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The Role of Molecular Analyses in the Era of Personalized Therapy for Advanced NSCLC

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Abstract

Platinum-based doublet chemotherapy is the traditional treatment of choice for advanced non-small cell lung cancer (NSCLC); however, the efficacy of these regimens has reached a plateau. Increasing evidence demonstrates that patients with sensitizing mutations in the *epidermal growth factor receptor (EGFR)* experience improved progression-free survival and response rates with first-line gefitinib or erlotinib therapy relative to traditional platinum-based chemotherapy, while patients with *EGFR*-mutation negative tumors gain greater benefit from platinum-based chemotherapy. These results highlight the importance of molecular testing prior to the initiation of first-line therapy for advanced NSCLC. Routine molecular testing of tumor samples represents an important paradigm shift in NSCLC therapy and would allow for individualized therapy in specific subsets of patients. As these and other advances in personalized treatment are integrated into everyday clinical practice, pulmonologists will play a vital role in ensuring that tumor samples of adequate quality and quantity are collected in order to perform appropriate molecular analyses to guide treatment decisions. This article provides an overview of clinical trial data supporting molecular analysis of NSCLC, describes specimen acquisition and testing methods currently in use, and discusses future directions of personalized therapy for patients with NSCLC.

Keywords

Epidermal growth factor receptor (EGFR); *Kirsten rat sarcoma viral oncogene homolog (KRAS)*; molecular testing; personalized medicine; tyrosine kinase inhibitors; non-small cell lung cancer

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Introduction

Lung cancer represents the leading cause of cancer-related deaths in the United States and worldwide, with an estimated 5-year survival rate of approximately 16% for all stages [1,2]. While platinum-based doublet therapy is the traditional treatment of choice for advanced/metastatic (stage IIIB/IV) non-small cell lung cancer (NSCLC), no specific regimen is clearly superior, and efficacy with these regimens has reached a plateau in terms of overall response rate (RR; ~25%-35%) and median overall survival (OS; 8-10 months) [2]. Molecular testing for *epidermal growth factor receptor (EGFR)* and *Kirsten rat sarcoma viral oncogene homolog (KRAS)* mutations has allowed for the identification of subsets of patients who will be more responsive to certain therapies. Consequently, the treatment of patients with NSCLC is evolving toward a more personalized approach that utilizes specific molecular and genetic tumor profiles in treatment decisions. In addition to oncologists, radiologists, and pathologists, pulmonologists are essential team members in this quest for individualized treatment, playing a critical role in obtaining material for cytologic and histologic studies as well as ensuring that tissue is submitted for appropriate molecular investigation. This article reviews the clinical evidence supporting molecular typing of NSCLC, describes approaches for tissue sampling and molecular analysis, and discusses future directions of molecular profiling and the personalized treatment of patients with NSCLC.

Rationale for the Routine Testing of *EGFR* in NSCLC

The pathologic role of the EGFR pathway in the initiation and progression of NSCLC is well established (Figure 1) [3,4]. Retrospective analyses in patients with NSCLC have reported increased EGFR expression in 40% to 80% of tumors and demonstrated a correlation between increased expression and poor prognosis [5,6]. Based on the role of EGFR in the pathogenesis of NSCLC, inhibitors of EGFR signaling have been developed as a therapeutic strategy for NSCLC, including monoclonal antibodies that block ligand binding [5] and small molecule tyrosine kinase inhibitors (TKIs). Reversible EGFR TKIs, such as gefitinib and erlotinib, competitively bind to EGFR and are approved for NSCLC in various settings [7,8], while investigational irreversible EGFR TKIs (eg, afatinib [BIBW 2992], PF00299804), which target multiple human epidermal growth factor receptor (HER) family members simultaneously, are undergoing clinical evaluation for NSCLC. Approximately 90% of patients with genetic *EGFR* aberrations harbor either a 15-base pair nucleotide in-frame deletion in exon 19 (E746-A750del) or a L858R point mutation in exon 21 (Figure 2) [6,9,10]. These aberrations lead to ligand-independent constitutive activation of EGFR and have been shown to confer sensitivity to EGFR TKIs.

Relationship of EGFR Mutations and Response to EGFR TKIs

Early clinical studies first showed improved clinical benefit with gefitinib and erlotinib in certain patient populations, including those with adenocarcinoma, never smokers, women, and those from East Asia [11]. In the double-blind phase III ISEL study in unselected patients with relapsed/refractory NSCLC, those with *EGFR* mutations had higher RR than patients without *EGFR* mutations (37.5% vs 2.6%), but data were insufficient for survival analysis [12]. In the randomized phase III BR.21 trial [13], again in an unselected population that had relapsed/refractory disease (following at least 1 chemotherapy regimen), those who received erlotinib had a longer progression-free survival (PFS) and OS compared with placebo ($P < 0.001$ for each). However, *EGFR* mutational status was not significantly associated with survival benefit with erlotinib [14], perhaps due to the sequential use of erlotinib in those that had already failed at least 1 conventional chemotherapeutic regimen.

Results from the Spanish Lung Cancer Group showed the feasibility of prospectively screening for *EGFR* mutation prior to EGFR TKI therapy [15]. Moreover, several phase III trials support the importance of EGFR testing prior to the initiation of first-line therapy for advanced NSCLC (Table 1). Two phase III trials (IPASS and First-SIGNAL) evaluated first-line gefitinib versus chemotherapy in Asian patients selected based on clinical factors known to be associated with higher prevalence of *EGFR* mutation (adenocarcinoma histology, never or former light smokers) [16,17]. The IPASS mutation subanalysis provided evidence that patients with *EGFR* mutations respond significantly better to gefitinib than standard platinum-based chemotherapy and those without *EGFR* mutations respond significantly better to standard chemotherapy [16], highlighting the importance of molecular selection rather than clinical selection for guiding first-line treatment for NSCLC. Two additional phase III Asian trials (WJTOG3405 and NEJ002) utilized molecular selection and included only patients with *EGFR*-mutation positive tumors, with results confirming those from IPASS and First-SIGNAL [18,19,20]. The OPTIMAL phase III trial conducted in China was the first to compare erlotinib with chemotherapy in patients with *EGFR*-mutation positive tumors [21,22]. Similar to results observed in the gefitinib trials, first-line erlotinib significantly prolonged PFS versus platinum-based chemotherapy. Interim results from the European phase III EORTC study also show significantly longer PFS with first-line erlotinib versus platinum-based chemotherapy in patients with *EGFR*-mutation positive NSCLC [23], providing further support for molecular testing prior to initiating therapy for advanced NSCLC. Results of clinical trials evaluating investigational irreversible EGFR TKIs may also provide further support for molecular testing in NSCLC. An open-label phase II trial is evaluating PF00299804 in the first-line setting, in patients with advanced adenocarcinoma of the lung harboring an *EGFR* mutation [24]. Preliminary results indicated that overall best responses were 1 complete response, 29 partial responses, 28 with stable disease, and 6 with progressive disease. In addition, an open-label, randomized phase III trial (LUX-Lung 3; NCT00949650) is evaluating afatinib versus pemetrexed/cisplatin in the first-line setting, in patients with stage IIIB/IV adenocarcinoma of the lung harboring an *EGFR*-activating mutation.

EGFR Gene Copy Number, EGFR Protein Overexpression, and Response to EGFR TKIs

EGFR gene amplification and EGFR protein overexpression have also been implicated in predicting response to EGFR TKIs. In the double-blind phase III ISEL study, high *EGFR* gene copy number was associated with a lower risk of death with gefitinib versus placebo (hazard ratio [HR] for death, 0.61; $P = 0.067$), and patients with tumors expressing EGFR had significantly improved OS with gefitinib versus those whose tumors did not express EGFR ($P = 0.049$) [12]. Similarly, univariate analysis from the phase III BR.21 study showed that OS was longer with erlotinib versus placebo in patients whose tumors expressed EGFR (HR, 0.68; $P = 0.02$) or had high *EGFR* copy number (HR, 0.44; $P = 0.008$) [14]. However, multivariate analysis revealed that EGFR expression or *EGFR* copy number was not significantly associated with survival benefit in either treatment group.

The issue of whether *EGFR* mutations or increased *EGFR* copy number (detected via fluorescent in situ hybridization [FISH]) is the better biomarker for EGFR-TKI response was clarified by exploratory analysis of the IPASS trial [25]. Patients with high *EGFR* copy number had improved PFS with gefitinib versus chemotherapy (HR = 0.66, 95% confidence interval, 0.50-0.88; $P = 0.005$). However, post hoc exploratory analyses showed this effect was primarily driven by overlap of high *EGFR* copy number with positive *EGFR*-mutation status (among the 249 patients who were FISH positive, 190 (78%) also harbored an *EGFR* mutation). Patients who were FISH positive/mutation negative derived little or no benefit from gefitinib; however, patients with *EGFR* mutations overall had significantly longer PFS with gefitinib ($P < 0.001$) [16], suggesting that *EGFR*-mutation status is the primary

determinant of response to gefitinib and consequently the preferred biomarker to predict EGFR-TKI benefit.

Secondary EGFR Mutations

While patients with *EGFR* mutations typically respond to erlotinib or gefitinib, additional mutations in the *EGFR* gene have been associated with primary and acquired resistance to EGFR-TKI therapy. For example, the T790M point mutation in *EGFR* exon 20 is present in approximately 50% of patients who initially respond to reversible EGFR TKIs and then develop resistance [26,27,28]. It is noteworthy that the T790M mutation can also occur de novo in EGFR TKI treatment-naïve patients [29,30,31,32], suggesting its potential utility as a predictive biomarker.

KRAS Mutations and NSCLC

Increasing evidence indicates that signaling pathways downstream of EGFR (Figure 1) are also involved in the progression of NSCLC and other malignancies [33]. While activating *KRAS* mutations are uncommon in NSCLCs with squamous histology, they have been identified in 15% to 30% of those with adenocarcinoma histology [34,35,36]. There are no convincing data, however, that *KRAS* mutations confer resistance to EGFR inhibitors. Instead, because *EGFR* and *KRAS* mutations are mutually exclusive in the vast majority of patients, it seems that *KRAS* positivity may predict resistance to EGFR TKIs (since practically all of these patients are *EGFR* mutation negative). Furthermore, there are no data to demonstrate that those patients who are *KRAS* positive are any more resistant to EGFR TKIs than patients who are *EGFR* negative or *KRAS* negative. The reason then, to test for *KRAS* mutation status, is twofold: 1) those who are *KRAS* positive do not require testing for *EML4/ALK* translocation and 2) these patients can be entered into clinical trials designed for *KRAS*-positive patients.

Implications for Pulmonologists

While additional studies are necessary to further define the role of these candidate predictive biomarkers in NSCLC, accumulating evidence suggests that *EGFR* and *KRAS* mutations have therapeutic implications and thus, mutational analysis is finding wider application in the clinic. Routine molecular testing of tumor tissue for use in treatment determination represents a significant paradigm shift in NSCLC therapy and therefore calls for a more streamlined and standardized approach to specimen acquisition and processing. It is essential that pulmonologists obtain samples of both sufficient quality and quantity so that adequate specimen is available for both diagnosis and molecular analyses. Some of the invasive and non-invasive approaches that can be adopted to obtain adequate amounts of tissue and appropriate ways to process them are described below.

Tissue Sampling Techniques and Considerations

Tissue for tumor typing and molecular studies can be obtained by a number of techniques ranging from noninvasive to surgical. Because the majority of patients with NSCLC have advanced disease and are not eligible for surgery, nonsurgical procedures are usually a better choice. Several factors must be considered when determining appropriate diagnostic tests, including sensitivity and specificity, false-negative and false-positive rates, procedure morbidity, and tumor location and characteristics [37]. It is important to optimize tissue recovery while decreasing procedure-related morbidity [38].

Sputum cytology is a noninvasive procedure particularly valuable in patients with centrally located tumors [38]; however, tumor location and size affect sensitivity of this method, and rigorous specimen sampling is required to ensure diagnostic accuracy. Flexible

bronchoscopy is minimally invasive and most effective with central lesions, but may also be used in conjunction with guided imaging to evaluate peripheral lesions [38]. Bronchoscopy may be performed with or without needle aspiration, which typically obtains cytologic specimens. When larger-gauge needles are used, core samples and material for cell block may also be obtained. Another minimally invasive sampling method, transthoracic needle aspiration, may be used for peripheral, parenchymal lesions; however, there is a high false-negative rate and a risk of pneumothorax associated with this procedure [38,39,40,41]. Some of these techniques may be supplemented with medical imaging, such as endobronchial ultrasound, to guide sampling, avoid surgery, and improve yield and accuracy [37,38]. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a minimally invasive procedure that produces high diagnostic yield allowing for combined pathologic and molecular analysis of metastatic lymph nodes [42]. The ideal methodology of EBUS-TBNA sample handling has recently been published, suggesting rapid on-site cytologic examination to confirm adequate samples for both molecular testing and pathologic diagnosis [42]. If rapid on-site cytology is not available, 3 aspirations per lymph node station or 2 aspirations with 1 tissue core are recommended [43]. While EBUS-TBNA appears particularly promising, regardless of the approach used, it is essential for pulmonologists to confirm that sufficient sample is available to perform appropriate molecular analyses to guide treatment decisions.

Molecular Assays

While larger tissue samples are preferable, diagnostic approaches in NSCLC have shifted toward minimally invasive procedures [44]. Therefore, techniques have been developed whereby molecular testing can be performed on smaller amounts of tissue and specific criteria for sample type, size, collection, and storage have been published (Table 2) [44].

It is important to select a well-validated and robust method for assessing mutation status to minimize false positive (which would indicate EGFR-TKI therapy for a patient more likely to benefit from chemotherapy) or false negative (which would deny a mutation-positive patient from receiving optimal treatment) results. Direct sequencing of polymerase chain reaction (PCR)-amplified regions is the most common method of identifying *EGFR* and *KRAS* mutations [45], but because of issues with sensitivity and time involved, other techniques have been developed [45]. Sensitive and specific mutational testing kits for *EGFR* and *KRAS* are currently commercially available for research use (DxS TheraScreen® EGFR29, ResponseDX™: Lung), and mutational analysis has also been performed in clinical trials.

EGFR gene copy number can be assessed by a variety of methods, including FISH, chromogenic in situ hybridization (CISH), and real-time quantitative PCR [45]; however, *EGFR* amplification has fallen out of favor as a potential predictive marker in NSCLC. Measurement of *EGFR* expression by IHC is also not commonly used for diagnosis, but a recent analysis from the FLEX trial suggests value as a predictive marker with first-line cetuximab, an EGFR-targeted monoclonal antibody, in combination with chemotherapy in NSCLC [46].

While the histologic classification of lung cancer is generally not difficult to determine on surgical specimens, small biopsy samples or cytologic specimens may be inadequate for certain assays [47]. Guidelines for tumor tissue analysis by FISH, IHC, and other methods have been standardized and published (Table 2) [44]. Alternative methods of tissue acquisition are also under investigation. A recent publication reported 92% sensitivity (in 11 of 12 patients) for detection of *EGFR* mutations in circulating tumor cells isolated from patients with metastatic NSCLC [32].

Future Directions in Individualized Care for Patients with NSCLC

Further efforts toward individualizing therapy for NSCLC have led to the investigation of other candidate biomarkers, including *echinoderm microtubule-associated protein-like 4* (*EML4*) and *anaplastic lymphoma kinase* (*ALK*) fusion [48,49], although most of these are specific to nonsquamous histology. Isoforms of the *EML4-ALK* transforming gene and its product have been identified in approximately 5% of patients with NSCLC, who are predominantly younger and light or nonsmokers [50,51,52,53]. Patients with *EML4/ALK*-expressing NSCLC are generally resistant to EGFR-TKI therapy and have tumors with wild-type *EGFR* and *KRAS* [52,54]. Several novel selective ALK inhibitors are in various stages of development [53]. Most recently, crizotinib (Xalkori®), an oral ATP-competitive selective inhibitor of the ALK and cMet (also known as hepatocyte growth factor receptor) tyrosine kinases [55], was approved for the treatment of patients with ALK-positive (by an approved molecular test) locally advanced or metastatic NSCLC based on results from an expansion cohort of a phase I study [56,57] and a phase II study [58].

As our knowledge of molecular markers and their prognostic and therapeutic value in NSCLC has evolved, it is clear that incorporation of testing for more common biomarkers into the initial workup of patients with NSCLC is necessary. Horn and Pao have previously proposed sequential *KRAS*, *EGFR*, and *EML4-ALK* molecular testing based on the gene prevalence and implications of each in NSCLC (Figure 3) [53]. Currently, there are no data to support the initial testing of only *KRAS* or *EGFR* mutations, and we instead suggest the simultaneous testing of both *EGFR* and *KRAS*. If *EGFR* mutation testing is positive, this has first-line implications, and if *KRAS* is positive, it is unlikely that *EGFR* will also be positive as the 2 mutations are generally mutually exclusive. If *KRAS* is found to be positive, patients can then be considered for enrollment in clinical trials. Figure 3 shows a proposed molecular testing algorithm.

The Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) program is the first prospective biopsy-mandated study to assess candidate predictive biomarkers as a means of guiding treatment choice in heavily pretreated patients with advanced NSCLC [59]. Fresh core needle biopsy was performed upfront to test for 11 biomarkers, including *EGFR* and *KRAS* mutation by PCR, and results from these analyses were used to select treatment based on individual patient profile. Patients with *EGFR* mutation had an improved 8-week disease control rate when treated with erlotinib (5/7; 71%), while patients with *KRAS* mutation did not respond well to erlotinib (2/9; 22%). The BATTLE program represents the first trial to evaluate the efficacy of personalized therapy for NSCLC and final results are awaited.

As personalized medicine for NSCLC evolves, pulmonologists and oncologists need to effectively integrate cytologic, molecular, and histologic tests to determine the most appropriate therapy for each individual patient. In addition to the assays discussed, there is ongoing interest in developing and implementing new, less invasive techniques to determine prognosis and identify appropriate therapy for patients with NSCLC. Potential future approaches include utilizing advances in imaging technology as well as serum testing for cell-free tumor nucleic acids.

In conclusion, results from several randomized phase III trials emphasize the importance of molecular testing prior to initiating first-line therapy for advanced NSCLC. Increasing evidence demonstrates that patients with *EGFR* mutations experience significant benefit with gefitinib or erlotinib versus standard chemotherapy, with an opposite effect occurring in patients with *EGFR*-mutation negative tumors. As molecular testing becomes standard

clinical practice, pulmonologists will play a vital role in ensuring samples of adequate quality and quantity are available for these purposes.

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Figure 1. EGFR signal transduction pathways

In response to ligand binding, members of the EGFR family of receptor tyrosine kinases form dimers and are activated, resulting in downstream signaling which promote survival and proliferation.

Akt, protein kinase B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; P, phosphate; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; Raf, v-raf 1 murine leukemia viral oncogene homolog 1; Ras, retrovirus-associated DNA sequences; STAT, signal transducers and activators of transcription; TGF, transforming growth factor.

Figure 2. Gefitinib- and erlotinib-sensitizing mutations of EGFR in NSCLC

A cartoon representation of epidermal growth factor receptor (EGFR) showing the distribution of exons in the extracellular domain (EGF binding), transmembrane domain (TM), and intracellular domain (comprising the tyrosine kinase and autophosphorylation regions). Exons 18-21 in the tyrosine kinase region where the relevant mutations are located are expanded and a detailed list of EGFR mutations in these exons that are associated with sensitivity to gefitinib or erlotinib is shown. Percentages are denoted for some mutations and exons, and the main mutations in each class are shown in bold.

Figure 3. Suggested algorithm for the molecular testing of patients with NSCLC of adenocarcinoma histology

Suggested order of testing for *KRAS* and *EGFR* mutations and EML4-ALK translocations and potential treatments based on mutation status.

Table 1
Phase III Studies Comparing Reversible EGFR TKIs Versus Platinum-based
Chemotherapy As First-line Treatment of Advanced NSCLC

Study	Patient population	Treatment	HR for progression (95% CI)	RR, %
IPASS (N = 1,217) [16]	Asian, never or former light smokers, adenocarcinoma	Gefitinib vs carboplatin/gemcitabine	Overall: 0.74 (0.65-0.85) ^a <i>EGFR</i> -mut pos: 0.48 (0.36-0.64) ^a <i>EGFR</i> -mut neg: 2.85 (2.05-3.98) ^a	Overall: 43.0 vs 32.2 ^e <i>EGFR</i> -mut pos: 71.2 vs 47.3 ^e <i>EGFR</i> -mut neg: 1.1 vs 23.5 ^b
First-SIGNAL (N = 309) [17]	Asian, never or former light smokers, adenocarcinoma	Gefitinib vs cisplatin/gemcitabine	Overall: 0.737 (0.580-0.938) ^b Gefitinib (<i>EGFR</i> -mut neg vs <i>EGFR</i> -mut pos): 0.385 (0.208-0.711) ^b CT (<i>EGFR</i> -mut neg vs <i>EGFR</i> -mut pos): 1.223 (0.650-2.305) ^c	Overall: 53.5 vs 42.0 ^c
WJTOG3405 (N = 172) [18]	<i>EGFR</i> -mut pos	Gefitinib vs cisplatin/docetaxel	0.489 (0.336-0.710) ^a	62.1 vs 32.2 ^a
NEJ002 (N = 228) [19]	<i>EGFR</i> -mut pos	Gefitinib vs carboplatin/paclitaxel	0.30 (0.22-0.41) ^d	73.7 vs 30.7 ^d
OPTIMAL (N = 154) [21]	<i>EGFR</i> -mut pos	Erlotinib vs carboplatin/gemcitabine	0.164 (NR) ^a	83 vs 36 ^a
EURTAC (N = 174) [23]	<i>EGFR</i> -mut pos	Erlotinib vs platinum-based chemotherapy	0.42 (NR) ^a	54.5 vs 10.5 ^a

CI: confidence interval; CT: chemotherapy; EGFR: epidermal growth factor receptor; *EGFR*-mut pos: *EGFR*-mutation positive; *EGFR*-mut neg: *EGFR*-mutation negative; HR: hazard ratio; NR: not reported; TKIs: tyrosine kinase inhibitors.

^a $P < 0.0001$.

^b $P < 0.01$.

^c $P > 0.05$.

^d $P < 0.001$.

^e $P = 0.0001$.

Table 2
Specimen Sampling in the Preanalytical Phase

Parameter	Recommendations
Sample type	<ul style="list-style-type: none"> • Tissue block is preferred • Cytology smears are not acceptable for IHC or FISH • Records should be kept of sample type (eg, excision, biopsy, cytology)
Minimum sample size	<ul style="list-style-type: none"> • Required tumor tissue section area of ~1-2 mm², excluding nontumor cells and tissue areas • Cell number requirements: FISH, >100 assessable tumor cell nuclei; IHC/mutation, ~ 2000 cells • High tumor cell content (50%-70% tumor cells) required for direct sequencing
Sample collection and storage	<ul style="list-style-type: none"> • Standard fixation in 10% neutral buffered formalin and paraffin embedding; records should be kept of fixative • Bouin's or mercury-containing fixatives should be avoided • Sample should be stored as a block, not as precut slides • Sectioning dates should be recorded if slides are precut
Tumor heterogeneity	<ul style="list-style-type: none"> • Intertumoral and intratumoral heterogeneity may confound interpretation • Ideally, 3 representative areas should be assessed per tumor section

FISH: fluorescence in situ hybridization; IHC: immunohistochemistry.