Emerging Concepts in the Pathology and Molecular Biology of Advanced Non–Small Cell Lung Cancer

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Abstract

*Non–small cell lung cancer (NSCLC) is traditionally classified histologically, but until recently, the histologic subtype has had little impact on the selection of therapy. Drugs such as pemetrexed and bevacizumab are indicated for specific NSCLC subtypes, and this type of stratification represents the first step toward individualizing therapy in NSCLC. Beyond histologic features, the status of molecular targets, such as the epidermal growth factor receptor (*EGFR*) gene, has been shown to correlate with response to treatment with EGFR tyrosine kinase inhibitors in patients with relapsed or refractory disease and in the first-line therapy setting. New therapies targeting the EGFR and other molecular aberrations are under way to help define specific subsets of patients responsive to certain molecularly targeted treatments. The role of pathologists in guiding treatment decisions will increase because molecular profiling, together with pathologic and histologic analysis, represents the future of personalizing medicine for patients with NSCLC.*

Ideally, a tumor classification system should have predictive value; that is, it should provide information to influence therapeutic decision making and elucidate morphologic distinctions between tumor types.[1] The World Health Organization tumor classification system **Table 1** provides a basis for comprehensive diagnosis and provides a guide for therapeutic decisions.[2,3] Non–small cell lung cancer (NSCLC) is a heterogeneous aggregate of histologic subtypes, which traditionally have been grouped together because of similarities of treatment and outcome.[4] Pathologists have been classifying NSCLC by histologic subtype, most broadly into adenocarcinoma, squamous cell carcinoma, and "others."

Adenocarcinoma is the most frequently occurring subtype, constituting approximately 40% of all NSCLCs, with squamous cell carcinoma close behind, and "others" representing the remaining cases.[5] Refinements of the classification system have been made, such as distinct separation of bronchioloalveolar carcinoma (BAC) as a noninvasive lesion based on correlation of outcome to H&E staining features and the recognition of neuroendocrine origin within large cell carcinoma, based mostly on immunohistochemical markers.[2] The significance of these findings has been mostly prognostic, as considered in the narrow sense of indicating the lesions' innate behavior, rather than predictive, indicating the lesions' response to a therapeutic intervention. Thus, for many years, the histologic subtype was not taken into account in the management of patients with NSCLC.

Recently, however, histologic subtype has become an important consideration when selecting therapy. In 2009, for the first time, the National Comprehensive Cancer Network (NCCN) issued guidelines that include histologic subtype as a factor for recommending specific treatment options.[6] For

Table 1
Histologic Classification of Lung Epithelial Neoplasms[2,3]

Histologic Type	Variant
Squamous cell carcinoma	Papillary
	Clear cell
	Small cell
	Basaloid
Adenocarcinoma	Mixed
	Bronchioloalveolar
	Mucinous
	Nonmucinous
	Mixed
	Indeterminate
	Acinar
	Papillary
	Solid adenocarcinoma with mucin production
	Fetal adenocarcinoma
	Mucinous "colloid" carcinoma
	Mucinous cystadenocarcinoma
	Signet-ring adenocarcinoma
	Clear cell adenocarcinoma
Adenosquamous carcinoma	
Large cell carcinoma	Large cell neuroendocrine carcinoma
	Basaloid carcinoma
	Lymphoepithelioma-like carcinoma
	Clear cell carcinoma
	Large cell carcinoma with rhabdoid features
Small cell carcinoma	Combined small cell carcinoma
Sarcomatoid carcinoma	Pleomorphic carcinoma
	Spindle cell carcinoma
	Giant cell carcinoma
	Carcinosarcoma
	Pulmonary blastoma
	Others
Carcinoid tumor	Typical carcinoid
	Atypical carcinoid
Carcinoma of salivary-gland type	Mucoepidermoid carcinoma
	Adenoid cystic carcinoma
	Epithelial-myoepithelial carcinoma
Preinvasive lesions	Squamous carcinoma in situ
	Atypical adenomatous hyperplasia
	Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia

example, based on the results of pivotal phase 3 clinical trials, pemetrexed, alone or in combination with cisplatin, is indicated only for patients with advanced disease (chemotherapy-naive or chemotherapy-treated) and nonsquamous histology.[7-9] Bevacizumab (Avastin, Genentech, South San Francisco, CA), in combination with carboplatin and paclitaxel, is not recommended for patients with squamous cell carcinoma because pulmonary hemorrhage occurs with greater frequency with bevacizumab in patients with this histologic subtype.[10] Results such as these have served as a guide in personalizing therapy for patients with lung cancer.

Morphologic examination of tumor biopsy specimens is essential to NSCLC diagnosis and staging and is the first step toward personalizing treatment. To more accurately classify NSCLC subtypes, additional methods can be and are used. The next section discusses 2 markers that have revolutionized the impact of pathologists' assessment of tumor tissue and are likely examples of how diagnostics will evolve in the future.

Epidermal Growth Factor Receptor and V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog

Evaluating appropriate molecular targets is the next step in making clinical diagnoses that will help to individualize therapy. The significant association between certain epidermal growth factor receptor (*EGFR*) mutations, especially exon 19 deletions, and clinical benefit in response to treatment with EGFR tyrosine kinase inhibitors (TKIs) is well established in patients with relapsed or refractory disease. However, increasing evidence suggests that such molecular selection is warranted earlier in the course of treatment **Table 2**.[11-34] IPASS, a phase III trial, compared gefitinib (Iressa, AstraZeneca, Wilmington, DE) with standard chemotherapy for the first-line treatment of advanced NSCLC with adenocarcinoma histologic features in patients who were light or never smokers.[31] The study included more than 1,200 patients, with a retrospective biomarker analysis performed on specimens from 437 patients with evaluable *EGFR* mutation data.[31] Mutations in the *EGFR* gene were identified in 261 (59.7%) of these patients. Among this subgroup, progression-free survival (PFS) was significantly longer in the patients who received gefitinib compared with patients who received carboplatin/paclitaxel (hazard ratio [HR] for progression or death, 0.48; 95% confidence interval [CI], 0.36-0.64; $P < .001$). Furthermore, the mutation-negative patients experienced significantly diminished PFS with gefitinib compared with carboplatin/paclitaxel (HR for progression, 2.85; 95% CI, 2.05-3.98; $P < .001$). In a retrospective analysis, increased PFS was also observed in patients with *EGFR*-mutation-positive tumors who received first- or second-line gefitinib monotherapy.[35]

The role of *EGFR* mutational status in helping patients achieve prolonged clinical benefit in response to EGFR TKI therapy seems to extend to the maintenance therapy setting as well. The phase 3 SATURN study was designed to compare the effects of erlotinib (Tarceva, Genentech) and placebo in patients with advanced NSCLC who had experienced clinical benefit (response or disease stabilization) after 4 cycles of standard platinum-based chemotherapy.[34] Biomarker analyses, including determination of *EGFR* mutation status, were performed. PFS was significantly prolonged with erlotinib compared with placebo in all patients (HR, 0.71; 95% CI, 0.62-0.82; $P < .0001$).[34] Response rates (11.9% vs 5.4%) and disease control rates (objective response plus stable disease >12 weeks, 40.8% vs 27.4%; $P < .0001$) were also improved with erlotinib.[34] Significantly prolonged PFS was

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Table 2
Summary of Key Clinical Trials Testing the Effect of *EGFR* Mutational Status on Response to EGFR Tyrosine Kinase Inhibitor Therapy

		EGFR-Mutation Findings		
Trial Name	**Agent**	**Positive**	**Negative**	**Reference**
≥Second-line therapy				
—	Gefitinib	RR, 100% (5/5)	RR, 0% (0/4); $P = .0027$	Paez et al,[11] 2004
—	Erlotinib	RR, 71% (5/7)	RR, 0% (0/10); $P = .003$	Pao et al,[12] 2004
—*	Gefitinib	RR, 100% (8/8)	RR, 13% (1/8); $P < .001$	Lynch et al,[13] 2004
ISEL	Gefitinib	ORR, 38% (6/16)	ORR, 2.6% (3/116)	Thatcher et al,[14] 2005; Hirsch et al,[15] 2006
BR.21	Erlotinib	RR, 27% (4/15); HR for OS, 0.77 (95% CI, 0.40-1.50); $P = .45$†	RR, 6.9% (7/101); $P = .03$; HR for OS, 0.73 (95% CI, 0.49-1.10); $P = .13$†	Shepherd et al,[16] 2005; Tsao et al,[17] 2005; Zhu et al,[18] 2008
—*	Gefitinib	RR, 65% (11/17; 95% CI, 42.0%-87.4%); TTP, 21.7 mo; HR for TTP, 0.23 (95% CI, 0.093-0.57); OS, 30.5 mo; HR for OS, 0.16 (95% CI, 0.046-0.52)	RR, 14% (10/73; 95% CI, 5.8%-21.6%); $P < .001$; TTP, 1.8 mo; $P < .001$; OS, 6.6 mo; $P < .001$	Han et al,[19] 2005
INTEREST	Gefitinib	OS, 14.2 mo	OS, 6.4 mo; $P = .59$	Kim et al,[20] 2008
V-15-32	Gefitinib	ORR, 67% (6/9) PFS, HR for mutation-positive vs mutation-negative, 0.33 (95% CI, 0.11-0.97)	ORR, 0% (0/26)	Maruyama et al,[21] 2008
—	Erlotinib	RR, 83% (15/18; 95% CI, 65%-94%); PFS, 13 mo; OS, 23 mo	RR, 6% (4/63); $P < .01$; PFS, 2 mo; $P < .01$; OS, 17 mo; $P = .24$	Miller et al,[22] 2008
LUX-Lung 2‡	Afatinib	ORR, 60.5%§ (78/129)	NA, *EGFR*-mutation-selected patient population	Yang et al,[23] 2010
First-line therapy				
—	Erlotinib	RR, 80% (4/5); OS, >627 d	RR, 5% (1/22); OS, 377 d; $P = .15$	Giaccone et al,[24] 2006
ONCOBELL	Gefitinib	RR, 63% (15/24); median OS, NR	RR, 23% (3/13); $P = .02$; median OS, 11.1 mo; $P = .5$	Cappuzzo et al,[25] 2007
WJTOG 0403	Gefitinib	RR, 75% (21/28; 95% CI, 57.6%-91.0%); PFS, 11.5 mo (95% CI, 7.3-NR); median OS, NR; 1-y OS, 79% (22/28; 95% CI, 63.4%-93.8%)	NA, *EGFR*-mutation-selected patient population	Tamura et al,[26] 2008
—	Gefitinib	RR, 69% (38/55); median TTP, 8 mo; median OS, 24 mo	RR, 20% (7/35); median TTP, 3.4 mo; median OS, 12.9 mo	Yang et al,[27] 2008
—	Gefitinib	RR, 63% (12/19; 95% CI, 38.4%-83.7%); PFS, 7.1 mo; median OS, 20 mo	NA, *EGFR*-mutation-selected patient population	Sugio et al,[28] 2009
—	Gefitinib	RR, 55% (17/31; 95% CI, 33.0%-70.0%); PFS, 9.2 mo (95% CI, 6.2-11.8); median OS, 17.5 mo (95% CI, 13.5-21.3)	NA, *EGFR*-mutation-selected patient population	Sequist et al,[29] 2008
TRUST‖	Erlotinib	RR, 50% (2/4) HR for PFS mutation-positive vs mutation-negative, 0.31 (95% CI, 0.13-0.78); $P = .009$; HR for OS mutation-positive vs mutation-negative, 0.33 (95% CI, 0.12-0.91); $P = .025$	RR, 3% (2/68); $P = .014$	Schneider et al,[30] 2008
IPASS	Gefitinib	ORR, 71.2%; HR for OS, 0.78 (95% CI, 0.50-1.20); HR for PFS, 0.48 (95% CI, 0.36-0.64)	ORR, 1.1%; HR for OS, 1.38 (95% CI, 0.92-2.09); HR for PFS, 2.85 (95% CI, 2.05-3.98)	Mok et al,[31] 2009
First-SIGNAL	Gefitinib	PFS, 8.4 mo; OS, 30.6 mo	PFS, 2.1 mo; OS, 18.4 mo	Lee et al,[32] 2009
—	Gefitinib	ORR, 66% (95% CI, 51%-80%); median PFS, 6.5 mo; median OS, 17.8 mo	NA, *EGFR*-mutation-selected patient population	Inoue et al,[33] 2009
Maintenance therapy				
SATURN	Erlotinib	PFS, HR, 0.1 (95% CI, 0.04-0.25); $P < .0001$	PFS, HR, 0.78 (95% CI, 0.63-0.96); $P = .0185$	Cappuzzo et al,[34] 2010

CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; NA, not available; NR, not reported; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; RR, response rate; TTP, time to progression.
* Also included patients receiving first-line therapy.
† The comparison group is placebo.
‡ Interim analysis of pivotal phase 2 trials.
§ Investigator-determined response.
‖ Also included patients receiving second- and third-line therapy.

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observed with erlotinib in patients whose tumors had *EGFR* mutations (HR, 0.10; 95% CI, 0.04-0.25; $P < .0001$) and in patients whose tumors bore wild-type (WT) *EGFR* (HR, 0.78; 95% CI, 0.63-0.96; $P = .0185$).[34]

Amplification of the *EGFR* gene also has been shown to correlate with response to EGFR TKIs. In patients with advanced NSCLC, an increased *EGFR* gene copy number (GCN), as measured by fluorescence in situ hybridization (FISH), predicted improved survival and/or response to gefitinib[25,36,37] and erlotinib.[17,18] In a biomarker analysis from the randomized phase 3 BR.21 trial, which compared erlotinib with placebo in the second-line NSCLC setting, 61 (38.4%) of 159 tumors analyzed were positive for an increased *EGFR* GCN.[18] Response rates were 21% and 5% in patients who were positive and negative for increased *EGFR* GCN, respectively ($P = .02$). This benefit seemed to extend to survival (HR, 0.43; $P = .004$). In a multivariate analysis, an increased *EGFR* GCN showed a significant prognostic association with poorer survival ($P = .025$) and differential survival benefit with erlotinib ($P = .005$). Taken together, these data suggest that in addition to *EGFR* mutational status, an increased *EGFR* GCN may also influence patient response to EGFR TKIs.

The role of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) testing in patients with NSCLC remains controversial. KRAS is a downstream effector of the EGFR pathway. The *KRAS* gene is mutated in approximately 20% to 30% of NSCLCs, principally in patients with lung adenocarcinomas and patients with a history of smoking.[38,39] *KRAS* mutations are associated with intrinsic TKI resistance; patients with mutated *KRAS* experience better PFS with chemotherapy than with EGFR TKI therapy. This finding is not surprising, because *KRAS* mutations have been shown to be a poor predictor of response to EGFR inhibitor therapy in patients with NSCLC.[18,38-44] However, a subgroup analysis of 90 patients in the SATURN study who had the *KRAS* mutation showed no significant difference in PFS between patients treated with erlotinib vs placebo (HR, 0.77; 95% CI, 0.50-1.19; $P = .2246$).[45] Although *KRAS* mutation has been associated with clinical outcomes with cetuximab in colorectal cancer,[46] no association was reported from analyses of multiple trials of cetuximab in combination with chemotherapy in patients with NSCLC.[44,47] *KRAS* testing continues to evolve, and the clinical implications of *KRAS* mutations will likely remain an active area of research until the significance of these genetic abnormalities is elucidated.

Testing for *EGFR* mutations and/or gene amplification and *KRAS* mutations is recognized in the current NCCN guidelines; however, it is unclear whether this testing should be routine for all lung cancers because the prevalence of *EGFR* mutations is low in patients with a history of heavy smoking,[48] and approximately 90% of lung cancers in men and 80% of lung cancers in women are caused by exposure to cigarette smoke.[49] According to the NCCN guidelines, the purposes of pathologic evaluation include not only classification of the lung cancer by histologic and immunohistochemical studies but also the determination of molecular aberrations that may predict for sensitivity or resistance to EGFR TKIs.[50] The NCCN recognizes that the presence of *EGFR*-activating mutations represents a "critical" biomarker for appropriate patient selection for therapy. As such, in the first-line therapy setting, erlotinib, with or without chemotherapy, should be considered for patients with known *EGFR* activation mutations or gene amplification. The guidelines further recognize that, in many studies, *KRAS* mutations have been shown to be associated with resistance to EGFR TKI therapy; as such, they may be a useful marker in the selection of patients for EGFR TKI therapy. For patients with known *KRAS* mutations, therapy other than erlotinib should be considered in the first-line setting.[50]

In the absence of *EGFR* mutation status, the overexpression of EGFR protein levels as detected by immunohistochemical studies may, in combination with *EGFR* GCN analyses, enhance the selection of patients who may respond best to EGFR TKI therapy. Hirsch and colleagues[37] studied a number of genetic characteristics in tumor specimens from 204 patients with NSCLC treated with the first-generation EGFR TKI, gefitinib. They observed that increased EGFR protein expression, detected by immunohistochemical studies, and increased *EGFR* GCN, as assessed by FISH, predict patients likely to respond to gefitinib; the cohort of patients who were *EGFR* FISH+ and positive immunohistochemically for EGFR showed an overall response rate of 41% and a 1-year survival rate of 77%. In contrast, only 2% of patients with specimens negative for *EGFR* FISH and negative immunohistochemically for EGFR responded to gefitinib; their median survival was 6 months, with a 1-year survival rate of only 30%. Based on these data, Hirsch and colleagues[37] recommend that patients with negative results not receive EGFR TKI therapy.

In the SATURN trial described earlier, maintenance therapy with erlotinib after first-line platinum-based chemotherapy resulted in significantly prolonged PFS as compared with placebo among patients with NSCLC whose tumors showed EGFR overexpression by immunohistochemical analysis (HR, 0.69; 95% CI, 0.58-0.82; $P < .0001$).[34] However, the predictive usefulness of EGFR protein expression as evaluated immunohistochemically may not be consistent. Several studies have found that EGFR protein expression measured immunohistochemically does not correlate significantly with response to EGFR TKI therapy or survival.[22,51,52] For example, in their series of 101 patients with BAC or adenocarcinoma with BAC who had received no more than 1 prior chemotherapy regimen, Miller and colleagues[22] found that improved response rate

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and prolonged survival with erlotinib correlated with *EGFR* mutations, but EGFR immunohistochemical status provided no predictive information.

Tissue Acquisition

According to the NCCN, the initial evaluation of a patient with suspected NSCLC requires a complete pathologic review to classify the lung cancer, determine the extent of the invasion, establish the involvement of surgical margins, and identify molecular abnormalities to predict sensitivity to targeted therapies.[50] Many patients have advanced disease at diagnosis; for them, surgery with curative intent is not an option. If metastatic or locally advanced disease is present, the patient must undergo tissue sampling to confirm the diagnosis and provide tissue for molecular characterization.

There are several ways to obtain tissue for diagnostic and biomarker analyses in patients suspected of having NSCLC. According to the American College of Chest Physicians, fine-needle aspiration (FNA) or core-needle biopsy of a metastatic site will help confirm the diagnosis and stage, but in some cases, the metastatic site may be technically difficult to biopsy.[53] If biopsy of the suspected metastatic site is not feasible, sputum cytology, bronchoscopy, or transthoracic needle aspiration of the primary lung lesion can be used to confirm the diagnosis.[53] According to the NCCN, core needle, endobronchial, or transbronchial biopsy may be performed as well.[50]

There is considerable variability in the success rates for acquiring the correct amount of tissue on which to perform these tests. For example, in the BR.21 trial, 328 (69.5%) of 472 patients who consented to EGFR testing on biopsied tissue had usable tissue for the determination of *EGFR* mutational status or GCN.[17] The success rates for these tests were 89.8% (177/197 samples) and 56.6% (125/221 samples), respectively. The BATTLE program at the M.D. Anderson Cancer Center, Houston, TX, assessed biomarker-guided treatments in patients with previously treated, advanced NSCLC and biopsy-amenable disease.[54] Fresh core-needle biopsies were required of all enrolled patients, and 11 biomarkers were analyzed. Of 255 randomized patients, 39 had insufficient tissue and 2 had tumors negative for all 11 biomarkers.[54] *EGFR* mutations, *EGFR* GCN, and *KRAS* and *BRAF* mutations, among others, were examined as biomarkers in this study, and significant correlations were observed with specific biomarker results in the treatment groups.[54] Based on these results, it seems that with improved technique and routine use, adequate tissue for biomarker analyses can be acquired in most cases and may be used to guide treatment choice.

The size of the needle used to obtain biopsy specimens may also affect the tissue yield and potential complications. The use of smaller needles (19-gauge or smaller) has become more frequent.[55] A retrospective review of 846 consecutive procedures demonstrated that pneumothorax occurred more frequently in patients whose computed tomography (CT)–guided transthoracic needle aspirations were performed with 18-gauge needles than in patients who underwent biopsy with 19-gauge needles (38% vs 23%; $P < .001$). However, diagnostic accuracy (malignant vs benign) was similar between the 2 methods (96% vs 92%). More recently, Cheung and colleagues[56] reported lower and similar rates of pneumothorax associated with the use of 18- and 20-gauge needles (12.5% and 13.3%, respectively). Although 18- and 20-gauge needle sizes both provided sufficient samples for *EGFR* mutational analyses, the 18-gauge needle provided specimens that were larger (average, 10.15 vs 9.00 mg) and provided more DNA (average, 47.13 vs 35.92 ng/μL).

An additional benefit of the use of CT-guided needle biopsies is that wash fluid from the needles can provide adequate sample material for highly sensitive DNA analyses. In 1 study, DNA was extracted from the wash fluid of 53 CT-guided needle biopsies of lung tumors.[57] The DNA yield spanned 2 orders of magnitude (range, 35-2,360 ng). DNA analysis of the wash fluid yielded results consistent with those of DNA analyses from tumor specimens. Of the 34 tumors from patients with histologically confirmed NSCLC, *EGFR* exon 19 deletions and L858R activating mutations were observed in 12% and 38% of samples, respectively. In the non-NSCLC samples, no *EGFR* activating mutations were found.

Histologic Correlates of EGFR and KRAS Status

Unusually high sensitivity to EGFR TKIs (eg, gefitinib and erlotinib) was originally detected in patients with adenocarcinomas, as opposed to other subtypes of NSCLC.[12,13,58] Furthermore, the presence of any BAC features in the specimens conferred this apparent sensitivity; the majority of lesions also displayed activating *EGFR* mutations.[13,58] Sequencing analysis revealed that *EGFR* mutations are present in the nonmucinous histologic subtype, either in adenocarcinomas with BAC features or in pure BAC.[59] The latter finding, in combination with the *EGFR* mutation–based oncogene addiction hypothesis,[60] suggests a molecular basis for consideration of "minimally invasive" BAC as a distinct histopathologic entity. While some studies showed no association between *EGFR* mutations and BAC histologic features in Asian populations,[61,62] another line of evidence emerged that showed an association between *EGFR* mutations and a papillary subtype of adenocarcinoma.[63,64] It is not clear whether the explanation for these observations lies in different population genetics, different classification criteria used by pathologists, or in sampling; the latter 2

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explanations have been proposed by some investigators.[63,65] It is also notable that *EGFR* mutations are virtually absent in nonadenocarcinoma NSCLC, such as large cell and squamous cell carcinomas,[66] and are found only if some adenocarcinoma component is present.[67] Reflecting these data, a proposal was made to modify the 2004 World Health Organization lung adenocarcinoma classification to include the histologic pattern of mixed-subtype adenocarcinomas.[63]

In contrast with *EGFR* mutations, which are associated with nonsmoking status and sensitivity to small-molecule EGFR inhibitors, most *KRAS* mutations are smoking-related and associated with resistance to EGFR TKI therapy.[68] It is interesting that the G to T transversion (smoking-related, present in codon 12) can be found not only in invasive adenocarcinomas but also in hypothetically premalignant adenomatous hyperplasias and BACs.[68,69] While histologically BACs with *EGFR* mutations do not appear distinguishable from those with *KRAS* mutations, the mutations are mutually exclusive, as are the biologic behavior of the lesions and their sensitivity to erlotinib.[40,70] While the significance of *KRAS*[71] as an independent prognostic and predictive marker has been contradictory, 2 large meta-analyses support the association of *KRAS* mutation and lack of effect of EGFR TKIs.[72,73] It is also clear that *KRAS* mutations are only rarely found in squamous cell carcinoma of the lung (<5%), and those lesions do not appear histologically distinct.[38,74,75]

Methods Used to Determine *EGFR* and *KRAS* Mutation Status and Gene Copy Number

The most commonly used technique to detect mutations is direct sequencing of polymerase chain reaction (PCR)-amplified exon sequences. Using this technique, the target DNA sequence is first amplified without selection of mutated vs WT sequence, usually using primers located a few hundred base pairs outside of the putative mutation location. In the second step, the resulting amplified DNA fragment is sequenced directly. Such *EGFR* mutational analysis tests are available from many commercial laboratories (eg, Quest Diagnostics, Madison, NJ; Genzyme Genetics, Westborough, MA).

In all such tests, the issue of specimen purity (eg, the proportion of lesional material to the "contaminating" benign or nonlesional cells) is critical.[76,77] Typical dye-terminator sequencing[78] used in the majority of laboratories requires a minimum of 25% of lesional tissue in the sample because neither the PCR amplification step nor the DNA extension reaction favors the mutant template over WT. This limitation can be circumvented by implementing "single-strand" sequencing analyses available from Solexa (now Illumina, San Diego, CA) or the SOLiD sequencing platform (Applied Biosystems, Carlsbad, CA), in which a single DNA template is clonally expanded and each individual sequence is read by a high-resolution camera.[79,80] However, the SOLiD technique is currently more expensive than other alternatives. Alternatively, the abnormal sequence can be preferentially extended by using mutated *Taq* enzyme, which has varying affinities for nucleotide terminators; systems that use this technique include the Mutector *KRAS* mutation detection kits (TrimGen Genetic Diagnostics, Sparks, MD).[81]

In addition, technology using reverse transcriptase–PCR (using messenger RNA as the template) has been developed by Response Genetics, Los Angeles, CA, with applicability in a number of malignancies; for example, ResponseDX: Lung may be used to detect expression (eg, ERCC1 and EGFR) and/or mutation of genes (eg, *EGFR* and *KRAS*) in NSCLC.[82,83] Yet another approach is to use methods that "ignore" the WT sequence and preferentially amplify and detect using mutant allele–specific PCR and Scorpion primers (eg, TheraScreen K-RAS and EGFR29 mutation kits from QIAGEN Manchester [formerly DxS], Manchester, England).[84,85] The manufacturer of the TheraScreen kits claims that the EGFR29 mutation kit can detect 1% of mutant *EGFR* DNA in a background of WT genomic DNA.[86] Sample types that can be studied with this kit include human genomic DNA from fresh, frozen, and paraffin-embedded tissue.[86] Commercial laboratories (eg, Genzyme Genetics and Quest Diagnostics) accept cytologic specimens, such as aspirates and fluids. While such applications have rarely been validated in published studies,[87] they are increasingly used given that FNA samples are often the only diagnostic samples available. As an example of a current application of this technology, the TheraScreen EGFR29 mutation kit is currently being used to identify *EGFR* mutations in the LUX-Lung 3 trial (NCT00949650) of afatinib (BIBW 2992) (Boehringer Ingelheim, Ingelheim, Germany).

Mutations can also be identified in DNA obtained from serum and circulating tumor cells. Kimura and colleagues[88] examined 42 pairs of tumor samples and serum DNA for *EGFR* mutations; the identified *EGFR* mutational status in tumor and serum samples was consistent in 93% of cases. Mutational status in cells derived from both sources strongly correlated with response to EGFR TKIs ($P < .001$). Circulating tumor cells represent another source of DNA for analysis of *EGFR* mutations. Maheswaran and colleagues[89] identified the expected *EGFR* activating mutations in 11 (92%) of 12 samples obtained. These results suggest that it is feasible to use DNA isolated from serum or circulating tumor cells to detect *EGFR* mutations. Because the procedure to obtain these specimens is only minimally invasive, repeated testing following response to therapy may be possible. However, although this analysis may provide valuable predictive data, circulating tumor cells may be derived from multiple disease sites with different responses to therapy and may not reflect

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the status of the primary tumor. Additional studies will be necessary to validate this technique as a diagnostic or predictive tool; the determination of mutational status still requires tissue obtained using core-needle biopsy.

Other Methods for Assessing EGFR

Fluorescent labeling of nucleic acid probes via FISH allows for the simultaneous detection of multiple chromosomal regions, and use of thin sections of paraffin-embedded blocks maintains tissue architecture and permits correlation of FISH results with histologic findings.[90] A considerable body of data from clinical studies suggests that FISH is a viable method of measuring *EGFR* GCN as a predictive marker for EGFR TKI therapy.[17,18,25,36,37,91] However, controversies remain, and the use of FISH as the preferred technology for *EGFR* amplification may be falling out of favor. The MARVEL study (NCT00738881), which was designed to determine whether patients whose tumors are *EGFR* FISH+ experience greater benefit from EGFR TKI therapy, was recently discontinued owing to slow accrual and a growing consensus that mutational analysis may be more important for guiding therapy.

Chromogenic in situ hybridization (CISH) is a recent modification of FISH that addresses some of the limitations inherent in FISH, including the need for an expensive fluorescent microscope with multi-bandpass filters and the fact that the signal seems to fade within a few weeks.[92,93] CISH permits the use of a conventional bright-field microscope, and results can be observed in the context of tissue morphology when slides are counterstained with hematoxylin.[92,93] Several investigators have confirmed that *EGFR* CISH results show high concordance with FISH.[92-94] In addition, CISH may represent an effective and readily applicable technique for identifying patients with NSCLC likely to respond to EGFR TKI therapy.[95,96] Nevertheless, large-scale validation of CISH in the context of clinical trials is required.

Emerging New Targets for Personalized Therapy

Defining therapy based on histologic subtype and EGFR status is the beginning of a new era in personalized medicine for patients with NSCLC. As such, new targets are being examined in the hopes that treatments can be individualized further. Human echinoderm microtubule-associated protein like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*) is a fusion gene created by a small inversion within chromosome 2p. The N-terminal portion is identical to that of human *EML4*, and the C terminus is the same as the intracellular domain of human ALK.[97] Two variants of the *EML4-ALK* fusion gene have been characterized, both involving exons 20 to 29 of *ALK* fused to exons 1 to 13 (variant 1) or exons 1 to 20 (variant 2) of *EML4*. Two other variants include fusion points starting at *EML4* exon 6 and exon 18.[98] In mouse models, this fusion gene gives rise to tumors.[97]

Approximately 3% to 7% of patients with NSCLC have the *EML4-ALK* fusion gene,[97,99] which seems to be unique to NSCLC and not present in other solid tumors. It also is seen more commonly in younger, never-, or light-smoking men whose tumors have adenocarcinoma histology, and it is mutually exclusive with *EGFR* and *KRAS* status.[98-102] The characteristics of patients with the *EML4-ALK* fusion gene are similar to those of patients with the *EGFR* mutation. Based on these preliminary studies, *EML4-ALK* may represent a new molecular target in NSCLC. Like *EGFR*, it seems to be specific to certain subpopulations of patients.

Preliminary results suggest that variants of the EML4-ALK fusion protein are sensitive to ALK inhibitors. Koivunen and colleagues[101] found that TAE684 (Novartis, Cambridge, MA), a specific ALK inhibitor, inhibits the growth of 1 of 3 (H3122 [*EML4-ALK* variant 1]) *EML4-ALK*–containing cell lines in vitro and in vivo. Recent preliminary results presented at the 13th World Conference on Lung Cancer indicate that another ALK inhibitor, PF-02341066 (Pfizer, New London, CT), which inhibits mesenchymal-epithelial transition factor and ALK, is associated with an overall response rate of 59% (17/29 patients) and a disease control rate of 83% at 8 weeks (24/29 patients).[103] Based on these results, a phase 3 trial has been designed for a select group of patients who have the EML4-ALK fusion protein.

Detection of the *EML4-ALK* fusion gene has not been standardized. Several studies have used FISH with a break-apart probe for *ALK*; others have used reverse-transcriptase–PCR. Finally, immunohistochemical analysis has been used as a confirmatory test using a monoclonal antibody against ALK.[99,102,104] The optimal methods for detecting the fusion gene and its product, and concordance between techniques, remain active areas of investigation.

Conclusions

Historically, pathologists have become accustomed to histologic analysis of surgical specimens for the sole purpose of staging the disease. However, the paradigm is shifting because cytologic and needle-biopsy specimens are collected more often and protein and gene analyses assume an ever-increasing role. Routine testing for *EGFR* mutations and/or gene amplification and *KRAS* mutations should become the standard of care for the initial workup of patients newly diagnosed with NSCLC.[105] As the oncology clinician's armamentarium swells with more targeted agents, identification of the people most likely to respond to them will gain in prominence. The role of *EGFR* testing to determine

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appropriate treatment will likely increase during the coming years as the nuances regarding response and resistance to current and emerging EGFR-targeted therapies continue to be revealed. To optimize the "personalized medicine" approach to the treatment of patients with NSCLC, *EGFR* mutation testing is critical.

Currently, the NCCN NSCLC guidelines recommend treatment with erlotinib as first-line therapy only in patients whose tumors have *EGFR* mutations, and this criterion may eventually emerge as a requirement for the use of EGFR TKIs in this setting.[50] Pathologists will gain additional responsibility for guiding treatment decisions by determining *EGFR* mutation status and the histologic subtype in patients with NSCLC.

Optimally, multiple 18-gauge core biopsy samples are preferred if they can be obtained safely because they seem to yield the most amount of tissue amenable to molecular analyses. The methods used to detect gene mutations and copy number and the sources from which tissue is obtained are continually being improved. Reduced invasiveness and enhanced sensitivity and specificity will help increase patient compliance with these tests and improve their efficiency, such that routine use may be feasible in the foreseeable future. This type of evaluation necessitates the increased involvement of pathologists in clinical decision making, because the morphologic, immunologic, and gene-based assessments they perform provide invaluable information to help medical oncologists guide treatment.

New targets are being tested, such as the EML4-ALK fusion protein, which further creates a need for pathologists to become familiar with the molecular fingerprints of tumors together with their histologic subtypes and other distinguishing features. The review and interpretation of pulmonary surgical and cytologic specimens provided by pathologists will define the tumor subtype using histologic and molecular characteristics. The clinical outcome data and the evolution of diagnostics suggest that the combination of differentiation and genetic markers to subtype NSCLC and guide treatment decisions is likely to be the next step in personalized medicine.

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