New Advances in the Precision Medicine of Lung Cancer

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Abstract First-generation tyrosine kinase inhibitors (TKIs) effective against non-small cell lung cancers (NSCLC) with epidermal growth factor receptor mutations or anaplastic lymphoma kinase fusion genes were initial steps in the precision medicine of lung cancer, but have significant limitations. Only about 19 % of NSCLC demonstrate predictive biomarkers that indicate that they are likely to respond to first-generation TKIs, and virtually all NSCLC that initially respond develop acquired resistance after several months. Ongoing developments in this field are expected to provide patients with improved diagnosis and treatment options. Next-generation sequencing offers superior options for genotyping tumors. Immunohistochemistry promises to allow predictive biomarkers analysis by direct observation of malignant cells using conventional tools and procedures. Treatment options that include repurposing of drugs for new targets and investigation of new clinically actionable targets will require greater knowledge and participation of the pathologist in personalized healthcare of lung cancer patients.

Keywords Lung cancer · Predictive biomarker · Personalized healthcare · Targeted therapy · Next generation sequencing

Introduction

Lung cancer has long been the leading cause of cancer death in the United States, responsible for greater mortality than the combined total of the next three most common types (colon, breast and prostate cancers) [1]. Lung cancer is also the leading cause of cancer deaths worldwide [2]. The overall five-year survival has remained a dismal 10–15 % for decades, because most patients present with advanced disease and treatment options are limited [3, 4]. Thus, it is not surprising that the advent of targeted molecular therapies for lung cancer has generated tremendous excitement. Predictive biomarker testing on pathology specimens has substantially altered the role of pathologists in lung cancer patient care.

The first strides toward a successful specific molecular therapy in lung cancer have been achieved with tyrosine kinase inhibitors (TKIs) against the epidermal growth factor receptor (EGFR). These include gefitinib (Iressa; Astra-Zeneca, London, UK) and erlotinib (Tarceva; Genentech, South San Francisco, CA and OSI Pharmaceuticals, Long Island, NY). Approximately 85 % of lung malignancies are non-small cell lung cancers (NSCLC). EGFR overexpression in the majority of NSCLCs was first recognized in the 1980s [5], but early clinical trials with EGFR TKIs demonstrated only modest results. However, in 2004, several independent investigators reported that the presence of *EGFR* gene somatic mutations predicted a significant response of NSCLCs to EGFR TKIs [6–8]. Since 2009,

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multiple clinical trials have confirmed better response rates (RR) and progression-free survival (PFS) in patients receiving EGFR TKIs versus conventional therapy if their advanced stage NSCLC has *EGFR* mutations [9, 10, 11]. Importantly, clinical trials have demonstrated that *EGFR* mutation analysis, rather than copy number alterations by fluorescent in situ hybridization (FISH) or overexpression by immunohistochemistry (IHC), is the most reliable method of determining which NSCLC are likely to respond to TKI therapy [3, 4, 12, 13].

Another target for TKI therapy for NSCLC, anaplastic lymphoma kinase (ALK), was reported in 2007. ALK was first described with the fusion partner echinoderm microtubule-associated protein-like 4 (*EML4*) [14]. Subsequently, multiple variants of the *EML4*–*ALK* gene rearrangement, as well as other ALK fusion partners, have been described in NSCLC [15]. The food and drug administration (FDA) accelerated approval of the ALK TKI crizotinib or Xalkori (Pfizer, NY, NY) when clinical trials demonstrated that it resulted in improved RR and PFS in patients with advanced NSCLC with ALK rearrangements [16, 17]. Simultaneously, the FDA approved a specific companion test (Vysis ALK Break-Apart FISH Probe Kit from Abbott Molecular, Des Plaines, IL) used to select patients for therapy with crizotinib in the clinical trials [18]. For ALK testing, cytogenetic techniques such as FISH are generally preferred over reverse transcriptase polymerase chain reaction (RT-PCR) or other non-cytogenetic methods due to the number of variants of *ALK* fusions with *EML4* and the fusion of *ALK* with partner genes other than *EML4*. RT-PCR requires separate primers for each possible *ALK* fusion gene and, therefore, unless primers are used for every possible fusion, some variants will be missed [17, 19].

Despite the exciting initial clinical trial results, there are a number of significant shortcomings with these first generation TKIs. First, in the United States, only ~15 % of NSCLC have *EGFR* mutations and ~4 % of NSCLC have ALK rearrangements. That is, no targeted therapies are available for the remaining 81 % of NSCLC [13]. Second, *EGFR* mutations and ALK rearrangements occur almost exclusively in adenocarcinomas, including adenosquamous carcinomas, and uncommonly in pure squamous cell carcinomas or pure small cell carcinomas [3, 4, 12, 13, 20, 21]. Finally, after a period of initial response of several months, most NSCLC develop acquired resistance to *EGFR* TKIs or crizotinib due to secondary mutations in the *EGFR* or *ALK* gene or other mechanisms. These patients then require second- or third-line therapies for which second generation TKIs or drugs directed at other molecular targets might be considered [22, 23]. Therefore, these early successes in lung cancer targeted therapy and associated predictive biomarker testing are only initial steps towards lung cancer precision medicine. Advances in predictive biomarker technology, as

well as the search for new drugs and new clinically actionable targets, are rapidly progressing to address patient needs.

Advances in Predictive Biomarker Technology

Next Generation Sequencing

Until recently, clinical laboratories had limited options for genotyping tumor specimens. These mostly encompassed laboratory-developed tests using traditional molecular diagnostics technologies, such as polymerase chain reaction (PCR) coupled with sequence-specific hybridization probes or fragment analysis, Sanger dideoxynucleotide chain termination sequencing, and pyrosequencing [24]. Although these assays continue to perform well and have been crucial to improving patient care, they are hampered by a single-target-gene approach that requires a separate assay be performed for each biomarker. Thus, when specimen volumes are limited (i.e., transbronchial biopsies), there may be insufficient material to perform all needed gene mutation (i.e. *EGFR*, *ALK*, and *PIK3CA* gene mutations) and gene rearrangement (i.e., *ALK* and *ROS1* gene rearrangements) testing. In this context, “next-generation” sequencing technologies are revolutionizing the field of molecular diagnostics. The laboratory can now very quickly and inexpensively generate highly multiplexed tumor genotypes in a single reaction that uses as little as 10–250 ng of input genomic DNA [25].

Several “next-generation” DNA sequencing platforms are commercially available. In general, they can be categorized into two groups based on the type of DNA molecule used as a template. The instruments most commonly used in clinical laboratories produce libraries of clonally amplified targets. That is, each DNA fragment in the extracted specimen is first amplified and then sequenced in parallel, leading to the term “massively parallel sequencing”. These instruments include the 454 GS Junior (454 Life Sciences, Roche, Branford, CT), Ion Torrent Personal Genome Machine (Ion Torrent Systems, Life Technologies, San Francisco, CA), and MiSeq Personal Sequencer (Illumina Inc, San Diego, CA). Assay reagent kits licensed for Research Use Only (RUO) can be purchased to perform highly multiplexed tumor genotyping on each of these instruments. Single-molecule sequencing platforms such as the PacBio RS (Pacific Biosciences, Menlo Park, CA) have more recently emerged in the research environment. Although a powerful tool for whole-genome sequencing, their role in the molecular diagnostics laboratory remains uncertain. The MassARRAY Analyzer (Sequenom Inc, San Diego, CA) is another attractive option for clinical laboratories. Rather than sequencing DNA, the MassARRAY

Analyzer uses mass spectrometry of amplified gene fragments to determine their genotype. Beadling et al. [26] demonstrated the power of this approach by genotyping 820 tumors selected from a cancer registry, identifying several unexpected mutations that could be clinically actionable. In addition, investigators have recently developed novel strategies to detect gene rearrangements on the MassARRAY Analyzer, making it possible to concurrently evaluate gene mutations and gene rearrangements in a single run [27]. Although each of the instruments discussed herein generates high-quality sequence data, they vary in technical performance and laboratory operation characteristics including throughput, read length, error rate, hands-on time, run time, instrument cost and reagent cost. Based on the particular need and intended application, we recommend that each pathology laboratory carefully evaluate the options and direct the reader to a recent review on this complex analysis [28]. To assist pathologists in implementing these new technologies in the clinical environment, the College of American Pathologists (CAP) recently convened an expert panel and released a next-generation sequencing checklist (www.cap.org).

Although still in its infancy as a diagnostic tool, whole-genome sequencing has the potential to truly personalize patient care, moving us ever closer to the goal of precision medicine in lung cancer. The long-sought \$1,000 genome is nearly a reality, and advances in laboratory automation and bioinformatics analysis are making genomics an increasingly tractable tool for routine patient care [29]. Two large research studies recently published the whole genome sequences of 183 lung adenocarcinomas and 178 lung squamous cell carcinomas, respectively [30, 31]. These incredibly rich datasets revealed the tremendous level of genomic complexity in lung cancer. They also identified numerous opportunities for the application of existing and the development of new targeted therapies. For example, investigators not only confirmed the frequent presence of *EGFR*, *KRAS* and *BRAF* mutations in lung adenocarcinoma, but they also identified previously unrecognized recurrent mutations in several genes including *U2AF1*, *RBM10* and *ARID1A* [31]. In total, 25 genes were determined to be significantly mutated in lung adenocarcinoma. That is, whole-genome sequencing identified a very promising panel of adenocarcinoma genes for further study. In comparison, only 11 genes were shown to have statistically recurrent mutations in lung squamous cell carcinoma [30]. Among these, *P53*, *PIK3CA* and *NOTCH1* were already noted above. However, multiple significantly altered pathways were also identified, including pathways implicated in oxidative stress response and squamous cell differentiation. Of note, many of these alterations are shared with head and neck squamous cell carcinomas, suggesting a possible similarity in their underlying biology

and therapeutic vulnerabilities. Thus, new avenues for investigating biologic basis and therapeutic targeting in adenocarcinoma were discovered. Taken together, these studies reveal that potentially druggable targets can be found in nearly every lung tumor. Furthermore, as the comprehensive catalogue of somatic gene mutations associated with particular lung tumor types is complemented with epigenetic, transcriptomic, proteomic and metabolomic signatures, our understanding of malignant transformation and predictable clinical phenotypes will expand. This research is expected to produce increasingly innovative and effective therapeutic strategies that will profoundly affect patient care.

Inasmuch as pathologists can generate tremendous amounts of data using next-generation sequencing, information management and interpretation is an ongoing challenge. Given the magnitude of the human genome (approximately three billion base pair encoding ~20,000 genes), investigators are increasingly building bioinformatics and statistics support into their clinical laboratories. Improved bioinformatics pipelines and automated analysis algorithms must be developed before whole genome sequencing data can be evaluated in a timeframe that is optimal for patient care. A further challenge will be to learn which gene alterations identified in a particular tumor are driver mutations versus bystander mutations, emphasizing the need for continued molecular biology research. Coordinated efforts to generate a comprehensive reference human genome sequence and cancer genome databases (<http://cancergenome.nih.gov>, <http://cancercommons.org>, and <http://icgc.org>) have begun filling this knowledge gap. Results from these initiatives will be crucial for translating research discoveries to clinically meaningful interpretations. Moreover, investigators are increasingly learning that any one cancer is not homogenous, with concurrent existence of multiple subclonal lineages. To fully evaluate the possible efficacy of targeted therapy for a patient, the pathologist may need to analyze multiple samples collected at serial time points.

Predictive Biomarker Immunohistochemistry (IHC)

Until recently, the use of IHC to identify *EGFR* mutations and *ALK* rearrangements in lung cancers has been problematic. Traditional *EGFR* IHC detects generic protein expression without differentiating between wild-type and mutant epitopes, so it has not proven reliable in selecting candidates for *EGFR* TKI therapy. Antibodies to proteins associated with specific *EGFR* mutations are under investigation. Generally, these antibodies recognize the two primary mutations that account for approximately 90 % of *EGFR* mutations associated with a response to *EGFR* TKIs. Thus, IHC may be a useful screening tool, but it fails

to detect the 10 % of patients whose lung cancers have less commonly identified *EGFR* mutations. Investigations are ongoing in this field. [32–34]

ALK expression is not detected by IHC in the vast majority of benign tissues that lack an *ALK* fusion gene. However, it is detected by IHC when an *ALK* fusion gene is present, including in lymphoma, solitary fibrous tumor and other tumors. However, traditional ALK IHC has sometimes failed to detect ALK expression in lung cancers that were subsequently proven to have *ALK* fusion genes by other techniques, presumably due to the comparatively low expression of ALK in lung cancers. Various techniques have been proposed to enhance the sensitivity of ALK IHC for lung cancers, but an important development has been the recent availability of ALK antibodies that are highly specific and sensitive to ALK expression in lung cancers such as ALK (D5F3) XP[®] Rabbit mAb (Biotinylated) #8936 (Cell Signaling Technology, Danvers, MA). Although the correlation of ALK expression with ALK rearrangements is established, clinical trials directly comparing ALK IHC results to patient outcomes with crizotinib have not yet been performed [35–37].

New Drugs

Cetuximab

Cetuximab (Erbix; Bristol-Myers Squibb, NY, NY, and Eli Lilly and Company, Indianapolis, IN) is not a new drug, having been approved by the FDA for treatment of advanced colon adenocarcinoma and advanced head and neck squamous cell carcinoma [38–42]. However, it has been recently repurposed to lung cancer therapy [43]. Cetuximab is an anti-EGFR monoclonal antibody, offering a different approach to anti-EGFR therapy from the currently approved EGFR TKIs. In the first line Erbitux in lung cancer (FLEX) clinical trials, overall differences in patient response and outcomes for advanced NSCLC were not impressive. Further analysis disclosed a subgroup of patients who were likely to benefit from the addition of cetuximab with EGFR IHC as the predictive biomarker test. The level of EGFR expression in NSCLC correlated with an increased overall survival in advanced NSCLC patients receiving first-line chemotherapy plus cetuximab versus chemotherapy alone if the cancer had an EGFR IHC score of 200 or more using the Dako pharmDx kit (Glostrup, Denmark) [44–46]. The Southwest Oncology Group (SWOG) study SO342 and Phase III trial SO819 suggested that EGFR FISH might also be a predictive biomarker for advanced NSCLC patients receiving cetuximab plus chemotherapy [47, 48].

ERBB Family Blockers

Since first-generation EGFR TKIs eventually become ineffective due to acquired resistance, second-generation EGFR TKIs, which are actually ERBB family blockers, are under development. These agents are currently undergoing advanced clinical trials as adjuvant lines of therapy and/or first-line therapy. Second generation EGFR TKIs are potentially superior to first-generation EGFR TKIs, due to their higher affinity for the target, irreversible binding to the target, and inhibition of more than one target in the ERBB family of receptors. ERBB family blockers under study include dacomitinib [49], XL647 [50] and afatinib (BIBW2992: Boehringer Ingelheim, Ingelheim, Germany) [51–54]. Afatinib irreversibly binds to EGFR, even with the most common acquired resistance mutation (T790M) [51–54].

Drugs that Inhibit Multiple Targets

Some drugs inhibit more than one target and thus may be repurposed. It is anticipated that crizotinib, already approved for treatment of ALK-positive NSCLC, will be approved for treatment of ROS1 positive NSCLC. Crizotinib is not only an ALK TKI, but also inhibits other tyrosine kinases that may be present in NSCLC, including ROS1, MET and RON [55, 56]. ROS1 rearrangements are detected in a small percentage of NSCLC by FISH, and as with ALK, several different fusion partners have been identified [56, 57].

New Druggable Targets

Genotype Based Therapy Under Investigation

Additional druggable targets are needed to be discovered for the lung cancers that do not respond to or become resistant to TKIs. A number of targets, including the signaling pathways downstream of ERBB receptors are being investigated. However, no drug that is effective against the most frequently mutated oncogene in adenocarcinoma of the lung, KRAS, has been discovered [58].

Targets in the PI3K/AKT/mTOR pathway have received considerable recent attention. Several drugs impacting targets in this pathway are undergoing clinical trial, including the mTOR inhibitor everolimus, the PI3K and mTOR inhibitor BEZ235, the PI3K inhibitors GDC-0941 and XL147 and the AKT inhibitor MK-2206 [59–62]. Several inhibitors and antibodies are under investigation for c-MET and its ligand, hepatocyte growth factor or HGF [63]. The Met Inhibitor ARQ 197 plus Erlotinib vs. Erlotinib (MARQUEE plus placebo in NSCLC) trial is investigating the c-MET TKI ARQ 197 (also known as tivantinib) as

second-line therapy in patients with advanced non-squamous NSCLC [64, 65]. Potential drugs suggested for members of the IL-6/JAK/STAT pathway include the JAK inhibitors enzastaurin and AZD1480 and the STAT inhibitor NSC-743380 [66–68]. MEK has also been investigated as a potential downstream target with modest results to date [69–71]. Other proposed TKIs and their corresponding targets include vandetanib for RET [72, 73] and dasatinib for Src [74, 75].

Genotype Based Therapy in Cell Types Other than Adenocarcinoma

The initial success with first generation TKIs for EGFR mutations and ALK rearrangements has been confined to adenocarcinomas, including adenosquamous cell carcinomas, but has not been applicable to pure squamous cell carcinomas or pure small-cell carcinomas. The search for druggable targets and corresponding TKIs for squamous cell carcinoma has led to intense interest in members of the PI3K pathway, the fibroblast growth factor receptor and the discoidin domain receptor, accounting for about 20–30 %, 20 % and 4 % of squamous cell carcinomas, respectively [30, 76–78]. It remains to be seen if these likely targets will respond to TKI therapy. Another approach to “targeted therapy” for squamous cell carcinoma (as well as other cell types) may be the targeting of the abundant folate receptors on the cell surface of most squamous cell carcinomas. Novel delivery molecules are being designed to specifically deliver chemotherapeutic drugs attached to folate [79]. The mTOR inhibitors everolimus and temsirolimus are also under investigation as possible therapies for small-cell lung cancer [80–82].

Molecular Targeting of Lung Cancer Stem Cells

Lung cancer stem cells represent a cancer cell population that has the ability to self-renew. These cells frequently are chemoresistant. Post-chemoradiotherapy expression of lung cancer stem cell markers has been shown recently to significantly correlate with poor prognosis in lung cancer patients [83]. There is increasing evidence that cancer stem cells, including lung cancer stem cells, play a critical role in drug resistance, tumor regeneration, and metastasis [84]. Their asymmetric cell division and slow cycling makes identification and treatment targeting these cancer stem cells challenging [84]. Identification of molecular targets for effective lung cancer stem cell therapy requires the differentiation of lung cancer stem cells from normal multipotent stem cells. This differentiation might be accomplished using magnetic bead isolation or flow cytometry to identify unique cell type markers [85].

As cancers have traditionally been treated with the assumption that all cancer cells have equal malignant potential,

the concept that cancers are driven by cancer stem cells has significant clinical implications [86]. Researchers are actively seeking therapies that target stem cells, and cancer stem cell inhibitory mechanisms such as blocking stem cell factor, antagonizing ABCG2 pumping activity, and inhibition Notch activity are becoming increasingly studied [86]. Specifically, *Notch*, *Wnt*, and *Hedgehog* pathways are important in stem cell regulation [84]. An activated *Notch* pathway has been identified in approximately one-third of lung cancer cases and shown to indicate a significantly worse prognosis in patients lacking a concomitant *p53* mutation [84]. *Wnt* signaling activation produces significantly enhanced proliferation, colony formation, migration, and drug resistance. It is also vital to embryogenesis and homeostatic maintenance of adult tissues [84, 87]. The *Hedgehog* signaling pathway plays a key role in determining whether a cell undergoes differentiation or self-renewal [87]. Studies of lung cancer stem cell targeted therapy based on these pathways is ongoing, and researchers are actively pursuing targeted therapies [87].

Studies of additional possible lung cancer stem-cell-targeted therapies are ongoing. For example, Rac1 GTPase activity was recently shown to be significantly increased in cancer cells that have undergone epithelial-mesenchymal transition. Targeting Rac1 might provide a more effective lung cancer therapy by eliminating cancer stem cell subpopulations and by blocking non-cancer stem-cell-to-cancer stem cell transition [88]. Also, ALDH1A1 positivity in lung cancer stem cells may cause lung cancer resistance to EGFR-TKI [89]. Similarly, phenothiazine-like antipsychotic drug trifluoperazine was recently shown to down-regulate cancer stem cell markers CD44 and CD133, conferring anti-lung cancer stem cell properties that might overcome chemotherapy- and EGFR-TKI resistance [90].

Conclusion

An ever-increasing number of predictive biomarker tests will require greater knowledge and participation of the pathologist in personalized healthcare of the lung cancer patient. The pathologist must be prepared to advise clinicians on selection of the most appropriate tests and decide how to most effectively triage pathology samples, particularly small biopsies and cytology specimens. Pathologists must also continue developing highly multiplexed testing to efficiently use available tissue resources and reduce turnaround times. With the advent of additional lines of therapy, particularly in response to acquired resistance to initial therapy, the pathologist may potentially have an increased workload of sequential biopsies from a given patient over time. This may be particularly true as surveillance protocols are increasingly used to detect secondary mutations associated with acquired resistance prior to relapse [91, 92].

The role of the pathologist may also significantly evolve as IHC markers become better established. IHC is readily accessible to the community pathologist, enabling both diagnosis and predictive biomarkers analysis by direct observation of malignant cells using conventional tools and procedures. IHC appears to be the appropriate predictive biomarker methodology for cetuximab therapy. Also, the availability of antibodies to ALK overexpression and the ongoing validation of antibodies against other predictive biomarkers such as specific EGFR mutations makes IHC increasing tractable to the anatomic pathologist [4, 93, 94].

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