Molecular Pathology of Non–Small Cell Lung Cancer

A Practical Guide

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Abstract

The traditional distinction between small cell lung cancer and non–small cell lung cancer (NSCLC) is no longer sufficient for treatment planning. It is advised to handle small diagnostic specimens prudently because they are often the only specimen available for molecular analysis. Pathologists are experiencing pressure to subclassify lung carcinoma based on extremely small tumor samples, because NSCLC tumor subtyping is now essential to determine molecular testing strategies. Evaluation for EGFR mutations and ALK rearrangements are now considered to be the standard of care in advanced-stage pulmonary adenocarcinomas. Immunohistochemical stains can aid in subclassifying NSCLC, but performing these ancillary studies can significantly reduce the quantity of tissue available for molecular tests, requiring careful balancing of these 2 needs. The pathologist plays a pivotal role in facilitating clear and timely communication between the clinical oncology care team and the molecular laboratory to ensure that the appropriate tests are ordered and optimal material is submitted for testing.

Over the last several years, extensive research has investigated the reproducible molecular alterations in lung cancers, with marked success in identifying specific molecular cohorts of patients. This has contributed to a new paradigm of classification of lung cancer. The traditional distinction between small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC) is no longer sufficient for treatment planning. Further tumor subtyping is now essential to appropriately select therapy and determine molecular testing strategies. Increasingly, the incorporation of molecular testing results is an essential component of clinical decision making in NSCLC.2–7 Molecular characterization of lung carcinoma contributes valuable information in terms of the patient’s diagnosis, prognosis, and the potential for treatment with targeted therapy. With additional evidence that targeted therapy is a major improvement over conventional chemotherapy when applied to the appropriately selected patients, evaluation for epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements are now considered by many to be the standard of care in advanced-stage pulmonary adenocarcinomas.2–7 As the vision of “personalized medicine” increasingly becomes a day-to-day reality, having a clear understanding of the processes involved in molecular testing of tumor specimens will be paramount to the practicing pathologist. Furthermore, the pathologist will play a pivotal role in facilitating clear and timely communication between both the clinical oncology care team and the molecular laboratory.

This review is intended to serve as a practical guide to aid in the handling and management of surgical pathology and cytopathology specimens that may be used for molecular
testing in the setting of lung cancer. Comprehensive references that discuss the various methods of molecular testing are available elsewhere. Although molecular alterations have been described in various histologic types of lung carcinoma, the preponderance of the available data regarding therapeutically important molecular testing is focused on NSCLC; thus this review mainly describes testing in this cohort.

Several guideline documents on the implementation of molecular testing in lung cancer have been published, for specific target audiences. A collaborative expert panel, sponsored by a joint effort of the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association of Molecular Pathology (AMP) has issued a preliminary guideline document. The initial release of this preliminary document was accompanied by an opportunity for comment on the proposed guidelines. The preliminary document is available online; however, a date for finalization of this guideline has not been released.

Clinical and Diagnostic Features

Lung cancer is the most commonly diagnosed malignancy in the world. It is the leading cause of cancer-related death among both men and women in the United States. Cigarette smoking has been shown to be a strong risk factor for the subsequent development of lung cancer; as the incidence of smoking has declined in the United States, the incidence of lung carcinoma in men has also declined. The incidence of lung cancer continues to increase for US women, but the rate of this increase is slowing. This has been attributed at least in part to the observation that the peak incidence of smoking in US women occurred later than in men. Another potential contributor to this trend is the observation that a higher percentage of female lung cancer occurs in never-smokers, and epidemiologic associations that are not yet well defined may play a role in this growth trend.

Despite multiple studies to find a cost-effective screening method for the early detection of lung cancers in high-risk patients, more than 50% of patients with lung cancer are at stage IV at the time of their diagnosis. The recent finding of a 20% reduction in lung cancer–associated mortality with the use of low-dose CT screening in high-risk individuals is promising for possible reduction in late-stage disease identification; however, the associated cost and risks may inhibit the widespread implementation of this strategy. The prognosis for all stages of lung cancer remains poor: the 5-year survival for all combined stages is 15%. For patients with early-stage (localized) disease, 5-year survival is significantly higher at 52%, but reflects the high recurrence rate of this disease. Patients who present with advanced disease (stage IV) have a 5-year survival rate of less than 5%.

Traditionally, clinical treatment options were based on a distinction between SCLC and NSCLC. Although this distinction is still paramount, this basic categorization of tumor types is no longer considered sufficient, and pathologists are experiencing greater pressure to precisely subclassify lung carcinoma even in extremely small tumor samples. Although the diagnosis of NSCLC, not otherwise specified (NOS), is acceptable (but discouraged) for small biopsy specimens, a more specific classification is often possible based on the immunoprofile of the tumor. In addition to having an effect on downstream molecular testing, subtyping is increasingly important for the selection of treatment agents, because specific agents are contraindicated in squamous lesions.

Although a panel of immunohistochemical preparations can aid in subclassifying NSCLC, performing such ancillary studies will also significantly reduce the quantity of tissue that is available for molecular tests. Recent recommendations specifically advise preserving a maximal amount of tissue for potential molecular testing and consciously limiting the number of immunohistochemical preparations needed to subclassify a lesion.

Associations among tumor histology, patient demographics, and the presence of specific molecular alterations have been previously described. EGFR mutations have been found to be more common in female patients, never-smokers, and patients of Asian ethnicity. EGFR mutations are also most commonly seen in adenocarcinomas, and are rare in neuroendocrine carcinomas (including SCLC) or mucinous carcinomas. ALK rearrangements are most common in patients with pulmonary adenocarcinomas who are either never-smokers or light smokers. Despite the association of smoking status, ethnicity, and gender with molecular alterations, clinical features alone are not an appropriate basis for selecting patients for molecular testing. This is because combined clinical features have low sensitivity for selecting for targetable alterations. However, histologic subtype may constitute a rational basis for determining molecular testing strategies.

Clinical Significance of Molecular Testing

Only a subset of patients with NSCLC have a molecular alteration that is associated with responsiveness to a Food and Drug Administration (FDA)–approved targeted therapy; however, the detection of an activating EGFR mutation or of an ALK rearrangement changes the treatment plan for an individual patient.

EGFR mutations are present in 10% to 15% of NSCLC in the United States. Tyrosine kinase inhibitors (TKIs), which target the intracellular tyrosine kinase domain of EGFR, such as gefitinib and erlotinib, have shown dramatic...
efficacy in advanced-stage NSCLC, with the greatest treatment benefit concentrated in patients harboring a “sensitizing” mutation in EGFR. Furthermore, several studies have demonstrated a significant first-line benefit with treatment with EGFR TKIs compared with traditional combined platinum-based chemotherapy in patients with tumors harboring an EGFR mutation (reviewed in Langer41). This is the basis on which EGFR mutation status is key in determining optimal first-line therapy and the associated emphasis on rapid results of molecular testing.

A rearrangement in the ALK gene (most commonly resulting in an EML4-ALK fusion gene) is present in approximately 5% of NSCLC.42-44 Identification of patients with this rearrangement is of key importance, owing to the availability of crizotinib, a newly approved targeted therapy with activity against the kinases of the products of ALK, ROS1, and MET genes.45-48 Early studies evaluating the efficacy of crizotinib in patients shown to harbor ALK rearrangement demonstrated an overall response rate of 61%.49 This led to the rapid development of phase II and III trials, and based on preliminary data from those trials, accelerated FDA approval of crizotinib was granted in 2011. The most recently available data from a global phase II study evaluating response to crizotinib following relapse to first-line chemotherapy demonstrated an overall response rate of 53%.50 The majority of patients enrolled in prior clinical trials evaluating crizotinib have received at least one prior treatment, and clinical studies to investigate crizotinib as a first-line agent are currently under way. Although crizotinib therapy may not necessarily be in the first-line approach at this time, identification of ALK positivity can also immediately affect chemotherapy treatment selection as seen in recent studies that demonstrated improved progression-free survival in patients with ALK rearrangements treated with the antifolate agent pemetrexed.51,52

Kirsten rat sarcoma viral oncogene, or v-Ki-ras-2 (KRAS), mutations are present in approximately 30% of pulmonary adenocarcinomas and 5% of pulmonary squamous cell carcinomas.53 KRAS mutations are associated with carcinomas with mucinous histology. Although KRAS mutations have been described in both smokers and never-smokers, it appears that the types of mutations are different for these patient populations.54-56 The clinical implication of KRAS mutation detection is not clear, because the literature regarding the prognostic and predictive usefulness of this marker in NSCLC is contradictory, and currently no approved therapeutic agents are available that target KRAS. However, one potential role for KRAS mutational analysis is based on an algorithmic approach to testing, which recognizes that while KRAS mutations do occur simultaneously with other alterations in a small number of cases,57 the majority of the time the identification of a KRAS mutation is a strong negative predictor for identifying an additional alteration in either EGFR or ALK. Because it is more commonly present overall in NSCLC, one potential role for KRAS testing is as a first-line “screening” test, allowing for exclusion of other testing if positive.53

Mesenchymal epithelial transition factor (MET) encodes a receptor tyrosine kinase named hepatocyte growth factor receptor (HGFR). Like other receptor tyrosine kinases, it is involved in signal transduction and mediates cell growth. A common mechanism of activation of MET in NSCLC is through amplification of the MET gene. MET gene amplification is occasionally observed in specimens before treatment with targeted therapy; however, a significant proportion of patients who develop acquired resistance to EGFR TKIs will have evidence of MET amplification on a resistance biopsy.58-61 MET amplification has been shown to result in resistance to EGFR TKIs in vitro.58 This suggests that selective pressure applied by targeted therapy results in selection for cells harboring this resistance factor. Interestingly, HGFR is also a target of crizotinib, and trials are currently under way to evaluate the effectiveness of this drug in this cohort.

Considerations for Testing

As part of an initial workup, the majority of patients with lung cancer are diagnosed after examination of a small biopsy (transbronchial or endobronchial) or cytology (fine-needle aspiration or body fluid) specimen of the primary lesion or a suspected metastasis.62,63 In most patients, complete clinical staging is not performed until after the cancer is definitively diagnosed. It is often unknown at the time of initial specimen submission whether the patient will have a future surgical resection or whether surgery will not be considered based on subsequent staging information. Therefore, it is prudent to handle small diagnostic specimens carefully, keeping in mind that they are often the only specimen available for molecular analysis.

Molecular testing for EGFR and ALK is indicated for all patients diagnosed with advanced-stage NSCLC with an appropriate histologic subtype (see following paragraphs) and for those who are candidates for targeted therapy.1,2,7,64,65 Communication between pathologist and treating physician is required to determine whether the patient would be a candidate for therapy if a positive finding were identified. In some cases, patients who are gravely ill may still be considered candidates for testing, because the detection of a treatable molecular alteration would make available therapies that can produce a clinical response in patients with poor performance status without inducing significant side effects.66

Histologic subtyping of NSCLC based on morphology alone can be a diagnostic challenge; however, the importance of such subclassification is underscored by its effect on decisions for molecular testing. All lesions classified as
advanced-stage adenocarcinoma should be tested for EGFR mutations and ALK rearrangements, unless specific features suggest that such testing is not indicated (eg, when the patient is not a candidate for therapy). Although the preponderance of mutations identified have been in the adenocarcinoma subset of NSCLC, additional histologic subtypes should also be considered for testing, including all cases with a component of adenocarcinomatous differentiation, or those in which an adenocarcinomatous component cannot be excluded. Studies have demonstrated that adenosquamous lesions do harbor EGFR mutations and ALK rearrangements. Testing should also be considered in cases diagnosed as large cell carcinoma, poorly differentiated carcinoma, and NSCLC-NOS. Lesions diagnosed on a small sample with histologic subtypes other than adenocarcinoma may be considered for testing, because limited sampling does not effectively exclude an adenocarcinomatous component.

Determination of appropriate testing patterns in squamous cell carcinoma can be more challenging. Although large studies have not shown a significant proportion of squamous cell carcinoma to demonstrate EGFR mutations or ALK rearrangements, isolated reports have described EGFR mutations in squamous cell carcinoma. If all lesions classified as squamous cell carcinoma are automatically excluded from EGFR and ALK testing, patients could be excluded from potentially beneficial therapies, especially in light of some important caveats regarding exclusion of testing in all cases diagnosed as squamous cell carcinoma. First, a small biopsy sample showing squamous morphology does not exclude the possibility of an adenocarcinomatous component elsewhere in the lesion.

Second, the distinction between adenocarcinoma and squamous cell carcinoma can be extremely challenging in some cases. The solid variant of adenocarcinoma can have significant morphologic overlap with nonkeratinizing squamous cell carcinoma. Although the keratin pearls and intracellular bridges serve as reliable indicators of squamous differentiation, and gland formation and intracellular mucin serve as reliable indicators of adenocarcinoma, these identifying features are not always present. This highlights the importance of selective immunohistochemical evaluation, especially in cases without clear morphologic differentiation. It has been noted that squamous cell carcinoma cases with EGFR mutations may be improperly classified and that retrospective evaluation demonstrates at least a small adenocarcinomatous component in these lesions. Retrospective reclassification may aid in understanding underlying pathways in subtypes of NSCLC, but it reveals the possibility that in the “real-time” diagnosis of lesions, it is possible for small areas of adenocarcinomatous differentiation to go unrecognized, thereby influencing which patients are considered eligible for molecular testing. A determination to not test a specimen based on squamous morphology should include a meticulous evaluation for even a focal adenocarcinomatous component, even if not quantitatively sufficient to render a diagnosis of adenosquamous carcinoma. It is unknown what quantity of adenocarcinoma differentiation is sufficient to increase the risk of a molecular alteration. Therefore many would suggest that if the histologic subtype is uncertain it is best to err on the side of caution and submit the sample for molecular testing.

Finally, the associations between tumor histology and molecular alterations may not be as discrete as previously thought. One recent case report demonstrated a lesion in which a treated pulmonary mass showed adenocarcinoma histology. However, the corresponding lymph nodes demonstrated a metastasis with “pure” squamous morphology and immunophenotype. Both areas were found to be positive for ALK rearrangement. In this case, the pure squamous lesion represents one component of an adenocarcinomatous carcinoma, in line with the assessment that these alterations are essentially restricted to lesions with some component of adenocarcinomatous differentiation. However, this case exemplifies the importance of recognizing the limited ability of a small sampling to exclude an adenocarcinomatous component, as well as the importance of thorough histologic and immunohistochemical evaluation. Furthermore, it underscores the clinical reality that some lesions classified as squamous cell carcinoma may carry an alteration in EGFR or ALK, especially if they derive from a lesion with an unrecognized adenocarcinomatous component. In some situations, clinical features relating to an individual patient may help to identify cases with high suspicion for such alterations in a “squamous” lesion (eg, young age or limited smoking history) and may help to guide testing patterns in lesions diagnosed as squamous cell carcinoma.

A significant issue confronting many pathology practices is the question of whether to implement pathologist-directed reflex molecular testing for newly diagnosed NSCLC. There are distinct advantages and disadvantages to reflex testing. In the setting of diagnoses made on a small sampling of a primary lung lesion, the pathologist may not know whether the patient is considered a surgical candidate. If those patients go on to have a resection, testing the initial small diagnostic sample, while feasible, may not be the optimal testing approach, because a large resection specimen will subsequently be available for testing. For diagnoses made on a metastatic lesion, the pathologist may not know whether the patient is a viable candidate for targeted therapy or whether any prior specimens exist on the same patient that might be more suitable for testing (or may have already been tested). Conversely, deciding against a reflex approach in newly diagnosed NSCLC has the potential to delay therapy for patients who are found to harbor a treatable molecular alteration. These issues further emphasize the need for effective communication regarding
the need for molecular testing. One approach to consider is the addition of a field for molecular testing instructions on the routine pathology requisition form.

The determination of whether to implement reflex molecular testing on resection specimens from patients with early-stage disease is similarly complex. Although they represent a minority of all patients with lung cancer (because of the high incidence of cases initially diagnosed with advanced stage disease), a significant number of patients undergo resection for early-stage NSCLC. In this situation, a decision on whether to test is not based on the availability of material, but rather on a clinical indication to perform such tests. There is extensive investigation into the implementation of neoadjuvant treatments using targeted therapy, and universal testing may help to identify patients who are best suited for this approach. In addition, because of the high recurrence rate of NSCLC, routine testing of resection specimens would make these results available at the time of a future recurrence. The most obvious downside is related to the high cost of molecular testing; although the recurrence rate is high, many patients with

Image II Subtyping of non–small cell lung cancer (NSCLC). These images demonstrate the significant degree of morphologic overlap between nonkeratinizing squamous cell carcinoma and the solid variant of adenocarcinoma. A, Tumor cells with prominent nucleoli growing in solid sheets. B, Tumor cells entrapping an airway structure, mimicking gland formation. This tumor was diagnosed as squamous cell carcinoma based on immunohistochemical staining that showed the cells to be cytokeratin 5/6–positive and thyroid transcription factor 1 (TTF-1)–negative. C and D, Tumor cells growing in solid sheets, with discrete cell-cell borders and focal cytoplasmic alterations mimicking keratinization. This tumor was diagnosed as an adenocarcinoma based on strong and diffuse immunohistochemical staining with TTF-1 (H&E, ×400).
early-stage disease are cured by surgical intervention. An additional argument against reflexive testing for early-stage specimens is the realization that the molecular targets for testing are evolving rapidly over time, and new markers may be identified in the interval between resection and recurrence. A collaborative consensus decision should be reached between pathologists and their oncologist colleagues to determine whether patients with earlier-stage disease should be routinely tested for EGFR and ALK alterations, because the effect of these analyses varies in local practice. Possible algorithms for specimen management are demonstrated in Figure II.

If multiple primary lesions are identified in a single patient, the rationale is that each lesion should be tested separately. Molecular analysis of synchronous lesions can

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**Figure II** Potential algorithms for specimen management and decisions to perform molecular testing on non–small cell lung cancer specimens for small biopsy and cytopathology specimens (A) and resection specimens (B), with special emphasis on the need for communication between the pathologist and the clinical oncology team. Unique clinical scenarios may require different handling from that suggested in these algorithms.
help to determine whether the lesions are likely to be clonally related, especially if different alterations are identified in the lesions. In the setting of synchronous lung primaries, the presence of a therapeutically actionable alteration in only one of the lesions does not preclude targeted therapy. A recent study has demonstrated a marked degree of genetic heterogeneity in a single tumor using next-generation sequencing methods. The question of intratumoral heterogeneity with respect to actionable mutations in NSCLC has also been investigated. In several studies, when multiple areas of a tumor were evaluated for key driver mutations, qualitatively identical results were found. Thus, in the absence of further data to the contrary, these results suggest that it is not necessary to test multiple areas from the same tumor.

In patients who have not been previously treated with a targeted therapy, it is currently acceptable to test EGFR and ALK on either a primary lung tumor or metastatic lesion specimen. Although studies have demonstrated no evidence of intratumoral heterogeneity with respect to driver mutations, multiple reports have shown discordance between the primary lesion and metastasis. The biological basis of such discordance is not well understood, and it is not clear if the potential for discordance should affect decisions on which specimen to test at this time. Thus until these issues are resolved, there are no definitive guidelines as to whether the primary lesion or metastasis should be preferentially tested.

### Specimens and Specimen Handling

Molecular testing of oncologic specimens, by definition, assumes that specimens consist of a mixture of tumor cells and nontumor cells. While resection specimens have abundant tissue available for testing, they are available only in a small subset of patients with NSCLC. Thus it is necessary to consider other types of specimens that might require testing.

It has been well documented that small biopsy specimens, cytology cell blocks, and cytology touch imprints or aspirate slides are suitable for molecular testing. The type of fixation may differ with each of these specimen types and could include formalin or alcohol fixation. Biopsy specimens and cell blocks generated from cytology specimens are usually processed into paraffin blocks, while fine-needle aspirates, fluids, and imprints may be preferentially placed onto slides. Each of these specimen types is potentially suitable for testing, and although testing directly from smears is possible, it is not widely available in laboratories at this time. In addition, testing directly from a smeared or imprinted slide will nearly always prevent that slide’s inclusion in the diagnostic archive.

Bronchial washes, bronchoalveolar lavage fluid, and bronchial brushes often will not be suitable specimens for molecular testing, owing to the low overall number and low concentration of tumor cells commonly observed in these specimen types.

The requirement for clear and effective communication between the clinical team and pathologist cannot be overemphasized. For instance, if a pathologist or cytopathologist is asked to provide an on-site statement of adequacy during an aspiration of a lung tumor or a suspected metastasis from a lung tumor it is important to know whether the specimen may be sent for possible molecular testing or if the sample is being collected for the sole purpose of molecular testing. In some situations, the radiologist performing the procedure may not be aware of the intention to perform molecular assays, therefore it may be advisable to contact the oncologist or ordering clinician directly. If only a confirmation of malignancy is required, then a minimal number of passes and slides are needed, but this material may be insufficient in quality or volume if ancillary testing is requested “after the fact.” Along the same line, if a pathologist evaluates a cytology specimen in a patient with NSCLC, it is often helpful to note in a comment whether the cell block material “contains abundant tumor cells” or “contains single, isolated tumor cells in low numbers and density.” Ideally these comments will assist the oncologist in deciding whether suitable material is available for molecular testing. Likewise, such comments may be helpful for the pathologist who is asked to review archived material after the original diagnosis is issued, to assess the tumor content, and determine suitability for molecular testing.

If a small surgical specimen or cytology specimen is obtained from a patient with suspected lung carcinoma, it is beneficial to preserve as much material as possible for molecular testing. Because the currently available targeted therapies are indicated for pulmonary adenocarcinomas, it seems an obvious but critical task to confirm that the tumor in the sample is of pulmonary origin and not a metastasis from another anatomic site. A limited number of immunohistochemical preparations (rather than a “shotgun” panel) is preferable to confirm a pulmonary origin and identify an adenocarcinomatous component. Although it is important to ensure that the specimen is appropriately indicated for molecular testing, performing numerous immunostains is likely to exhaust the sample and might in fact preclude critical molecular testing. For example, a single stain showing a morphologic adenocarcinoma in a cytology cell block to be reactive for thyroid transcription factor 1 (TTF-1) or novel aspartic proteinase of the pepsin family (Napsin-A) would be sufficient evidence to send the specimen for EGFR and ALK testing. If a biopsy sample shows an obvious architectural pattern of adenocarcinoma (ie, lepidic pattern), then immunohistochemical studies are likely unnecessary and the material can be prioritized for molecular testing. For tumors that do not show distinct morphologic features of either adenocarcinoma or squamous cell carcinoma (NSCLC-NOS) it has been recommended that only
a single immunostain for each subtype be performed.\textsuperscript{24} TTF-1 and Napsin-A have been recommended to identify areas of adenocarcinoma, while p63 and cytokeratin 5/6 have been shown to highlight areas of squamous differentiation.\textsuperscript{102,103} The use of dual-color immunostains is another strategy to conserve tumor tissue, although some laboratories may not have this capability. If the staining results do not allow for a more specific diagnosis than NSCLC-NOS, then the remaining tissue should be sent for molecular testing, rather than extensive immunohistochemical staining. In addition, recommendations that NSCLC-NOS be avoided as a diagnostic category can be accommodated in a significant number of cases by using terminology in which one subtype is favored.

If ancillary studies are requested in a piecemeal fashion, valuable tissue samples are lost with each block facing. Cutting the block only once would be ideal to save as much tumor tissue as possible for molecular testing. Specific methods that can be used to preserve tissue include cutting 15 to 20 unstained slides up front (which may provide enough slides for molecular testing and leave several blanks available if immunostains are needed). Although it requires additional time and effort in the histologic processing laboratory, all tissue cut from the block should be picked up onto glass slides (no sections should be discarded from the water bath). Limiting the use of negative control slides run on tissue samples is another option to increase the amount of tissue available for molecular testing.

In sending material to a molecular laboratory, it is preferable to send the entire paraffin block with the original H&E (rather than a recut) slide, as well as any unused precut slides. Forwarding the block is preferable because some laboratories use specific processes to cut blocks using a “clean cut” protocol to minimize case-to-case contamination, or may have other approaches that are best implemented on blocks. If a pathology practice is unable to release a paraffin block, unstained glass slides with a corresponding H&E slide should be sent. For very small specimens, the best practice is to include an H&E-stained section that has already been cut from the block to minimize the amount of material that is cut from the specimen and that cannot be used for molecular analysis. It is important to ensure that material is cut onto glass slides that are compatible with fluorescence in situ hybridization (FISH), if that is the method that will be used for ALK or other testing. Specifically, sections for FISH should be placed on charged slides, and the use of Snowcoat or X-tra slides (Leica Biosystems, Buffalo Grove, IL) should be avoided because they are poorly compatible with FISH procedures. Determining the number of unstained slides to forward can be challenging, because different molecular laboratories have different approaches to specimen evaluation and different acceptable minimum tumor quantity. When uncertain, it is best to include the maximum number of slides that is feasible.

Bone is a common site for metastatic spread of lung cancer. If a biopsy is performed on a suspected bony metastasis, it is critical that decalcification be avoided because some methods of decalcification can preclude any subsequent molecular testing. A bone that is infiltrated by metastatic carcinoma often does not require decalcification to be successfully sectioned. Alerting the processing laboratory to avoid automatically decalifying a biopsy submitted as “bone” is critical, and careful tactile examination of the specimen is helpful to determine whether decalcification is needed at all.

Specimens With Molecular Prioritization

Increasingly, patients in whom the histologic diagnosis of NSCLC has already been established are undergoing additional biopsies for the express purpose of acquiring material for molecular testing. Numerous anecdotal reports describe the frustration encountered when a specimen submitted with the intent of molecular testing is inadvertently exhaustively because of numerous immunohistochemical stains performed to classify the tumor (nearly always because the pathologist was not informed of the previous diagnosis and prioritization for the specimen). This unfortunate scenario can often be avoided with effective communication, and it is suggested that clear lines of communication be established for clinicians to indicate that molecular testing is a priority for the specimen. In these circumstances, immunohistochemical staining can often be avoided by either comparing the current material to the previously evaluated specimen or by issuing a diagnosis clearly stating that molecular testing is prioritized over immunohistochemistry.

Rebiopsy Specimens for Resistance Testing

Although there is a significant clinical and/or radiographic response to targeted therapy in most patients with EGFR mutations or ALK rearrangements, the disease commonly progresses as a result of acquired resistance.\textsuperscript{104-107} Several mechanisms of acquired resistance have been discovered and can be detected with molecular assays, though the implications for treatment are still evolving.\textsuperscript{105,106,108-110} For patients with a documented prior actionable alteration in whom the disease has progressed with therapy, retesting should be done exclusively on rebiopsy specimens of a progressing lesion. For the practicing pathologist, that has a specific logistical implication—if a request for testing is received on a patient with both a recent biopsy and prior specimens, it is prudent to seek clarification from the clinical team whether testing is indicated only for the most recent specimen based on disease progression.

Specimen Selection

The pathologist plays a critical role in communicating between the molecular and histology laboratories to ensure
that the appropriate material is submitted. When evaluating a specimen for potential molecular testing, a critical parameter is the percentage of total nucleated cells that are tumor cells (tumor cellularity). Estimations of tumor cellularity can vary widely among pathologists, and those pathologists who do not spend significant time evaluating specimens specifically for molecular testing may underestimate the number of nontumor cells in a specimen, thereby overestimating tumor cellularity. This has particular relevance, because the analytic sensitivity of molecular tests relies on the relative proportion of tumor and nontumor cells to have confidence in the results. Each molecular laboratory will have a minimal amount and concentration of tumor cells required for accurate detection of molecular alterations, based on the specific tumor enrichment protocols available and assay platforms used for testing; the pathologist should be knowledgeable about such requirements to limit the number of specimens rejected by the molecular laboratory.

If molecular testing is requested on a patient with multiple specimens available, several criteria should be considered when selecting the best one for testing. First, only specimen types that are validated in the testing laboratory should be considered. Second, a specimen with limited tumor quantity but a high tumor cellularity may be favorable over an abundant specimen with a low tumor cellularity, depending on methods used by the molecular laboratory. Ultimately, when uncertain as to which is the best specimen, all available specimens should be forwarded to the laboratory. Molecular laboratories should have a pathologist who is highly trained in selecting the best specimen while considering the specific methods used by that particular laboratory.

On occasion, a specimen will be so limited that not all desired ancillary studies can be performed. In these situations the order of testing needs to be prioritized. This exemplifies why it is critical that the pathologist have open communication with the treating oncologist to determine the most important test for the individual patient.

Selection of a Reference Laboratory

As the arena of testing for personalized medicine grows, so too does the number of reference laboratories. Several key parameters should be considered in determining what laboratory to use for requested testing. First, and perhaps most importantly for laboratories in the United States, is that the laboratory have Clinical Laboratory Improvement Amendment (CLIA) accreditation. Numerous inspecting agencies can allow a laboratory to maintain this accreditation; however, because the testing has numerous areas of overlap with anatomic pathology, it is recommended that the laboratory be inspected by CAP as well. An additional critical feature is the involvement of an appropriately trained pathologist in the laboratory. The evaluation of anatomic pathology specimens is a key element of solid tumor testing, therefore it is highly recommended that only those laboratories be considered in which an anatomic pathology–certified pathologist is involved in specimen evaluation.

As mentioned previously, a key element of assessing specimen suitability for testing is based on tumor cellularity. In many cases, tumor cellularity of a specimen is not adequate for testing without taking specific steps for tumor enrichment. Numerous tumor enrichment methods are available, of which macrodissection and microdissection are 2 common ones. Macrodissection involves a pathologist evaluating a slide and indicating a broad area of the paraffin profile with tumor. Subsequently, unstained slides are aligned and the designated area is isolated from the slides. Alternatively, the indicated tumor-rich areas may be isolated directly from the block. Microdissection, especially when performed under a dissecting microscope with a counterstain, often allows for a higher degree of tumor enrichment. Knowledge of tumor enrichment methods used by the laboratory is an important consideration, especially in the context of the testing method used. A laboratory that uses a molecular testing with low analytic sensitivity (ie, Sanger sequencing, which requires a high proportion of tumor cells to detect a mutation) should be paired only with methods that result in the highest degree of tumor enrichment. Important questions to ask a candidate laboratory include “What method is used for mutation testing?” and “What is the minimum tumor percentage required to accept a specimen?” It is also beneficial to determine if the laboratory routinely microdissects samples, and the method of microdissection used.

Another key issue to consider is the specimen types accepted for testing. Most laboratories will accept formalin-fixed paraffin-embedded blocks of biopsy and surgical resection specimens. Although less commonly seen, some laboratories are not validated on cytology cell blocks. An additional option that has not yet reached widespread adoption is the use of cells isolated directly from a smeared or imprinted slide. It is important that any specimen type you may want to have tested be validated in the candidate reference laboratory.

Testing method is yet another issue to consider. The vast majority of mutation testing uses polymerase chain reaction (PCR)–based methods. The analytic sensitivity of the assay dictates the burden of tumor that must be present in the tested sample. However, some methods with very high analytic sensitivity (ie, when a low tumor burden can be effectively tested) evaluate only a limited subset of known mutations. Thus, understanding whether the method used has sufficient coverage for a spectrum of mutations is important, and this decision is likely to require input from the clinical team. Testing for rearrangements and amplification is typically performed using FISH methods, but other approaches are possible. The
Emerging Molecular Markers

At present, EGFR mutation and ALK rearrangement constitute the 2 best-characterized molecular alterations in NSCLC associated with targeted therapy. However, the frequency of these 2 findings combined is less than 50% in most patient populations. Thus, extensive efforts are ongoing to identify additional recurrent aberrations that can be exploited using targeted therapy.112,113 Based on these efforts, numerous molecular markers may become part of routine analysis of NSCLC. Of particular importance, there is currently a dearth of molecular markers with known therapeutic implications in squamous cell carcinoma, and many of the emerging markers may have implications in this subtype. In addition to a growing list of new markers that may be associated with outcome on targeted therapy, some existing markers are finding new indications in investigational therapies. Although the list of emerging markers and new indications is too extensive to review comprehensively here, a few selected examples are presented; readers are directed to other reviews highlighting additional markers and indications.114-118

referring pathologist should be aware that the method used for testing rearrangements can affect the detectability of all possible rearrangements.

ⅡImage 2Ⅱ Representative images of 4 separate pleural fluid cytology cell blocks, each containing metastatic carcinoma. In all cases, the tumor cells are conspicuously visible but constitute a low percentage of the total nucleated cells. Although the tumor cellularity is low, many molecular laboratories would accept specimens like those shown, based on a combination of tumor enrichment and testing methods (H&E; A, B, D, ×200; C, ×400).
ROS1

ROS1 is another receptor tyrosine kinase in which a rearrangement has been identified in NSCLC. A recent study demonstrated detection in biomarker-verified squamous cell carcinoma.119,120 The small molecule inhibitor crizotinib has activity against ROS1. Early studies have demonstrated a marked response of tumors harboring ROS1 rearrangement to crizotinib.121

BRAF

Mutations in BRAF have gained widespread attention based on the association of the mutation with responsiveness to targeted therapy in malignant melanoma. Approximately 3% of NSCLCs harbor a BRAF mutation.122 However, unlike melanoma, BRAF mutations in NSCLC are not overwhelmingly concentrated in a single point mutation (resulting in amino acid substitution at position 600, p.V600E).122,123 Numerous agents are being investigated in clinical trials for activity in patients with NSCLC harboring BRAF mutations.

PIK3CA

Mutations in PIK3CA are seen in approximately 5% of squamous cell carcinomas of the lung,130 and numerous agents are under active investigation, with patients with tumors harboring mutations receiving higher priority.

KRAS

Although testing for KRAS mutation is variably implemented based on the early suggestion that it serves as a negative predictor of responsiveness to targeted therapy in NSCLC, and because it can play a role in algorithmic testing strategies, emerging indications suggest that KRAS mutational testing may be used for selecting patients for inhibitors that affect the pathway downstream of KRAS (ie, MEK inhibitors).118,131,132

Conclusion

In cases of NSCLC, the pivotal role of the pathologist as a bridge of communication between the clinical oncology team and molecular laboratory will become increasingly important in the era of personalized medicine. In current practice, it seems that more information is being required from ever smaller samples obtained with minimally invasive techniques. Effective communication regarding a patient’s history and clinical presentation will allow for judicious use of immunohistochemistry in the diagnosis of NSCLC, and will allow a maximum volume of tumor to be prioritized for molecular testing. EGFR and ALK testing is indicated in NSCLC with any adenocarcinomatous component, and the results of these assays form the basis of a patient’s candidacy for targeted therapy. Familiarity with the molecular laboratory’s testing methods and tumor enrichment protocols will allow the pathologist to send optimal specimens for testing. This in turn will result in the incorporation of accurate and timely results into the clinical treatment of individual patients. Which molecular markers to test, in what order, and whether reflex testing of newly diagnosed NSCLC should be implemented are collaborative decisions to be made in a local practice setting. The pathologist’s role in the treatment of patients with lung cancer and the relationships and communication required for optimal care will only increase in importance as additional discoveries into this common disease come to light.

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