IHC</th>
<th>MET inhibitor</th>
<th>Response</th>
<th>PFS (months)</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Smoking, pack-year</th>
<th>Histology</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>NA</td>
<td>Crizotinib</td>
<td>PR</td>
<td>≥6</td>
<td>73</td>
<td>M</td>
<td>F, 45</td>
<td>Sq</td>
<td>(15)</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>+*</td>
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<td>PR</td>
<td>8</td>
<td>76</td>
<td>W</td>
<td>F, 12</td>
<td>Sq</td>
<td>(20)</td>
</tr>
<tr>
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<td>PR</td>
<td>11</td>
<td>84</td>
<td>W</td>
<td>N, –</td>
<td>Sarcoma¹</td>
<td>(16)</td>
</tr>
<tr>
<td>4</td>
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<td>3+</td>
<td>Capmatinib</td>
<td>PR</td>
<td>≥5</td>
<td>82</td>
<td>W</td>
<td>F, 25</td>
<td>La</td>
<td>(16)</td>
</tr>
<tr>
<td>5</td>
<td>2.3 (MET:CEP7)</td>
<td>3+</td>
<td>Capmatinib</td>
<td>PR</td>
<td>13</td>
<td>66</td>
<td>W</td>
<td>F, 45</td>
<td>Sq</td>
<td>(16)</td>
</tr>
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<td>6</td>
<td>6 copy gain</td>
<td>3+</td>
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<td>SD</td>
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<td>80</td>
<td>W</td>
<td>N, –</td>
<td>Ad</td>
<td>(17)</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>3+</td>
<td>Crizotinib</td>
<td>PD**</td>
<td>3.6</td>
<td>80</td>
<td>M</td>
<td>F, 20</td>
<td>Ad</td>
<td>(17)</td>
</tr>
<tr>
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<td></td>
<td>Crizotinib</td>
<td>PR</td>
<td>≥4.6</td>
<td>65</td>
<td>M</td>
<td>C, 20</td>
<td>Ad</td>
<td>(17)</td>
</tr>
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<td>–</td>
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<td>Crizotinib</td>
<td>PR</td>
<td>≥3.1</td>
<td>90</td>
<td>W</td>
<td>N, –</td>
<td>Ad</td>
<td>(17)</td>
</tr>
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<td>PR</td>
<td>8</td>
<td>64</td>
<td>W</td>
<td>N, –</td>
<td>Ad</td>
<td>(7)</td>
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<td>PR</td>
<td>≥3</td>
<td>74</td>
<td>W</td>
<td>F, –</td>
<td>Sarcoma¹</td>
<td>(11)</td>
</tr>
<tr>
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<td>–</td>
<td>3+</td>
<td>Crizotinib</td>
<td>PD</td>
<td>1</td>
<td>45</td>
<td>M</td>
<td>C, –</td>
<td>Ad</td>
<td>(12)</td>
</tr>
<tr>
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<td>76</td>
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<td>N, –</td>
<td>Ad</td>
<td>(12)</td>
</tr>
<tr>
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<td>Crizotinib</td>
<td>NE³</td>
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<td>86</td>
<td>M</td>
<td>N, –</td>
<td>Ad</td>
<td>(21)</td>
</tr>
<tr>
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<td>Crizotinib</td>
<td>PR</td>
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<td>68</td>
<td>W</td>
<td>F, 24</td>
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<td>(22)</td>
</tr>
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<td>PR</td>
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<td>71</td>
<td>M</td>
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<td>PR</td>
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<td>F, –</td>
<td>Sq</td>
<td>(24)</td>
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<td>Crizotinib</td>
<td>PR</td>
<td>24</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>(18)</td>
</tr>
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<td>PR</td>
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<td>(18)</td>
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<td>(18)</td>
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<tr>
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<td>NA</td>
<td>Crizotinib</td>
<td>SD</td>
<td>≥4</td>
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<td>NA</td>
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<td>(18)</td>
</tr>
<tr>
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<td>NA</td>
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<td>PR</td>
<td>NA</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>(18)</td>
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<tr>
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<td>CR</td>
<td>≥3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>(18)</td>
</tr>
<tr>
<td>25</td>
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<td>NE¹</td>
<td>NA</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>(18)</td>
</tr>
<tr>
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<td>PR</td>
<td>13</td>
<td>67</td>
<td>W</td>
<td>N, –</td>
<td>Ad</td>
<td>(25)</td>
</tr>
</tbody>
</table>

*, after treatment with crizotinib; **, PR in primary and PD in liver metastasis; ††, tumor shrinkage +; †, pathological CR; ‡, histiocytic sarcoma; †, pulmonary sarcoma. NA, not available; IHC, immunohistochemistry; PR, partial response; SD, stable disease; PD, progressive disease; CR, complete response; NE, not evaluable; M, man; W, woman; F, former smoker; N, never smoker; C, current smoker; Sq, squamous cell carcinoma; Ad, adenocarcinoma; La, large cell carcinoma. MET, mesenchymal-to-epithelial transition.

BRAF, or ERBB2 (15), 3% (131/4,402) (16), 3% (8/178) (17), 2.8% (205/7,140) (18), and 2.9% (2/70) (19) in lung adenocarcinoma. Thus, overall 1–4% of lung adenocarcinoma may have MET exon 14 mutation, which should be investigated in all NSCLC subtypes including squamous cell, large cell, and sarcomatoid carcinomas (Table 1), especially without other druggable mutations.

Next-generation sequencing (NGS) also clarified genomic alterations such as KRAS, EGFR, ERBB2, BRAF and TP53 mutations; ALK, ROS1, and RET fusions; MET and MDM2 amplifications in the same specimens (7). Although none of the 28 patients with MET exon 14...
After we have found MET exon 14 mutations in NSCLC with accuracy, we should elucidate whether MET-TKI was effective in such patients or not. The characteristics of MET-TKI-treated NSCLC patients with MET exon 14 mutation were summarized in Table 1, to my knowledge in the literatures. The response to MET-TKIs is shown in Figure 1. Twenty-six patients were treated with MET-TKIs (23 crizotinib, 2 capmatinib; 1 cabozantinib). The responses, complete response (CR)/partial response (PR)/stable disease (SD)/progressive disease (PD)/not evaluable (NE), were observed in 2/18/2/2/2, respectively. Overall response rate was 77% (20/26). MET, mesenchymal-to-epithelial transition; TKI, tyrosine kinase inhibitors.

Figure 1 The response to MET-TKIs is shown. Twenty-six patients were treated with MET-TKIs. The responses, complete response (CR)/partial response (PR)/stable disease (SD)/progressive disease (PD)/not evaluable (NE), were observed in 2/18/2/2/2, respectively. Overall response rate was 77% (20/26). MET, mesenchymal-to-epithelial transition; TKI, tyrosine kinase inhibitors.
are needed in order to define the activities of various MET-TKIs for central nervous system metastasis.

Clinical trials of MET-TKIs in NSCLC with MET exon 14 mutations have been conducted. Current studies are available from ClinicalTrials.gov (https://clinicaltrials.gov/ct2/search/index). In the study of ‘Targeted therapy directed by genetic testing in treating patients with advanced refractory solid tumors or lymphomas (NCT02465060; NCI-MATCH)’ is using crizotinib for MET exon 14 mutations. A study of capmatinib (INC280) in NSCLC patients with MET exon 14 alterations who have received prior MET inhibitor is for MET inhibitor-resistant NSCLC (NCT02750215). A phase II study of HMPL-504 (AZD6094, savolitinib) in lung sarcomatoid carcinoma is for MET exon 14 mutation who has failed prior systemic therapy (NCT02897479). A phase II study in lung adenocarcinoma harboring MET exon 14 skipping alterations is using tepotinib (MSC2156119J) (NCT02864992). A phase II study of glesatinib (MGCD265) in patients with NSCLC is for activating genetic alterations in MET (mutation or amplification) (NCT02544633). The studies will clarify whether a variety of MET-TKIs may be useful for NSCLC with MET exon 14 mutations in various situations.

One of the EGFR or ALK-TKI resistant mechanisms is composed of hepatocyte growth factor (HGF)/MET signal activation (29-31). MET activation is induced by binding to its ligand, HGF and mediates cell scatter, growth, proliferation, transformation, and morphogenesis (32,33). MET interacts with several molecules including PI3K and SRC. Thus, excess ligand or bypass signals can abolish the targeted drugs blocking the original oncogene driver and MET signaling is very important in drug resistance. In addition, alterations of MET itself were expected to participate in MET-TKI resistant mechanisms (34,35). Two reports of crizotinib-resistant MET exon 14 mutant NSCLC were described (20,25). An acquired mutation, D1228N in exon 19 of MET, was found at time of progression on crizotinib in a patient with the original exon 14 skipping D1010H mutation (20). Analysis of circulating tumor DNA revealed that Y1230C resistance mutation in MET activation loop occurred in MET D1010H mutant NSCLC post-progression on crizotinib (25). Most MET-TKIs are categorized as three types differing in their mode of binding site in ATP binding pocket in MET kinase. Type I (e.g., crizotinib, capmatinib, tepotinib), type II (e.g., merestinib, cabozantinib, glesatinib) and type III (e.g., MT3) are all ATP competitive inhibitors although tivantinib inhibits ATP binding to the MET kinase in a non-competitive manner (1). Thus, MET-TKI resistance mutations in ATP binding sites (34,35) were expected as T790M in EGFR, and L1196M and G1269A in ALK. MET kinase sites bound to type I MET inhibitors were important interaction sites in Y1230 and D1228 (1,20). Because type II MET inhibitors occupy the ATP binding pocket but also extend into a second pocket that is formed when the side chain of D1222 instead points away from the ATP binding pocket (1), they may be useful for MET secondary mutant NSCLC (20,25). In preclinical tests, a newly developed MET antibody (KTN0073-IgG2), was identified as a potential therapeutic for the treatment of NSCLC with MET exon 14 mutation (36) although it has not been investigated in MET-TKI resistant circumstances. Drugs to overcome MET-TKI resistant NSCLC with MET exon 14 mutation should be developed.

In conclusion, discovery of MET exon 14 mutation in NSCLC similarly to EGFR mutation and ALK fusion was breakthrough because it was targetable oncogenic driver. NSCLC harboring MET exon 14 mutation occupy approximately 1–4% containing adenocarcinoma and other histologic subtypes. Although MET-TKIs have been useful in such a situation, we should wait for the results of ongoing clinical trials for the selected patients. Also, we should clarify the mechanisms of acquired resistance to MET-TKIs which are just beginning to be understood. The therapeutic strategies against drug resistance and intracranial metastasis in NSCLC with MET exon 14 mutation patients should be investigated.

Acknowledgements

None.

Footnote

Conflicts of Interest: The author has received honoraria from Pfizer Inc., Japan.

References


27. Dempke WC, Edvardsen K, Lu S, et al. Brain Metastases in NSCLC - are TKIs Changing the Treatment Strategy?

Cite this article as: Takigawa N. MET-inhibitors meet MET mutations in lung cancer. Transl Cancer Res 2016;5(Suppl 6):S1248-S1254. doi: 10.21037/tcr.2016.11.61
In The Journal of Clinical Oncology, Ying Cheng and colleagues (1) have recently reported the results of a phase II randomized trial comparing pemetrexed plus gefitinib vs. gefitinib in treatment-naive, East Asian patients, with advanced non-squamous non-small cell lung cancer (NSCLC) and activating epidermal growth factor receptor (EGFR) mutations. The study met its primary end-point in the intent-to-treat population, showing a significantly longer median progression free survival (PFS) in favors of the combination arm (15.8 months) compared to single agent arm (10.9 months) [hazard ratio (HR): 0.68; 95% CI, 0.48 to 0.96; one-sided P=0.014; two-sided P=0.029]. The significant improvement in PFS was independent from the specific type of mutation (EGFR exon 19 deletion vs. EGFR exon 21 L858R point mutation). The addition of pemetrexed to gefitinib resulted also in a significantly longer time to progressive disease (16.2 vs. 10.9 months; HR: 0.66; 95% CI, 0.47 to 0.93) and duration of response (15.4 vs. 11.3 months; HR: 0.74; 95% CI, 0.50 to 1.08), while no differences in response rate (RR: 80% vs. 74%) were observed between the two treatment arms. As attended, the percentage of patients who reported grade 3–4 drug-related adverse-events (AEs) was significantly higher (42% vs. 19%) in the combination arm, as well as the proportion of patients who discontinued treatment because of AEs nearly doubled with pemetrexed plus gefitinib compared to single agent arm.

Several randomized phase III studies (2-10) previously showed that EGFR-tyrosine kinase inhibitors (TKIs) significantly improve both RR, PFS and quality of life (QoL) compared to platinum-based doublets chemotherapy as first-line treatment of EGFR-mutated NSCLC patients. Subsequently a pooled analysis of both LuxLung3 and LuxLung6 trials has also shown an overall survival (OS) benefit in favor of the EGFR-TKI Afatinib, even if it was limited to the subgroup of patients with EGFR exon 19 deletion (9). Overall, the results of all such studies convincingly and consistently demonstrated that for the subgroup of patients whose tumors harbor an EGFR activating mutation, the optimal strategy is starting with an EGFR-TKI, including gefitinib, erlotinib, or afatinib (11,12).

The trial conducted by Ying Cheng and colleagues (1) suggests that the addition of chemotherapy to the EGFR-TKI may further improve the outcomes of EGFR-mutated, non-squamous NSCLC patients. Pre-clinical studies have shown a potential synergism between the EGFR-TKI, erlotinib, and the multi-targeted antifolate pemetrexed in NSCLC cell-lines (13,14). The modulation of both EGFR and Akt phosphorylation, together with a significant decrease of thymidylate synthase (TS) expression and activity in all NSCLC cells, represent the molecular mechanisms underlying such synergistic interaction. Later, early phase I-II studies demonstrated both a promising activity and a tolerable safety profile of EGFR-
TKI plus pemetrexed combination in pre-treated NSCLC patients (15), with a significantly longer PFS compared to either drug alone in a clinically selected population of never-smokers with non-squamous histology (16).

Several phase III studies investigated the efficacy of EGFR-TKI in combination with chemotherapy in first-line treatment (17-20), showing no survival benefit with combinations, likely because wild-type patients were also enrolled. Among these, CALGB30406 study (21) evaluated erlotinib with and without platinum-chemotherapy in clinically selected patients with advanced lung adenocarcinoma who were never or light former smokers, showing similar efficacy between the two treatment arms in the overall study population. A subsequent EGFR-mutation analysis revealed that patients with EGFR-positive tumors were most likely to benefit, reaching a median PFS of 14.1 months, and OS of 31.3 months with erlotinib, even higher (PFS: 17.2 months, OS: 38.1 months) in the combination arm. Such data suggested that EGFR-TKIs synchronously combined with chemotherapy could improve survival in molecular selected subsets of patients. Similarly the FASTACT2 randomized phase III study (22) also showed a survival benefit of a first-line intercalated regimen of chemotherapy and erlotinib in EGFR-mutated NSCLC patients.

Recently the NEJ005 randomized phase II study (23) prospectively compared concurrent gefitinib plus carboplatin/pemetrexed regimen vs. sequential alternating regimen in East-Asian, EGFR-mutated NSCLC patients. The results of such study showed a favorable trend in PFS (18.3 vs. 15.3 months; HR: 0.71; 95% CI, 0.42–1.20; P=0.20) and a significant improvement in OS (41.9 vs. 30.7 months; HR: 0.51; 95% CI, 0.26–0.99; P=0.042), in favor of the concurrent regimen arm, first demonstrating the superiority of the upfront combination of gefitinib and carboplatin/pemetrexed, which is currently investigated in the ongoing phase III NEJ009 study. The trial conducted by Ying Cheng and colleagues (1) suggested that adding single-agent chemotherapy to EGFR-TKI in first-line may be sufficient to improve outcomes of EGFR-mutated patients. The results are intriguing, but need to be interpreted in light of the recent NEJ005 study. The PFS improvement is consistent across both studies, and is more favorable in comparison to the 9–10 months PFS observed in previous studies of first-line gefitinib monotherapy in EGFR-mutated NSCLC patients (2). It could be likely related to the activity of early concurrent use of cytotoxic agents against de-novo resistance alterations, but the lack of tissue samples collection for biomarker analysis, limited the possibility to evaluate molecular data. However, it will be important to see whether the addition of pemetrexed to gefitinib will also lead to an OS benefit.

Indeed, OS improvement is crucial in order to evaluate the optimal treatment sequence in this setting of patients, and ultimately accept the increased adverse events and cost of a potential upfront combination. Even if authors declare that “platinum-based therapies may still be used after progression”, the patients included in the experimental arm will never receive the standard treatment option, which is platinum-pemetrexed combination followed by pemetrexed maintenance therapy (24), and this could negatively affect their final OS.

Furthermore we need to discuss the clinical benefit obtained with chemotherapy plus gefitinib combination considering the other promising treatment option emerging in this setting.

The addition of bevacizumab to the EGFR-TKI, Erlotinib, reached a median PFS: 16 vs. 9.7 months of erlotinib monotherapy, with about 50% significant reduction of the risk of progression [HR: 0.54 (0.36–0.79)], in East-Asian, EGFR-mutated patients (25). Waiting for the randomized phase III studies currently ongoing both in Asian and Caucasian populations, such combination has recently received the approval by both Food and Drug Administration (FDA) and European Medical Agency (EMA) as first-line treatment. Even more exciting are the data emerging from the first-line cohort of AURA phase I trial (26), which showed an impressive activity of the third generation EGFR-TKI osimertinib, with a median PFS: 19 months and an ORR: 77%, leading to the ongoing phase III randomized FLAURA trial comparing osimertinib vs. gefitinib/erlotinib in first-line. Despite immunotherapy with anti-PD1/PDL1 single agent seems to be not effective in NSCLC harboring EGFR-mutations (27), several trials are currently investigating potential combinations of checkpoint-inhibitors with EGFR-TKI, in order to further improve the outcomes of these patients.

In conclusion the study of Cheng et al. represent a significant attempt to the improvement of first-line treatments for EGFR-mutated NSCLC patients. The PFS benefit together with a modest increase in toxicity suggest that adding chemotherapy to EGFR-TKI may represent an effective treatment option in this setting. However, as mentioned before, OS benefit is crucial in order to confirm the effectiveness of Pemetrexed plus Gefitinib upfront combination. Finally, considering the advent of new
promising drugs/combinations, the main challenge will be how to combine all these agents and ultimately define the optimal treatment sequence for EGFR-mutant NSCLC patients.

**Acknowledgements**

None.

**Footnote**

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

**References**

ALK rearrangements in lung cancer (LC) were discovered in the year 2007 upon the systematic search for novel LC-associated oncogenes (1,2). Fortunately, an experimental MET inhibitor, PF-2341066 (crizotinib), was by then known to have a concurrent ALK-inhibiting activity and its clinical profile was already under phase I evaluation (3-6). It was quickly revealed that the status of ALK, but not MET, is a primary determinant of tumor sensitivity to crizotinib (5), and a number of subsequent studies heralded a real breakthrough in the treatment of ALK-rearranged cancers (6-9).

Almost all pivotal trials involving ALK inhibitors relied on a companion fluorescent hybridization in situ (FISH) break-apart assay for the detection of ALK rearrangements. FISH is perfectly compatible with the routine of histopathological diagnosis of LC and is capable to detect all variants of ALK translocations. However, FISH is cumbersome and prohibitively expensive, therefore many laboratories now utilize immunohistochemical (IHC) prescreening for ALK-overexpressing LC in order to reduce the number of tumors forwarded to FISH-testing. For the time being, the majority of clinical decisions regarding the administration of ALK inhibitors is based on FISH or IHC/FISH testing, with thousands of patients receiving ALK-specific treatment worldwide. It is important to bear in mind that IHC/FISH, being proficient in establishing the mere fact of the presence of ALK translocation in the tumor, are unable to inform on the exact molecular structure of the detected ALK rearrangements (10-14).

There are a few dozen of distinct variants of ALK fusions and the novel types of chimeras continue to be identified (6,15-17). All ALK rearrangements preserve tyrosine kinase domain, with the breakpoint usually occurring before the exon 20. However, the gene partners and the composition of 5'-terminal part of the chimeric protein vary substantially, and at least some translocation variants demonstrate significant differences in sensitivity to crizotinib in laboratory experiments (18). The potential clinical significance of these differences remains largely uncertain, owing to the fact that ALK-specific inhibitors are usually prescribed solely on the basis of FISH-test result, and the ALK variant subtyping is not required for the drug administration (10-14).

Recently published study of Yoshida et al. (19) demonstrates that the diagnostic attitude towards ALK translocations has to change, at least on the level of clinical investigations. Yoshida et al. (19) analyzed crizotinib treatment outcomes in 35 patients with distinct EML4-ALK translocations. The median progression-free survival (PFS) in 19 patients with the variant 1 fusion (E13;A20) approached to 11.0 months, while PFS in 16 patients carrying other EML4-ALK rearrangements was only 4.2 months. Statistical analysis...
confirmed the significance of this difference. These data have potential practical importance, as they may impact the sequence of targeted and cytotoxic therapies. For example, there are two major types of EGFR mutations in LC, ex19del and L858R, with the former rendering more pronounced tumor response to EGFR inhibitors than the latter. Accordingly, patients with EGFR ex19del survive significantly longer when afatinib is administered in the first line, whereas a chemotherapy may be considered as an upfront treatment option for the patients carrying the L858R (20). It remains to be addressed whether similar trend is applicable to the patients with distinct ALK translocations.

The study of Yoshida et al. (19) considered only known EML4-ALK fusions, while some other gene partners may be involved in ALK rearrangements as well (6,15-17). The mechanistic basis for the distinct duration of clinical response to crizotinib for LC carrying distinct ALK translocations is unknown. One hypothesis relies on the role of 5'-terminal portion of ALK chimeras in the protein oligomerization. It is also possible that the genetic variants of ALK translocations may have distinct propensity to acquire secondary mutations or provoke the bypass signaling pathways associated with the drug resistance. In addition, there is a question whether the correlations described by Yoshida et al. (19) are applicable to the novel ALK inhibitors, such as alectinib, ceritinib, brigatinib, lorlatinib, etc. (17).

The study of Yoshida et al. (19) illustrates an important gap in current diagnostic practices towards ALK translocations. Although polymerase chain reaction (PCR)-driven detection of ALK fusions is appreciated by many investigators due to its high sensitivity and ability to identify the translocation variant, its use in clinical routine is somehow discouraged (10-14). To our knowledge, Japan is the only country where the use of PCR for ALK detection is considered non-inferior to other testing methods (21); therefore it is not surprising that the first study emphasizing the significance of ALK genotyping came from this country (19). It is fair to acknowledge that commercial PCR kits usually target only the most common variants of ALK rearrangements, therefore, in contrast to FISH, rare ALK translocations are likely to be missed [for example, see descriptions for the Entrogene EML4-ALK Fusion Gene Detection Kit (http://entrogene.com/web3/eml4-alk-fusion-gene-detection-kit/), AmoyDx® EML4-ALK Fusion Gene Detection Kit (http://www.mobitec.com/cms/products/bio/09_iva/Real-Time_ PCR_Cancer_Diagnostic_Kits.html?pdf=ADx-AE02.pdf), QuanDx EML4-ALK Fusion Gene Detection Kit (http://www.quandx.com/sites/quandx.com/files/images/EML4-ALK%20flyer%20v3.0.pdf), Diacarta QFusion™ EML4-ALK and KIF5B-ALK Fusion Gene Detection Kit (http://www.diacarta.com/products/fusion-gene-tests/alk-fusion-gene-detection-kit/), etc.]. This limitation, however, can be overcome by PCR test for unbalanced ALK 5'/3'-end expression, which detects all types of rearrangements (15). Opponents of PCR-based ALK testing also frequently state that this methodology is less standardized as compared to the FISH analysis. Furthermore, FISH, but not PCR, was used as a companion test in the registration trials of ALK inhibitors, therefore some commercial agreements between diagnostic companies and drug manufacturers are also likely to play a role.

As a result, there is a drastic difference in the knowledge on clinical use of EGFR and ALK inhibitors. Ample experience has been accumulated for LC carrying distinct EGFR mutations and their response to distinct EGFR inhibitors (22,23). In contrast, despite the fact that ALK variant typing is no more complicated than EGFR mutation analysis, the data on genotype-response correlations for ALK-specific drugs remain very scarce. Similar limitations apply to the newly approved indication for crizotinib, i.e., ROS1-rearranged LC (24,25). We call to reconsider current approaches to the diagnostic translocation testing in human tumors and to encourage the identification of the involved gene fusion variants.

Acknowledgements

Funding: This work has been supported by the Russian Scientific Fund (grant number 16-15-10396).

Footnote

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

References


At the time of acquired resistance to the first generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), gefitinib, erlotinib or afatinib (1), 50–60% of EGFR mutant non-small cell lung cancer (NSCLC) patients have developed the secondary gatekeeper T790M point mutation on exon 20 of the EGFR gene (2,3). Osimertinib (AZD9291) is a third-generation EGFR TKI that targets EGFR mutant (including T790M positive) tumors (4). It was initially evaluated in EGFR mutant NSCLC patients who had disease progression after previous treatment with an EGFR TKI, in the phase I part of the phase I/II AURA study (5). A response rate of 51% was achieved for all patients treated across all dose levels. Among the 222 patients of the expansion cohorts, the response rate to osimertinib was 61% for EGFR T790M-positive patients while the EGFR T790M-negative patients were not able to achieve a response rate greater than 21% (Table 1). No dose limiting toxicities were observed at any dose level and, based on tumor growth inhibition, the dose of 80 mg once daily was selected for being further evaluated for the treatment of EGFR T790M-positive NSCLC patients (5). On November 13th 2015, and on February 3rd 2016, FDA and EMA approved osimertinib 80 mg once daily for the treatment of EGFR T790M-positive NSCLC patients, respectively, based on the data from two phase II studies (AURA extension and AURA2) and the AURA phase I expansion study. Sixty-three EGFR T790M-positive, dose expansion cohort patients receiving 80 mg of osimertinib once daily in the phase I part of the AURA study demonstrated an objective response rate (ORR) of 71% [95% confidence interval (CI), 57–82] and a median progression-free survival (PFS) of 9.7 months (95% CI, 8.3–13.6). In a pre-planned pooled analysis of the AURA extension phase II study and the AURA2 phase II study with a total of 411 EGFR T790M-positive patients, ORR was 66% and median PFS was 11.0 months (95% CI, 9.6–12.4) (6).

Goss et al., published in *Lancet Oncology* the final results of the phase II, open-label, single-arm AURA2 study, which assessed the efficacy and safety of osimertinib in patients with EGFR T790M-positive NSCLC, who had progressed after previous therapy with EGFR TKIs. In less than 6 months, more than 472 patients were screened, 210 EGFR T790M-positive patients were treated with osimertinib and 199 patients were evaluable for response analysis in the AURA II study (7). The FDA approved Cobas EGFR mutation test v2 was used for the central confirmation of the EGFR T790M mutation. An ORR of 70% and a disease control rate of 92% were achieved, as evaluated by blinded independent central review. Six
(3%) patients achieved complete response with osimertinib treatment and 134 (67%) achieved partial response. The median duration of response was 11.4 months (95% CI, 9.0–not calculable). There was a high concordance between the responses obtained by investigator assessment and by the blinded independent central review. The median PFS was 9.9 months (95% CI, 8.5–12.3) (7). The treatment was well tolerated with the most common treatment related grade 3–4 adverse events being prolonged electrocardiogram QT, decreased neutrophil count, and thrombocytopenia. Interstitial lung disease occurred in 2% of the patients.

Only 2 months after the publication of the AURA2 study, the results of the phase III AURA3 clinical trial became available (8). In this study, 419 EGFR T790M-positive NSCLC patients who had progressed during first-line were assigned in a 2:1 ratio to receive osimertinib or platinum-pemetrexed chemotherapy. Median PFS was significantly longer with osimertinib compared with chemotherapy [10.1 vs. 4.4 months; hazard ratio (HR) 0.30; 95% CI, 0.23–0.41; P<0.001] (8). Osimertinib-treated patients achieved an ORR of 71% (95% CI, 65–76) in comparison to an ORR of 31% (95% CI, 24–40) for those who received chemotherapy (odds ratio 5.39; 95% CI, 3.47–8.48; P<0.001) (8).

It was previously demonstrated that patients with the T790M mutation detected by plasma ctDNA respond equally to osimertinib as those whose mutation is detected in a tumor tissue biopsy (9-11). Similar findings were reported in the AURA3 trial (8). On September 29th, 2016, the FDA approved an expansion of the Cobas EGFR blood mutation test v2 to include testing of the T790M mutation in order to confirm the presence of the EGFR T790M mutation and qualify patients for treatment with osimertinib. Due to the relatively high false negative rates with plasma T790M testing, it is highly recommended that patients with a negative liquid biopsy for the presence of the T790M to be reevaluated for the feasibility of a tissue biopsy (10).

More than 30% of EGFR mutant NSCLC patients, whose disease progressed during or after first line EGFR TKI, have brain metastases (12). In comparison with other EGFR TKIs, including gefitinib, rociletinib or afatinib, osimertinib has demonstrated greater blood-brain barrier penetration in preclinical models (13). In the AURA2 trial, patients with brain metastases obtained an ORR of 69% (95% CI, 58–79) and a median PFS of 9.2 months (95% CI, 7.7–11.1) (7). In the AURA3 study, among the 144 patients who had central nervous system (CNS) metastases, median PFS was 8.5 months (95% CI, 6.8–12.3) for those treated with osimertinib and 4.2 months (95% CI, 4.1–5.6); HR 0.30; 95% CI, 0.21–0.49) (8). Interestingly, due to the high risk of development CNS metastases in EGFR mutant NSCLC, an agent with high blood-brain barrier penetration, AZD3759, has been recently developed and is currently being evaluated in a phase I clinical trial (14). Unavoidably, as happens with the first generation EGFR TKIs, osimertinib treated patients develop resistance to treatment after less than one year (8). An additional EGFR mutation, the C797S, can cause resistance to third generation EGFR TKIs and its allelic

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<tr>
<td>AURA: expansion cohort treated with 80 mg of osimertinib once daily</td>
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<td>AURA2</td>
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NR, not reached; DoR, duration of response; Ref., reference; vs. versus; HR, hazard ratio; OS, overall survival.
context can define sensitivity to subsequent treatments (15,16) (Figure 1).

Osimertinib is now being evaluated in the first line setting of EGFR mutant NSCLC patients. According to the results of two (80 and 160 mg) phase I expansion cohorts of the AURA study including 6 EGFR mutant patients, an overall median PFS of 19.3 months (95% CI, 13.7–not reached) and an ORR of 77% (95% CI, 64–87) were obtained (17). The phase III FLAURA study (NCT02296125) compares osimertinib with gefitinib or erlotinib in treatment-naïve EGFR mutant NSCLC patients. PFS in patients with tumors harboring T790M is a key secondary objective of the study (18). The coexistence of the pretreatment T790M mutation has been under appreciated, in spite of accumulative evidence that it is present in a frequency of 35–60%, using different detection methods (19-21). In the Spanish Lung Cancer Group (SLCG) and the European Thoracic Oncology Platform (ETOP) BELIEF trial, pretreatment T790M mutations were centrally identified in 34% of the patients who reached a median PFS of 16 months (95% CI, 13.1–not reached) with the combination of erlotinib plus bevacizumab (21). We have recently started the AZENT study (NCT02841579), an investigator initiated study that explores the safety and efficacy of osimertinib as first line therapy for patients with metastatic EGFR mutant NSCLC and concomitant pretreatment T790M mutation (Figure 1).

Without any doubt, osimertinib has made a breakthrough
in lung cancer therapy. Other third generation EGFR TKIs are in clinical development, including olmutinib (Hanmi Pharmaceutical Company), EGF816 (Novartis Pharmaceuticals), naquotinib (Astellas Pharma Global Development), PF06747775 (Pfizer) and avitinib (Hangzhou ACEA Pharmaceutical Research) (22,23). Still, there are several issues to be resolved in the treatment of EGFR mutant patients. One important issue is the discovery of the best therapeutic approach, besides chemotherapy, for those patients who are not EGFR T790M-positive at the time of progression to first and second generation EGFR TKIs. A second issue is that, even if osimertinib is an efficient treatment for T790M driven acquired resistance to initial EGFR inhibition, still the number of complete responses reported in the phase II and III clinical trials is very small, indicating that we are far from the cure of this disease. We have shown that co-targeting STAT3 and Src with EGFR can more efficiently abrogate tumor growth than EGFR inhibition alone (24). Resistance to first, second and third EGFR TKIs are heterogeneous and complex, evolving dynamically under the pressure of each generation’s inhibitor and is a challenge for the development of novel targeted combinations.

Acknowledgements

Funding: This work was funded by La Caixa Foundation and Red Tematica de Investigacion Cooperativa en Cancer (RTICC; grant RD12/0036/ 0072).

Footnote

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

References


Considering that, as recently as 10 years ago, physicians caring for patients with advanced lung cancer had only a handful of conventional cytotoxic agents from which to choose, the field’s recent development and competition seem truly remarkable. A prime example of this shifting and crowded landscape is immunotherapy. Since 2015, three different checkpoint inhibitors targeting programmed death 1 (PD-1) or PD-1 ligand (PD-L1) have received U.S. FDA approval, with several others currently in clinical trials. While these drugs may differ by specific target (PD-1 versus PD-L1), antibody species (humanized versus fully human), and IgG subclass (IgG1 versus IgG4), it remains unclear whether there are clinically meaningful differences in efficacy or toxicity between these agents.

The treatment of anaplastic lymphoma kinase (ALK)-positive lung cancer has seen similar developments. Although these cases represent only 3–5% of non-small cell lung cancer (NSCLC), researchers and pharmaceutical companies have devoted intense effort to this disease subset. The field received an initial boost by the rapidity of drug development. Largely because the first-generation ALK inhibitor crizotinib was already under clinical development as a MET inhibitor, the interval between discovery of the ALK target and evidence of a clinically effective drug was a remarkably short 3 years (1-4), compared to 41 years between the discovery of BCR-ABL and approval of imatinib and 26 years between the discovery of epidermal growth factor receptor (EGFR) and approval of erlotinib (4).

For ALK-positive lung cancer, the pace of development has not slowed. Within 3–5 years, so-called second-generation ALK inhibitors such as ceritinib and alectinib, both of which have clear activity in crizotinib-resistant cases, were available. By contrast, it took more than a decade to develop and approve a late-generation EGFR inhibitor that had meaningful efficacy in erlotinib- and gefitinib-resistant cases (5).

This time period also saw increased understanding of the heterogeneous and complex science of crizotinib resistance in ALK-positive lung cancer. Broadly, mechanisms can be characterized as pharmacologic or biologic. Pharmacologic reasons may include patient non-adherence, reduced absorption, drug interactions, and most importantly inadequate blood-brain barrier penetration. Indeed, up to 40% of progression on crizotinib occurs in the central nervous system (6). Biologic mechanisms include bypass tracks with alternate oncogenes such as EGFR and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (35% of cases) (7,8), ALK gene copy number gain (20% of cases) (9), and ALK resistance mutations (35% of cases). To date, more than a dozen ALK resistance mutations have been identified, including gatekeepers analogous to T790M in EGFR mutant NSCLC (10) and T315I in chronic myeloid leukemia, which reduce crizotinib binding and enhance ATP affinity (8,11-14). A potential explanation...
for contemporary late-generation ALK inhibitors. Among the eight crizotinib-naive ALK-rearranged cases, all responded [median progression-free survival (PFS) not reached]. Response rate was 74% for crizotinib-treated cases (median PFS 14.5 months). Intracranial response rate was 50%. Median intracranial PFS was 15.6 months for all assessable patients and 22.3 months for assessable patients with no prior brain radiotherapy. Activity was also noted in ROS1-positive NSCLC, as well as other ALK-rearranged malignancies including inflammatory myofibroblastic tumor and neuroendocrine tumor. Despite encouraging preclinical data, only 5% of EGFR-mutant NSCLC cases had an objective response.

These results lead to as many questions as they answer. Are second-generation ALK inhibitors best used as initial treatment or following crizotinib failure? It would seem that reserving second-generation ALK inhibitors for post-crizotinib failure would yield the greatest overall period of disease control. However, first-line use of drugs such as ceritinib or alectinib may have greater PFS than the overall combined PFS when they follow crizotinib (18,24). Which cases of ALK-positive brain metastases may be treated medically, and which are best approached initially with resection or radiation therapy? In this trial, enrollment of previously untreated brain metastases was limited to those that were neurologically stable and not requiring escalating steroid doses or anticonvulsants. Could brigatinib use be extended to those patients with symptomatic intracranial disease? At what point should disease progression on an ALK inhibitor be addressed with a change in systemic therapy, and when can local treatments be employed to prolong disease control? Small series have demonstrated that surgical resection or stereotactic ablative radiation in cases of oligo-progression, with continuation of the initial systemic targeted therapy, may extend disease control for several months (25). Such an approach is particularly effective against intracranial progression, presumably because it may represent failure of drug delivery rather than emergence of systemic resistance. Perhaps the most relevant to the trial under discussion: which second-generation ALK inhibitor has greatest efficacy? Which has the least toxicity?
<table>
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<td>L1151T ins, L1152R, C1156Y, F1174L, L1196M, L1198F, G1202R, S1206Y, G1269A</td>
<td>Visual changes orthostatic hypotension, elevated creatinine, peripheral edema, nausea, diarrhea, fatigue, constipation</td>
<td></td>
<td>(18,26)</td>
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<td>Alectinib</td>
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<td>L1196M, C1156Y, F1174L, G1269A, S1206Y, L1152R, 1151T-ins</td>
<td>G1202, V1180L, I1171T</td>
<td>Dysgeusia, elevated AST, elevated ALT, elevated bilirubin, elevated creatinine, rash, constipation, fatigue, myalgia, edema, elevated CPK, nausea, photosensitivity</td>
<td>Neutropenia, elevated creatinine, elevated CPK</td>
<td>(20)</td>
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<td>Entrectinib</td>
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<td>L1196M, C1156Y</td>
<td></td>
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<td>Asthenia, muscle weakness, cognitive impairment</td>
<td>(27)</td>
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<td>Lorlatinib</td>
<td>ALK, ROS1</td>
<td>L1196M, G1202R, G1269A</td>
<td>L1198F</td>
<td>Hypercholesterolemia, peripheral edema, peripheral neuropathy</td>
<td>Hypercholesterolemia</td>
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</tbody>
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*, preclinical evidence of activity against activating and resistance EGFR mutations but minimal clinical efficacy. InsR, insulin receptor; IGF-R1, insulin like growth factor; MET, proto-oncogene protein c-MET; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; ROS1, ROS proto-oncogene 1; LTK, leukocyte receptor tyrosine kinase; GAK, cyclin G-associated kinase; ALT, alanine transaminase; AST, aspartate transaminase; LFTs, liver function tests; CPK, creatine phosphokinase; NTRK1, neurotrophic tyrosine kinase 1.
efficacy against ROS1-positive NSCLC, but alectinib does not. Unique among clinically available ALK inhibitors, crizotinib also has activity against NSCLC harboring tyrosine-protein kinase Met (cMET) exon 14 mutations (29,30). Activity also differs across the spectrum of secondary ALK resistance mutations. Indeed, some rare cases of molecular resistance to late-generation ALK inhibitors regain sensitivity to crizotinib (31). Realistically, there are too many mutations and too many drugs for clinicians to remember these associations. Awareness of and access to these data are critical to optimal patient care. Similarly, physicians must thoroughly understand each drug's monitoring requirements and toxicity profile. Crizotinib may cause hypotension, while brigatinib may cause hypertension. Ceritinib may cause diarrhea, while alectinib may cause constipation. The visual changes associated with crizotinib may be striking. However, they do not impact visual acuity and resolve spontaneously in most cases despite continued drug administration. Oncologists unfamiliar with this clinical pattern may inappropriately reduce or discontinue dosing. Brigatinib pulmonary toxicity suggests that combinations with immune checkpoint inhibitors be approached with caution.

While the addition of brigatinib strengthens our anti-ALK armamentarium, it represents an incremental rather than revolutionary advance. ALK inhibitors and other molecularly targeted therapies requiring daily administration convey chronic toxicities that may rarely be severe but frequently impact quality of life. And clinical outcomes remain suboptimal. We continue to measure survival in intervals of several months. Particularly given the relatively young age of many ALK-positive patients cancer, in 2017 a diagnosis of advanced ALK-rearranged NSCLC remains tragic, with decades of life lost. Let us hope that forthcoming discoveries can truly change that.

Acknowledgements

Funding: This article is funded in part by a National Cancer Institute Midcareer Investigator Award in Patient-Oriented Research (K24CA201543-01) (to DE Gerber).

Footnote

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


29. Ou SH, Greenbowe J, Khan ZU, et al. I1171 missense mutation (particularly I1171N) is a common resistance mutation in ALK-positive NSCLC patients who have progressive disease while on alectinib and is sensitive to ceritinib. Lung Cancer 2015;88:231-234.


Patients with advanced epidermal growth factor receptor (EGFR) mutation-positive non-small cell lung cancer (NSCLC) receive first-line therapy with an EGFR tyrosine kinase inhibitor (TKI). This treatment strategy is based on the results of several phase 3 trials which have demonstrated superiority of EGFR TKIs over first-line chemotherapy in terms of progression-free survival and quality of life in these patients (1). This strategy also mandates EGFR mutation analysis in patients with advanced NSCLC, particularly in those with adenocarcinomas (2). A study in the real-world setting in Central European countries, where lung cancer incidence rates are high, confirmed that EGFR mutation testing has been implemented and that patients with advanced EGFR mutation-positive NSCLC preferentially receive first-line therapy with EGFR TKIs in routine clinical practice (3).

While patients with EGFR mutation-positive NSCLC respond well to first- or second-generation EGFR TKIs, they will eventually develop resistance to these agents after median progression-free survival times of 9–13 months (1). Acquired resistance may be caused by pharmacological changes and molecular changes of the tumor (4,5). Molecular alterations include EGFR target alterations in about 60%, non-EGFR bypass track alterations in about 20% of the patients, histological transformation to small cell lung cancer, epithelial-mesenchymal transformation, and yet to be identified mechanisms (4,5). T790M mutations within exon 20 are the main EGFR target alterations and occur alone in 40–55% and in combination with EGFR amplification in 10% of the patients with acquired resistance. The T790M mutation increases the affinity of EGFR for adenosine triphosphate (ATP), thereby causing resistance to EGFR TKIs (6).

Several treatment options have been studied in EGFR mutation-positive patients with acquired resistance to EGFR TKIs. Treatment decisions have considered slow versus rapid progression, single versus multiple sites of progression, cancer-related symptoms, and other factors. Switch to chemotherapy has been considered as standard treatment. Continuing treatment with TKIs has been considered as an option for patients with only slowly progressing tumors in the absence of symptomatic deterioration. In case of progression at a single site, the addition of local radiotherapy may also be an option. Afatinib combined with cetuximab has also been studied (7,8). The most promising strategy, however, has focused on third-generation EGFR TKIs.

Third-generation TKIs are active against EGFR mutations and the T790M resistance mutation and have only limited efficacy against wild-type EGFR (9,10). Thus these drugs may overcome TKI resistance and result in fewer side effects, particularly in terms of diarrhea and skin rash. Third-generation TKIs that entered clinical development include osimertinib, rociletinib and olmutinib (11-15). While the clinical development of rociletinib and olmutinib have been halted in the meantime due to...
insufficient efficacy and/or unexpected toxicity, osimertinib has been successfully evaluated in phase 2 and 3 trials in patients with advanced EGFR mutation-positive NSCLC.

**Osimertinib**

Osimertinib is an oral, irreversible EGFR TKI which is active against both EGFR mutations and the T790M resistance mutation (9,11). It also has activity in the central nervous system, has only little activity against wild-type EGFR and does not bind to the insulin receptor or the insulin-like growth factor receptor (9,11). This favorable drug profile suggested that osimertinib should result in greater clinical efficacy at less toxicity in comparison to first- and second-generation TKIs. Therefore, osimertinib was further evaluated in the AURA program (11,12,15).

In a phase I-II trial in patients who had progressed after pre-treatment with EGFR TKIs, osimertinib resulted in a response rate of 51% (11). The response rate was 61% among patients with T790M mutations and 21% among those without T790M mutations. Median progression-free survival times were 9.6 and 2.8 months in T790M-positive patients and T790M-negative patients, respectively. Adverse events were diarrhea in 47% of patients, rash in 40%, nausea in 22%, and decreased appetite in 21% of the patients. The trial suggested an osimertinib dose of 80 mg once daily for further clinical development.

The AURA2 phase II trial confirmed the efficacy of osimertinib in patients with EGFR T790M mutation-positive NSCLC who had developed resistance to frontline therapy with EGFR TKIs (12). The objective response rate was 70% and the disease control rate was 92%. Median progression-free survival was 8.6 months and median duration of response was 7.8 months.

**AURA3**

Recently, the results of the AURA3 trial have been published (15). This open-label phase 3 trial compared osimertinib with platinum-based chemotherapy in patients with advanced T790M-positive NSCLC who had disease progression after first-line EGFR TKI therapy. The primary endpoint of the trial was progression-free survival assessed by the investigators. Secondary endpoints included response rate according to investigator assessment, response duration, disease control rate, patient-reported outcomes, overall survival, safety, and side-effect profiles. Patients had to have documented presence of an EGFR mutation and central confirmation of the T790M mutation on the cobas EGFR Mutation Test (Roche Molecular Systems) (15).

A total of 419 patients with T790M-positive locally advanced or metastatic NSCLC and disease progression after first-line EGFR TKI therapy were randomized in a 2:1 ratio to either osimertinib (80 mg once daily) or pemetrexed (500 mg per square meter of body-surface area) plus either carboplatin (target area under the curve 5) or cisplatin (75 mg per square meter) every three weeks for up to six cycles (15). The major findings of the trial are summarized in Table 1. Patient characteristics were well balanced between the two treatment arms, in particular in terms of age, race, never-smokers, histology, central nervous system metastases, and previous treatments. The distribution of EGFR mutations was similar to those of other studies with exon 19 deletions in about two thirds and L858R mutations in about one third of the patients. T790M mutations were present in 99% of the patients. All patients except one had been pretreated

<table>
<thead>
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<th>Items</th>
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<tr>
<td>Patients, n</td>
<td>279</td>
<td>140</td>
</tr>
<tr>
<td>T790M positive patients</td>
<td>275 (99%)</td>
<td>138 (99%)</td>
</tr>
<tr>
<td>Objective response rate</td>
<td>71%</td>
<td>31%</td>
</tr>
<tr>
<td>Median progression-free survival, months</td>
<td>10.1</td>
<td>4.4</td>
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<td>Diarrhea, any grade (grade 3)</td>
<td>41% (1%)</td>
<td>11% (1%)</td>
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<tr>
<td>Rash, any grade (grade 3)</td>
<td>34% (1%)</td>
<td>6% (0)</td>
</tr>
<tr>
<td>Nausea, any grade (grade 3)</td>
<td>16% (1%)</td>
<td>49% (4%)</td>
</tr>
<tr>
<td>Vomiting, any grade (grade 3)</td>
<td>11% (&lt;1%)</td>
<td>30% (2%)</td>
</tr>
</tbody>
</table>

Table 1 Results of AURA3 trial (15)
Patients receiving osimertinib had superior progression-free survival compared to patients treated with chemotherapy (15). The hazard ratio was 0.3 (95% CI, 0.23–0.41) and median progression-free survival times were 10.1 and 4.4 months, respectively. The benefit in progression-free survival was seen across all major subgroups including patients with brain metastases in whom duration of progression-free survival was longer for patients treated with osimertinib than for patients treated with chemotherapy. Patient-reported outcomes were better in patients treated with osimertinib than in patients treated with chemotherapy. The response rate was 71% with osimertinib compared to 31% with chemotherapy. With regard to post-study treatments, 60% of patients in the chemotherapy arm crossed over to osimertinib. Survival data have not been presented yet.

The side effects were different between the two treatment arms (15). Overall, osimertinib was better tolerated as underlined by the lower frequency of grade 3 adverse events compared to chemotherapy (23% vs. 47%). Adverse events more commonly seen with osimertinib were diarrhea, skin toxicity (rash, dry skin, paronychia). Nausea, vomiting, constipation, fatigue, and hematotoxicity were more commonly seen in the chemotherapy arm.

Impact of AURA3

The AURA3 trial demonstrated improved outcome for osimertinib compared to chemotherapy in patients with EGFR mutation-positive NSCLC who had developed T790M-mediated resistance to first- or second-generation EGFR TKIs (15). Improvements with osimertinib over chemotherapy include prolonged progression-free survival, higher response rate and better drug tolerability. Because progression-free survival was the primary endpoint, it was important that improvements in progression-free survival were also accompanied by better patient-reported outcomes. Thus the AURA3 trial established osimertinib as the preferential treatment for patients with EGFR mutation-positive NSCLC at the time of T790M-mediated resistance.

The AURA3 trial was a well-designed phase III trial with progression-free survival as the primary endpoint. The control arm of the trial was adequate as patients received pemetrexed-based chemotherapy. The fact that progression in the AURA3 trial was assessed by investigators is of relevance for the use of osimertinib in the real-world setting in the future when clinicians (and not independent radiological review boards) will monitor treatment response.

The clinical use of osimertinib also requires the determination of T790M mutations in routine practice in either tumor tissues or circulating tumor DNA. The study also proofs that characterization of resistance mechanisms followed by development of drugs to overcome these mechanisms can lead to therapeutic advances. Finally, important questions yet to be answered include the impact of osimertinib on overall survival and the characterization of drugs that will be able to overcome resistance to osimertinib.

Detection of EGFR T790M

The establishment of osimertinib as a treatment for patients with T790M-mediated resistance now also requires proof of the presence of EGFR T790M mutation in tumor cells by either tissue or plasma genotyping. Tissue genotyping remains a clinical challenge because tumor re-biopsy at the time of TKI resistance may be difficult due to poor performance status of the patients and/or limited tumor access. In addition, re-biopsy may also result in insufficient tumor material for genetic analyses. Therefore, minimally invasive plasma genotyping (liquid biopsy) represents an attractive alternative for detection of EGFR T790M. Blood samples are easily obtainable, can be taken repeatedly, and may provide a better picture of the tumor genome than tissue analysis. Furthermore, blood-based analytic approaches also allow real-time monitoring of the total tumor burden and the detection of mutations that will arise during clinical treatment through serial blood sampling and analysis. While the cobas EGFR Mutation Test is currently the only FDA-approved test, highly sensitive digital genotyping assays such as ddPCR or BEAMing can also accurately detect mutations in cell-free plasma DNA (16-19).

Impact of osimertinib on overall survival

The question whether osimertinib also improves overall survival of patients remains to be answered. Until a survival benefit will have been proven, postponing the use of osimertinib after chemotherapy may be considered as an option in selected patients, particularly at times when economic pressure increasingly limits access to novel but expensive drugs. Therefore, the proof of a survival benefit by osimertinib is paramount for establishing osimertinib as the only standard treatment for patients who have developed T790M-mediated resistance to EGFR TKIs.
Resistance to third-generation EGFR TKIs

Patients treated with osimertinib will eventually develop resistance against this drug. Resistance mechanisms that have already been described are EGFR C797S mutations (20,21). A C797S positivity rate of 40% (6 of 15 cases) was recently reported in patients with acquired resistance to osimertinib (21). In cell lines and mouse models, NRAS mutations have been shown to mediate acquired resistance to osimertinib (22). These mutations include NRAS Q61K, E63K, and G12V point mutations as well as a gain of copy number in wild-type NRAS. Whether these molecular alterations are also involved in the osimertinib resistance in patients remains to be determined.

Research on the characterization of drugs that will overcome osimertinib resistance in patients is ongoing. In mouse models, C797S-mediated resistance can be overcome by EAI045 (23). EAI045 targets specific drug-resistant EGFR mutants but spares the wild-type receptor. EAI045 in combination with cetuximab has been shown to be active in EGFR L858R/T790M- and EGFR L858R/T790M/C797S-mutated NSCLC. Although the efficacy of these drugs has yet to be confirmed in clinical trials, these findings are encouraging and further indicate that stepwise improvement in the outcome of treatment with targeted agents has become a clinical reality.

Acknowledgements

None.

Footnote

Conflicts of Interest: R.P. has received speaker’s fees from AstraZeneca and Boehringer Ingelheim and honoraria for advisory boards from AstraZeneca, Boehringer Ingelheim and Clovis; M.F. has received speaker’s fees and/or honoraria for advisory boards from AstraZeneca, Boehringer Ingelheim, Eli Lilly, MSD, Novartis, Ratiopharm, and Roche; A.B. declares no conflict of interest.

References

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with EGFR mutations who had received EGFR tyrosine kinase inhibitors (TKIs). J Clin Oncol 2014;32:5s.


Targeted therapy in small cell lung cancer: can DLL3 notch up a victory?

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Provenance: This is an invited Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Mar 08, 2017. Accepted for publication Mar 20, 2017.
doi: 10.21037/tcr.2017.03.70
View this article at: http://dx.doi.org/10.21037/tcr.2017.03.70

Introduction

Small cell lung cancer (SCLC) is the most lethal and aggressive subtype of lung carcinoma, responsible for ~13–18% of lung cancer death with no appreciable improvements in outcomes or treatment options for the last 30 years. The clinical behavior of SCLC is tailor made for nihilism with excellent initial overall response rates transforming to inevitable chemotherapy resistant recurrence in the majority of patients. Targeted therapies to date have failed with little to no efficacy in unselected populations. Naturally, this state of affairs has led to an underfunded SCLC research community, and historical pharmaceutical disinterest in this “graveyard of drug development”. The National Cancer Institute (NCI) and worldwide refocus upon “recalcitrant” carcinomas has led to renewed interest in SCLC making this the perfect opportunity to consider how and why targeted therapy in unselected SCLC has failed so consistently. The critical factors are both biological factors and structural limitations to previous targeted therapy studies in SCLC. (I) The rapid recurrence after initial response to chemotherapy of SCLC is suggestive of biological features consistent with stem cell biology. This strongly suggests a stem cell like phenotype, or a resistant subclonal expansion (1). Stem cell signaling is complex and redundant which limits signaling interference as a monotherapy; (II) the lack of mutational drivers and the mutational signature of SCLC appears to be principally driven by changes in tumor suppressor or transcription factors. These targets are challenging to drug and this has hampered targeted therapy options; (III) the related issue of inadequate biomarkers for the delineation of SCLC subpopulations. SCLC has long been known to be a heterogeneous disease (2), but previously the tools were unavailable to further characterize potential subpopulations by single cell based methods. The study “Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study” recently published in Lancet Oncology constitutes an attempt to address these critical factors in SCLC biology: stem cell targeting, lack of a novel druggable target, and biomarker driven clinical trials. This study is a promising theoretical approach using an antibody drug conjugate (ADC) to target DLL3 labeled putative stem cell populations in SCLC and incorporates an intrinsic biomarker of response.

Theoretical underpinnings: SCLC and the stem cell hypothesis

The putative cell of origin for SCLC is the pulmonary neuroendocrine cell (PNEC) which participates in oxygen sensing and lung morphogenesis. This cell of origin has not been definitively established in human cancers, but multiple SCLC murine models implicate p53 and Rb loss in the neuroendocrine cell niche (3). These PNECs have a substantial stem cell and injury repair role in normal
physiology and have stem cell like properties including transdifferentiation capability (4). The maintenance of this injury repair capability relies on the contribution of multiple signaling pathways including the Hedgehog (Hh) pathway (5) and Notch activation inhibits the related processes of epithelial-mesenchymal transition (EMT) and invasion (6). These same signaling pathways along with SOX2 and MYCL1 are vital to the maintenance and growth of SCLC tumors (7).

**SCLC, druggable targets and biomarkers**

Recent genetic analyses from multiple groups have expanded our understanding of the underlying gene expression associated with SCLC and have identified putative “stemness” signaling targets in SCLC (7,8). These studies have uncovered changes in multiple pathways with readouts amenable to biomarker or mutational analysis including SHH, PTEN, NOTCH, EZH2, FGFR and others (Table 1). However, given the high mutational burden in SCLC, it remains unclear the relative contributions of each biomarker and the precise delineation of passenger and driver mutations in SCLC. The largest analysis to date involved sequencing data from 152 primary tumor specimens and RNAseq analysis on a subset of 81 primary tumors (8). One notable pathway implicated from this study was notch signaling which is downregulated in 77% of SCLC tumors (8). NOTCH family genes had genomic alterations in 25% of SCLC tumors. Additional studies found reduced tumor formation, metastatic capability, cell cycle inhibition, and reduced neuroendocrine markers with Notch activation thus demonstrating NOTCH as a tumor suppressor in SCLC (6,8).

Saunders et al.’s pre-clinical findings expand upon this work by focusing on DLL3, an inhibitory notch ligand which was found to be over expressed in both patient derived xenografts (PDX) and a cohort of primary SCLC tumors (11). This inhibitory ligand is downstream of the ASCL1 neuroendocrine differentiation pathway and has high level surface expression in SCLC and LCNEC tumors, but low expression in normal lung tissue and normal expression confined largely to the brain. This combination of characteristics made DLL3 an ideal candidate for an ADC with the advantage that DLL3 expression thereby formed an intrinsic predictive biomarker for response. Pre-clinical results supported this hypothesis and showed that DLL3 expression in the PDX model was predictive of response with multiple high DLL3 expressing PDX showing complete responses and xenograft rejection (11). Additional work has also described a potential role for future

<table>
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<th>Potential biomarkers/correlatives</th>
<th>Clinical trial number</th>
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<td>NCT00682981</td>
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<td>FGF pathway inhibition</td>
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This table summarizes some recent promising active trials in SCLC with biomarkers and preclinical data suggesting correlatives which may predict response to specific targeted agents in SCLC. We call particular attention to recent preclinical findings showing c-Myc status in SCLC determines susceptibility to Aurora kinase inhibition (9,10). We note the need for biomarkers to assess more than one step in downstream pathways to ensure inhibition (i.e., Hh signaling).
“theragnostic” approaches allowing for the noninvasive imaging of DLL3 status (12). These data provided compelling pre-clinical evidence for efficacy leading to the first successful targeted therapy study in SCLC.

Clinical/practice impact of this study

It is important to note that this is a phase 1 study with a primary focus on safety and tolerability. The expansion cohort and planned phase 2 arm of the study branched off into NCT 02674568. Toxicity was not insignificant at the intended phase 2 dose with a relatively novel toxicity pattern of serosal effusions including serious pleural and pericardial effusion requiring paracentesis. Grade 3–4 thrombocytopenia was also noted in 11% of patients.

The etiology most likely is an off-target effect from the conjugate toxin based on what is currently known about the expression pattern of DLL3. However, these toxicities are manageable with clinical awareness and compare reasonably with alternative potential agents (13), although they may be a concern in an already frail patient population receiving third line therapy.

In this phase I trial, of the 60 patients who received therapeutic dose levels of Rova-T, there was an 18% response rate which is comparable with existing second line agents. However, it is worth noting that among patients with at least 50% DLL3+ tumor tissue there was a more impressive 38% response rate with a disease control rate of 83% and a PFS of 4-6 months. For this extensively treated patient population with limited therapeutic options, this could be considered clinically significant. Moreover, among the responding patients, there are multiple patients who had responses of greater than 12 months with multiple patients still alive post study completion.

This is very exciting and suggests both a strong predictive effect of the DLL3 expression and strong clinical potential for Rova-T given the lack of options beyond first line therapy for SCLC patients. We should note the obligatory caveats of preliminary results from a small study with a select patient population, but overall this study is well designed with an excellent predictive intrinsic biomarker and promising clinical activity. We await confirmation from larger phase II/III trials where careful monitoring of the novel toxicities associated with this agent will be needed.

Additionally, future phase II and III studies should incorporate post treatment DLL3 analysis or other Notch/ neuroendocrine identity components to better identify mechanisms of resistance including DLL3 downregulation or alternative pathways. Similarly to the approach with targeted therapy in NSCLC patients, the acquisition of rational molecular correlates and clinical samples upfront in studies is increasingly important in order to anticipate future mechanisms of resistance and design trials appropriately to treat this highly heterogeneous and challenging carcinoma.

Conclusions

SCLC is a highly aggressive and heterogeneous lung cancer where targeted therapies have lagged behind. However, the primary clinical approach to date has used unselected SCLC patient populations. This is suboptimal and has stemmed from the lack of genetic and expression information on SCLC and the extreme heterogeneity of this tumor. The recent study “Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study”, demonstrates how a trial approach incorporating an intrinsic biomarker targeting a specific stem cell like population can have efficacy, even in 3rd line+ therapy in SCLC. SCLC clinical research needs to move in the direction of biomarker driven selected population or unselected populations with appropriate and extensive correlates in order to identify and treat the right patient with the right drug at the right time.

Acknowledgements

JM Lehman is provided by a Career Development Award from the LUNGevity foundation.

Footnote

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

References


Concomitant ALK/KRAS and ALK/EGFR mutations in non small cell lung cancer: different profile of response to target therapies

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Provenance: This is an invited Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Mar 14, 2017. Accepted for publication Mar 24, 2017.
doi: 10.21037/tcr.2017.03.77

View this article at: http://dx.doi.org/10.21037/tcr.2017.03.77

The development of therapeutic agents targeting products of epidermal growth factor receptor (EGFR) gene mutation and anaplastic lymphoma kinase (ALK) rearrangements has significantly improved survival in patients with non small cell lung cancer (NSCLC). Thus, the patients eligible for the treatment with EGFR or ALK inhibitors should be selected through appropriate molecular tests (1). On the other hand, although representing the most frequent genic alteration in NSCLC patients, Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation-specific therapy has not been validated in clinical practice (2). Indeed KRAS mutations are described in approximately 20–30% of NSCLC, commonly observed in smokers and associated with a poor prognosis (2).

Although driver genes mutations were reported to be mutually exclusive in NSCLC (3,4), however in several series driver genes mutations seem to occur particularly associated to EGFR mutations (5,6). In wide series of NSCLC, rare cases of concomitant mutations were reported with different frequency, however the TKI response data were conflicting (6-8). In particular, the frequency of concurrent EGFR/ALK mutations was reported in a range of 0.0% to 6% (6,9). Recently, in a large series of Chinese NSCLC patients, the concomitant EGFR and ALK mutations was observed in 1.9% of the cases (6). In a total of 977 NSCLCs, EGFR mutations was found in 336 (32.7%), ALK rearrangements in 70 (6.8%), KRAS mutation in 40 (3.9%) patients and concomitant EGFR and ALK aberrations were observed in 13 patients (1.3%). Although the overall rate of EGFR/ALK co-alterations was only of 1.3% (13/977), however the prevalence of co-alterations was 3.9% (13/336) in EGFR mutant patients and 18.6% (13/70) in ALK-positive patients (6). These results suggested that driver mutations of EGFR and ALK genes could occur in a small group of NSCLC, but more frequently in ALK-positive tumors. In literature, the concomitant EGFR/KRAS mutations were described mainly in case reports, but lately in a large cohort of 5,125 Chinese NSCLCs 30 cases harboring concomitant aberrations were reported (5).

Besides ALK and KRAS alterations, several mutations in various oncogenes were described as concomitant with EGFR mutations. Rarely occurrence of other driver genes mutations were reported associated to EGFR mutations, such as HER2, RET, KRAS and ROS1 genes mutations, while no BRAF and NRAS were found coexisting with EGFR mutations (6). Furthermore, the phosphatidylinositol 3-kinase (PI3K), playing a critical role in cancer cell proliferation, is mutated in approximately 2–4% of NSCLCs (10), often associated to KRAS mutations and less frequently with EGFR and ALK mutations (11).

The concomitant EGFR mutations and other driver genes might decrease substantially the efficacy of EGFR-TKIs (6). The median PFS of patients with concurrent EGFR/ALK mutations treated with EGFR-TKI ranged from 5.0 to 11.2 months, relatively lower than patients harboring only EGFR mutation (6,7). Ulivi et al. observed disease control rate (DCR) in 67% of co-altered patients, that is lower than the 81.7% in patients with an EGFR-mutation only (12). Particularly Yang et al. attributed the
efficacy of EGFR-TKI to relative levels of phospho-EGFR in patients with concomitant EGFR/ALK mutations (7). Indeed, of the ten patients receiving first-line EGFR-TKIs, eight achieved objective response with a median PFS of 11.2 months. Of the four patients treated with crizotinib, three cases were previously treated with EGFR-TKI, particularly one case was not responsive to EGFR-TKI, but sensitive to crizotinib, whilst two cases were responsive to EGFR-TKI, but not to crizotinib. Finally, one case showed partial response to crizotinib, but no response to subsequent treatment with EGFR-TKI. Immunohistochemistry showed in all examined cases co-expression of EGFR mutant protein and ALK fusion proteins in the same cancer cells, indicating that different driver oncogenes could act in the same cell population. Moreover, different levels of receptors phosphorylation were observed using specific antibodies. Thus, three patterns of phosphorylated proteins were documented: high p-EGFR and high p-ALK, high p-EGFR and low p-ALK, and low p-EGFR and high p-ALK. High levels of p-EGFR correspond to partial responses to EGFR-TKI, while two patients with low levels of p-EGFR had progressive or stable disease. Of the four cases treated with crizotinib, two had low p-EGFR and high p-ALK; one of them was not responder to EGFR-TKI, but sensitive to crizotinib, and the other was highly responsive to crizotinib, but resistant to subsequent EGFR-TKI. On the other hand, two cases had high p-EGFR levels and low p-ALK levels, corresponding to partial responsiveness to EGFR-TKI, but with poor results when treated with crizotinib (7).

Generally, the results of subsequent treatment with crizotinib in NSCLC patients with concomitant EGFR/ALK mutations after failure of EGFR-TKI treatment are conflicting (6). Lee et al. observed that two ALK-positive/EGFR-mutant NSCLC patients non-responder to EGFR-TKI showed a durable partial response to ALK inhibitors (13). Therefore, in non-responded to EGFR-TKI patients, ALK gene status test should be investigated, since it might be responsible for unsuccessful treatment. In parallel, acquired EGFR mutations are also described as a mechanism of resistance to ALK inhibitor (14). However, in a series of 1,683 NSCLCs, all 25 ALK-positive patients crizotinib-resistant were both KRAS and EGFR wild type (3).

Finally, in NSCLC patients harboring ALK/EGFR co-alterations, EGFR-TKIs seem to be more active compared to ALK-TKIs. Schmid et al. identified five patients with EGFR/ALK co-alteration, four out of five received one or more lines of EGFR-TKIs and three patients received one or more lines of ALK-TKI. In particular, patients showed different response to TKI: one out of three patients responding to ALK-TKI and three out of four patients responding to EGFR-TKI. Median PFS were slightly better in patients treated with EGFR-TKIs than in patients treated with ALK-TKIs (8).

Different response rate might be explained considering intratumor heterogeneity of both genes, strictly related to gene mutation tumor burden (9,15). Therefore, the mutation tumor burden of each mutation could affect lack of response to EGFR-TKIs in these patients (16). Thus, since highly sensitive EGFR tests have widely been introducing in practical diagnosis of NCSLC, an increasingly high number of cases with concomitant alterations in different oncogenes could be identified in the future. It is calculated a significant increased rate of concomitant EGFR and ALK mutations in NSCLC—from 4.4% to 15.4%—using targeted next-generation sequencing (NGS) (16).

As regards KRAS mutations, since they are responsible for secondary resistance to ALK TKIs such as crizotinib (17), the concomitant ALK and KRAS co-alteration may be associated with primary resistance to ALK-TKI treatment. Indeed, for the first time Schmid et al. reported that six out of seven patients treated with crizotinib were non-responder patients (8). In the clinical setting of concomitant EGFR/KRAS mutations, KRAS mutations seems to be related to a reduced response to EGFR TKI (2). On the contrary Lee et al. reported that response rate to EGFR-TKI in concomitant EGFR/KRAS mutation patients is superimposable to only EGFR mutant patients. This observation might be attributable to EGFR mutation as driver dominant role, even if the tumor cells harbored an additional KRAS mutation (14).

In conclusion, most NSCLC patients harboring concomitant EGFR/KRAS mutations partially responded to EGFR TKI, while NSCLC patients harboring concomitant EGFR/ALK mutations slightly responded to specific ALK or EGFR TKI. EGFR and ALK alterations play an important role in the oncogenesis of NSCLC, however their interaction in terms of synergism versus the possible dominance of one rather than the other oncogene are currently not completely clarified. The dominance of one oncogenic alteration over the other could be explained...
Concomitant ALK/R and EGFRm

Response to ALK inhibitor

A

High phospho-ALK levels

Response to EGFR inhibitor

B

High phospho-EGFR levels

Concomitant ALK-R and EGFRm

Cells with ALK-R

Cells with EGFRm

**Figure 1** Different tumor mutations burden in concomitant EGFR/ALK mutations NSCLC related to the response to target therapies. In (A) most tumoral cells harboring ALK rearrangement (ALK-R) with better response to ALK-TKI with respect to EGFR-TKI and in (B) most tumoral cells harboring EGFR mutation (EGFRm) with better response to EGFR-TKI with respect to ALK-TKI. EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; NSCLC, non small cell lung cancer.

Concomitant ALK-R and EGFRm

**Figure 2** Different level of phosphorylation of ALK and EGFR in concomitant EGFR/ALK mutations NSCLC related to the response to target therapies. In (A) higher level of phosphorylation of ALK than EGFR, with better response to EGFR-TKI with respect to ALK-TKI; in (B) higher level of phosphorylation of ALK with better response to ALK-TKI with respect to EGFR-TKI. EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; NSCLC, non small cell lung cancer.

essentially through two mechanisms, a different mutation tumor burden of each driver gene (*Figure 1*) and differential phosphorylation of the single mutated proteins (*Figure 2*). Different mutation tumor burden could justify the inconsistency of TKI response in patients investigated through cytology or small biopsies, clearly representing only a small portion of the entire tumor. On the other hand the presence of concomitant EGFR/ALK mutation...
could have a little value, if not associated to the evaluation of altered protein phosphorylation. Finally, the alternative over-phosphorylation of altered EGFR and ALK proteins needs more studies of validation in order to address the patients to the better treatment.

Acknowledgements

None.

Footnote

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

References


Activating gene rearrangements in the anaplastic lymphoma kinase are present in approximately 2–7% of lung adenocarcinomas (ALK + cancers) (1,2). Patients with ALK + lung adenocarcinoma often benefit from treatment with an ALK tyrosine kinase inhibitor (TKI), such as crizotinib, ceritinib, and alectinib (2). However, acquired ALK TKI resistance remains an obstacle to long-term patient survival in patients who do respond to initial therapy and a distinct subset of ALK + patients fails to experience an initial tumor regression, exhibiting intrinsic resistance (2). Identifying the basis of both innate and acquired resistance is essential to improve clinical outcomes.

The ALK gene rearrangements present in lung adenocarcinoma typically involve a 5’ fusion of the echinoderm microtubule-associated protein-like 4 (EML4) gene to the ALK kinase domain (2). Several variants of the EML4-ALK fusion have been observed in lung adenocarcinoma patients. These different variants result from translocations at different points within the EML4 gene: variant 1, variant 2, and variant 3a/b are the most common fusion variants (2). EML4 contains several protein domains that may be critical to protein folding, stability, and function (3,4): N-terminal coiled-coil region, a basic region, a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) region, and tryptophan-aspartic acid repeats (WD). The HELP-WD region forms a tandem atypical β-propeller (TAPE) structure (3). The EML4 TAPE domain is variably present in the different EML4-ALK fusion proteins. The absence of the full TAPE domain in EML4-ALK variants 1, 2, 7 may render the protein less stable than EML4-ALK variants 3a/b and 5a/b, which contain the full TAPE domain (3,4). Whether the different variants of EML4-ALK as they relate to the presence or absence of the full TAPE domain impact clinical response to ALK TKI treatment has remained an important unresolved question.

A new study now begins to address this question (5). The authors conducted a retrospective analysis of EML4-ALK lung adenocarcinoma patients treated with an ALK TKI to determine whether the different variants that either contained or lacked the full TAPE domain were associated with differential treatment response. They report that patients with variants 3a/b showed decrease response to ALK TKI treatment, compared to patients with variants 1 and 2. In vitro studies further showed that cells expressing variant 1 or 2 were more sensitive to ALK TKI treatment and showed lower kinase activity than cells expressing variant 3a or 5a.

Together, these findings provide important evidence suggesting that the degree of kinase activity and/or stability of the EML4-ALK fusion protein, as dictated by determinants within EML4, influence ALK TKI response in patients. The data, if further confirmed in additional clinical cohorts, could establish EML4-ALK variant status as a novel biomarker by which to stratify patients for treatment with an ALK TKI and/or additional treatment strategies.
such as combination therapies (6,7)). On the basis of these intriguing findings, additional retrospective analyses and, more importantly, prospective studies are warranted to confirm these new findings.

Overall, we are just beginning to understand the role of non-kinase fusion partners in oncogenesis in kinase fusion driven cancers, such as EML4-ALK lung adenocarcinoma. This study is an important step forward. Another recent study by our group revealed an important role for the HELP domain within EML4 in the EML4-ALK fusion protein in downstream signaling pathway activation and RAS-mitogen activated protein kinase (MAPK) pathway signaling (7). More detailed studies are necessary to determine how the different domains within kinase fusion partners such as EML4 influence the signaling, oncogenic, and biomarker roles of this class of oncogene driver. Studies such as this recent report (5) are essential to fuel both basic and translational research efforts that hold promise to improve the molecular precision with which we diagnose and treat patients with ALK + lung adenocarcinoma, and potentially other malignancies driven by kinase gene fusions in the future.

Acknowledgements

Funding: The author has received funding from NIH (R01CA211052).

Footnote

Conflicts of Interest: The author is a consultant or advisory board member to Novartis, Astrazeneca, Revolution Medicines, and Array Biopharma.

References

The discovery of ALK rearrangements in non-small cell lung cancer (NSCLC) and their oncogenic properties by Soda et al. in 2007 gave the start to one of the most famous stories in the treatment of lung cancer (1). Diagnosed in around 5% of patients with NSCLC, ALK rearrangements are effectively treated with the ALK-directed tyrosine kinase inhibitor (ALK-TKI) crizotinib (2,3). Two phase III trials have demonstrated that crizotinib is superior to standard chemotherapy in first- and second-line settings, the objective response rates ranging from 65% to 74% with median progression-free survivals (PFS) of 7.7 to 10.9 months, and crizotinib is thus currently the standard of care in advanced ALK-positive NSCLC, in first-line setting (3). This justifies the search for ALK-rearrangements in all advanced non-squamous NSCLCs, especially in adenocarcinomas of cribriform/signet-ring cells subtypes, non-smoker and young patients (4). Identification of ALK-positive NSCLCs is based on immunohistochemistry (antibodies 5A4 Novocastra™ and D5F3 Cell Signaling™), break-apart fluorescent in situ hybridization (FISH), fusion variants specific multiplex RT-PCR, Nanostring nCounter® technology and targeted RNA-sequencing assays (5,6). By these latter techniques several variants can be identified for which prognostic or predictive values remain to be elucidated.

Nevertheless, individual responses and PFS observed with crizotinib in clinical trials and in routine practice are heterogeneous (3,7). Some patients experience a progressive disease as best response, others relapse within a few months after crizotinib initiation, and the last remain with persistent objective responses after years of treatment. The inability to achieve a therapeutic concentration of crizotinib in the central nervous system is probably the main mechanism of early brain oligometastatic progression observed in some patients (3). On the other hand, the molecular mechanisms underlying early diffuse progression are poorly understood, even if uncommon false positive ALK diagnosis, lack of observance and pharmacological interactions may be in cause for a few patients (3). One of the main hypotheses that have been raised is based on the different protein stabilities of EML4-ALK fusion variants (v1, v2, v3a and v3b) in a BaF3 cell line model and showed that the cell line sensitivity to crizotinib and the tool compound ALK-TKI TAE684 correlated with the expected protein stability of the EML4-ALK variants (8). EML4-ALK v2 was the...
most sensitive to ALK-TKI, EML4-ALK v1 and v3b had intermediate sensitivity and V3a was less sensitive. Until the recent article of Woo et al. published in *Annals of Oncology* accompanying this editorial, these *in vitro* data suggesting that ALK rearrangements are not created equal had never been corroborated by clinical observations (9). The authors report the results of a single institution, retrospective analysis of crizotinib efficacy according to EML4-ALK fusion variants among a population of 54 Korean patients with ALK-positive advanced NSCLC. Using a multiplex RT-PCR, the authors identified 28 known EML4-ALK variants in tumor samples. An EML4-ALK v3a/b was identified in 24 cases (44.4%), a v1 in 18 (33.3%) and a v2 in 6 (11.1%). Rare ALK transcripts were detected in the 6 remaining cases (v7 in one, two new EML4-ALK variants in two and non-EML4 variants in three). According to the expected greater instability of EML4-ALK v1 and v2 compared to v3a/b, Woo et al. cleverly compared ALK-TKI efficacy between a v1/v2/others group (n=24) and a v3a/b group (n=24), excluding from the analysis the non-EML4 variants. In the 44 patients treated with crizotinib, the 2-year PFS rate was clearly improved in the v1/v2/others group compared to the v3a/b group but this did not reach statistical significance (69.0% vs. 32.7%, P=0.108). Objective response rates and disease control rates were slightly superior in the v1/v2/others group, especially when considering all ALK-TKIs, but with no statistical significance. In IL-3-dependent Ba/F3 cells that stably expressed v1, v2, v3a or v5a, ALK tyrosine kinase activity was shown to be higher in v3a- and V5a-expressing cells. The v3a- and v5a-expressing cells were also resistant to crizotinib, ceritinib and alectinib (IC50 >500nM), as were v3a-expressing H2228 and v5a-expressing BEAS-2B cell lines, whereas v1- and v2-expressing cells were sensitive to ALK-TKIs.

To date, this is the first study that supports the notion that EML4-ALK variants are able to condition the clinical benefit of ALK-TKIs according to the different stabilities of the protein products in ALK-positive NSCLC. However, apparent conflicting data have emerged from the literature (*Table 1*). Indeed, a single-institution retrospective study of 35 ALK-positive patients recently published by Yoshida et al. suggested that crizotinib was more effective in EML4-ALK v1 versus non-v1 variants (12). Apart from the small size of the cohort studied, the comparison of EML4-ALK v1 versus non-v1 was not supported by any biological rationale. A careful analysis of the individual PFS provided in this paper shows that the PFS was always inferior to 5 months in the four EML4-ALK v3a/b patients, which is indeed lower than

### Table 1 Crizotinib efficacy according to EML4-ALK variants

<table>
<thead>
<tr>
<th>Study</th>
<th>End-point</th>
<th>Clinical outcomes according to variants stratification</th>
<th>Statistical significance</th>
<th>Reference</th>
</tr>
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<tr>
<td>Lei et al.</td>
<td>v1 (n=22)</td>
<td>v3a/b (n=18) Others (10)</td>
<td>No</td>
<td>(11)</td>
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<tr>
<td></td>
<td>Response rate (%)</td>
<td>72.7 55.6 81</td>
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<td></td>
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<tr>
<td></td>
<td>Median PFS (mo.)</td>
<td>11 10.9 7.4</td>
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<td></td>
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<tr>
<td>Yoshida et al.</td>
<td>v1 (n=19)</td>
<td>Non-v1 (n=16)</td>
<td>No</td>
<td>(12)</td>
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<tr>
<td></td>
<td>Response rate (%)</td>
<td>74 63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median PFS (mo.)</td>
<td>11.0 4.2</td>
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<td></td>
</tr>
<tr>
<td>Cha et al.</td>
<td>v1 (n=12)</td>
<td>v3a/b (n=10) v2 (n=3)</td>
<td>No</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td>Response rate (%)</td>
<td>50 66.7 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median PFS (mo.)</td>
<td>=31 =11 =6</td>
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<td></td>
</tr>
<tr>
<td>Woo et al.</td>
<td>v1/v2/others (n=24)</td>
<td>v3a/b (n=20)</td>
<td>No</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>Response rate (%)</td>
<td>83.3 75.0</td>
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<td></td>
<td>2-year PFS rate (%)</td>
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PFS, progression free-survival; mo., month.
what is expected with crizotinib in ALK-positive NSCLC. Another retrospective study in 61 ALK-positive patients showed lower response rates in EML4-ALK v3a/b patients compared to EML4-ALK v1 but no differences in PFS were observed (11). Finally, a retrospective study reported clinical outcomes with crizotinib in 52 ALK-positive patients and showed no differences in response rates between EML4-ALK v1 and EML4-ALK v3a/b, but a better PFS in EML4-ALK v1 compared to EML4-ALK v3a/b (13). Considering these scarce data available in the literature, the findings from the paper of Woo et al., together with the work from Heuckmann et al., seem to be relevant. Are these new data strong enough to suggest that ALK rearrangements should not be considered equal in the clinic?

Some important limitations have to be underlined in this work (9). First, a selection bias is suspected that is inherent to the retrospective nature of this study. From the 182 patients initially diagnosed with an ALK-rearranged NSCLC, only 54 patients were finally analyzed. Importantly, 81 samples from patients treated with ALK-TKI were available and among them, 24 were excluded because of RT-PCR failure. In numerous studies that report the frequency distribution of EML4-ALK variants, the v1 is the more common (around 50% of the cases), followed by v3a/b (around 25%) and v2 (around 10%) (3). The distribution reported herein shows a predominance of v3a/b (44.4%) followed by v1 (33.3%) and v2 (11.1%). This probably does not reflect the distribution in the overall ALK-positive NSCLC population and therefore questions the representativity of the cohort that has been analyzed.

Second, the 2-year PFS rate was improved in the v1/v2/others group compared to the v3a/b group when considering the 44 patients treated with crizotinib. This difference did not translate into overall survival (OS), with stackable curves in the two groups. The authors argued that this could be due to the low mortality rate and a larger proportion of patients treated with front-line ALK-TKI in the v3a/b group. These assumptions are right but can only partially explain the lack of OS differences. The progression was defined according to RECIST criteria, as recommended in clinical trials. However, it is very well known that RECIST-defined progression is not always a marker of treatment failure in the field of oncogene-addiction (14). In ALK-positive NSCLC, oligoprogression as first progression event is not uncommon and could be effectively treated with local ablative therapy and continuing ALK-TKI (15). Continuing crizotinib beyond progression is also a widely used strategy when a clinical benefit of the treatment is still observed (7,16). ALK-positive NSCLC patients that could be managed by this “treatment beyond progression” exhibit a very good prognosis (7,16). As no data are shown about the profile of progression, it cannot be excluded that this subgroup of ALK-positive NSCLC with oligoprogressive disease and/or treated with crizotinib beyond progressive disease is over-represented in the v3a/b group, therefore explaining the lack of OS differences. Furthermore, no information was provided about next-generation ALK-TKI that some patients probably received after crizotinib failure. Next-generation ALK-TKIs are effective in this setting, and imbalance of next-generation ALK-TKI treatment after crizotinib failure favoring the v3a/b group could also explain the lack of OS differences (3). The 2-year PFS rate improvement in the v1/v2/others group was no longer observed when the seven patients treated with next-generation ALK-TKI ceritinib and alectinib were added to the analysis, rising the hypothesis that the efficacy of these ALK inhibitors is less impacted by the EML4-ALK different variants. As ceritinib and alectinib have a better ALK tyrosine kinase inhibitor activity than crizotinib, one could postulate that these former drugs inhibit more effectively v3a/b ALK variants despite a more stable protein product. However, in vitro experiments reported by Woo et al. did not support this hypothesis. Interestingly, preliminary data from ASCEND-1 clinical trial testing ceritinib after crizotinib failure or in ALK-TKI naïve-patients also suggest that PFS with ceritinib is higher in v1 patients than in v3 patients (17). More data are needed to elucidate whether next-generation ALK-TKI affect differently EML4-ALK variants, or not.

Third, the multiplex RT-PCR assay used to detect the EML4-ALK variants is not able to distinguish the variant 3a from the variant 3b. In the original study that suggested the role of different EML4-ALK variants stability in crizotinib efficacy, v2 was the most sensitive, v1 and v3b had intermediate sensitivities and 3a was the least sensitive to crizotinib (8). Pooling v1 and v2 variants then v3a and v3b variants in the same group could be considered somewhat artificial in regard of these previous results. However, experiment correlates from the paper of Woo et al. suggest that v1-expressing BEAS-2B cells were more sensitive to crizotinib than v2- and v3a-variants (9). Unfortunately, no v3b-expressing BEAS-2B was generated but the H2228 cell line which contains a v3b variant was resistant to crizotinib. These results are in line with the clinical correlates but conflicting with the original study from Heuckmann et al. (8).
Non-EML4-ALK rearrangements are not detectable with the assay used by Woo et al. To date, at least 21 non-EML4 partner genes have been described in NSCLC and very few data about sensitivity to ALK-TKI in the clinic are available (Table 2) (1,10,17-30). Partial responses to crizotinib are commonly reported in clinical cases but PFS are very heterogeneous, ranging from 6 months to 28 months (Table 2). In RET-rearranged NSCLC, differences in vandetanib RET-TKI efficacy has been shown according to RET rearrangement variants (31). Responses and PFS were more favorable in CCDC6-RET than in KIF5B-RET fusion. Despite the fact that these

<table>
<thead>
<tr>
<th>ALK fusion partner</th>
<th>Location of fusion partner</th>
<th>Crizotinib efficacy</th>
<th>Next generation ALK-TKI efficacy</th>
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</tbody>
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ALK-TKI, ALK tyrosine kinase inhibitors; PR, partial response; CR, complete response; PD, progressive disease. HIP1, huntingtin interacting protein 1; EIF2AK3, eukaryotic translation initiation factor 2 alpha kinase 3; PRKAR1A, protein kinase CAMP-dependent type i regulatory subunit alpha; PPM1B, protein phosphatase, Mg2+/Mn2+ dependent 1B; FAM179A, family with sequence similarity 179 member A; COL25A1, collagen type xxv alpha 1 chain; KIF5B, kinesin family member 5B; SPTBN1, spectrin beta, non-erythrocytic 1; BIRC6, baculoviral iap repeat containing 6; SEC31A, SEC31 homolog A, copii coat complex component; DCTN1, dynactin subunit 1; SQSTM1, sequestosome 1; KL1, kinesin light chain 1; PTPN3, protein tyrosine phosphatase, non-receptor type 3; TFG, TRK-fused gene; CLTC, clathrin heavy chain; TPR, translocated promoter region, nuclear basket protein; CRIM1, cysteine rich transmembrane BMP regulator 1; STRN, striatin; PICALM, phosphatidylinositol binding clathrin assembly protein; CEBP, CCAAT/enhancer binding protein beta.

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kinds of rearrangements are probably uncommon, more data are needed to evaluate the impact of non-\textit{EML4-ALK} rearrangements on ALK-TKI efficacy.

Finally, the findings of Woo \textit{et al.} raise the question whether all ALK rearrangements are created equal. Considering the conflicting results in the literature and the limitations that have been highlighted herein, these data are too preliminary to answer this question and impact our treatment decision in \textit{ALK}-rearranged NSCLC. However, this hypothesis should be better elucidated using NGS technology to explore all variants, including non-\textit{EML4-ALK} variants, in a more scalable way than multiplex RT-PCR. This NGS diagnostic approach should be considered in clinical trials to enhance the understanding of ALK variants biology, clinical course and impact in treatment efficacy and make a step forward in its clinical application as a relevant predictive biomarker.

\textbf{Acknowledgements}

MD has received research funding from Novartis and Pfizer. He has been reimbursed for travel, accommodation, and/or other expenses by Novartis, Pfizer and Roche; DMS has received research funding from Pfizer; JC has received research funding from Novartis and Pfizer.

\textbf{Footnote}

\textit{Conflicts of Interest}: MD has received research funding from Novartis and Pfizer. He has been reimbursed for travel, accommodation, and/or other expenses by Novartis, Pfizer and Roche; AMF has declared no conflicts of interest; DMS has received research funding from Pfizer. He has served as a consultant (advisory board) for Novartis, Pfizer and Roche; JC has served as a consultant (advisory board) for Novartis, Pfizer and Roche.

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Cite this article as: Duruisseaux M, Mc Leer-Florin A, Moro-Sibilot D, Cadranel J. Are all ALK rearrangements created equal? Transl Cancer Res 2017;6(Suppl 2):S270-S275. doi: 10.21037/tcr.2017.03.10
In the era of precision medicine, rearrangement of the anaplastic lymphoma kinase (ALK) gene has been proven to be a targetable oncogenic driver in 3–7% of patients with advanced non-small cell lung cancer (NSCLC) (1). Multiple clinical trials have demonstrated the superiority of ALK inhibitors compared with chemotherapy for treating patients with ALK-rearranged NSCLC; however, the responses to ALK inhibitors have varied in each study (2-7). Fluorescence in situ hybridization (FISH) or VENTANA anti-ALK (D5F3) immunohistochemistry, which are widely used as standard tests for ALK detection for enrollment in clinical trials, are unable to distinguish between the different variants or fusion partners of the ALK gene. The impact of ALK variants on the heterogeneity of the response to ALK inhibitors has not been fully elucidated.

One major mechanism may be that various portions of the echinoderm microtubule-associated protein-like 4 (EML4) are fused to ALK in different variants, which may be identified by real time-polymerase chain reaction (RT-PCR) or next-generation sequencing (NGS). More than a dozen different variants of EML4-ALK variants and non-EML4 fusion genes have been detected in NSCLC (8-12). Among the variants known thus far, three of the EML4-ALK variants identified in NSCLCs are most commonly reported, including variant 1(V1), variant 2 (V2), and variant 3a/3b (V3a/b) (13-15).

The biological basis for the differential activity of EML4-ALK has been typically correlated with the distinct stability of the EML4-ALK protein. The primary sequence of the EML4 portion comprises different domains, including a hydrophobic EMAP-like protein (HELP) domain that is linked to a variable number of tryptophan-aspartic acid (WD) repeats separated from an N-terminal coiled coil by a basic region consisting of serine, threonine, and basic residues. The tertiary structure of the HELP-WD region creates a tandem atypical propeller EML (TAPE) domain in which the HELP motif is part of the hydrophobic core and is crucial for maintaining the folding of the TAPE region. The TAPE domain influences protein stability. Variants 1 and 2 in which the break point occurs within the N-terminal and the C-terminal β-propeller, respectively, include only a partial TAPE domain. This domain determines the exposure of the hydrophobic core, thus rendering the protein unstable and requiring binding with a chaperone to avoid the protein misfolding. By contrast, variants 3a/b and 5 lack the TAPE domain and are more stable (16). The protein stability of the EML4-ALK variants influence the overall fusion protein stability, inhibitor-induced protein degradation, and drug sensitivity (17).

One highlight of the recent study by Woo et al. published in Annals of Oncology was categorization of EML4-ALK variants based of differential protein stability rather than clinical frequency (15). A total of 51 patients with advanced NSCLC harboring an EML4-ALK fusion were subdivided into two groups: variants 1/2/others (27, 52.9%) and variants 3a/b (24, 47.1%). Among the patients treated
with crizotinib, the 2-year progression-free survival rate (PFSR) was 76.0% (95% CI: 56.8–100) for the EML4-ALK variants 1/2/others group, and this was significantly higher than the 26.4% (95% CI: 10.5–66.6) for the variants 3a/b group (P=0.034). Of note, this report also established specific EML4-ALK variant-expressing cell lines for evaluating the response of various ALK inhibitors. In line with the clinical findings, the in vitro results have demonstrated that all three ALK inhibitors suppressed the growth of V1- or V2-expressing Ba/F3 cells, but had weak inhibition in V3a- or V5a-expressing cells. Contrary to the abovementioned results, another retrospective study in which patients were categorized based on the frequency of ALK variants, no statistically significant correlation between the ALK variants and median PFS of crizotinib was demonstrated by two types of categorization (EML4-ALK V1 vs. EML4-ALK V3a/b vs. other uncommon ALK variants or common EML4-ALK variants including V1 and V3a/b vs. other rare ALK variants) (18). Recently, Yoshida et al. retrospectively analyzed the efficacy of crizotinib in 35 patients with ALK-positive NSCLC categorized by the presence of EML4-ALK V1 versus non-V1 variants. Although there was a statistically significant difference in the disease control rate (95% vs. 63%, respectively; P=0.0318), and median PFS (11 vs. 4.2 months, respectively; P<0.05) (14), the biological rationale for categorizing patients based on the presence of EML4-ALK V1 is somewhat artificial (19). According to an in vitro study using the EML4-ALK variant-expressing Ba/F3 cell line, variants 1 and 3b exhibited intermediate sensitivity, V3a was least sensitive, and V2 was most sensitive to ALK inhibitors (17). To take it a step further, Hrustanovic et al. also discovered differential sensitivity to EML4-ALK V1 and V3b in cell lines. Compared with H3122 (harboring V1), crizotinib failed to suppress RAS-GTP, p-ERK, or cell viability in H2228 cells harboring V3b), and thus the half-maximal growth inhibitory concentration for crizotinib was higher in H2228 than in H3122 cells (20). This difference was caused by the lack of a HELP domain in EML4 variant 3, which enhances activation of the RAS-MAPK signaling pathway. These findings suggested that EML4-ALK V1 and EML4-ALK variant 3a/b might represent two distinct diseases, and patients with EML4-ALK V1 achieved a longer PFS from crizotinib than that found with the EML4-ALK variant 3a/b; thus, the type of ALK fusion may partially determine the initial sensitivity to ALK inhibition.

In addition to the abovementioned progress in determining the correlation between EML4-ALK variants and response to ALK inhibitors, some limitations of this study need to be addressed. First, the small enrollment size might not reflect the true landscape of EML4-ALK variants. With more than ten different EML4-ALK variants identified, the genetic landscape of EML4-ALK variants could be characterized by distinct mountains and hills. Data from earlier studies have demonstrated that EML4-ALK V1 and V3a/b are the most frequent variants, and they have been detected in 33% and 29% of NSCLCs respectively (13), suggesting that both are mountains in the heterogeneous landscape of ALK variants, while other ALK variants, such as V2 and V7, account for 9% and 3%, respectively, and might be categorized as hills. Such a complicated landscape for ALK variants has posed a tough challenge for discriminating various variants in retrospective analysis of small sample sizes.

In addition to the analysis by Woo et al. (15), there were three other retrospective studies analyzing the correlation between ALK variants and the efficacy of ALK inhibitors (14,18,21). It was intriguing to find that distinct ALK variants demonstrated heterogeneous landscapes across these studies, particularly for the common EML4-ALK variants 1 and 3a/b. In addition to the EML4-ALK variants, the percentage of non-EML4 variants also remains controversial, ranging from 3.3% to 36.5% across these four studies. Due to the small sample size of each study, whether patients enrolled with a specific subtype of ALK variants could represent the true genetic landscape of this subpopulation deserves further investigation.

Consequently, results from these retrospective analyses have to be carefully interpreted. With regards to the complexity of ALK variant subtypes and small sample sizes of enrollment, whether such controversial results could be simply attributed to the different categorizations in each study and/or the small sample sizes, which might not represent the true genetic landscape of ALK variants, is largely unsettled. A multi-center, prospective study with a larger cohort is warranted to provide answers to this question.

Second, whether EML4-ALK V3a/b is truly important for the resistance to ALK inhibitors deserves further investigation. The study by Woo et al. draws the conclusion that EML4-ALK V3a/b might be a major source of resistance to ALK inhibitors, which was supported by clinical efficacy analyses and viability tests using established in vitro cell lines (15). It appears that this is the first report on clinical data that recognizes the impact of ALK variants in generating resistance to ALK inhibitors. Previous
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retrospective analyses have mostly demonstrated the differential or similar role of ALK variants in predicting response to crizotinib or ALK inhibitors (14,18,21). Whether such a conclusion could be directly drawn is still worth discussing.

Multiple acquired resistance mechanisms to ALK inhibitors have been identified, including ALK gene alterations, such as ALK point mutations and copy number gain (22,23) and the bypass activation of other oncogenic genes (24,25). In this study, we noticed that only a small percentage of patients (7/23) underwent rebiopsies at disease progression, and there were none with ALK mutations. Thus, without comprehensive data on the ALK mutations that have been considered as a major resistance mechanism to ALK inhibitors, it still needs to validate the role of EML4-ALK V3a/3b in modulating resistance to ALK inhibitors despite evidence from in vitro tests. The emergence of next-generation sequencing techniques will possibly allow for the detection of various ALK variants and mutation screening in a single test in the near future. Further studies employing NGS-based tests might help determine a more precise correlation between specific ALK variants and the efficacy of ALK inhibitors.

Acknowledgements

None.

Footnote

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

References


Lung cancer is the most common cancer worldwide and the leading cause of cancer-related deaths (1,2). About 50% of patients with non-small cell lung cancer (NSCLC) are older than 65 year-old, and median ages at diagnosis are currently 63–70 in Western countries (3). Due to demographic trends and CT-based screening, incidence of NSCLC in elderly is expected to increase. Therefore, the management of these patients is a challenge for the medical community.

Difficulties in management of elderly might be explained by different problems. On the one hand, despite increasing evidence of chemotherapy benefit (4), elderly are often undertreated, because of the nihilism of both doctors, families and patients. On the second hand, elderly are more prone to toxicities and treatment-related mortality, and overtreatment needs to be prevented. Finally, they are too often excluded from clinical trials (5) and there are very few studies dedicated to this population, which makes guidelines difficult to establish. For all these reasons, it is crucial to optimize treatment in elderly patients to better assess the risk-benefice ratio, identifying those who are likely to benefit from chemotherapy of those who are likely to have too much toxicity.

The first trial demonstrating the benefit of single-agent chemotherapy in elderly patients was the Elderly Lung Cancer Italian Study (ELVIS) (6). After that, international guidelines have recommended single-agent therapy as the treatment of choice for elderly population (7,8). Some sub-groups or retrospective analyses from randomized trials then suggested that a platinium-based doublet was feasible and efficient in fit elderly patients (9,10). The current evidence-based for a carboplatin-based doublet as a standard of care in elderly patients was demonstrated by the Intergroupe Francophone de Cancérologie Thoracique (IFCT)-0501 phase III trial. Monthly carboplatin and weekly paclitaxel doublet chemotherapy regimen have been compared with single-agent regimen (either vinorelbine or gemcitabine) in 451 elderly patients with a PS of 0 to 2 with advanced NSCLC (11). Despite increased but manageable toxic effects, doublet chemotherapy was associated with survival benefits compared to monotherapy, with a median overall survival (OS) of 10.3 vs. 6.2 months respectively (HR, 0.64; 95% CI, 0.52–0.78; P<0.0001). Two other phase III studies have confirmed these findings (12,13). Therefore, today’s guidelines recommend carboplatin-based doublet as first-line treatment for fit elderly NSCLC patients, whereas single-agent treatment (gemcitabine, vinorelbine, taxanes) represents a valid option for less fit patients (14).

But what is a fit patient? How can we precisely define a fit patient? Age and Performance Status (PS) are not sufficient to assess the capacity of an elderly to receive CT: comorbidities, age-related physiological variations of the main body functions, long-term treatments, polypharmacy, and social setting must also be considered for the therapeutic algorithm. Basic and reproducible geriatric assessment tools have to be developed in this way. Comprehensive Geriatric Assessment (CGA) is a multidisciplinary and global scale evaluating comorbidities, functional status, cognition, emotional status, social and...
environmental situation, nutritional status, mental health, polypharmacy, and geriatric syndromes. Its objectives are multiple: detecting unknown health problems, evaluating patients vulnerability, preventing iatrogenic effects and functional decline, managing pain and offering psychological support to elderly patients. It aims to reduce both undertreatment as well as overtreatment. CGA have been shown to predict morbidity and mortality in elderly patient treated for cancer (15) and to prevent treatment toxicity in solid cancers (16). Balducci and Extermann used a CGA-based approach to stratify patients in three groups (fit, vulnerable and frail patients) with three adapted treatment options [standard therapy, adjusted therapy, and best supportive care (BSC) respectively] (17).

Until now, even if the use of CGA is encouraged in guidelines, this is mainly on the bases of retrospective studies and no instrument has been shown to improve treatment selection when added to the routine geriatric oncology patient evaluation.

Corre et al. have tried to answer to the question in the Elderly Selection on Geriatric Index Assessment (ESOGIA)-Groupe Français de Pneumo-Cancérologie (GFPC)—Grupo Español de Cáncer de Pulmón (GEPC) 08-02 study (18). This is the first phase III randomized trial comparing in first line a standard strategy of treatment allocation (carboplatin-based doublet or single agent on the basis of PS and age) with experimental CGA-based allocation of the same chemotherapies or BSC. The choice of the chemotherapy regimen is somewhat strange because it adds some complexity to the analysis and is not part of the usual ones, but study was designed before the IFCT-0501 trial and the corresponding recommendations about carboplatin plus weekly-paclitaxel regimen.

The primary endpoint was treatment failure-free survival (TFFS), defined as the time of elapsing between randomization and treatment discontinuation resulting of any reason (disease progression, treatment toxicity, and death). This combined primary endpoint was particularly adapted to elderly patients, taking into account not only progression but also tolerability and death from other causes than cancer. CGA-based treatment allocation failed to improve the TFFS or OS: median TFFS was 3.1 months (2.7–4.4 months) for CGA arm versus 3.2 months (2.9–4.1 months) for standard arm (P=0.32); median OS was 6.1 versus 6.4 months respectively (P=0.87). Nevertheless, patients in CGA arm seemed to be better oriented and to receive a more appropriate treatment: more patients received doublet chemotherapy (45.7% vs. 35.1% in the standard group), and 23% were assigned to BSC. As a result, patients in the CGA arm experienced significantly less all grade toxicity (85.6% vs. 93.4% respectively, P=0.015) and less toxicity-related treatment failure (4.8% vs. 11.8%, P=0.007). Furthermore, CGA identified patients with a poor natural prognosis: median OS BSC was only 2.8 months, which is significantly lower than in other studies (11).

Several geriatric indexes have been shown as independent prognostic factors in lung cancer, such as ADL in the IFCT-0501 study (11), IADL (19) or BMI (20). But the main problem is that none of these factors has ever demonstrated any predictive value. So how relevant the use of CGA is in lung cancer? This tool is time consuming and hard to apply in routine care (approximately one supplementary hour per patient, which will require more medical time or more physicians). Should it be of no help to predict outcomes, maybe it does not make sense to use it for each patient. The cutoffs used to define fit, vulnerable, and frail patients may not be the most appropriate in advanced NSCLC, probably because most of patients die of cancer rather than comorbidities (11,18).

The authors conclude saying that the use of CGA in this setting cannot be routinely advised in clinical practice. Waiting for this, simplified geriatric assessment adding to PS, such as body mass index, Charlson comorbidity index, or ADL would be of interest and their predictive value have to be studied.

Acknowledgements
None.

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

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Cite this article as: Leduc C, Quoix E. Is there really a role for the comprehensive geriatric assessment in metastatic non-small cell lung cancer? Transl Cancer Res 2016;5(S1):S44-S46. doi: 10.21037/tcr.2016.04.11
We thank Dr. Leduc and Pr. Quoix (1) for their interest in and valuable comments about the role of the comprehensive geriatric assessment (CGA) in metastatic non-small cell lung cancer (NSCLC).

We share their comments about the difficulties to manage elderly with advanced NSCLC. Today’s guidelines recommend carboplatin-based doublet as first-line treatment for fit elderly NSCLC patients, whereas single-agent treatment (gemcitabine, vinorelbine, taxanes) represents a valid option for less fit patients (2). But as a matter of fact, no consensual definition of fit elderly patients exists.

The main goals of a CGA are to provide a comprehensive health appraisal to guide appropriate cancer treatment selection and to target geriatric interventions.

ESOGIA trial objective was to evaluate prospectively the first part of this definition: the relevance of CGA to guide the treatment selection for elderly patients with stage IV NSCLC. We compared in first line setting a standard strategy of treatment allocation (carboplatin-based doublet or single agent on the basis of PS and age) with experimental CGA-based allocation of the same chemotherapies or BSC (3). Carboplatin-based doublets were allocated according to histology, squamous and non-squamous histologies were well-balanced between the two arms. The association carboplatin-weekly paclitaxel was successfully tested in the IFCT 05-01 trial (4) and conducted to modifications of international recommendations. Consequently, ESMO guidelines recommend a carboplatin based-doublet as first-line treatment for fit elderly NSCLC patients but without naming the second drug. Carboplatin-pemetrexed doublet has also been successfully tested in a phase II trial (5) and in a large phase III trial dedicated to PS2 (36% of the patients were ≥70 years old) (6). Carboplatin-gemcitabine doublet was compared to carboplatin-paclitaxel in a large phase III trial published by Treat et al. in 2010 with no difference in terms of overall survival, the median age of the patients enrolled was 64 years old (7). According to ESMO recommendations, a single-agent treatment represents a valid option for less fit patients: gemcitabine, vinorelbine and taxanes are the most evaluated. Every three weeks administration schedule of docetaxel has been more evaluated than weekly schedule.

Even if ESOGIA trial provides interesting data concerning efficacy and safety of carboplatin-pemetrexed and carboplatine-gemcitabine in non-squamous and squamous histologies respectively, it is important to note that this trial compared two strategies of allocation of treatment and not chemotherapy regimen. So it was crucial to have the same chemotherapy regimen in the two arms, to answer correctly to the primary objective of the study.

CGA-based treatment allocation failed to improve the TFFS or OS. Can we conclude that CGA is useless in advanced NSCLC?

We consider that CGA can be relevant because, in our study, CGA-based treatment allocation allowed to reduce all grade toxicities and toxicity-related treatment failures. Moreover the CGA identified a sub-group of patients with a very poor prognosis (median OS BSC was 2.8 months),
even if further studies are needed to determine how to treat the best these frail patients. Moreover, as mentioned by Dr. Leduc, several geriatric indexes have been shown as independent prognostic factors in lung cancer, such as ADL in the IFCT-0501 study, IADL or BMI (1). We can add that in ESOGIA trial a BMI \( \leq 20 \text{ kg/m}^2 \), a Charlson comorbidity index \( \geq 2 \), and the existence of a geriatric syndrome were associated to a worse TFFS in multivariate analysis. Published studies that included various types of cancer and among them NSCLC demonstrated that the ADL score and malnutrition were independently associated with changes in cancer treatment (8), and that in advanced solid cancers, a low MNA score (\( \leq 23.5 \)) and a poor mobility predicted early death (<6 months) after initiation of chemotherapy treatment (9).

Moreover, it’s important to remember that ESOGIA trial did not evaluate the second part of CGA’s definition: its ability to guide appropriate targeted geriatric interventions. CGA reveals deficits that are not routinely captured in standard history and physical examination. Geriatric interventions adapted to these deficits can be planned. The impact on outcomes of such interventions has not been prospectively evaluated in the elderly with advanced NSCLC. But in a study comparing the impact of early palliative cares to standard care in patients with metastatic NSCLC, the early palliative interventions improved quality of life and also overall survival (10). Early palliative cares consisted on specific attention to assessing physical and psychosocial symptoms, establishing goals of care, assisting with decision making regarding treatment, and coordinating care on the basis of the individual needs of the patient. This management is probably not so far from what are the geriatric interventions proposed to an elderly population. The precise impact remains nevertheless to evaluate.

Does simplified and less time consuming geriatric assessment adding to other few scales (PS, ADL, BMI…) would be of more interest? Probably not because it appears difficult to summarize a complex status like frailty through very few questions. The strategy that consists to select the patients that could justify a CGA through a previous shorter geriatric screening tool seems to be more relevant.

A lot of progress remains to do in geriatric oncology, we move forward together slowly but surely.

**Acknowledgements**

ESOGIA trial was supported by grants from Eli Lilly, Sanofi, Roche and Chugai.

### Footnote

**Conflicts of Interest:** Romain Corre—consulting or advisory role: Eli Lilly, Roche, Bristol-Myers Squibb; Travel, Accommodations, Expenses: Bristol-Myers Squibb, Amgen, Boehringer Ingelheim, Roche. Christos Chouaid—consulting or advisory role: Eli Lilly, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Roche, Sanofi, Bristol-Myers Squibb, Novartis, Amgen; Travel, Accommodations, Expenses: AstraZeneca, Roche.

### References


Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors. Unfortunately, a majority of patients will experience relapse of their disease within 1 year of completing treatment. Median survival for patients with relapsed disease is dismal at about 5–6 months even with best available therapy. Good performance status and sensitivity to first-line chemotherapy are significant prognostic factors of survival in patients treated for relapsed SCLC (1). Therapy options for relapsed SCLC remain limited, due to poor efficacy of most chemotherapy regimens and the poor performance status of many of these patients at relapse. Currently, topotecan is the only FDA-approved agent for the treatment of relapsed SCLC based on a phase III trial that demonstrated improvement in survival and quality of life (QOL) compared to best supportive care (2).

The proportion of patients who had disease response was also higher in the combination group (84% vs. 27%; risk ratio 0.32; P<0.0001). Based on the results of this study, the authors suggested that combination chemotherapy with cisplatin, etoposide, and irinotecan could be considered as the standard second-line treatment for sensitive relapsed SCLC.

While the authors should be commended for the randomized nature of their trial and the relevance of the question they addressed, there are several aspects of the patient selection and outcomes of this study that raise concerns regarding the general applicability of their results. Greater than 70% of patients in both groups (72% in the topotecan group and 78% in the combination group) had extensive-stage SCLC at entry into the study. The goal of chemotherapy in this setting is palliative and therefore, QOL becomes an even more important consideration. The combination regimen that Goto et al. utilized in their study was very intensive—cisplatin given on days 1 and 8, etoposide on days 1–3, and irinotecan on day 8 of a 21 day cycle. Given the myelosuppressive nature of this regimen, G-CSF support was given daily starting on day 9. QOL was not formally assessed. However, the toxicity of this combination regimen was significant; febrile neutropenia occurred in 31% of patients in the combination group compared to only 7% in the single-agent topotecan group, and more patients experienced a serious adverse event (10% vs. 4%). In addition, 50% of patients in the combination regimens received G-CSF support.
group required a dose reduction and 84% had a dose delay. Overall, the toxicity profile of cisplatin, etoposide, and irinotecan raises significant concerns about the tolerability of this regimen.

The authors did not collect information on the number of patients screened, number of patients who were not eligible, and number of patients who declined participation in the study. This introduces the possibility of enrollment bias, which is supported by the low overall enrollment rate of < two patients per institution per year.

Imbalances in baseline characteristics between the two groups may have skewed results to favor survival in the combination group. In general, over 90% of patients in each group had an ECOG performance status of 0–1, which does not reflect the typical patient with relapsed SCLC. However, 58% of patients in the combination group had an ECOG performance status of 0 compared to 44% of patients in the topotecan group. The impressive performance status of these patients is likely a significant contributor to the relatively good survival noted in both arms of this study. In fact, the results of a study by Sundstrøm et al. showed that performance status at recurrence was the only independent predictor of survival in patients with relapsed SCLC (4). Goto et al. also did not report the number of patients with extensive stage disease in each group nor the proportion that received prophylactic cranial irradiation, which has also been shown to improve overall survival (5).

The median time to relapse in the combination group was substantially greater than in the topotecan group (181 vs. 148 days, respectively). Increased time to recurrence is also a positive prognostic factor for survival (1). In current practice, the time to relapse in SCLC also influences the chemotherapy that is recommended. Per NCCN guidelines, a platinum and etoposide doublet is the recommended first-line therapy. If relapse occurs more than 6 months after completion of first-line therapy, reuse of the initial regimen should be considered in patients with eligible performance status (6). The interquartile range for time to relapse in the topotecan group was 113–228 days (7.8 months) with a range of 92–2,318 days (6.4 years). This, in addition to their overall excellent performance status, suggests that a subset of the patients randomized to the topotecan group were eligible to receive a platinum-etoposide doublet and were therefore undertreated. In their study, Goto et al. administered topotecan at 1.0 mg/m² IV on days 1–5 of a 21 day cycle, which is lower than 1.5 mg/m² that is the approved dose in the United States. Huber et al. found that that a topotecan dose of 1.25 mg/m² is equally efficacious to the 1.5 mg/m² dose (7) and a phase II Japanese study showed continued efficacy of topotecan at 1.0 mg/m² (8). Therefore, the lower topotecan dose likely did not contribute to the improved overall survival of the combination chemotherapy group.

Unfortunately, efficacious treatment options for relapsed SCLC remain limited. Although the results of JCOG0605 are provocative, they cannot be generalized to the average patient with relapsed SCLC. Therefore, while a select few patients who are very fit could be considered for combination chemotherapy with cisplatin, etoposide, and irinotecan, the regimen will likely not be tolerated by most and should not be considered as standard second-line treatment.

Acknowledgements

None.

Footnote

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

References


Combination chemotherapy for relapsed small-cell lung cancer—perspective on mechanisms of chemoresistance

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Provenance: This is an invited Perspective commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Abstract: Small cell lung cancer (SCLC) has a dismal prognosis due to early dissemination and aggressive growth. Despite high response rates to initial chemotherapy, SCLC relapses fast and exhibits broad chemoresistance. The JCOG0605 Japanese trial reported increased survival for a regimen combining cisplatin with etoposide and irinotecan compared to topotecan in chemosensitive patients and proposed this treatment as standard chemotherapy. Analysis of the trial data indicates an enrichment of patients with favorable prognosis in the combination chemotherapy arm, questioning the feasibility of this highly aggressive regimen in typical SCLC patients of higher age and afflicted by comorbidities. Considering the modest prolongation of life with current therapies, quality of life should be traded against extension of survival rated in months. Circulating tumor cell (CTC) lines established from relapsed SCLC patients suggest chemoresistance due to formation of large spheroidal multicellular aggregates, termed tumorospheres, which restrict drug access and contain quiescent and hypoxic cells. With the possible exception of metformin, clinical means to eliminate such tumor spheroids are confined to experimental research with cell lines and xenografts, but this new insight into chemoresistance of SCLC discloses entirely new modes of efficient treatment of SCLC.

Keywords: Small cell lung cancer (SCLC); combination chemotherapy; quality of life; chemoresistance; metformin

doi: 10.21037/tcr.2016.11.51
View this article at: http://dx.doi.org/10.21037/tcr.2016.11.51

Introduction

Small cell lung cancer (SCLC) comprises approximately 15% of lung cancers and is found disseminated in the great majority of patients at first presentation (1). After confirmation of the diagnosis by biopsy, chemotherapy is started and, with few examples of refractory disease, patients respond well to platinum-based combination therapy, with response rates to first-line treatment on the order of 70–90% in limited disease and 50–60% in extended disease (2). Etoposide-platinum (EP) was shown to be superior to cyclophosphamide, epirubicin, and vincristine (CEV), with significantly higher 2- and 5-year survival rates of 14% and 5% in the EP arm versus 6% and 2% in the CEV arm, respectively (3). Platinum-based chemotherapy regimens did not offer a statistically significant benefit in survival or overall tumor response but increase complete response rates, at the cost of higher adverse events (4). Trials of three- and four-drug regimens, dose-intensifying regimens, the addition of third generation cytotoxic agents (e.g., gemcitabine, taxanes, topotecan), and high-dose chemotherapy have all failed to
improve outcomes (5).

However, despite high response rates to initial chemotherapy, nearly all patients with SCLC eventually relapse with relatively chemoresistant tumors which are difficult to treat and have a dismal prognosis (1,6). Patients with “sensitive” disease, that is, who have relapsed beyond 60 or 90 days of completing first-line treatment, are regarded to benefit most from second-line treatment. Low performance status and weight loss at the time of relapse relate to a poorer prognosis. Efficacy of second-line chemotherapy is much lower than that of first-line treatment, but it can provide significant palliation and prolongation of survival for many patients (7). For patients who relapse >6 months after initial treatment, retreatment with the original regimen may be applied but for patients who relapse within 6 months, therapy is more controversial, because many patients have a poorer performance status, and the benefit of second-line chemotherapy over best supportive care was not clear (8).

The single drug approved for second-line treatment of SCLC is topotecan and an anthracycline-based regimen consisting of cyclophosphamide, adriamycin (doxorubicin or epirubicin), and vincristine (CAV/CEV) represents an alternative. Topotecan proved to result in prolonged survival compared to best supportive care (median 26 versus 14 weeks) and offered better tolerability with equal efficacy compared to the CAV scheme (9-11). However, all second line treatments resulted in poor response rates and short-lived stabilization of the disease. In general, attempts to use more aggressive regimens have resulted in larger proportions of patients achieving responses without significant prolongation of survival (12). Unfortunately, all trials to achieve better therapeutic responses with a host of alternative drugs failed so far, as well as trials employing targeted agents (13,14). The genomic makeup of SCLCs was characterized in great detail, but in the presence of a universal inactivation of the two tumor suppressor proteins p53 and retinoblastoma RB1, a range of diverse and interchangeable drivers are responsible for aggressive tumor growth (1,15). Thus, in contrast to NSCLC, where targeted agents against mutated driver proteins proved highly effective, similar kinase addictions could not be found for most SCLCs. Numerous attempts are ongoing to improve survival of these patients in order to overcome the poor progress in therapy for SCLC for the last decades. Furthermore, the definite mechanisms producing general chemoresistance to a host of unrelated drugs in relapsed SCLC has not been defined so far (16).

The JCOG0605 trial of the Japan Clinical Oncology Group

The JCOG0605 trial investigated combined chemotherapy with cisplatin, etoposide, and irinotecan versus topotecan alone as second-line treatment for patients with sensitive relapsed SCLC in a multicenter (n=29), open-label and randomized phase III trial (17). This study included 180 patients and sensitive relapsed SCLC is defined as a recurrence that occurred ≥90 days after completion of first-line therapy. The term “sensitive” indicates that patients were not refractory from beginning and may be susceptible to further chemotherapy but does not suggest that the relapsing tumors are actual chemosensitive at a cellular or tumor physiological level. Randomization was done via the minimization method with biased-coin balancing for Eastern Cooperative Oncology Group (ECOG) performance status, disease stage at recruitment, and institution. Combination chemotherapy consisted of five 2-week courses of intravenous cisplatin 25 mg/m² on days 1 and 8, intravenous etoposide 60 mg/m² on days 1–3, and intravenous irinotecan 90 mg/m² on day 8, with granulocyte-colony stimulating factor (G-CSF) support. Topotecan therapy consisted of four courses of intravenous topotecan 1.0 mg/m² on days 1–5, every 3 weeks. The primary endpoint was overall survival (OS) in the intention-to-treat population, which was analyzed with a one-sided significance level of 5%, and safety was assessed in all patients who received at least one dose of medication.

This study reported a significant improvement in OS with the combination therapy in relapsed SCLC [median 18.2 months (95% CI, 15.7–20.6) with combination therapy vs. 12.5 months (95% CI, 10.8–14.9) with topotecan; HR, 0.67 (95% CI, 0.51–0.88); P=0.0079]. Both the proportion of patients achieving an objective response (84% vs. 27%; P<0.0001) and progression-free survival [5.7 months (95% CI, 5.2–6.2) vs. 3.6 months (95% CI, 3.0–4.4); P<0.0001] were better with combination therapy than with topotecan alone. The authors concluded that this combination chemotherapy should become the standard treatment for selected patients with sensitive relapsed SCLC.

Patient characteristics of the JCOG0605 study arms

In a critical accompanying commentary to the trial report, Kalemkerian criticized the patient selection of the JCOG0605 as severely biased (18). This study enrolled subjects who were younger and healthier than the usual
population of patients with SCLC. The great majority of patients had performance status of 0–1, a very long first remission and a frequent administration of third- and fourth-line chemotherapy. Furthermore, the interval between progression and death was unusual long and both study groups showed a much better than expected survival. In particular, patients receiving the combination therapy had a better performance status than those assigned to topotecan (58% vs. 44% with performance status 0) and median duration of initial response to first-line chemotherapy likewise favored the combination therapy group (181 vs. 148 days). Some patients in the control group received suboptimum therapy, since combination chemotherapy, rather than single-agent therapy, is regarded as the most appropriate option for patients who have a relapse more than 180 days from initial treatment (19).

The previous phase II trial of the cisplatin, etoposide, and irinotecan combination led by the same group, in which sensitive relapse was defined as more than 56 days after the end of treatment (rather than ≥90 days), reported a much shorter median survival than did JCOG0605 (11.8 vs. 18.2 months), despite a similar objective response rate (78% vs. 84%) suggesting an important impact of the long median duration of initial response (20). Another Japanese study reported that re-induction with the first-line combination regimen yielded a favorable median OS of 15.7 months in patients who had relapse beyond 180 days (21). In JCOG0605, a lower-than-standard dose of topotecan was used, but attenuated-dose topotecan is commonly used in practice (22). Finally, 50% of patients treated with the combination therapy required dose-reductions and 22% stopped treatment because of adverse events consisting of grade 3–4 neutropenia and anaemia in more than 80% of patients, and febrile neutropenia occurred in 31% of patients, raising serious concerns about the tolerability of this regimen. Unfortunately, quality of life was not analyzed. In these patients, with limited survival expectations, symptom palliation, quality of life, and convenience of therapy are especially important end points. Moreover, symptom palliation correlates well with QoL and survival duration, providing further rationale for therapy selection based on these parameters (23). The survival reported in JCOG0605 is encouraging for the highly selected patients enrolled in the trial, but previous experience suggests that promising initial results might not be reproducible in other populations (24,25). Especially, these study participants do not represent the average patient with SCLC in the USA including elderly people who smoke and have impaired performance status due to comorbidities and the aggressiveness of the disease. Further study is needed before the cisplatin, etoposide, and irinotecan combination can be accepted as the standard treatment for patients with relapsed SCLC.

**Chemoresistance of relapsed SCLC**

Although topotecan has been approved by many countries for the monotherapy of relapsed SCLC, its low response rate and short median survival time is disappointing. Compared with topotecan, irinotecan and etoposide did not show any advantages as single agents (26). However, the combination of cisplatin with etoposide and irinotecan represents a potentiation of the cytotoxicity of the DNA-damaging agent cisplatin and the inhibition of the subsequent startup for DNA repair by both topoisomerases I and II by irinotecan and etoposide, respectively. In this manner, basic processes of every cell in the body are affected, such taking into account severe side effects in the hope of a small differential impact on malignant versus normal tissues. The combination of several agents with high toxicity is of course thus contrary to the aim of targeted therapy to avoid chemotherapeutics with poor specificity and to develop agents against key proteins of the tumors which are indispensable for tumor growth and progression. However, SCLC exhibits no oncogene addiction which can be suppressed for broader subpopulations of the patients and, consequently, all attempts to apply precision medicine failed so far (1,13). Furthermore, the mechanisms behind chemoradioresistance in relapsed SCLCs were not elucidated so far and, therefore, specific agents to resensitize the tumor cells could not be formulated. Chemoresistance of relapsed SCLC proved to be universal and new camptothecins, platinums and other drugs with novel targets failed (27). Moreover, research investigated SCLC was hampered by scarcity of tumor material, since after drawing of a small biopsy therapy is initiated by chemotherapy without any further invasive procedure.

A unique feature of SCLC, namely the occurrence of excessive numbers of circulating tumor cells (CTCs) provided an opportunity to study tumor dissemination and evolution of chemoresistance. In contrast to breast, colon and prostate patients who have a negative prognosis with a CTC count of several cells/7.5 mL of blood as detected with the CellSearch system, CTC counts in SCLC patients may exceed more the 400 cells in the same volume of blood (28,29). CTCs are shed by tumors and are responsible for induction of secondary lesions at distal
sites (30). The high CTC counts of SCLC recurrences allowed us to set up permanent CTC SCLC lines and to study their cell biologic characteristics (31,32). The CTCs as single cells proved to be chemo-sensitive to second-line chemotherapeutics toptotecan and epirubicin (33). However, all six lines established from relapsed SCLC patients so far formed large multicellular spheroidal structures, termed tumorospheres, which exhibited marked resistance to a range of chemotherapeutic drugs in vitro (34). The tumorospheres reach diameters of 1–2 millimeters and they assemble spontaneously in tissue culture (35). Such structures are known to contain interior layers of quiescent cells and hypoxic core regions. Chemo-resistance is caused by limited penetration of drugs, low proliferative activity, cell-cell contact-mediated resistance and resistance to irradiation by lack of oxygen radical formation (36). Cell death in response to chemotherapeutics only occurs in outer spheroid regions, as a viable multicellular tumor spheroids (MCTS) core could be isolated after recovery from cytostatic treatment and removal of the dead cell layer. Such protection from cytotoxic drugs in form of a physical barrier which limits access of agents, nutrients and oxygen leaves a host of unrelated compounds ineffective without referring to individual cellular pathways of drug inactivation (28,37).

Unfortunately, at present most means to eliminate tumor spheroids are in early preclinical development. The efforts to improve cancer therapy largely rested upon massive work to fully characterize the genome of cancer cell and decipher their transcriptomes. However, tumors have been described as “organs” with three-dimensional structures and specific microenvironmental characteristics (38). To be most effective anticancer drugs must penetrate tissue efficiently, reaching all the cancer cells in a concentration sufficient to exert a therapeutic effect. Most research into the resistance of cancers to chemotherapy has concentrated on molecular mechanisms of resistance, whereas the role of limited drug distribution within tumors or spheroids has remained largely unattended (39). Around 95% of new anticancer drugs eventually fail in clinical trial, despite robust indications of activity in existing in vitro preclinical models (40). Innovative models are required that better capture tumor biology, instead of reductionist 2D-culture or artificial cluster models. Techniques to grow 3D-cultures include aggregating cells at the bottom of a drop, different methods to prevent cell from attaching to substrates or growing cells in stirred culture systems. 3D-spheroid closely resembled avascular tumor nodules, micrometastases, and inter-vascular regions of large solid tumors (41). Resistance to cytotoxic agents is due to insufficient distribution of the drugs, non-proliferative and hypoxic cells in the core of the spheroid, cell-cell interactions mediated by E-cadherin, and production of extracellular matrix (ECM) proteins. Comparison of 3D- with 2D-cultures suggested up-regulation of E-cadherin, downregulation of vimentin, decreased expression of the proliferation marker Ki-67 and increased expression of the apoptotic marker caspase-3 in spheroids (42).

Several approaches may be promising to target multicellular tumor structures. Drug formulations with lipids or nanomaterials which accumulate at tumors or penetrate cellular aggregates are in development. Junction openers are investigated in order to open cell-cell connections in order to improve drug diffusion. Furthermore, ECM components can be attacked enzymatically but most enzymes are rapidly inactivated in the circulation. Special formulations like in the case of pegylated recombinant hyaluronidase (PEGPH20) overcome this limitation and seem to have a therapeutic benefit in patients with hyaluronic acid-rich pancreatic ductal adenocarcinomas (43). Treatment led to re-expansion of the tumor vasculature, reduction in tumor hypoxia, and increased penetration of drugs into the tumor as well as reduced signaling via CD44 (44).

Nine substances that specifically target cells in inner MCTS core regions were identified in a screen of drugs in 3D-cell cultures (45). These compounds act as inhibitors of the respiratory chain in dependence of extracellular glucose concentrations and showed synergistic cytotoxicity with chemotherapeutics against spheroids. Outer MCTS cells (or cells cultured in 2D), with direct access to glucose resort to glycolysis while cells in inner MCTS regions with lower glucose levels become sensitive to inhibitors or uncouplers of the respiratory chain. Sequential treatment with chemotherapeutics and metformin targeted the dormant cell population in the MCTS core (45). The reported cancer-protective effect of metformin could be induced, in addition to other mechanisms, by a combination complex I respiratory chain inhibition and concomitant lowering of blood glucose levels. The beneficial effect of metformin medication in diabetic patients for treatment of SCLC has been documented in several studies. A trial in 259 SCLC patients showed that the use of metformin decreased SCLC recurrence rate (46). Median OS and DFS were significantly better in the metformin group (OS 19.0 vs. 11.5 months, DFS 10.5 vs. 7.0 months). In another study with 79 diabetic patients, median OS and DFS were again significantly better in the metformin group (OS 18.0 vs. 11.5 months, DFS 10.8 vs. 6.5 months) (47). Metformin
might be considered a potential useful anticancer drug in treating SCLC patients. Metformin could enhance CP treatment in SCLC cells, likely through promoting further IGF-1R down-regulation (48). Trials of metformin in combination with (radio)chemotherapy are ongoing for NSCLC (49,50).

Conclusions

This study confirms the previous finding that a higher dose intensity of chemotherapy can be delivered to SCLC patients with a good performance status which the typical patient with lower performance status is unable to tolerate. Chemoresistance in SCLC seems to be related to CTC-derived tumorospheres which resemble highly organized multicellular structures which differ from most spheroidal cell aggregates induced by prevention of cellular attachment. This type of physiological resistance requires completely new strategies to eliminate tumor cells and to prolong survival of SCLC patients.

Acknowledgements

None.

Footnote

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

References


Small cell lung cancer (SCLC) makes up approximately 13–15% of all lung cancer cases (1). Despite initial response to treatment, a large majority of patients with extensive stage SCLC relapse within 6 months (2). Improved outcome for SCLC patients remains stunted in major part because of lack of effective therapies for progressive disease following frontline therapy. Topotecan is the only salvage therapy with worldwide approval but its efficacy is quite modest and may be ineffective in patients with platinum insensitive disease (3-5). Contemporary comparative phase III studies of cytotoxic agents such as cabazitaxel and amrubicin against topotecan for relapsed SCLC have been negative especially in Western patient populations (6,7). It is therefore intriguing and interesting to observe that the randomized phase III JCOG0605 trial recently reported by Goto et al. showed an impressive benefit of the combination of cisplatin, etoposide and irinotecan, which significantly outperformed topotecan as second line therapy for patients with sensitive relapsed SCLC (8).

The study compared topotecan as standard therapy to the investigational regimen of cisplatin, etoposide and irinotecan in an open label, multicenter randomized trial that enrolled 180 patients with 90 patients per arm. Treatment was administered along with growth factor support as five 2-week cycles of combination chemotherapy (cisplatin 25 mg/m² on days 1 and 8, etoposide 60 mg/m² on days 1–3 and irinotecan 90 mg/m² on day 8) versus single agent topotecan (1.0 mg/m² on day 1–5 every 3 weeks) for four cycles. An impressive median overall survival of 18.2 months (95% CI, 15.7–20.6) versus 12.5 months (95% CI, 10.8–14.9) with more than 30% reduction in the risk of death (stratified HR, 0.67; 90% CI, 0.51–0.88; P=0.0079) was recorded in favor of the experimental arm. This is an unprecedented result in this disease especially in the relapsed setting. An astounding result like this therefore warrants a critical appraisal of various aspects of the study design, the selection of the experimental and comparator treatments, as well as the patient population for proper contextualization of the data. Several prognostic factors are associated with improved outcome in SCLC including, performance status, gender, burden of disease and response to platinum-based frontline therapy (9). The JCOG0605 study was designed to compare efficacy of two regimens in patients who progressed following frontline therapy with restriction to patients with sensitive relapse. While topotecan is an acceptable regimen for this population, retreatment with platinum doublet is also an established and perhaps preferable option for those with treatment free interval of more than 180 days, as observed in a significant proportion of patients enrolled on the JCOG0605 study.
27%, which is comparable to historical data, makes one fact that the response rate for the topotecan arm was only even for platinum sensitive disease. Moreover, the modest JCOG0605 study in the relapsed setting is quite unusual response rate for the triplet chemotherapy regimen in the double the rate for resistant disease (16). However, 80% chemotherapy in sensitive relapse SCLC patients is showed in a meta-analysis of results of clinical studies in to warrant a definitive phase III study. We previously the two regimens were therefore deemed uninteresting be expected for platinum doublet chemotherapy and and 11.0 months for both arms was no better than would be expected for platinum doublet chemotherapy and the two regimens were therefore deemed uninteresting to warrant a definitive phase III study. We previously showed in a meta-analysis of results of clinical studies in relapsed SCLC that objective response rate to salvage chemotherapy in sensitive relapse SCLC patients is double the rate for resistant disease (16). However, 80% response rate for the triplet chemotherapy regimen in the JCOG0605 study in the relapsed setting is quite unusual even for platinum sensitive disease. Moreover, the modest efficacy of a similar regimen in the E5501 study and the fact that the response rate for the topotecan arm was only 27%, which is comparable to historical data, makes one wonder about other factors beyond the chemotherapy that could have contributed to this outcome.

The study population is another factor to consider as possible contributor to the survival benefit of the triplet chemotherapy in the JCOG0605 study. Ethnic based differences in the effectiveness and adverse event profiles of topoisomerase inhibitors are well recognized. It is also well demonstrated that irinotecan may be more effective in Japanese population in part due to pharmacogenomic differences but the magnitude of benefit of irinotecan in the frontline or post frontline setting for Japanese patients quite modest and not sufficient to explain the survival benefit observed in the JCOG0605 study (17-20). Finally, the study population was defined as those with sensitive relapsed SCLC, which on face value implies that most of these patients were extensive stage disease patients who have progressed and need second line treatment. However, a quarter of the patients were originally diagnosed with limited stage disease and more than 40% of the patients received radiation along with chemotherapy for the frontline therapy. It is unclear how many of these patients progressed outside the original site of disease. This study population should therefore not be taken as fully representative of the typical second line extensive stage SCLC patient population. Perhaps the enrichment for patients with limited stage disease and those with low volume extensive stage disease contributed to the improved survival recorded in this study. Additionally, since this population is already preselected for platinum sensitivity, one could speculate that retreatment with an intensified platinum-based regimen really amplified the efficacy out of proportion to what would be expected in an unselected patient population as was the case with the E5501 study. Regardless of the reason for this impressive survival benefit, this approach highlights a potential opportunity to exploit for improved outcome for SCLC patients. It is conceivable that a similar strategy to intensify platinum doublet chemotherapy in platinum sensitive relapse using biologically rational agents such as PARP inhibitors and mTOR inhibitors without overlapping toxicity could lead to comparative or even greater survival benefit and without additive toxicity.

In conclusion, the JCOG0605 trial demonstrated a significant advantage to a three-drug chemotherapy combination and identified another salvage therapy option for sensitive relapse SCLC. Real world application of this regimen will be limited by the significant hematologic toxicity and careful patient selection focusing on those with small volume disease who achieved objective response...
to frontline platinum doublet chemotherapy. Moreover, whether this regimen is applicable to Western population of patients would require additional investigation given the known differences in topoisomerase inhibitor efficacy and toxicity between Japanese and non-Japanese patients of North America and Europe.

Acknowledgements

None.

Footnote

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

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Cite this article as: Patel NT, Owonikoko TK. Thriving where others have faltered—a critical appraisal of the role of patient factors versus treatment effect in JCOG0605 trial in relapsed small cell lung cancer. Transl Cancer Res 2016;5(Suppl 6):S1082-S1084. doi: 10.21037/tcr.2016.11.49
Small cell lung cancer (SCLC) is a very aggressive and complex disease representing approximately 12% to 15% of all lung cancers (1). More than 90% of patients diagnosed with this disease are elderly, current or former heavy smokers (2). SCLC is characterized by rapid growth, early metastasis, and excellent initial response to chemotherapy and radiation (3). The dramatic response to frontline chemotherapy and radiation, unfortunately, contrasts with its subsequent disappointing responses in the relapsed setting. Patients with recurrent disease have a dismal survival of approximately 5 months when treated with chemotherapy (4). Topotecan is the only second-line drug approved by the Food and Drug Administration (FDA) in the United States. Response rate (RR) to topotecan are highly dependent on the progression-free survival (PFS) after frontline platinum-based therapy, reaching 25% in patients who relapsed ≥3 months (sensitive disease) after front-line therapy and <10% for those whose disease relapsed <3 months from initial platinum-based treatment (5).


doio: 10.21037/tcr.2016.12.74
View this article at: http://dx.doi.org/10.21037/tcr.2016.12.74

The JCOG0605 study published in Lancet Oncology was a multicenter phase III randomized trial, comparing cisplatin plus etoposide plus irinotecan with the standard topotecan monotherapy in patients with SCLC with a sensitive relapse (6). The major eligibility criteria included: sensitive relapse (>90 days from the initial platinum-based chemotherapy or chemoradiotherapy); Eastern Cooperative Oncology Group (ECOG) performance status 0–2; and adequate organ function. Patients were randomized 1:1 to receive either topotecan or cisplatin plus etoposide plus irinotecan with growth factor support (combination chemotherapy). A total of 180 patients were enrolled, 90 assigned to each treatment group. The primary endpoint of overall survival (OS) was significantly longer in the combination chemotherapy group (median 18.2 months) compared to the topotecan group [12.5 months; hazard ratio (HR) 0.67; 90% CI, 0.51–0.88; P=0.0079]. RR was dramatically higher in the combination chemotherapy group (84% vs. 27%; 95% CI, 0.22–0.46; P<0.0001). The most common grade 3 or 4 adverse events were neutropenia (83% in the combination chemotherapy group vs. 86% in the topotecan group), anemia (84% vs. 28%), leucopenia (80% vs. 51%), febrile neutropenia (31% vs. 7%), and thrombocytopenia (41% vs. 28%). Serious adverse events were reported in 4% of patients in the topotecan group and 10% in the combination chemotherapy group. The results of this trial led the authors to conclude that the combination of cisplatin, etoposide, and irinotecan should be the new standard of care for selected patients with sensitive relapsed SCLC.

The study presented by Goto et al. met the primary endpoint of OS in patients with sensitive relapse SCLC (6). However, these results need to be analyzed closely prior to making a generalized recommendation in all relapsed SCLC patients. The first aspect to highlight is the highly selective population enrolled in this study, as demonstrated by almost 60% of the patients in the combination arm
having an ECOG performance status of 0, compared to 44% in the topotecan arm. In addition, in the combination arm patients had a longer time to relapse/progression after platinum-based therapy compared to the patients in the topotecan arm (181 vs. 148 days, respectively). Even after selecting healthier patients, the toxicity associated with the combination arm was very concerning. Of note, grade 3 or worse neutropenia and febrile neutropenia were reported in 83% and 31% patients receiving combination chemotherapy, respectively.

Lastly, can the results of this study be applied to the Caucasian population? In 2002, Noda et al. published the results of a phase III trial performed in Japan that compared irinotecan plus cisplatin to etoposide plus cisplatin in patients with newly diagnosed ES-SCLC (7). The median survival was 12.8 months in the irinotecan plus cisplatin and 9.4 months in the etoposide plus cisplatin arm (P=0.002). Subsequently, 2 large randomized trials done in the United States comparing cisplatin/etoposide to cisplatin/irinotecan in treatment naïve ES-SCLC failed to demonstrate a significant survival difference between the arms (8,9). A plausible explanation for the different outcomes in the Japanese and North America results is the genetic variability, and pharmacodynamics between these ethnic groups.

Therefore, although there is a significant survival advantage seen with the combination of cisplatin, etoposide and irinotecan, the combination appears to be associated with increased toxicity; nonetheless it could still be an option for highly selected, young, fit, Asian patients with sensitive-relapse SCLC. Given previous experiences with discordant results using an irinotecan based regimen, caution should be taken to generalize the results into a standard second-line treatment for sensitive-relapse.

Unfortunately, the therapeutic options for SCLC have remained unchanged over the last 30 years (10). Despite the heterogeneity and high incidence of mutations in SCLC, no targeted therapy has shown to benefit these patients. More recently, however, the use of immunotherapy has entered into the treatment arsenal to tackle cancer. A phase I/II trial (CheckMate 032) assessed the activity and safety of nivolumab and ipilimumab in 216 patients with SCLC who progressed after one or more lines of therapy. RR was 18% with nivolumab monotherapy and 23% with nivolumab/ipilimumab. The median OS was 4.4 months with monotherapy (95% CI, 2.9–9.4) and 8.2 months with combination therapy (95% CI, 3.7–not reached). Treatment was well tolerated with safety profiles similar to that observed in other diseases (11). Another exciting study presented at ASCO by Rudin et al. evaluated a first-in-human antibody-drug conjugate against delta-like protein 3 (DLL3), rovalpituzumab tesirine (Rova-T) (12). The trial included 74 patients with SCLC that had progressed on at least one prior therapy. In DLL3 overexpressors (≥50% of cells expressing DLL3), the RR was 55%. The most common grade 3 and higher toxicities were thrombocytopenia 12%, serosal effusions 11%, and skin reactions 8%. A phase II trial using Rova-T in the 3rd line setting is currently enrolling (TRINITY trial). The combination of Rova-T and nivolumab in the front-line setting is also on the horizon and will be explored in the near future.

In summary, after 30 years of dismal progress in the treatment of SCLC, we are finally starting to see some light at the end of the tunnel. The checkpoint inhibitors (nivolumab and ipilimumab) and Rova-T are exciting novel agents studied in the second-line and beyond. They are also characterized by manageable toxicity profiles, which is essential in the palliative scenario. For now, initial management for SCLC continues to be driven by platinum based-therapy and second-line remains topotecan, but hopefully not for much longer.

Acknowledgements

None.

Footnote

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

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Treatment of small cell lung cancer (SCLC) remains a significant challenge for the oncologists. Attempts to improve the results of first- and second-line treatment have all failed so far and no real progress has been made in last years, emphasizing the need for novel strategies of treatment. Patients with relapsed SCLC are usually classified into different categories, according to the time elapsed from the end of previous treatment: sensitive, if tumor progression is documented at least 3 months after the completion of initial treatment, or resistant if tumor progression occurs within 3 months. In sensitive patients, the same platinum-based treatment used as first-line can be re-administered, although there are no randomized trials definitely demonstrating the efficacy of this rechallenge strategy (1). Moreover, the chance of obtaining a new response is higher in patients which had previously obtained a complete response and a long treatment free interval (2,3). In a non-randomized study in Japanese patients, the rechallenge did not demonstrate progression-free survival (PFS) or overall survival (OS) superiority compared to other regimens, but the small number of patients and the retrospective nature of the study did not allow a definitive conclusion on this topic (4). Several agents have shown modest activity in phase II trials, and to date, topotecan is the only approved drug for the second-line treatment of SCLC patients (5). In four randomized clinical studies conducted with topotecan in patients with relapsed SCLC, intravenous topotecan was compared with best supportive care (BSC), combined chemotherapy with cyclophosphamide, doxorubicin and vincristine (CAV), oral topotecan and amrubicin: topotecan improved OS and quality of life compared with BSC, while CAV and amrubicin did not show any survival benefit compared with topotecan (6-9). Although the efficacy of topotecan was low, with response rates from 7% to 24% and OS from 5.8 to 9.9 months, no regimen showed superiority over topotecan that continues to be considered as the standard second-line chemotherapy for patients with relapsed SCLC. Irinotecan showed promising activity in patients with relapsed SCLC and it was used as single agent or in combination with etoposide, with the aim to enable the synergistic effects of a topoisomerase II inhibitor (etoposide) and a topoisomerase I inhibitor (irinotecan) (10-12). The feasibility and the activity of a weekly chemotherapy regimen consisting of cisplatin plus etoposide plus irinotecan, with granulocyte colony-stimulating factor (G-CSF) support was first evaluated in a phase I trial (JCOG9507) and then in a phase II study, where this combination chemotherapy regimen showed a 78% of responses and a median OS of 11.8 months, supporting the further development of the combination (13,14). JCOG0605 is a large, multicentre, open-label, randomized clinical trial comparing combined chemotherapy with cisplatin, etoposide and irinotecan versus topotecan alone as second-line treatment for patients with sensitive relapsed small-cell lung cancer (JCOG0605): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2016;17:1147-57.
A phase III trial that evaluated a combination chemotherapy with cisplatin, etoposide, and irinotecan versus topotecan alone as second-line treatment for Japanese patients with sensitive relapsed SCLC (15). The study met the primary and secondary endpoints: combination chemotherapy with cisplatin plus etoposide plus irinotecan improved OS compared with topotecan (18.2 vs. 12.5 months; HR: 0.67; P=0.0079). Moreover, PFS was significantly longer (5.7 vs. 3.6 months; HR: 0.50; P<0.0001) and the proportion of patients who achieved an objective response was significantly higher (84% vs. 27%; RR: 0.32; P<0.0001) in the combination chemotherapy group than in the topotecan group. Combination chemotherapy was associated with a worst toxicity profile, in terms of grade 3 or 4 anemia, febrile neutropenia and thrombocytopenia, without difference in treatment-related deaths (1 in the combination chemotherapy group and 2 in the topotecan group). Other strengths of the study are the statistical design, allowing the detection of a 33% prolongation in OS (primary objective), the large sample size (180 patients), and the balance of the subsequent regimens of chemotherapy between the two groups. Limitations of the study, as highlighted by the authors themselves, are the lack of quality of life as endpoint, considering the palliative aim of the treatment, and the chosen dose of topotecan (1.0 mg/m\(^2\)), lower than the approved dose (1.5 mg/m\(^2\)), commonly considered very toxic.

The authors concluded that this is the first time that any regimen has shown a survival benefit compared with single-agent topotecan in SCLC and that combination chemotherapy with cisplatin plus etoposide plus irinotecan could be considered the new standard second-line chemotherapy for selected patients with sensitive relapsed SCLC. We agree with the first statement, but we think that there is less data to support the second conclusion, at least in patients of Western countries. In fact, the results obtained with this irinotecan based regimen in Japanese patients can't be generalized to patients of Western countries, considering the contrasting results observed in first line with irinotecan combinations between trials conducted in Japan and in North America, probably due to the presence of inherent genetic differences that exist between North American and Japanese populations, resulting in different outcomes with the same cytotoxic agents (16-19). Moreover, if this is the first time that a regimen has shown a survival benefit compared with single-agent topotecan in relapsed SCLC, actually we don't know if this benefit is due to the addition of irinotecan to a platinum-based regimen or just to the rechallenge with a platinum-based regimen. Only a dedicated phase III study could answer this question that, to date, seems to be less crucial than in the past, in consideration of the recent development also for SCLC of new promising drugs, including immune checkpoints inhibitors or rovalpituzumab, an antibody-drug conjugate recognizing DLL3.

In conclusion, the JCOG0605 study showed that combined chemotherapy with cisplatin, etoposide and irinotecan is an effective treatment for selected Japanese patients with sensitive relapsed SCLC, but it could be also considered more generally as evidence supporting a rechallenge strategy with platinum and etoposide in this setting of patients. The results of ongoing trials with immune checkpoints inhibitors or rovalpituzumab could represent a significant advance in the treatment of patients with relapsed SCLC, radically changing the current therapeutic scenario that remains unsatisfactory.

**Acknowledgements**

The Thoracic Medical Oncology of the National Cancer Institute of Naples is partially supported by Associazione Italiana per la Ricerca sul Cancro (AIRC).

**Footnote**

*Conflicts of Interest:* The author has no conflicts of interest to declare.

**References**


More than 85% of lung cancer cases are classified as non-small-cell lung cancer (NSCLC), with predicted 5-year survival of 16% (1). However, after many years of dismal prognosis we are now experiencing a revolution in lung cancer treatment. Apart from targeted therapies, the potential of immunotherapy has created great excitement in the oncology community and has ushered in a new era of optimism. Cancer immunotherapy encompasses different approaches designed either to boost or restore immune functions. Therapeutic cancer vaccines are designed to stimulate the immune system of a cancer patient to act against tumor antigens (2). Sipuleucel-T, a vaccine designed to stimulate an immune response to prostatic acid phosphatase (PAP), was the first cancer vaccine approved for treatment of metastatic prostate cancer (3). T alimogene laherparepvec (T-VEC) is the first oncolytic virus therapy approved for inoperable metastatic melanoma.

Unfortunately, other therapeutic cancer vaccines such as MAGE-A3 or tecemotide have not proved successful in the treatment of NSCLC (4,5). Checkpoint blockade therapy releases the ‘brakes’ of the immune system and enhances the anti-tumor T-cell response (6) and in 2015 two such therapies, nivolumab (7,8) and pembrolizumab (9) were approved for the treatment of NSCLC.

Mucin 1 (MUC1) is a high molecular weight mucin-like transmembrane glycoprotein belonging to the mucin family (21 members) that is abnormally expressed in over 80% of all cancers. MUC1 functions as a tumor-associated antigen that induces CD8⁺ and CD4⁺ T-cell responses (10), as an intracellular signal transduction molecule and as a regulator of transcription of growth factors, similar to connective tissue growth factor (CTGF), platelet-derived growth factor A and B (PDGF-A and PDGF-B) (11) and multidrug resistance genes (MDR) (12). MUC1 directly associates with the epidermal growth factor receptor (EGFR) gene and translocates to the nucleus (11) (Figure 1).

MUC1 has been defined as the second most promising target among 75 potential tumor associated antigens, but still there is no licensed product available against this target (13). Interesting results of disease stabilization have been described with the anti-MUC1 SP vaccine (ImMucin) in multiple myeloma patients (14,15). L-BLP25 (Stimuvax), developed by Merck, has now completed trials in NSCLC (16). TG4010 is a suspension of a modified vaccinia of Ankara that expresses the tumor antigen MUC1 and interleukin 2 (17). In a phase I clinical trial, TG4010 was shown to be safe and to have clinical activity (18). Two randomized studies have shown that the combination of TG4010 with chemotherapy in NSCLC patients is feasible and safe (19-21). The vaccine has also been tested in other types of tumors and the association between clinical activity of TG4010 and the cellular immune response against MUC1 has been demonstrated (22,23).

The TIME trial is a phase 2b/3 randomized, double-
blind, placebo-controlled study evaluating TG4010 in combination with chemotherapy as first-line treatment of advanced NSCLC patients. Two hundred and twenty two patients from 45 centers in France, Belgium, the UK, Italy, Spain, Hungary, Poland, Israel and the USA were included. All patients had MUC1 expression by immunohistochemistry in at least 50% of the tumoral cells. The primary objective of the phase 2b part of the study was to analyze progression-free survival (PFS) and Quoix and colleagues demonstrated that adding TG4010 to first line chemotherapy improves PFS in comparison to placebo plus chemotherapy in advanced NSCLC patients (24). Overall, the TIME study demonstrated a significant benefit in PFS with the addition of TG4010 to first line chemotherapy. However, although the results were statistically significant, they were not notable, with an increase in PFS of only 5.9 months for patients in the TG4010 plus chemotherapy arm, compared to 5.1 months for the placebo plus chemotherapy arm. Interestingly, almost 50% of patients had unknown status of EGFR mutations at baseline. TG4010 was well tolerated and the trial is set to continue to phase 3. Low baseline values of triple positive lymphocytes (TrPAL) for CD16, CD56 and CD69, corresponding to a phenotype of activated natural killer cells, were found to be predictive of TG4010 activity in combination with chemotherapy. In fact, the TrPAL test, developed as a companion diagnostic for TG4010, would be validated in the TIME trial. Validation was planned for if patients with baseline TrPAL values less or equal to the upper limit of normal (ULN) had a more than 95% probability of hazard ratio (HR) for PFS being less than 1. Also, and if patients with baseline TrPAL values greater than ULN had a more than 80% probability of HR for PFS of more than 1. The probability of HR being greater than 1 was 31.3%, and the primary endpoint was not met. From the clinical point of view, the most interesting results were in the 127 patients with non squamous histology and TrPAL values less than the third quartile (Q3). These patients had the highest benefit in terms of PFS and overall survival (OS) with the addition of TG4010 to chemotherapy (HR, 0.59, 95%CI 0.40–0.87; P=0.0033 and HR, 0.59 95%CI, 0.39–0.91; P=0.0072, respectively).

The pretreatment normal levels of activated natural killer cells (TrPAL) defined a subgroup of patients who derived significant benefit in several parameters including OS (20). But is there a reliable way to define the heterogeneous and plastic natural killer cells repertoire? There are two primary phenotypically defined subsets of natural killer cells. The majority has low-density expression of CD56 (CD56dim) and high levels of Fcγ receptor III (FcγRIII, CD16) (CD56dimCD16+), while others are CD56brightCD16dim or CD56brightCD16−. CD56dim natural killer cells predominate in the blood and are more cytotoxic than the CD56bright, which produce abundant cytokines following activation.
of monocytes but have low cytotoxicity (25). Therefore, natural killer cells should be treated as distinct CD56^{bright} and CD56^{dim} subsets, rather than as a homogenous population.

In a post-hoc exploratory analysis, programmed cell death 1 ligand 1 (PD-L1) expression was assessed by immunohistochemistry in pretreatment tumor specimens; both in tumoral cells and the immune infiltrate (24). Quoix and colleagues did not find improvement in PFS in the TG4010 group compared with placebo according to PD-L1 expression in tumoral cells. However, there was significant improvement in PFS in the TG4010 group compared with placebo according to PD-L1 expression in the immune infiltrate. Those patients with low PD-L1 expression in their immune infiltrate derived greater benefit from TG4010 (24) but whether these results are relevant is a matter of debate (26,27).

Immunotherapy trials targeting tumor antigens have yet to meet expectations. To better define patients who can benefit from immunotherapy, other factors such as tumor-infiltrating immune cells and gene signatures are now being evaluated. Hong at al. described a transcriptional signature related to innate anti-PD-1 resistance (28). CD8+ T cells play a significant role in antitumor immunity in many types of cancers (29) and it would be reasonable to assume that good prognosis is associated with CD8+ T cell infiltrate into the tumor, while accumulations of regulatory T cells (Treg) or myeloid-derived suppressor cells (MDSC) correlate with worse outcome. Signal transducer and activator of transcription 3 (STAT3) has been found to promote an immunosuppressive tumor microenvironment (30) (Figure 1). The S100A8 peptide belongs to the transcriptional network of STAT3 and decreases tumor infiltration of CD8+ T-cells, promoting a tumor growth-enhancing immune microenvironment through activation of the toll like receptor 4 (TLR4)/MD2 pathway (31) (Figure 1). Inhibition of acetyl-CoA acetyltransferase (ACAT1), a cholesterol esterification enzyme, significantly enhances the function and proliferation of CD8+ T-cells (32). The immunoproteosome (formed by proteasome subunits B; PSMB) generates peptides that bind to human leukocyte antigen (HLA) molecules, facilitating CD8+ T cell response. Reduced immunoproteosome expression has been linked to NSCLC with mesenchymal phenotype and reduced repertoire of HLA-bound peptides (33). STAT3 acts as an antagonist for STAT1, a key regulator of immunoproteosome and antigen presenting machinery (Figure 1) (33). STAT3 activates DNA methyltransferase 1 (DNMT1) which methylates the promoter region of interferon regulatory factor 1 (IRF1), PSMB8 and PSMB9 and HLA molecules that are crucial components of the antigen-presenting mechanism (33) (Figure 1).

A major impediment to cancer vaccines is that tumors with inherent resistance to T cell-mediated immunotherapy may never respond to therapies that target tumor antigens. Therefore, new strategies combining a MUC1 vaccine with antagonists of tumor-induced immune suppression are warranted.

Acknowledgements
None.

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

References


Immunotherapy has proven to be a major breakthrough in the treatment of a variety of cancers, having been called the major oncologic achievement in 2015 by the American Society of Clinic Oncology. Two immune checkpoint inhibitors, antibodies to the programmed cell death protein-1 receptor (PD-1), received FDA approval in 2015 for the treatment of advanced non-small cell lung cancer, and others similar agents are actively being studied. Advancements in the treatment of lung cancer have been desperately needed as treatment strategies utilizing platinum-based doublet therapy result in modest improvements in overall survival with a median of 8–10 months and 2-year survival rates of 10–15% in patients with metastatic disease (1). The benefit of immune checkpoint inhibitors as monotherapy after progression on platinum-based chemotherapy is significant in that durable responses can be achieved. Despite this, a number of important questions remain about the optimal utilization of immune checkpoint inhibitors in lung cancer. The feasibility of combined immune checkpoint blockade, namely inhibition of the PD-1/programmed cell death ligand-1 (PD-L1) pathway together with inhibition of the cytotoxic T-lymphocyte antigen (CTLA-4), has been proven in malignant melanoma and is now being tested in non-small cell lung cancer (2).

Cancer cells have multiple mechanisms to evade the immune system. As the understanding of the complex relationship between the immune system and cancer continues to be defined, immune checkpoint inhibitors can mediate reversal of T-cell exhaustion, that is caused by the activation of PD-1 pathway. In the normal function of the immune system, T-cell activity is modulated by a balance of interplaying stimulatory and inhibitory signals (3,4). Immune checkpoints are responsible for controlling the intensity of the T-cell response by serving as inhibitory signals, maintaining homeostatic balance and preventing autoimmunity. Two of these important checkpoints are CTLA-4 and the PD-1 receptor. CTLA-4 is an inhibitory T-cell receptor that is involved in regulating T-cell activation, acting in the lymphoid compartment during the initial stages of the immune response. It competes with the co-stimulatory T-cell receptor, CD28, for binding to ligands on antigen presenting cells, thereby halting T-cell activation. Additionally, it serves an important role in the function of regulatory T-cells. The PD-1 receptor works in the tumor microenvironment to regulate T-cell response. It is expressed on the cell surface of activated T-cells and has two ligands, PD-L1 and programmed cell death ligand-2 (PD-L2). When bound to these ligands, the inhibitory signal leads to reduced cytokine production and suppression of proliferation. Cancer cells use these checkpoints to evade the anticancer effects of the immune system by increasing the activity of these two inhibitory pathways. Cancer cells also up regulate PD-L1 expression, further increasing the inhibitory signal after interacting with PD-1 on T-cells. Antibodies to these immune checkpoints, inhibitors of CTLA-4 and the PD-1/PD-L1 pathway, unleash these inhibitory signals and allow the generation of a T-cell antitumor response, enabling the patient’s immune system...
to recognize and kill cancer cells.

The immune checkpoint inhibitors for the treatment of non-small cell lung cancer that are currently commercially available are nivolumab and pembrolizumab. Others are currently in clinical trials. Nivolumab, a fully human monoclonal antibody against PD-1, was approved by the US Food and Drug Administration (FDA) in 2015 for the treatment of patients with advanced metastatic squamous and non-squamous non-small cell lung cancer who have progressed after platinum-based chemotherapy (5). The approval was based on two Phase 3 clinical trials. The CheckMate 017 trial compared nivolumab to docetaxel in 272 squamous cell lung cancer patients who had disease progression after platinum-based chemotherapy (6). Median overall survival, the primary endpoint, was significantly improved to 9.2 months with nivolumab compared to 6 months with docetaxel (HR = 0.59). One year overall survival was also higher with nivolumab (42% vs. 24%). Nivolumab also led to improvements in median progression-free survival (3.5 vs. 2.8 months, HR = 0.62) and increase in objective response rate (20% vs. 9%). Nivolumab was also evaluated in 582 non-squamous non-small cell lung cancer (NSCLC) patients in the CheckMate 057 trial, a trial that mirrored CheckMate 017 in design (7). Nivolumab also proved effective in this group showing improvements in median overall survival of 12.2 vs. 9.4 months with docetaxel (HR = 0.73). Median progression-free survival (PFS) was similar between the groups (2.3 vs. 4.2 months, HR = 0.92), but 1 year PFS was greater with nivolumab (18.5% vs. 8.1%). The objective response rate (ORR) was also improved with nivolumab (19.2% vs. 12.4%). Pembrolizumab, a humanized IgG4 monoclonal antibody to PD-L1, has received accelerated approval by the FDA for the treatment of advanced non-small cell lung cancer after progression on platinum-based chemotherapy (8). The Phase 1 KEYNOTE-001 study included 495 NSCLC patients who received pembrolizumab at 2 or 10 mg/kg (9). The ORR was 19.4% with a median duration of response of 12.5 months (median follow-up 10.9 months). A randomized study that included approximately 1,000 patients confirmed the superior of pembrolizumab over docetaxel in PD-L1 expressing patients with advanced stage NSCLC. In this study, KEYNOTE-010, pembrolizumab significantly improved both median overall and progression-free survival compared to docetaxel (10). Median overall survival was 10.4 and 12.7 months, respectively, with pembrolizumab 2 and 10 mg/kg vs. 8.5 months with docetaxel. Pembrolizumab further increased overall survival among patients with at least 50% PD-L1 expression; median overall survival was 8.2 months with docetaxel compared to 14.9 months with pembrolizumab 2 mg/kg (HR = 0.54) and 17.3 months with pembrolizumab 10 mg/kg (HR = 0.59). While median PFS with the two therapies was similar among the entire cohort, pembrolizumab led to improved median PFS in patients with at least 50% PD-L1 expression (pembrolizumab 2 mg/kg: 5 months, pembrolizumab 10 mg/kg: 5.2 months, docetaxel 4.1 months) (Table 1).

In NSCLC, immune checkpoint inhibitor monotherapy

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<th>Table 1 PD-L1 inhibitors in NSCLC: phase III clinical trials</th>
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PD-L1, programmed cell death ligand-1; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; ORR, objective response rate.
targeting the PD-1/PD-L1 pathway has shown remarkable results with improvements in OS, PFS and with ORR of 15–25% with some patients achieving durable responses lasting years. Agents blocking the PD-1/PD-L1 pathway work in the tumor microenvironment regulating the T-cell response, while CTLA-4 inhibitors provide a different mechanism targeting T-cell activation in the lymphoid compartment. Simultaneous blockade of these two pathways may provide greater antitumor activity and lead to improved outcomes in non-small cell lung cancer patients. The results of an early phase 1b trial evaluating the combination of durvalumab, an anti-PD-L1 antibody, and tremelimumab, an anti-CTLA-4 antibody, was recently published by Antonia et al. (11). This multicenter, non-randomized, open-label study enrolled 102 immunotherapy-naïve patients with locally advanced or metastatic non-small cell lung cancer. Varying dosing combinations were examined, including durvalumab at doses 3 to 20 mg/kg every 4 weeks for 13 doses, or 10 mg/kg every 2 weeks for 26 doses. Tremelimumab was administered at doses of 1, 3, or 5 mg/kg every 4 weeks for six doses followed by every 12 weeks for three doses. In 84 evaluable patients, the overall response rate was 25% across all cohorts.

Lung cancer patients are often advanced in age with many co-morbidities and limited performance status, thus tolerability to any therapy is of utmost importance. Anti-PD-1/PD-L1 therapy has been shown to be well tolerated. In the Checkmate trials, the rate of grade 3 or 4 adverse events was 7–10% with nivolumab versus 53–55% with docetaxel. There are however risks for severe life-threatening immune-related adverse events requiring close monitoring and early intervention. In the durvalumab/tremelimumab combination study by Antonia et al., adverse events resulted in 28% of patients discontinuing therapy. The maximum tolerated dose was exceeded in the cohort receiving durvalumab 20 mg/kg every 4 weeks with tremelimumab 3 mg/kg with 30% of patients (2 of 6 patients) experiencing dose-limiting toxicity. The most common grade 3 or 4 toxicities were diarrhea (11%), colitis (9%) and increased lipase (8%). Durvalumab 20 mg/kg with tremelimumab 1 mg/kg had a manageable tolerability profile and will be used as the dose moving forward in Phase 3 studies.

Simultaneous blockade of PD-1/PD-L1 and CTLA-4 has proven effective in melanoma, and the study by Antonia et al. is encouraging for the potential benefit of this strategy in non-small cell lung cancer. Higher response rates were seen relative to those seen with single-agent PD-1/PD-L1 inhibition. The clinical characteristics of many lung cancer patients can lead to poorer tolerability of more intensive therapies. Trial data revealed that doses higher than 1 mg/kg of tremelimumab resulted in greater toxicities with no improved activity; the toxicity associated with inhibition of this pathway may be a limiting factor in the combination approach. Further evaluation of the balance between efficacy and toxicity is needed. The study identified a dose with a reasonable tolerability profile, which should now be studied in larger patient cohorts. Interestingly, the combination was found to improve response rates regardless of PD-L1 status, differing from most trials with PD-1/PD-L1 monotherapy in NSCLC. Further studies will aid in determining if the PD-L1 negative population will receive the greatest benefit from the addition of CTLA-4 inhibition. Further support of the combination strategy comes from early results of CheckMate-012, a multi-arm Phase 1b trial in chemotherapy-naïve advanced non-small cell lung cancer evaluating the combination of the PD-1 inhibitor, nivolumab, and the anti-CTLA-4 antibody, ipilimumab (12). The combination was tested using four different dosing regimens in 148 patients and led to objective response rates of 13% to 39%. Clinical activity was observed regardless of PD-L1 expression. Toxicity was acceptable with 10% of fewer patients discontinuing treatment due to toxicities. The outcomes of Phase 3 studies with combination therapy will further enlighten our understanding of these agents and potentially shape the future of non-small cell lung cancer management.

Acknowledgements
None.

Footnote
Conflicts of Interest: Dr. Ramalingam has served on scientific advisory board meetings and received honoraria from Astra Zeneca, Bristol Myers Squibb, Merck and Genentech. The other author has no conflicts of interest to declare.

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Cite this article as: Eubanks JT, Ramalingam SS. Immune checkpoint inhibitors in non-small cell lung cancer: is simultaneous blockade better? Transl Cancer Res 2016;5(S3):S532-S535. doi: 10.21037/tcr.2016.08.18
Non-small cell lung cancer (NSCLC) is a dismal disease with a significant death toll, since, at the time of diagnosis, the disease is frequently disseminated (Stage IV), or locally advanced, and rapidly evolves to metastatic disease. In spite of adjuvant therapy, recurrence after surgery often occurs. Response to cisplatin-based chemotherapy is meager and radiographic response only reaches 30% (incomplete or partial response), with a short progression free survival (PFS) of 4–5 months and median survival of 10–12 months, with or without the addition of bevacizumab or EGFR monoclonal antibodies. Immune checkpoint blockade (ICB) or immune checkpoint antibody inhibitors have revolutionized the treatment of lung cancer, as well as other tumors. Recent data show that ICB and pembrolizumab (a PD-1 inhibitor) induce response rate in brain metastases of melanoma and NSCLC patients in a similar proportion found in systemic disease, between 20–30% (1,2). Fifty two patients received nivolumab at the standard dose of 3 mg/kg intravenously every 2 weeks. This was the first time that four ongoing complete responses were observed and the response was not associated with the degree of PD-L1 expression, although, numerically, the response rate was higher in patients with positive PD-L1 expression, 28%, than in those with no PD-L1 expression, 14%. Even though the median PFS was 3.6 months, the median overall survival of 19.4 months constitutes a new landmark in survival of patients with advanced NSCLC (3). The median survival was 16.8 months for patients with squamous NSCLC and was not reached for patients with non-squamous NSCLC. The 18 month overall survival rate was 57%. Tumor PD-L1 expression was not quantifiable in 12% of the patients. There was no clear association between PFS or overall survival and baseline PD-L1 expression. Tumor biopsies taken from patients before treatment with ICB may indicate a lack of PD-L1 expression, however, immune checkpoint antibody blockade enhances T-cell response and infiltration into tumor tissue. Therefore, ICB allows reinvigoration of T cells that release interferon-γ, inducing PD-L1 expression in tumor cells. Henceforth, a biopsy after ICB would show PD-L1 positive tumor cells. This observation leads to the conclusion that the expression of PD-L1 in tumor tissue should not be used as a predictive biomarker for selection or exclusion of patients for treatment with ICB (4,5). Two phase III trials have evaluated the efficacy of nivolumab in first line therapy (CheckMate 026 and CheckMate 227). CheckMate 227 assesses nivolumab alone or in combination with ipilimumab or cisplatin based chemotherapy with or without nivolumab [reviewed in (3)]. Whether or not PD-L1 expression and response rate are related to the type of immune checkpoint inhibitor is at
present unknown. However, pembrolizumab, also an anti-PD-1 antibody, has been approved for use only in PD-L1 positive previously treated NSCLC patients. The great advantage of nivolumab is that it does not require tumor PD-L1 expression for prescription. Are the response rates of nivolumab and pembrolizumab really different according to PD-L1 expression? Not really, if we compare the results of CheckMate 012 to those of pembrolizumab in the KEYNOTE-001 trial (6). In the latter, PD-L1 positivity was defined as a membranous staining in at least 1% of cells (neoplastic and intercalated mono-nuclear cells) between tumor nests or a distinctive staining pattern caused by infiltration of mono-nuclear inflammatory cells in the stroma, forming a banding pattern adjacent to tumor nests (6). Membranous PD-L1 expression in at least 50% of tumor cells (proportion score, >50) was selected as the cutoff (6). The pembrolizumab response rate was 55.2%, with a proportion score of 50%, including 43.9% in previously treated patients and 50% in previously untreated patients (6). The fact that nivolumab response was 50% in patients with 50% PD-L1 expression in the CheckMate 012 trial is of interest (3). Median PFS among patients with a proportion score of 50% was 12.5 months for previously untreated patients in the KEYNOTE 001 study (6) and 8.3 months in the CheckMate 012 study (3). It is true that, numerically, response rate declines according to the level of PD-L1 expression, (See sup Table S7 and S8 in the KEYNOTE study, as well as Table 4 in CheckMate 012), however response is also observed in PD-L1 negative tumors. In addition, the prevalence of patients with a proportion score of 50% is around 23% (6). In both studies, CheckMate 012 and KEYNOTE 001, responses were higher in patients with KRAS mutations and KRAS mutations had increased PD-L1 staining (6). Patients with EGFR mutations responded less to nivolumab and pembrolizumab (3,6). It has been clearly announced that other predictive biomarkers should be kept in mind, including the presence of pre-existing CD8+ T cells and cytokines in tumor samples which could supplement PD-L1 expression in order to better identify patients that could respond to ICB (4-6).

It is rather interesting that interferon-γ related genes, including signal transducer and activation of transcription 1 (STAT1), have been associated with better clinical outcome in pembrolizumab treated metastatic squamous cell carcinoma of head and neck (KEYNOTE 012) (7). Along the same lines, in melanoma, resistance to PD-1 blockade has been seen to be related to a lack of response to interferon-γ. Western blot analysis shows that one baseline cell line responded to interferon-γ with the expected signal transduction, including an increase in STAT1, an interferon regulatory factor (IRF), expression, STAT1 phosphorylation and the production of downstream interferon targets, such as PD-L1 and major histocompatibility complex (MHC) class I. However, the cell line from the progression lesion shows a lack of response to interferon-γ (8). Of interest is the fact that in NSCLC the activation of STAT3 leads to activation of DNMTs, which further methylate the promoter region of STAT1 and key molecules such as IRF1 and proteasome subunits, PSMB8, PSMB9 and HLA molecules (9). It is well known that chemotherapy and radiotherapy can enhance response to ICB by the release of damage associated molecular patterns (DAMPS) [Reviewed in (5)]. Calreticulin is considered an essential DAMP and recent evidence shows that calreticulin expression in NSCLCs is associated with intra-tumoral infiltration of CD8+ T lymphocytes and predicts favorable response to ICB (10).

Of interest is the fact that other investigators in the POPLAR study, comparing atezolizumab (PD-L1 antibody) with docetaxel in previously treated NSCLC patients, showed that patients with pre-existing immunity, defined by high T-effector-interferon-γ-associated gene expression, had improved overall survival with atezolizumab. Survival benefit from atezolizumab increased with increasing PD-L1 expression on tumor cells, tumor infiltrating immune cells, or both. Median overall survival was 15.5 months for patients with a proportion score of 50% or more of PD-L1 expressing tumor cells or tumor infiltrating immune cells (11). Other anti-PD-L1 antibodies, such as avelumab, are very promising, since, in addition to anti-PD-L1 activity, avelumab mediates antibody-dependent cell-mediated cytotoxicity (ADCC), contributing to the lysis of tumor cells (12). Purified natural killer (NK) cells are potent effectors for avelumab (12). Intriguingly, NK cells are tightly regulated by the JAK-STAT signaling pathways and cannot survive in the absence of STAT5 (13). At the same time, STAT5 repressed the transcription of VEGFA in NK cells, providing new clues for developing specific biomarkers for the assessment of avelumab therapy.

In summary, CheckMate 012 paved the way for the use of ICB as a novel therapy in NSCLC patients, mainly in smokers and those harboring KRAS mutations, with the first hints of complete responses in metastatic NSCLC and the observation that responses are durable and median survival exceeds, by far, those obtained by chemotherapy.
The concept of DAMPS released by chemotherapy encourages the promotion of studies with the combination of chemotherapy and ICB. Several layers of evidence further pave the way for a more accurate predictive biomarker scenario. Some new PD-L1 antibodies, such as avelumab with ADCC activity, could provide further advantages.

Acknowledgements

None.

Footnote

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

References

Despite advances in diagnostic, surgical/interventional, and supportive care strategies, lung cancer remains a lethal entity representing the most common cause of cancer-related mortality worldwide (1). Non-small cell lung cancers (NSCLCs) represent the vast majority of these cases. With more than half of all patients presenting with advanced stage disease at initial diagnosis, there has been a persistent and pressing need for improved systemic therapies—both with regards to efficacy and toxicity. Even so, platinum doublets have remained the mainstay of palliative therapy for the past several decades. Based on a number of randomized trials, platinum doublet chemotherapy administered to fit and willing patients achieves improved survival and quality of life (QoL) as compared to best supportive care alone and has long remained the unchallenged standard of care (2). Though many chemotherapeutic agents have been studied in combination with a platinum agent, none has demonstrated superior outcomes in unselected cohorts (3).

In recent years, there have been key developments in our understanding of this heterogeneous disease, with growing appreciation for the impact of tumor-specific histopathology and molecular characterization on the clinical course and response to various systemic therapies. Specifically, this includes demonstration of a survival benefit in patients with nonsquamous histology receiving the antimetabolite pemetrexed as part of the platinum doublet (4), pemetrexed maintenance therapy in patients with adenocarcinoma histology and stable disease/treatment response following four to six cycles of first line platinum doublet therapy (5), and addition of bevacizumab to platinum doublet in patients with nonsquamous disease (6).

The recognition and characterization of molecularly defined subsets of patients with oncogene-addicted advanced NSCLC and actionable therapeutic targets has further transformed the landscape of this disease. Identification of oncogenic driver mutations or gene rearrangements in the epidermal growth factor receptor (EGFR) (10–15% of advanced NSCLC), anaplastic lymphoma kinase (ALK) (3–5% of advanced NSCLC), and ROS proto-oncogene 1 (ROS1) (1–2% of advanced NSCLC) and application of precision tyrosine kinase inhibitors (TKIs) have rendered the ability to optimally match targeted systemic therapies with tumor-specific abnormalities—particularly in lung adenocarcinomas.

To date, seven oral targeted therapies have been approved by the United States Food and Drug Administration (FDA) for use in molecularly defined subsets of advanced NSCLC: erlotinib, gefitinib, and afatinib for tumors with sensitizing EGFR mutations; osimertinib for tumors with the EGFR T790M mutation; crizotinib, ceritinib, and alectinib for tumors with ALK gene rearrangements; and crizotinib for tumors with ROS1 gene rearrangements. Across multiple randomized studies comparing these TKIs with conventional cytotoxic chemotherapy, a consistent theme has emerged: brisk [objective response rates (ORRs) on the order of 60–80%] and durable improvements in clinical outcomes [progression-free survival (PFS) on the order of 9–12 months] with lesser toxicity and better QoL as compared to chemotherapy (7-14). Thus, since 2013, expert guidelines have recommended routine testing for
EGFR mutations and ALK gene rearrangements on all tumor specimens for patients with advanced NSCLC and an adenocarcinoma component (or inability to exclude adenocarcinoma)—regardless of clinical, demographic, or other characteristics (15).

Taken together, the standard of care for management of advanced NSCLC in recent years has emphasized upfront stratification in medically fit patients on the basis of: (I) actionable molecular targets (i.e., EGFR mutations or ALK/ROS1 gene rearrangements) and (II) histology (i.e., nonsquamous vs. squamous). In patients with an identified actionable molecular target, the use of an upfront oral palliative TKI is the evidence-based standard. For those patients with no actionable molecular target, first line intravenous (IV) palliative chemotherapy with a platinum doublet is recommended; addition of bevacizumab and maintenance chemotherapy are added considerations in these patients (Figure 1).

Even despite such advances, however, the median overall survival (OS) for advanced NSCLC treated with palliative chemotherapy has not been moved beyond 9–12 months. Further, availability of an actionable, FDA-approved targeted therapy will only be relevant in some 20–25% of all patients with advanced NSCLC—and primarily in patients with adenocarcinoma histology. More tailored paradigms for management of squamous cell lung cancers is an area of unmet need, as use of pemetrexed, bevacizumab, or oral TKIs is generally not indicated/relevant in this tumor histology. Thus, moving beyond conventional chemotherapy to identify more broadly applicable, durably efficacious, and less toxic systemic therapies has remained a dire unmet need in advanced NSCLC—perhaps until now.

Immune checkpoint inhibitors have afforded a novel approach to antineoplastic therapy. By impeding inhibitory signals affecting cancer-targeting T lymphocytes, the host anticancer immune response is reignited. Monoclonal antibodies inhibiting both programmed death 1 (PD-1) (nivolumab and pembrolizumab) and programmed death ligand 1 (PD-L1) (atezolizumab) have demonstrated significant promise in the management of advanced NSCLC. Notable and durable responses were observed in the early phase trials of these drugs in heavily pretreated, treatment-refractory patients with advanced NSCLC (16). Subsequent large randomized studies have demonstrated the superiority of the immune checkpoint inhibitors nivolumab, pembrolizumab, and atezolizumab as compared to palliative docetaxel in the second line setting with regards to OS, magnitude and durability of response, and treatment-related toxicity (17-20). Since October 2015, three immune checkpoint inhibitors (nivolumab, pembrolizumab, and atezolizumab) have garnered FDA approval for use in advanced NSCLC without actionable molecular targets.

**Figure 1** Stratification for frontline therapy by histology, molecular, and immune profile. NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; IHC, immunohistochemistry; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS1, ROS proto-oncogene 1; TPS, tumor proportion score; PD-1, programmed death 1.
EGFR/ALK aberrations in the first (pembrolizumab) or second (nivolumab, pembrolizumab, and atezolizumab) line settings—regardless of squamous/non-squamous histology.

Identifying determinants of therapeutic benefit by way of predictive biomarkers has been an ongoing era of investigation and debate. PD-L1 status—either on tumor cells, tumor-infiltrating immune cells, or both—has been the major emphasis. However, clinical trials of PD-1 and PD-L1 inhibitors in advanced NSCLC to date have shown conflicting results with regard to the predictive impact of PD-L1 immunohistochemistry (IHC). Definitions of PD-L1 “positivity” (i.e., staining of tumor cells vs. tumor-infiltrating immune cells or both and quantitative thresholds) have varied considerably across studies as have methods of PD-L1 testing (i.e., different diagnostic antibodies, scoring systems, and technical platforms). Not unsurprisingly, therefore, correlation between biomarker positivity and treatment response rates has varied widely (13–83% depending on the study in question) (21). Moreover, rates of therapeutic response in patients deemed PD-L1 IHC “negative” have not been insignificant (3–20%)—especially given that responses to second line palliative docetaxel have historically been on the order of ≤10% and with far greater toxicity than seen with immune checkpoint inhibitors (21). To date, only pembrolizumab has acquired an FDA-approved companion diagnostic, the PD-L1 IHC 22C3 pharmDx assay (Dako North America, Inc.). Further, it is the only one of the immune checkpoint inhibitors that has been FDA approved in advanced NSCLC for use selectively in patients with PD-L1 positive tumors—though thresholds for PD-L1 tumor proportion score (TPS) “positivity” are defined differently in the first line (PD-L1 TPS ≥50%) vs. second line (PD-L1 TPS ≥1%) settings.

Findings from the four major phase III randomized trials of the FDA-approved immune checkpoint inhibitors for previously treated advanced NSCLC are summarized in Table 1.

It is amidst this burgeoning landscape that Reck and colleagues published KEYNOTE-024, a phase III randomized study of first line pembrolizumab vs. platinum doublet for previously untreated, PD-L1 positive (TPS ≥50%) stage IV NSCLC (22). In this study, 305 patients were randomly assigned to either pembrolizumab administered IV at a flat dose of 200 mg every 3 weeks for 35 weeks or platinum doublet (carboplatin/pemetrexed, cisplatin/pemetrexed, carboplatin/gemcitabine, cisplatin/gemcitabine, or carboplatin/paclitaxel at the investigator’s discretion) given IV every 3 weeks for four to six cycles. The most common regimen for the group randomized to conventional chemotherapy was pemetrexed (44.4%), more than half of whom went on to receive pemetrexed maintenance. The majority of patients were male, current/former tobacco users, and with nonsquamous histology. Tumor PD-L1 IHC was assessed using the FDA-approved companion diagnostic (pharmDx 22C3) and was performed on core/excisional biopsies obtained at the time that metastatic disease was diagnosed; fine needle aspirates or archival specimens obtained from sites treated with any intervening radiation therapy or chemotherapy were not permitted. Of 1,653 patients whose samples were evaluable for PD-L1, 30.2% had a PD-L1 TPS of ≥50%, thus meeting the threshold for positivity for entry into the trial.

With a median follow-up of 11.2 months, the primary endpoint of PFS in the pembrolizumab vs. chemotherapy arms was a significant 10.3 vs. 6.0 months [hazard ratio (HR) for disease progression/death =0.50, P<0.001]. The estimated rate of OS at 6 months was also increased in the pembrolizumab vs. chemotherapy group (80.2% vs. 72.4%, P=0.005). Response rates were 44.8% vs. 27.8% in the pembrolizumab vs. chemotherapy groups, respectively, consistent with response rates reported for platinum doublet therapy in this setting previously in the literature. Further, median duration of response (DoR) was notably longer in the pembrolizumab vs. chemotherapy group: not reached vs. 6.3 months, respectively. Crossover from chemotherapy to pembrolizumab was allowed, and 43.7% of patients initially receiving chemotherapy subsequently crossed over to the immunotherapy arm. Additionally, the study was stopped early at the recommendation of the external data and safety monitoring committee due to evidence of superior OS with pembrolizumab vs. chemotherapy, thus allowing patients receiving chemotherapy the opportunity to receive pembrolizumab.

The toxicity profile noted with pembrolizumab was consistent with previous reports of PD-1/PD-L1 antibodies and favorable as compared with chemotherapy: grade 3–5 treatment-related adverse events (AEs) were 26.6% vs. 53.3%, respectively. The most common treatment-related AEs in the pembrolizumab group were diarrhea (14.3%), fatigue (10.4%), and pyrexia (10.4%). Immune-mediated AEs were noted in 29.2% of patients receiving pembrolizumab; however, grade 3–4 immune-related AEs occurred infrequently and included: severe skin reactions (3.9%), pneumonitis (2.6%), and colitis (1.3%).

Notably, preliminary results have also recently been
### Table 1: Completed phase III trials of immune checkpoint inhibitors in previously treated advanced NSCLC

<table>
<thead>
<tr>
<th>Trial</th>
<th>Histology, PD-L1 details</th>
<th>OS (mos)</th>
<th>PFS (mos)</th>
<th>ORR (%)</th>
<th>IO ORR by PD-L1 (%)</th>
<th>DoR (mos)</th>
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<tr>
<td><strong>CHECKMATE-017: nivolumab vs. docetaxel (17)</strong></td>
<td>Squamous</td>
<td>9.2 vs. 6.0</td>
<td>3.5 vs. 2.8</td>
<td>20 vs. 9</td>
<td>17 (&lt;1)</td>
<td>NRE vs. 8.4</td>
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<td></td>
<td>Epitomics 28-8 PD-L1 IHC</td>
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<td>17 (≥1)</td>
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<tr>
<td></td>
<td>PD-L1 stratification: 1%, 5%, 10%</td>
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<td></td>
<td></td>
<td>21 (≥5)</td>
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<tr>
<td><strong>CHECKMATE-057: nivolumab vs. docetaxel (18)</strong></td>
<td>Non-squamous</td>
<td>12.2 vs. 9.4</td>
<td>2.3 vs. 4.2</td>
<td>19 vs. 12</td>
<td>9 (&lt;1)</td>
<td>17.2 vs. 5.6</td>
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<tr>
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<td>31 (≥1)</td>
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<tr>
<td></td>
<td>Epitomics 28-8 PD-L1 IHC</td>
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<td>36 (≥5)</td>
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<tr>
<td></td>
<td>PD-L1 stratification: 1%, 5%, 10%</td>
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<td>37 (≥10)</td>
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<td><strong>KEYNOTE-010: pembrolizumab vs. docetaxel (19)</strong></td>
<td>Squamous and non-squamous</td>
<td>10.4 vs. 12.7 vs. 8.5</td>
<td>3.9 vs. 4.0 vs. 18 vs. 18 vs. 9</td>
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<td>PD-L1 + (≥1%)</td>
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<td>29–30 (≥50)</td>
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<td>Pembrolizumab 2 vs. 10 mg/kg</td>
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<td>PharmDx 22C3 PD-L1 IHC</td>
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<td><strong>OAK: atezolizumab vs. docetaxel (20)</strong></td>
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<td>13.8 vs. 9.6</td>
<td>2.8 vs. 4.0</td>
<td>14 vs. 13</td>
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<td>16.3 vs. 6.2</td>
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<td>Any PD-L1</td>
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<td>Ventana SP142 PD-L1 IHC</td>
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<td>18 (TC/IC ≥1)</td>
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<td>31 (TC ≥50 or IC ≥10)</td>
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NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand 1; OS, overall survival; PFS, progression free survival; ORR, objective response rate; IO, immunotherapy; DoR, duration of response; NRE, not reached; IHC, immunohistochemistry; NR, not reported; TC, tumor cells; IC, tumor-infiltrating tumor cells.
reported for CheckMate-026, a phase III study of nivolumab vs. platinum doublet chemotherapy in patients with previously untreated, PD-L1 positive (defined as present in ≥1% of tumor cells) advanced NSCLC (23). A total of 541 patients were randomized in a 1:1 fashion to receive weight-based nivolumab 3 mg/kg IV every 3 weeks or investigator’s choice of platinum doublet chemotherapy (same as in KEYNOTE-024) IV every 3 weeks for up to six cycles. Patients progressing on chemotherapy were allowed to crossover to nivolumab. OS was 14.4 vs. 15.2 months for nivolumab vs. chemotherapy (HR 1.02). The primary endpoint of improved PFS in patients whose tumors were “strongly” PD-L1 positive (i.e., PD-L1 ≥5% by IHC) was not met. No new safety signals were observed with nivolumab, and serious AEs were seen in 18% vs. 51% of patients receiving nivolumab vs. chemotherapy, respectively.

The conflicting outcomes of these two rigorously conducted phase III studies of frontline immunotherapy in advanced NSCLC have raised important questions regarding optimal patient selection and perpetuate the controversies pertaining to PD-L1 as a predictive biomarker. To date, there have been no head to head comparisons of the various PD-1 or PD-L1 targeting agents, though we have generally considered that they are equally efficacious. Though PD-L1 positivity has in numerous studies now been associated with improved response rates and survival outcomes, multiple questions persist. Were different thresholds for defining PD-L1 “positivity” (i.e., TPS ≥50% in KEYNOTE-024 vs. ≥1% in CheckMate-026) enough to explain these divergent outcomes? How much PD-L1 is “enough”? What is the optimal method for assessing PD-L1 status—tumor cells, tumor-infiltrating immune cells, both? What is the optimal platform for PD-L1 testing? How should the existing platforms best be harmonized? Addressing the latter issue has become an important priority as this therapeutic domain has evolved. Initial results from the Blueprint PD-L1 IHC Assay Comparison Project suggest that 3 of the 4 most commonly utilized PD-L1 IHC assays in the key trials of immune checkpoint inhibitors in NSCLC to date [22C3 (pembrolizumab), 28-8 (nivolumab), and SP263 PD-L1 IHC as opposed to the SP142 assay (atezolizumab)] demonstrate PD-L1 expression to a similar degree, though interchanging assays and cut-offs may still lead to “misclassification” of PD-L1 status in some cases (24).

In sum, the experience of immune checkpoint inhibitors in the care of patients with advanced NSCLC has given credence to some recurring themes: (I) ORRs are generally in the 10–30% range, regardless of PD-L1 status (though patients whose specimens express higher PD-L1 may experience a greater likelihood of response and long-term outcomes); (II) in those patients achieving a response, the response is often durable (i.e., lasting many months and often superseding the more limited DoR seen with conventional chemotherapy); and (III) toxicity profiles with the immunotherapeutic agents are generally less severe than those historically seen with conventional chemotherapy—though the identification and management of immune-mediated AEs requires heightened awareness on the part of patients and providers alike to permit early intervention.

In the second line setting and beyond, conventional chemotherapy has proven inferior to immune checkpoint inhibitors both with regards to outcomes and toxicity—regardless of PD-L1 status and other patient selection factors—in patients who are otherwise deemed fit to continue with cancer-directed therapy. This reflects the hugely unsatisfying outcomes for patients with this difficult disease and the heretofore modest options available to patients whose disease has progressed on first line platinum-based therapy. With the approval of EGFR-(~10–15%), ALK- (~3–5%), and ROS1- (~1–2%) targeting TKIs and pembrolizumab (~30%) in defined subsets of patients, some 50% of patients with advanced NSCLC will now have an option for a frontline, tumor-specific systemic palliative therapy (25). Additional needed exploration is ongoing to see if combining immunotherapies (either with themselves or concurrently/sequentially with chemotherapy) will allow us to further improve outcomes for the vast majority of patients whose tumors lack an actionable biomarker. Decades after platinum-based therapy established itself as the standard of care, these tumor-specific therapies finally offer our patients a more efficacious, durable, and less toxic approach to care for their dire disease.

Acknowledgements

Funding: This work was funded in part through an American Cancer Society grant RSG 11-186 (to Daniel B. Costa) and a National Cancer Institute grant CA090578 (to Daniel B. Costa).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.
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Should optimal supportive care alone be the standard of care for brain metastases patients from non-small cell lung cancer, who are not eligible for radiosurgery or surgery?

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Provenance: This is an invited Editorial commissioned by Section Editor Shaohua Cui (Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China).


Submitted Nov 05, 2016. Accepted for publication Nov 16, 2016.
doi: 10.21037/tcr.2016.12.03

View this article at: http://dx.doi.org/10.21037/tcr.2016.12.03

The Quality of Life after Treatment for Brain Metastases (QUARTZ) trial was a non-inferiority, phase III randomized trial comparing optimal supportive care (OSC) including dexamethasone versus OSC including dexamethasone and whole brain radiotherapy (WBRT). In this trial, 538 patients were recruited from 69 United Kingdom and three Australian centres. The primary outcome was quality-adjusted life-years (QALYs), generated from overall survival and patient completed EuroQOL EQ-5D questionnaires. The mean QALYs was 46.4 days for the OSC and WBRT arm versus 41.7 days for the OSC arm, with a mean difference of 4.7 days (90% CI: 12.7 to 3.3 days). In addition, there was no difference in overall survival (hazard ratio 1.06, 95% CI: 0.90–1.26), overall quality of life or dexamethasone use between the two groups (1).

The researchers for the QUARTZ trial are to be congratulated for formally examining and reporting the age old question as to whether WBRT adds benefit in terms of quality of life or survival. This multi-centre trial was well designed with excellent follow-up (90% of the expected follow-up forms were received and 80% expected quality of life forms completely filled). Only one patient was lost to follow-up (1).

Despite difficulties in accrual (2,3), the trial was ultimately successful in its completion and final publication. Why was accrual challenging? Perhaps the main reason is that management biases exist from the perspective of the patient/family and from the perspective of the treating medical team. Clinical equipoise may not exist for all eligible patients with respect to the QUARTZ trial.

Numerous studies have reported that palliative cancer patients have misconceptions regarding the intention of treatment and prognosis (4). Patients and families may expect treatment and it may be harder to accept that treatment may have little benefit (5-8). On the other hand, a patient who has very poor performance status may not want to return for daily brain radiation.

Physicians may also be biased. For patients with estimated good prognosis and/or quiescent extracranial disease, there may be reluctance in randomizing such patients to OSC. On the other hand, for patients with very poor performance status and uncontrolled extracranial disease, there may be reluctance in randomizing such patients to WBRT (9-11).

The challenges for participation in this trial may have also arisen because the overall concept of the QUARTZ trial goes against the grain of moving forward and testing innovative treatments.

The median survival in the QUARTZ trial was not statistically different between the two treatment arms: 9.2 weeks (95% CI: 7.2–11.1 weeks) for patients receiving OSC and WBRT versus 8.5 weeks (95% CI: 7.1–9.9 weeks) for patients receiving OSC alone. Due to poor survival,
53% of enrolled patients were assessed for quality of life at 4 weeks, 31% at 8 weeks only 18% were assessed at 12 weeks. It has been hypothesized that in patients with better survival, the benefit of WBRT (including reduction in steroid requirements) may occur more than 4 weeks after treatment. As such, the trial was criticized for the possibility that WBRT might benefit a subgroup of patients with better prognoses (12).

The QUARTZ trial did explore the effect of WBRT on different subgroups (1). For younger patients (age less than 60 years) WBRT may provide survival benefit. The median survival for patients younger than 60 years was 10.4 weeks (95% CI: 6.3–13.4 weeks) for the WBRT and OSC arm versus 7.6 weeks (95% CI: 4.6–10.1 weeks) for the OSC arm, hazard ratio 1.48 (95% CI: 1.01–2.16). The p value for interaction between age group and treatment arm was 0.0061 (P=0.0043 with age as a linear trend). The association between KPS, P=0.0964 and primary non-small cell lung cancer (NSCLC) status (controlled or uncontrolled), P=0.0941 suggested a potential survival benefit with WBRT for patients with KPS of at least 70 and those with controlled NSCLC. A potential survival benefit with WBRT may also exist for better prognostic groups (P=0.0843 for RPA and P=0.0812 for GPA).

On the other hand, is there a subset of patients who are unlikely to benefit from WBRT and OSC and who may be better managed, instead, with OSC? The majority of patients in the QUARTZ trial (94%) were categorized into either Recursive Partitioning Analysis (RPA) class 2 or 3. Only 6% of enrolled patients were classified as good prognosis (RPA class 1). Furthermore, median survival for patients in the QUARTZ trial was disappointingly short. Median survival for those who received WBRT and OSC was 9.2 weeks (95% CI: 7.2–11.1 weeks) versus 8.5 weeks (95% CI: 7.1–9.9 weeks) for patients receiving OSC (1). As such, the QUARTZ trial provides evidence to support withholding WBRT and managing with palliative care alone for NSCLC brain metastases patients with poor performance status, progressive extracranial disease and anticipated survival of less than 3 months. There is debate as to whether OSC is the best management for better prognosis NSCLC brain metastases patients, who are not eligible for radiosurgery or surgery.

It is also important to note that the study period for accrual of patients in the QUARTZ trial was from March 2, 2007 to August 29, 2014. During this time, the benefit of molecular targeted therapy for certain NSCLC mutations emerged (13-24). Out of the 538 patients who were recruited into the QUARTZ trial, 36 received a tyrosine kinase inhibitor (1). Epidermal growth factor receptor (EGFR) mutated or anaplastic lymphoma kinase (ALK) rearranged molecular subtypes of NSCLC were not captured, due to the era in which the QUARTZ protocol was being developed. The QUARTZ trial was not designed to ascertain the role for WBRT, if any, in the present era of molecular targeted therapies for NSCLC patients (13-24).

The management of brain metastases is an example of personalized medicine where medical decisions are tailored to the individual. The evidence does not support a best supportive care alone approach for all brain metastases patients. Focal radiation (SRS, surgery, focal fractionated radiation) is also not supported by level I evidence for all brain metastases patients. Furthermore, there may be patients not represented by the QUARTZ trial who may benefit from WBRT.

The art of caring for these brain metastases patients takes into account the science learned from high quality trials and involves applying the therapeutic tools available (best supportive care including the use of dexamethasone, WBRT, SRS, surgery, alone or in combination). Management decisions are guided by risks of toxicity and takes into account prognosis with the goals to optimize survival, quality of life, neurocognition, and neurologic function.

While the QUARTZ trial illuminates the limitations of WBRT in certain subsets of NSCLC patients with brain metastases, the future of brain metastases management looks promising with respect to the development of new drugs and the advancement of radiation, surgical and imaging techniques.

Acknowledgements
None.

Footnote
Conflicts of Interest: The author has no conflicts of interest to declare.

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Cite this article as: Tsao MN. Should optimal supportive care alone be the standard of care for brain metastases patients from non-small cell lung cancer, who are not eligible for radiosurgery or surgery? Transl Cancer Res 2016;5(Suppl 7):S1320-S1322. doi: 10.21037/tcr.2016.12.03
We read with interest the editorial by Tsao (1), and thank the author for this well considered response to our recent paper describing the results of the QUARTZ trial of whole brain radiotherapy for patients with inoperable brain metastases from non-small cell lung cancer (2). We continue to be encouraged by the amount of discussion taking place surrounding treatment options for these patients.

Tsao rightly highlights that it was very challenging to recruit patients into QUARTZ. Diagnoses such as inoperable brain metastases are clearly very distressing and present a difficult setting in which to conduct a clinical trial. We would like to express our sincere thanks and admiration for the patients and clinicians who persevered with the trial and made it a success. We largely agree with the author that the lack of clinical equipoise in individual cases was a major reason for the slower than expected recruitment rate. We collected screening logs during the trial, and they highlighted that the most common reason for not entering the trial was that the clinician and/or the patient wanted to either receive or avoid whole brain radiotherapy. Whilst we were unable to record any characteristics of these patients it does point to a lack of clinical equipoise, and it would be reasonable to think that patients with better prognoses were being selected for WBRT, and those with poorer prognoses were avoiding WBRT.

One of the unusual steps undertaken in QUARTZ was to release interim trial data to investigators (3,4). We believed that the lack of existing data was one of the main reasons for the lack of clinical equipoise, and having access to some data might make clinicians and patients more comfortable with the trial randomisation. It was interesting to note that after the presentation of these data to investigators, the rate at which poor performance status patients (KPS <70) were randomised into the trial dropped slightly (from 2.9 patients per month to 2.2 per month), whereas the rate that good performance status patients were randomised increased significantly (from 3.2 patients per month to 5.3 per month). This perhaps suggests that having viewed the interim data and seen the small size of any potential benefit, clinicians/patients became more comfortable with the possibility of omitting WBRT.

This links to another important point raised by...
Tsao, that patients often have misconceptions about the intentions and potential outcomes of treatment. This was something described in this specific patient population by Dorman et al. (5), who interviewed nine QUARTZ patients from a single centre, several of whom demonstrated a misunderstanding of both the practical requirements of WBRT and their likely prognosis. In order for patients and clinicians to make fully informed treatment decisions, they need access to accurate estimates of likely treatment effects, and trials such as QUARTZ are the best source of this information.

The author also notes the emergence of several targeted agents during the life of QUARTZ. This is an important point, and these agents appear to be good options for patients with the appropriate molecular make-up (6). However at present only a small percentage of patients have a driver mutation targetable with approved treatment (in the UK approximately 10% of NSCLC patients have an EGFR mutation and 5% an ALK-rearrangement). Nonetheless it seems reasonable to believe that this will increase as our knowledge increases and more targets are identified.

Two important outcomes from QUARTZ are: firstly that it is possible to conduct trials in this patient group; and secondly, that for the majority of patients, future trials of systemic agents can be conducted without also having to include WBRT. We agree with Tsao’s closing statement that the future of brain metastases research and treatment is promising, with increasing options and hopefully more opportunities for well conducted clinical trials.

Acknowledgements

None.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.
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<td>NOTCH, ASCL1, p53 and RB alterations define an alternative pathway driving neuroendocrine and small cell lung carcinomas</td>
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<td><em>Nature Medicine</em></td>
<td>Combined inhibition of DDR1 and Notch signaling is a therapeutic strategy for KRAS-driven lung adenocarcinoma</td>
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<td><em>Cell</em></td>
<td>Metabolic heterogeneity in human lung tumors</td>
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<td><em>Clinical Cancer Research</em></td>
<td>Genomic profiling of large-cell neuroendocrine carcinoma of the lung</td>
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<td><em>Cancer Discovery</em></td>
<td>Molecular mechanisms of resistance to first- and second-generation ALK inhibitors in ALK-rearranged lung cancer</td>
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<td><em>Oncotarget</em></td>
<td>Lung cancer exosomes as drivers of epithelial mesenchymal transition</td>
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<td><em>Nature Medicine</em></td>
<td>Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition</td>
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<td><em>PLoS Genetics</em></td>
<td>Genomic landscape survey identifies SRSF1 as a key oncodriver in small cell lung cancer</td>
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<td><em>Journal of Thoracic Oncology</em></td>
<td>Comprehensive characterization of oncogenic drivers in Asian lung adenocarcinoma</td>
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<td>Whole-exome sequencing and immune profiling of early-stage lung adenocarcinoma with fully annotated clinical follow-up</td>
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<td><em>Cancer Cell</em></td>
<td>Tumor exosomal RNAs promote lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils</td>
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<td><em>Journal of Thoracic Oncology</em></td>
<td>Exosomal proteins as diagnostic biomarkers in lung cancer</td>
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<td>Routine molecular profiling of patients with advanced non-small-cell lung cancer results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT)</td>
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<td>Prospective analysis of oncogenic driver mutations and environmental factors: Japan molecular epidemiology for lung cancer study</td>
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<td><em>JAMA</em></td>
<td>Development and validation of risk models to select ever-smokers for CT lung cancer screening</td>
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<td><em>The Lancet. Oncology</em></td>
<td>Occurrence and lung cancer probability of new solid nodules at incidence screening with low-dose CT: analysis of data from the randomised, controlled NELSON trial</td>
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<td>Liquid Biopsy</td>
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<td>Molecular analysis of circulating tumor cells identifies distinct copy-number profiles in patients with chemosensitive and chemorefractory small-cell lung cancer</td>
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<td>A prospective evaluation of circulating tumor cells and cell-free DNA in EGFR mutant non-small cell lung cancer patients treated with erlotinib on a phase II trial</td>
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<td>A prospective study of total plasma cell-free DNA as a predictive biomarker for response to systemic therapy in patients with advanced non-small-cell lung cancers</td>
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<td><em>JAMA Oncology</em></td>
<td>Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer</td>
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<td><em>Nature Communications</em></td>
<td>Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients</td>
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<td><em>Clinical Cancer Research</em></td>
<td>Detection of therapeutically targetable driver and resistance mutations in lung cancer patients by next generation sequencing of cell-free circulating tumor DNA</td>
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<td>Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase 2B, open-label, randomised controlled trial</td>
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<td>Dabrafenib in patients with BRAFV600E-positive advanced non-small-cell lung cancer: a single-arm, multicentre, open-label, phase 2 trial</td>
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<td>Intracranial efficacy of crizotinib versus chemotherapy in patients with advanced ALK-positive non-small-cell lung cancer: results from PROFILE 1014</td>
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<td>Activity and safety of ceritinib in patients with ALK-rearranged non-small-cell lung cancer (ASCEND-1): updated results from the multicentre, open-label, phase 1 trial</td>
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<td>MET Exon 14 mutations in non-small-cell lung cancer are associated with advanced age and stage-dependent MET genomic amplification and c-Met overexpression</td>
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<td>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</td>
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<td>Differential crizotinib response duration among ALK fusion variants in ALK-positive non-small-cell lung cancer</td>
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<td>Osimertinib for pretreated EGFR Thr790Met-positive advanced non-small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study</td>
<td>The Lancet. Oncology</td>
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<td>Activity and safety of brigatinib in ALK-rearranged non-small-cell lung cancer and other malignancies: a single-arm, open-label, phase 1/2 trial</td>
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<td>Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer</td>
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<td>Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study</td>
<td>The Lancet. Oncology</td>
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<td>Clinical outcome of ALK-positive non-small cell lung cancer (NSCLC) patients with de novo EGFR or KRAS co-mutations receiving tyrosine kinase inhibitors (TKI)</td>
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<td>Differential protein stability and clinical responses of EML4-ALK fusion variants to various ALK inhibitors in advanced ALK-rearranged non-small cell lung cancer</td>
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<td>First-line erlotinib therapy until and beyond response evaluation criteria in solid tumors progression in Asian patients with epidermal growth factor receptor mutation-positive non-small-cell lung cancer: the ASPIRATION study</td>
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<td>Crizotinib-resistant ROS1 mutations reveal a predictive kinase inhibitor sensitivity model for ROS1- and ALK-rearranged lung cancers</td>
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<td>Multicenter phase II study of whole-body and intracranial activity with ceritinib in patients With ALK-rearranged non-small-cell lung cancer previously treated with chemotherapy and crizotinib: results from ASCEND-2</td>
<td>Journal of Clinical Oncology</td>
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<td>Use of a comprehensive geriatric assessment for the management of elderly patients with advanced non-small-cell lung cancer: the phase III randomized ESOGIA-GFPC-GECP 08-02 study</td>
<td>Journal of Clinical Oncology</td>
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<td>Combined chemotherapy with cisplatin, etoposide, and irinotecan versus topotecan alone as second-line treatment for patients with sensitive relapsed small-cell lung cancer (JCOG0605): a multicentre, open-label, randomised phase 3 trial</td>
<td>The Lancet. Oncology</td>
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<td>Clinical outcomes with pemetrexed-based systemic therapiies in RET-rearranged lung cancers</td>
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<td>TG4010 immunotherapy and first-line chemotherapy for advanced non-small-cell lung cancer (TIME) results from the phase 2b part of a randomised, double-blind, placebo-controlled, phase 2b/3 trial</td>
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<td>Safety and antitumour activity of durvalumab plus tremelimumab in non-small cell lung cancer: a multicentre, phase 1b study</td>
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<td>Nivolumab monotherapy for first-line treatment of advanced non-small-cell lung cancer</td>
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<td>The Lancet. Oncology</td>
<td>Nivolumab plus ipilimumab as first-line treatment for advanced non-small-cell lung cancer (CheckMate 012): results of an open-label, phase 1, multicohort study</td>
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<td>Lancet</td>
<td>Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial</td>
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