Biomarkers in Lung Adenocarcinoma

A Decade of Progress

Lynette M. Sholl, MD

Context.—The analysis of molecular biomarkers in lung adenocarcinoma (ACA) is now a central component of pathologic diagnosis and oncologic care. The identification of an EGFR mutation or ALK rearrangement in advanced-stage lung ACA will dictate a change in first-line treatment from standard chemotherapy to targeted inhibition of these oncogenic alterations. Viable approaches to therapeutic targeting of KRAS-mutated ACA are now under investigation, raising the possibility that this too will become an important predictive marker in this tumor type. The recognized array of less common oncogenic alterations in lung ACA, including in the ROS1, RET, BRAF, and ERBB2 genes, is growing rapidly. The therapeutic implications of these findings are, in many cases, still under investigation.

Objective.—To focus on the major molecular biomarkers in lung ACA, recommended testing strategies, the implications for targeted therapies, and the mechanisms that drive development of resistance.

Data Sources.—Our current understanding of predictive and prognostic markers in lung ACA is derived from a decade of technical advances, clinical trials, and epidemiologic studies. Many of the newest discoveries have emerged from application of high-throughput next-generation sequencing and gene expression analyses in clinically and pathologically defined cohorts of human lung tumors.

Conclusions.—Best practices require a solid understanding of relevant biomarkers for diagnosis and treatment of patients with lung ACA.

Lung carcinoma is one of the most common and lethal diseases afflicting the global population. Despite decades of efforts to improve outcomes through multimodality therapy, including surgery, radiotherapy, and chemotherapy, survival rates have remained dismal. However, in the last decade, recognition of specific molecular alterations in certain lung cancer subtypes has facilitated tailored therapy targeting these alterations and has ushered in the era of “personalized” oncologic practice.

Within the family of lung carcinomas, the molecular underpinnings of lung adenocarcinoma (ACA) are best understood at this time; approximately 60% of lung ACAs have an oncogenic driver mutation that in many cases predicts treatment response and correlates with certain clinicopathologic features. The advent of high-throughput sequencing technologies has enabled the simultaneous identification of mutational and copy number alterations that may modify pathway activity downstream or in parallel with these driver alterations. This more sophisticated understanding of altered cell signaling pathways promises to enhance our ability to tailor therapy and anticipate treatment failure. The molecular subclassification of more uncommon and/or genetically complex tumors has lagged behind that of lung ACA; however, large sequencing efforts are beginning to identify putative therapeutic targets in carcinoid tumors, small cell carcinomas, and squamous cell carcinomas.

This review will focus on the evolution of biomarker identification in lung ACA, beginning with the seminal studies identifying EGFR-activating mutations published in 2004, examining the discoveries made during the last decade that have translated into major shifts in the standard of cancer care, and highlighting the emerging biomarkers likely to influence pathology and oncology practice in the near future.

BIOMARKERS IN LUNG ADENOCARCINOMA

Today, lung ACA is the most common form of lung cancer in the United States. In the 1950s, squamous cell carcinoma was 18-fold more common than ACA; the shift in tumor epidemiology is not entirely understood but is thought to reflect changing practices and patterns of cigarette smoking. Smoking remains the leading cause of lung ACA and tends to give rise to genomically complex tumors as a result of the mutagenic effects of tobacco-associated carcinogens. Adenocarcinoma arising in nonsmokers or light smokers represents a significant and unique subset of lung cancers and tends to be genomically less complex, often with only a
handful of identifiable DNA sequence mutations or chromosomal translocations. Alterations in 3 particular genes appear to define, or at least inform, the biology of at least half of lung ACAs; KRAS, EGFR, and ALK. EGFR and ALK are now widely recognized as therapeutic targets, and thus routine testing for alterations in these genes is standard of care for advanced lung ACA. Alterations in many other oncogenes have also been identified, most of which occur in less than 5% of lung ACAs (Figure 1).

**EGFR Mutations**

The epidermal growth factor receptor gene (EGFR) is a member of the ErbB transmembrane growth factor receptor family. It shares homology with other family members including ERBB2 (HER2/neu), ERBB3, and ERBB4. Extracellular ligand binding triggers homodimerization or heterodimerization of ErbB family receptors, phosphorylating active sites in the cytoplasmic tyrosine kinase, and activating intracellular PI3K/AKT/mTOR and RAS/RAF/MAPK pathways. EGFR signaling is critical in development and cellular homeostasis, proliferation, and growth.7 Recognition that EGFR protein is overexpressed in most lung cancers dates back to the 1990s,8 thus EGFR has long been implicated as an oncogene and putative therapeutic target in lung ACA. Attempts to inhibit EGFR signaling in lung ACA with monoclonal antibody–based approaches, such as those used in colon ACA, have generally yielded disappointing results.9 Small-molecule inhibitors designed to block the adenosine triphosphate (ATP)–binding groove in the EGFR tyrosine kinase domain appeared to effectively kill lung ACA cells in vitro and showed promise in early clinical studies.10,11 However, in large phase 3 trials examining unselected patients with advanced-stage non–small cell lung carcinoma, the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib showed little to no benefit as compared to conventional chemotherapy.12 A subset of patients, however, enriched for female sex, ACA histology, of smoking history, female sex, ACA histology, and Asian ethnicity.6 However, EGFR mutations have been identified in smokers and in tumors with other histologic profiles, including adenocarcinomas, “large cell carcinomas,” and, rarely, in squamous cell carcinomas. The presence of EGFR mutations in these latter 2 entities likely reflects misclassification of solid subtype ACA, in the case of large cell carcinoma, and possibly undersampling of an adenocarcinoma, in the case of squamous cell carcinomas.22–24 Most large studies22,25 suggest that EGFR mutations virtually never occur in tumors lacking at least some ACA features. The predictive value of lung cancer subtyping for the presence of an underlying EGFR mutation has driven into relative obsolescence the term non–small cell lung carcinoma and, in fact, current recommendations generally discourage

![Figure 1. The distribution of oncogenic driver alterations in lung adenocarcinoma.](image-url)
use of this term unless a tumor eludes classification despite histologic and immunophenotypic evaluation.26

EGFR Copy Number and Protein Expression

In the mid 2000s, some clinical trial results suggested an association between EGFR copy number gain as detected by fluorescence in situ hybridization (FISH) and response to EGFR-TKIs.27 However, in subsequent analysis of phase 3 trials, EGFR copy number gain failed to predict response as effectively as mutation status, with response rates of approximately 30% in the EGFR copy number gain versus 70% in the mutated cohorts.6 Tumors with activating mutations may also show EGFR copy number gain,28 with the mutation typically located on the chromosomal allele showing gain. EGFR gain is in fact a frequent event in lung ACA, often in the context of balanced polysomy of chromosome 7, and thus is a relatively nonspecific phenomenon.29 Focused, high-level amplification of the EGFR gene is less common in EGFR-mutated tumors; when present in pretreatment tumors, it predicts enhanced response to EGFR-TKIs.30 It is also seen in tumors at the time of relapse in association with the p.Thr790Met (T790M) resistance mutation31 (see below) (Figure 2, A). The presence of high-level amplification may be heterogeneous within the tumor (Figure 2, B), correlates with more advanced-stage and higher-grade disease (suggesting a role in promoting tumorigenesis), and is associated with increased EGFR protein expression.32

High expression of total EGFR is not specific to tumors with EGFR-activating mutations and thus is an inadequate predictor of response to EGFR-TKIs.33 Mutation-specific antibodies against EGFR L858R–mutated protein are highly sensitive and specific (Figure 3); however, this alteration accounts for less than half of those seen in targetable lung ACA.34 Mutation-specific antibodies against the EGFR exon 19 deletion are also highly specific but lack adequate sensitivity to the full range of small insertion/deletion mutations that can affect the LREA motif of the kinase domain.35–36 As a result, these tools have limited utility in clinical practice, but may be useful in confirming or refuting unexpected or equivocal molecular results, such as in patients with multiple oncogenic driver alterations. Clinical practice guidelines for EGFR testing in lung ACA recommend use of DNA-level mutation analysis, but not copy number or protein expression, for selection of patients for targeted therapies.6

Mechanisms of Resistance to EGFR Inhibitors

Patients with EGFR-mutated lung ACA typically have a relapse within 9 to 14 months after initiation of EGFR-TKI therapy.37 At relapse, the oncologist may choose to transition a patient to standard chemotherapy or may pursue tumor rebiopsy in an effort to identify a resistance mechanism and potentially select a second-line targeted therapy.38 The most common resistance mechanism in EGFR-mutated lung ACA is acquisition of the T790M substitution in EGFR exon 20, in addition to the original activating mutation. This can be found in approximately 60% of tumors that are rebiopsied at relapse.39,40 The T790M mutation leads to an increased affinity of the receptor for ATP, thereby reducing the potency of ATP-competitive kinase inhibitors such as gefitinib and erlotinib.21 However, T790M activity is abrogated by irreversible inhibitors of EGFR, the efficacy of this family of covalently bound inhibitors in the blockade of EGFR kinase domain signaling, Figure 2. Heterogeneous epidermal growth factor receptor (EGFR) copy number gain in a lung adenocarcinoma. A, Targeted hybrid-capture next-generation sequencing permits evaluation of copy number alterations on a gene level. This image shows the coverage over select genes on the short arm of chromosome 7; a normal diploid sample will align at a log2 ratio of sample per median of zero; copy loss is reflected by a negative log2 ratio, and gain, by a positive log2 ratio. In this case, most genes on 7p show some degree of copy number gain, with a suggestion of additional gain of EGFR, without frank evidence of amplification. B, Paired EGFR silver in situ hybridization showing heterogeneous, high-level amplification of EGFR silver in situ hybridization showing heterogeneous, high-level amplification of EGFR in scattered larger cells. This finding demonstrates the effect of copy number dilution by tumor heterogeneity and normal contaminating tissue on the next-generation sequencing output (original magnification ×600 [B]).

Figure 3. EGFR mutation-specific immunohistochemistry (clone 43B2) for the Leu858Arg mutation. Diffuse cytoplasmic positivity is typical (original magnification ×400).
even in the presence of this resistance mutation, has been recognized for many years. However, the toxicity profile of such inhibitors, which is driven by their effects on wild-type EGFR receptor activity, has slowed their adoption into clinical practice. Third-generation irreversible inhibitors (including dacomitinib, afatinib, and AZD9291) have shown efficacy against T790M-mutated tumors in cell culture and mouse xenograft models. In phase 1 trials, these drugs appear to be well tolerated and effective against tumors containing the resistance mutation. Phase 2 trials of these drugs are now underway; these promising clinical developments are likely to lead to increased demand for tumor testing at relapse in order to determine the mechanism of first-line EGFR-TKI resistance.

**MET**, the gene encoding the receptor for hepatocyte growth factor that is critical to cell growth and proliferation across many organs, is amplified in 5% to 20% of relapsed lung ACAs after EGFR-TKI therapy. This may occur in concert with or independently of other resistance mechanisms, including T790M. Early in vitro studies suggested that MET amplification may be present within a very small subclone of the primary lung ACA, with its outgrowth reflecting drug selection. Indeed, MET amplification is significantly more common in posttreatment as compared to pretreatment lung tumors (21% versus 3% in one report). It appears to overcome the growth inhibitory effects of EGFR signaling blockade by activating PIK3 pathways. Combined use of EGFR- and MET-targeted inhibitors appears effective in cell culture studies. Phase 2 studies of combined erlotinib and Met inhibition using a monovalent anti-Met receptor antibody (MetMAb) have shown some benefit in patients with Met protein overexpression by immunohistochemistry; phase 3 trials of combined Met and EGFR inhibition are now underway. MET gene copy number and protein expression are significantly correlated; however, in exceptional cases, strong Met protein expression may be seen in the absence of MET copy number gain. To date, the best biomarker for assessing Met activation status has not been established; indeed, the definition of positivity based on expression level or copy number is still a matter of debate and will likely not be resolved until larger studies of outcomes following treatment with Met-targeted agents come to fruition.

Other, less common forms of EGFR-TKI resistance have been described in the form of ERBB2 (HER2/neu) amplification, PIK3CA mutation, and evolution to small cell carcinoma. ERBB2 amplification has been described in 10% to 15% of relapsed lung ACAs, providing rationale for second-line use of HER2/neu or pan-HER inhibitors. Acquired PIK3CA mutations have been described in 5% of relapsed lung ACAs, but are also seen in concert with EGFR (and other driver gene) mutations in untreated samples. The mechanisms underpinning a morphologic transformation to small cell carcinoma are unknown.

**KRAS Mutations**

KRAS (Kirsten rat sarcoma 2 viral oncogene homolog) is the most commonly mutated driver oncogene in lung ACA. It is a member of the RAS family of membrane-associated G proteins and acts downstream of a number of receptor tyrosine kinases including EGFR. RAS protein signaling activates RAF then MEK family members, ultimately driving cell proliferation and growth pathways. KRAS is one of the best-studied oncogenes, in part because it was one of the first discovered and is frequently mutated in a similar fashion across multiple common tumor types. In lung ACA, more than 90% of the mutations in KRAS occur at codons 12 and 13, in the form of single-nucleotide missense variants.

KRAS mutation is more strongly associated with smoking history than most other known driver oncogenic alterations described in lung ACA. Large genotyping series have identified KRAS mutations in 34% of smokers and 6% of never smokers with lung ACA. Among ACA with solid-subtype histology, the frequency of KRAS mutation is 40%. The most common KRAS alteration in current and former smokers is a codon 12 G→T transversion mutation leading to p.Gly12Cys; transversions reflect the typical DNA-level alterations induced by tobacco carcinogens.

To date, there is no established approach to targeted inhibition of KRAS-mediated signaling in carcinoma. KRAS mutations essentially occur in a mutually exclusive fashion with other oncogenic drivers such as EGFR mutation and ALK rearrangement. Thus, the greatest utility of testing for KRAS mutations at this time may be to exclude the presence of other, less common targetable alterations. Current recommendations do not mandate KRAS testing; indeed, they recommend against it as a determinant of eligibility for EGFR-TKI therapy. However, they do recognize the value of a testing algorithm that incorporates KRAS testing as a cost-effective approach to excluding cases from further, potentially more expensive and technically demanding assays to detect EGFR and ALK alterations.

Clinical trials are currently underway testing a variety of inhibitors in KRAS-mutated lung ACA, including with compounds targeting the RAS/RAF/MEK and PI3K/AKT/mTOR pathways and cyclin-dependent kinases. Phase 2 trial results using an inhibitor of MEK1/MEK2 (selumetinib) in patients with advanced, KRAS-mutated lung ACA demonstrated improved progression-free and overall survival in patients receiving a combination of selumetinib and docetaxel versus docetaxel alone, albeit with a more significant side effect profile in the combined-therapy arm. In what likely represents a paradigm shift for the study of clinical therapeutics, a “coclinal trial” carried out in genetically engineered mouse models highlighted the effects of genetic modifiers of response to MEK inhibition. The study design mimicked that of the human clinical trial, but had the advantage of prospectively randomizing mice whose tumors had concomitant tumor suppressor gene alterations. The study authors thereby determined that tumors containing KRAS-activating mutations and TP53 and LKB1/SKT11 loss-of-function mutations were less likely to respond to docetaxel monotherapy. Interestingly, the mice with combined KRAS and TP53 mutations benefited from selumetinib and docetaxel, but those with KRAS + LKB1 mutations did not. In the absence of a next-generation sequencing approach, comprehensive TP53 mutation and deletion analysis is challenging owing to the breadth of possible alterations that can lead to loss of function. Immunohistochemical reagents are currently available; however, to assess LKB1 expression status (Figure 4, A), and loss of expression appears to correlate well with gene-level loss-of-function alterations including mutations and deletions (Figure 4, B) (P.A. Jänne and L.M.S., unpublished data, April 2014).

**ALK Rearrangement in Lung Adenocarcinoma**

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase whose overactivation secondary to oncogenic fusion with a...
variety of partners was originally described in anaplastic large cell lymphoma, after which the gene is named, nearly 20 years ago.\textsuperscript{70} ALK rearrangements were subsequently described in inflammatory myofibroblastic tumor,\textsuperscript{71} and, finally, in 2007, in a subset of lung carcinomas harboring a fusion of ALK to EML4 via a small intrachromosomal inversion event on chromosome 2.\textsuperscript{72} In vitro studies\textsuperscript{73} showed that ALK-rearranged lung ACA cells were sensitive to treatment with the multitargeted TKI originally designed to target Met activity, namely, crizotinib. Within 3 years of the discovery of ALK rearrangements in lung ACA, a high-profile clinical trial using FISH to select candidates for therapy with crizotinib was published. This trial identified ALK rearrangements in approximately 5\% of lung cancers and demonstrated a 57\% response rate to crizotinib in ALK-positive patients, with many experiencing dramatic shrinkage of their tumor burden.\textsuperscript{74} Crizotinib was approved for treatment of advanced-stage ALK-positive non–small cell lung cancer by the US Food and Drug Administration (FDA) less than 1 year later.\textsuperscript{75} These original reports, and many subsequent retrospective analyses, described ALK rearrangements almost exclusively in ACAs. Demographically, patients tend to be younger, nonsmokers who often present at more advanced stages of disease than those with ALK-negative tumors.\textsuperscript{76–78} Randomized phase 3 trials demonstrated the superiority of crizotinib as compared to conventional chemotherapy in ALK-positive lung cancer, with response rates of 65\% versus 20\%, respectively.\textsuperscript{79} As a result, current clinical practice guidelines recommend upfront testing for ALK rearrangements in patients with advanced lung ACA.\textsuperscript{6}

**Testing for ALK Rearrangement in Lung Adenocarcinoma**

Fluorescence in situ hybridization is considered the gold standard for detection of ALK rearrangements in lung ACA, as a result of its use in the selection of patients for crizotinib therapy in the original clinical trials and because the FDA has approved the ALK Break Apart FISH Probe Kit (Abbott

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**Figure 4.** LKB1 immunohistochemistry in Kirsten rat viral sarcoma oncogene homolog (KRAS)–mutated lung adenocarcinoma. A, An acinar-pattern tumor with diffuse cytoplasmic staining by LKB1, considered to have “intact” expression. B, A solid-pattern tumor with absent staining by LKB1 in tumor cells. Infiltrating inflammatory cells are characteristically strongly positive (original magnification \( \times 400 \) [A and B]).

**Figure 5.** An anaplastic lymphoma kinase (ALK)–rearranged lung adenocarcinoma with positive ALK protein expression. The intensity of staining varies relative to detection system and antibody. A, Clone 5A4 at 1:50 dilution without an amplification step and using an Envision Plus Detection Kit (Dako, Carpinteria, California). B, Staining of the same case performed on the Ventana BenchMark XT automated system, using clone D5F3 with the OptiView Amplification Kit (Ventana Medical Systems, Inc, Tucson, Arizona) (original magnification \( \times 400 \) [A and B]).
Molecular, Des Plaines, Illinois) as a companion diagnostic for use of this drug in lung cancers.\textsuperscript{80} This break-apart FISH design places a fluorescent probe on either side of the common breakpoint area in ALK (intron/exon 20). Red and green fluorescent probe signals are most commonly used; when visualized on a normal ALK gene they appear fused, giving rise to a yellow signal. A rearrangement involving ALK will cause the signals to split apart, so that distinct red and green signals appear.\textsuperscript{74} The most common rearrangement event, a small paracentric intrachromosomal inversion fusing EML4 and ALK, may be difficult to perceive because it may lead to a subtle split in the probes, leading to false-negative results if overlooked, and to false-positive results as a result of assay noise due to aberrant probe hybridization or high background signal.\textsuperscript{83} As a result, a relatively high split signal cutoff of 15\% is required to achieve a positive result. Less commonly, the rearrangement pattern will show a single red (3') probe signal, reflecting loss of the 5' portion of the gene. The type of rearrangement pattern does not appear to predict differential response to crizotinib therapy, although occasional cases harboring single green (5') probe signals should not be considered “ALK positive,” as this event reflects loss of the ALK kinase domain and is therefore unlikely to be activating.\textsuperscript{82}

As well documented in studies of anaplastic large cell lymphoma, ALK fusion events lead to increased ALK transcription and protein expression by immunohistochemistry (IHC).\textsuperscript{76,85} However, the antibodies traditionally used to detect ALK overexpression in anaplastic large cell lymphoma are insufficiently sensitive for use in ALK-rearranged lung cancers.\textsuperscript{86} More sensitive IHC antibodies were ultimately developed; commercially available clones 5A4 and D5F3 have sensitivities and specificities ranging from 83\% to 100\% for the detection of ALK-rearranged lung tumors, when compared to FISH (Figure 5, A).\textsuperscript{84–87} The variable sensitivity, in particular, appears to be driven in part by variable expression of the protein in ALK-rearranged tumors and in part by technical variation including use of signal amplification and specialized detection systems. A fully automated IHC assay that has been shown to have 100\% sensitivity and 95\% specificity for detection of ALK rearrangements, when compared to FISH and reverse transcription–polymerase chain reaction (RT-PCR) (Figure 5, B),\textsuperscript{88} has been approved as a companion diagnostic in China for use in selecting patients for therapy with crizotinib.\textsuperscript{89}

Several series suggest that IHC and FISH may serve as complementary tools; IHC may be carried out successfully in cases with suboptimal DNA preservation that may fail FISH testing, or may be used to adjudicate equivocal FISH results. In some cases, IHC may ferret out those cases with positive FISH results that actually harbor a nonfunctional ALK rearrangement and thus are unlikely to respond to targeted therapy.\textsuperscript{84} However, on-label use of crizotinib in ALK-rearranged lung ACA requires documentation of a positive FISH result; consequently, aside from occasional case reports, outcome data from a systematic IHC-based selection approach are lacking. Clinical practice guidelines recommend use of FISH for selection of patients for ALK-targeted therapy; however, the use of carefully validated IHC may be used to screen out negative cases.\textsuperscript{90} Similarly, other approaches to FISH detection, such as RT-PCR and next-generation sequencing, are in use but have not been examined systematically relative to FISH in terms of their utility for predicting response to ALK inhibitors.\textsuperscript{90,91}

Most of the published literature, with few exceptions,\textsuperscript{92} supports the original observations that ALK rearrangements and other oncogenic driver mutations occur in a mutually exclusive fashion.\textsuperscript{93} On occasions where both ALK rearrangements and independent driver mutations are detected, multimodality evaluation (i.e., using IHC, next-generation sequencing) may help to clarify false-positive results.\textsuperscript{94} However, larger series suggest that ALK and EGFR alterations may occur concomitantly in approximately 1\% of selected populations but these dually altered tumors are differentially responsive to EGFR and/or ALK inhibition; phosphorylation status of EGFR and ALK may help to guide selection of the more appropriate inhibitor.\textsuperscript{95}

**ALK Inhibitor Resistance**

Resistance to the ALK inhibitor crizotinib inevitably emerges, driving disease relapse usually within a year of starting treatment. The mechanisms of resistance are diverse and include secondary mutations in the ALK tyrosine kinase domain, fusion gene amplification, ErbB family activation, and KIT gene amplification.\textsuperscript{96} In cell culture studies, dual ALK and EGFR inhibition show activity against crizotinib-resistant tumor cells driven by EGFR pathway activation.\textsuperscript{94} Similar studies combining crizotinib with the KIT inhibitor imatinib have demonstrated their ability to overcome KIT amplification–driven resistance.\textsuperscript{95} In vitro studies\textsuperscript{88} have shown that second-generation ALK inhibitors are effective against both crizotinib-sensitive and crizotinib-resistant ALK-rearranged tumors. Phase 1 clinical trials of the 20-fold more potent ALK inhibitor ceritinib demonstrated an overall response rate of 58\% among patients with non-small cell lung carcinoma who had previously received crizotinib.\textsuperscript{97}

In the small subset of patients in this trial who underwent rebiopsy and molecular analysis at disease recurrence, the mechanism of resistance to crizotinib did not influence the efficacy of this second-generation inhibitor. Other in vitro studies\textsuperscript{88} have shown, however, that a subset of secondary ALK mutations, including G1202R and F1174C, confer resistance to both crizotinib and ceritinib.

**ROS1 Rearrangements**

Oncogenic ROS1 fusions were first identified in 1987 in glioblastoma multiforme.\textsuperscript{99,100} Many years later, ROS1 fusions were detected in a subset of lung cancer cells lines by using phosphoproteomic screens.\textsuperscript{103} Subsequent FISH and IHC–based screening of primary lung tumors have reported ROS1 rearrangements involving a variety of fusion partners including CD74, SLC34A2, SDC4, EZR, and FIG1 in approximately 1\% to 2\% of lung ACAs.\textsuperscript{77,102–104} These fusion events trigger ROS1 overexpression with associated onco- genetic transformation in in vitro and in vivo studies.\textsuperscript{77,102} Fortuitously, the same multtargeted inhibitor effective in ALK-rearranged tumors has also shown efficacy in treating ROS1-translocated lung ACAs. Interestingly, ROS1 and ALK are phylogenetically related.\textsuperscript{105} Perhaps as a result of this relatedness, the clinicopathologic features of tumors harboring either of these rearrangements are similar. As with ALK, ROS1 rearrangements are significantly more common in young, never smokers whose tumors lack other oncogenic driver mutations.\textsuperscript{104} ROS1- and ALK-rearranged lung ACAs have overlapping histologic features, including solid and papillary-pattern growth with cribriforming, prominent mucin production, and frequent psammomatous calcifications.\textsuperscript{103,106} Studies examining cohorts enriched for patients fitting these characteristics identify ROS1-rearranged lung ACAs as tumors with overlapping pathological and clinicopathologic features with ALK-positive tumors.\textsuperscript{106}
ranged tumors at much higher frequencies (up to 10%) than in all comers. As will be discussed below, enrichment of study sets for otherwise wild-type tumors has led to the identification of a number of novel alterations in lung ACA that may have important therapeutic implications.

As with ALK, FISH may be considered the gold standard for detection of ROS1 fusions. A break-apart design with probes spanning the common breakpoint region (exons 32, 34, and 35) allows for identification of a rearrangement event without requiring a priori knowledge of the fusion partner. ROS1 translocations most commonly result from interchromosomal exchanges; a positive FISH result is characterized by split signals or by loss of the 5’ telomeric probe; when the translocation takes the form of split signals, the distance between the probes is typically greater than that seen in ALK-rearranged lung tumors, lending itself to relatively straightforward interpretation. However, in light of the rarity of this event, other, less costly and technically demanding screening approaches may be preferable to FISH for many laboratories. ROS1 immunohistochemistry using the commercially available D4D6 clone appears to have a sensitivity approaching 100% but variable specificity for many laboratories. ROS1 immunohistochemistry using the commercially available D4D6 clone appears to have a sensitivity approaching 100% but variable specificity for detection of ROS1-rearranged lung ACAs, with some groups reporting 92% to 97% specificity and others reporting an 8-fold relative frequency of immunohistochemical to FISH positivity. The variability in the analytic features of this IHC marker are likely related in part to the scoring algorithm, and obviously the sensitivity and specificity can be optimized by redefining criteria for positivity. Individual laboratories may choose alternative definitions for positivity depending on how they use ROS1 IHC; if a positive score is defined to optimize sensitivity in particular, IHC may be a cost-effective screening tool to integrate into an algorithmic biomarker testing strategy, limiting the volume of cases that require FISH screening. ROS1 rearrangements have been described almost exclusively in tumors that lack other driving molecular alterations in genes such as EGFR, KRAS, and ALK; however, some groups have reported ROS1 translocations in conjunction with other driver alterations. Additional data on the clinical significance of these combined alterations are needed; if these findings are validated and clinically relevant, more comprehensive testing (i.e., nonalgorithmic/sequential) strategies such as next-generation sequencing will be needed to provide optimal biomarker identification.

**RET Rearrangements**

Oncogenic RET gene rearrangements in lung cancer are thought to occur at a frequency similar to that of ROS1 rearrangements (approximately 1% of lung ACAs) and are more common in never smokers. Recent data suggest that, as in thyroid carcinomas, RET rearrangements may occur more commonly in patients with a prior history of locoregional radiation therapy. RET rearrangement is detectable by FISH; however, the most commonly described alteration, a small intrachromosomal inversion on chromosome 10, leading to a KIF5B-RET fusion, leads to only a subtle split in the FISH probe signals and thus may be difficult to consistently detect in practice. Although RET expression appears robust in other tumor types with RET alterations, reports on the use of RET IHC in lung ACA are sparse, with one group reporting marginal specificity for RET rearrangements when using 2 different commercially available antibodies and another group describing only 71% sensitivity when using clone EPR2871. Other approaches to detection may include RT-PCR or targeted next-generation sequencing. The relative rarity of RET-rearranged lung ACA and challenges to widespread screening have led to relatively limited literature on the clinicopathologic features of this tumor type. Preliminary data from phase 2 trials of the MET and VEGFR2 inhibitor cabozaatinib in RET-rearranged lung tumors are promising.

**BRAF Mutations**

Like KRAS, BRAF is mutated across multiple tumor types of strikingly different histogenesis, including melanoma, carcinomas, neural, and hematopoietic. In fact, BRAF-activating mutations, namely, the p.Val600Gly (V600E) mutation, is a diagnostic feature of some entities, such as hairy cell leukemia. BRAF is a critical member of the RAS/MAPK growth and proliferation signaling pathway, acting downstream of RAS family members. In melanoma, in which BRAF V600E occurs in upwards of 50% of cases, it is a critical biomarker of response to BRAF-targeted inhibitors such as vemurafenib. In colon carcinoma, in which BRAF alterations are much less common, occurring in 10% to 15% of cases, BRAF V600E is a negative prognostic factor, associated with markedly worse survival than tumors with KRAS-mutated or wild-type genotypes, and is a negative predictor of response to EGFR monoclonal antibody therapy.

BRAF mutations in lung carcinoma are uncommon, found in approximately 4% of lung ACAs. In contrast to other tumor types where V600E substitutions clearly predominate, the BRAF mutation profile in lung ACA is split about 50-50 between V600E in exon 15 and other alterations in exons 11 and 15. BRAF-mutated tumors appear to lack distinctive clinicopathologic features, but may occur slightly more frequently in smokers. Several of the BRAF alterations detected in lung ACA, including D594N and G496del, lack activating activity and in fact dampen kinase activity and fail to transform cells in vitro. Also, in contrast to other tumor types where BRAF and KRAS mutations appear to occur in a mutually exclusive fashion, BRAF exon 11 mutations may be found together with KRAS and EGFR mutations. Comprehensive data on the kinase activity of all known exon 11 mutations are lacking, thus these co-occurring mutations may simply be “passengers” that may have little bearing on the growth/proliferative effects resulting from better-understood upstream pathway alterations.

In light of the relative rarity of lung ACA containing BRAF-activating mutations, data on the predictive and prognostic significance of these alterations remain limited. BRAF mutations appear not to influence response to standard platinum-based chemotherapy, although V600E mutations may be associated with more aggressive disease. Efforts to combat these tumors with BRAF-specific inhibitors have led to generally disappointing results. Although patients with BRAF V600E mutations will initially respond to targeted inhibitors, their tumors tend to recur quickly. Resistance mechanisms include constitutive autocrine signaling through EGFR or loss of the full-length BRAF V600E protein.

**ERBB2 (HER2/neu) Mutations**

ERBB2, also known as HER2/neu, is, like EGFR, a member of the ErbB family of growth factor receptors, and a well-recognized oncogene in a variety of tumor types. In breast...
and gastrointestinal carcinomas, ERBB2 amplification is relatively common, leads to activation of downstream MEK and AKT pathways, and predicts response to targeted therapies such as trastuzumab and lapatinib. Therapeutic targeting of this event in breast carcinoma, in particular, has had a significant positive impact on clinical outcomes.

The best-defined mechanism of ERBB2 activation in lung ACA is in the form of small exon 20 in-frame insertion mutations occurring at or around codon 775 and leading to duplications of some or all of the YVMA amino acid sequence. Copy number changes have not been a component of regular mutation screening in lung ACA; however, selected studies suggest that ERBB2 amplification is a relatively infrequent event in this tumor type but appears to correlate with the presence of an ERBB2-activating mutation. ERBB2 exon 20 mutations have been reported in between 2% and 6% of lung ACAs and are more common in female nonsmokers. Aside from rare reports, ERBB2 mutations occur in a mutually exclusive fashion with other defined oncogenic alterations.

The largest series to date examining the efficacy of targeted therapies in this tumor type included a total of 65 patients with ERBB2-mutated lung ACA, of which 16 patients with stage IV disease received ERBB2-directed therapy. Four different targeted therapies were used in this series in addition to conventional platinum-based chemotherapy; ERBB2-targeted therapies were associated with an 82% disease control rate (stable disease or partial response) and 5.1-month median progression-free survival. Another small series reports an objective response rate of 100% in 3 ERBB2-mutated lung ACA treated with afatinib. Collectively, it appears that different ERBB2-targeted drugs are associated with variable degrees of response; however, the small number of patients treated with any individual agent limits the conclusions that can be drawn regarding relative efficacy.

Large-scale genomic profiling efforts have uncovered other rare extracellular domain mutations in ERBB2 in a variety of tumor types, including breast, bladder, and lung. These extracellular domain mutations occur as substitutions at codon S310 in exon 8 of ERBB2 and represent approximately 1% of all ERBB2 mutations reported and 0.2% of mutations in lung ACA overall. In vitro assays demonstrate that these S310 mutations are associated with oncogenic transformation in vitro. The implications of these mutations for ERBB2-targeted therapies are unknown.

**Evolving Directions: Discoveries in “Pan–Wild-Type” Lung Adenocarcinoma**

Despite the significant advances made during the last decade in our understanding of the spectrum of driver mutations in lung ACA and their utility in prediction and prognostication, 40% to 50% of lung ACAs remain poorly characterized. Of these, there is significant interest, in particular, in further defining the oncogenic alterations driving development of these “pan–wild-type” or “oncogene-negative” tumors arising in never or light smokers. As mentioned earlier, enrichment of study sets for otherwise mutation-negative tumors from patients who fit this demographic leads to an increased rate of detection of driver translocations involving ALK, RET, and ROS1. This observation has led to a series of studies in which discovery methods have been used in pan–wild-type tumors from nonsmoking patients. Recent data suggest this approach will be productive in identifying rare drivers of oncogenesis and have led to the discovery of several novel alterations in lung ACA.

**NTRK1 Rearrangements**

Tropomyosin receptor kinases (TRKA through TRKC) bind the neurotrophin family of ligands, including nerve growth factor, and have been implicated in tumorigenesis. TRKA (NTRK1) in particular is rearranged in thyroid and colon carcinomas and appears to undergo autocrine or paracrine activation in a variety of other tumor types including neuroblastoma, pancreatic carcinoma, and breast carcinoma. Targeted next-generation sequencing led to identification of novel NTRK1 rearrangements leading to oncogenic fusion products MPRIP-NTRK1 and CD74-NTRK1. Screening of cases, using a combination of next-generation sequencing and FISH, led to identification of NTRK1 fusions in 3 of 91 lung ACAs (3.3%) that were otherwise negative for known oncogenic driver alterations. All 3 tumors were derived from female patients, 2 of whom were never smokers. A variety of kinase inhibitors, including lestaurtinib and crizotinib, have shown activity against TRK proteins, leading to decreased autophosphorylation and reduced MAPK/AKT pathway activation. However, the single patient with an identified NTRK1 rearrangement who received treatment with crizotinib showed only a minor radiographic response and had disease progression 3 months after starting therapy. Novel TRK inhibitors are under investigation with some promising early-phase results with selective TRKA inhibitors.

**NRG1 Rearrangements**

NRG1 encodes a ligand for ERBB3 and ERBB4 tyrosine kinase receptors; in normal tissues its expression is restricted to neurons. Whole transcriptome sequencing of driver mutation–negative lung ACA from never smokers identified a CD74-NRG1 fusion with associated high expression of NRG1 protein in 1 of 25 cases. Subsequent screening studies by RT-PCR identified this chimeric transcript in 4 of 102 tested pan–wild-type lung ACAs from never smokers. Interestingly, all of the identified cases showed features of invasive mucinous ACA on pathologic review, a histologic pattern that has previously been associated with KRAS mutations. CD74-NRG1 fusion–positive tumors showed high levels of ERBB3 phosphorylation, suggesting that this chimeric protein serves as a ligand for ERBB3 and leads to downstream PI3K-AKT pathway activation. To date, there are no data on the therapeutic implications of this fusion event, in particular the efficacy of targeted ERBB3 inhibitors.

**RIT1 Mutation**

RIT1 (Ras-like without CAAX 1 or Ras-like in all tissues) is a recently described member of the RAS family that promotes cell survival via signaling through the p38 MAPK-dependent AKT pathway and appears to be a critical component of cell survival mechanisms in response to stress. RIT1 amplification is reported in hepatocellular carcinoma, and RIT1-activating mutations have been reported in slightly more than 1% of myeloid neoplasms. These activating mutations are present in the switch II domain in the vicinity of codon Q79, the homolog to codon Q61 in other RAS family members.

RIT1 mutations were recently described in an oncogene–wild-type cohort of 87 lung ACAs at a frequency approaching 6%, occurring as small insertion/deletion or substitution
mutations in a hot spot spanning codons 76 to 90. In a larger cohort of lung ACA, RIT1 mutations were present in 2.4% of samples overall and occurred in a mutually exclusive fashion with other oncogenic driver alterations. In vitro studies pose demonstrate that RIT1 mutations activate MEK and PI3K pathways, resulting in oncogenic transformation that is inhibited by PI3K/mTOR inhibitors.

FUTURE DIRECTIONS

As the cost of next-generation sequencing declines, more widespread adoption of this testing approach and less biased tumor genomic profiling will become possible. Next-generation sequencing permits analysis of sequence-level changes on a read-by-read basis, thus permitting more accurate quantitation of mutated alleles with implications for improved understanding of mutational heterogeneity and tumor evolution, particularly in the face of targeted therapies. In addition, the sensitivity of next-generation sequencing and other targeted genotyping approaches, such as digital droplet PCR, will facilitate analysis of specimens with low tumor content, whether they be small tumor biopsy specimens, cytology specimens, circulating tumor cells, or plasma specimens. These advances, coupled with improvements in the efficacy and tolerability of targeted therapies, have the potential to revolutionize the care of solid tumors, allowing oncologists and pathologists to anticipate tumor recurrences and associated resistance mechanisms before they become clinically or radiographically apparent. Coordinated approaches to pathologic review of biopsy specimens with appropriate annotation of fresh frozen tissue will facilitate introduction of more RNA-based assays into clinical care, thus accelerating detection of early clinical use of new technologies.

References

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