Molecular Pathology of Lung Cancer

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KEYWORDS

• Non–small-cell lung cancer • Adenocarcinoma • Molecular testing • Next-generation sequencing • Biomarkers • Lung cancer

Key points

- Molecular testing is standard of care in the clinical management of advanced-stage non–small-cell lung cancer.
- Molecular testing in lung cancer is a rapidly evolving field.
- Guideline recommendations from professional organizations have outlined the requirements for molecular testing.
- Guideline recommendations from professional organizations provide guidance for biomarkers to test and how to test them.
- Awareness of testing requirements is critical to judiciously triage small specimens and provide adequate testing results.

ABSTRACT

The identification of targetable genomic alterations in lung cancer is required as standard of care to guide optimal therapy selection. With a constantly evolving landscape of ancillary molecular and biomarker testing in lung cancer, pathologists need to be aware of what specimens to test, how the testing should be performed, and which targets to test for to provide the clinically relevant genomic information necessary to treat these patients. Several guideline statements on the topic are currently available to help pathologists and laboratory personnel best use the small specimens obtained from patients with lung cancer for ancillary molecular testing.

OVERVIEW

Lung cancer remains the leading cause of cancer death in the United States.[1] Over the past few decades, the management of patients with lung cancer, particularly non–small cell lung cancer (NSCLC), has increasingly relied on characterizing the oncogenic genomic alterations and biomarker phenotype that drive targeted therapies.[2] The rapid pace of identifying key biomarkers that drive oncogenesis in these tumors has led to an unprecedented number of new approvals by the US Food and Drug Administration (FDA) for NSCLC therapy in the past 1 year.[3] With a growing list of biomarkers currently recommended for testing in NSCLC and with emerging therapeutics targeting additional alterations, the list of biomarker-driven therapeutic options has expanded exponentially in recent years with targeted inhibitors, antibody conjugates, and combination therapies showing significantly improved patient outcomes.

Of the clinical practice guidelines available for managing patients with NSCLC, the National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology for NSCLC are the most frequently updated, widely adopted, and reflect the current standard of care for managing these patients.[4] The NCCN guidelines are also

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used by the health care payers in the United States, including the Centers for Medicare and Medicaid Services, to determine their coverage policies for NSCLC and therefore have financial implications for molecular laboratories and their NSCLC testing practices. The list of biomarkers that needs to be assessed for NSCLC has rapidly grown from just *EGFR, Anaplastic Lymphoma Kinase (ALK),* and *proto-oncogene tyrosine protein kinase ROS (ROS1)*, to now include *BRAF* mutations, *MET* exon 14 skipping mutations, *RET* and *NTRK* gene rearrangements, and programmed death (PD)-ligand 1 (L1) expression. Currently, most drug approvals are for the management of patients with advanced-stage disease; however, the promise of precision oncology is likely to drive biomarker testing into the realm of early-stage disease in the near future.

SPECIMENS FOR TESTING

The biggest strides in oncogenic characterization of lung cancer has been primarily in NSCLC, particularly lung adenocarcinomas. Therefore, the pathologic diagnosis based on morphologic and immunohistochemical (IHC) profiling is critical for decisions regarding biomarker testing for oncologic management. In general, it is considered standard of care to test all patients with advanced stage nonsquamous NSCLC for targetable alterations, whereas PD-L1 assessment by IHC is recommended in both patients with squamous carcinoma and those with adenocarcinoma.[4] However, due to the inherent limitation of adequately evaluating tumor heterogeneity in limited volume samples, such as cytology or small biopsy specimens, physicians may perform biomarker testing even in tumors that do not necessarily demonstrate an adenocarcinoma histology, if clinical features suggest a high probability of an oncogenic driver.[5]

Molecular testing is performed primarily on formalin-fixed paraffin-embedded (FFPE) tissue blocks of histology or cytology specimens; however, the updated College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) lung molecular testing guidelines and the CAP thoracic small specimen collection and handling for ancillary studies guideline recommend the use of any cytology specimen preparation (ie, non-FFPE material), provided the substrate has been appropriately validated.[5]

TESTING METHODOLOGY

In the past, molecular testing was performed using a single gene testing model, with separate tests for each target. However, with a limited amount of tumor in small biopsy and cytology specimens, a single-gene, single-test approach is not compatible with the multitarget biomarker testing needed for patients with NSCLC.[6] Hence, most molecular testing laboratories are rapidly moving toward a multiplexed sequencing approach as the preferred testing modality over separate single-gene tests. High-throughput multigene molecular profiling platforms, such as next-generation sequencing (NGS), have been gaining popularity because of their ability to provide the breadth of genomic information required for standard of care therapy, as well as identify additional therapeutic targets for enrollment in clinical trials.[7–9] Besides NGS and sequencing-based testing, some genomic targets, such as *ALK* and *ROS1* rearrangements, can be tested using alternative techniques, including fluorescence in situ hybridization (FISH) and IHC.[5] Evaluation of PD-L1 is currently performed by IHC alone.[10]

BIOMARKERS FOR TESTING

A comprehensive genomic profiling has become increasingly necessary in patients with NSCLC to make an optimal therapeutic selection. The currently approved targeted therapies in advanced stage NSCLC include actionable alterations in *EGFR, ALK, ROS1, BRAF, MET, RET*, and *NTRK*.[4] With a growing list of potential therapeutic targets, including *ERBB2* and *KRAS* mutations, high-level *MET* amplification, tumor mutational burden, and several other emerging genomic alterations, completion of successful clinical trial results followed by rapid FDA drug approvals will likely expand the armamentarium of effective therapeutic options for these patients.

In recent years, the field of immunotherapy has emerged as a major therapeutic choice for NSCLC tumors that do not harbor a targetable driver mutation. The FDA-approved immune checkpoint inhibitor drugs for NSCLC that target the PD-1/PD-L1 axis have demonstrated superior response rate and patient survival as compared with conventional chemotherapy.[11,12]

Clinically relevant biomarkers in NSCLC are briefly described in the following section and summarized in **Table 1**.

EPIDERMAL GROWTH FACTOR RECEPTOR

The discovery of a subset of patients with NSCLC harboring mutations in the epidermal growth factor receptor (*EGFR*) gene that sensitize them to tyrosine kinase inhibitor (TKI) therapy has led to a paradigm shift in the management of these

Table 1
Guideline recommended biomarkers required for clinical management of non–small-cell lung cancer

Biomarker	Evidence Level	Testing Methodology	Therapeutic Agent
EGFR Mutation	Required	PCR-based assays Sequencing (Sanger, NGS)	First-line therapy • Afatinib • Erlotinib • Dacomitinib • Gefitinib • Osimertinib • Erlotinib + ramucirumab • Erlotinib + bevacizumab (nonsquamous) Subsequent therapy • Osimertinib
ALK rearrangement	Required	FISH break-apart probe assay IHC NGS Real-time PCR	First-line therapy • Alectinib • Brigatinib • Crizotinib • Ceritinib Subsequent therapy • Alectinib • Brigatinib • Ceritinib • Lorlatinib
ROS1 rearrangement	Required	FISH break-apart probe assay IHC, with confirmation if positive NGS Real time PCR	First-line therapy • Crizotinib • Ceritinib • Entrectinib Subsequent therapy • Lorlatinib
BRAF V600E Mutation	Required	PCR-based assays Sequencing (Sanger, NGS) IHC (limited data)	First-line therapy • Dabrafenib/trametinib
MET exon 14 skipping mutation	Required	NGS, preferably RNA based	First-line therapy • Capmatinib • Crizotinib
RET rearrangement	Required	NGS RT-PCR FISH	First-line therapy • Selpercatinib • Pralsetinib • Cabozantinib • Vandetanib

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Table 1
(continued)

Biomarker	Evidence Level	Testing Methodology	Therapeutic Agent
PD-L1 ≥1%	Required	IHC	First-line therapy • Carboplatin or cisplatin/ pemetrexed + pembrolizumab (non-squamous) • Carboplatin + paclitaxel + bevacizumab + atezolizumab (non-squamous) • Carboplatin + albumin-bound paclitaxel + atezolizumab (nonsquamous) • Nivolumab + ipilimumab + pemetrexed + carboplatin or cisplatin (nonsquamous) • Carboplatin + paclitaxel or albumin-bound paclitaxel + pembrolizumab (squamous) • Nivolumab + ipilimumab + paclitaxel + carboplatin (squamous) • Pembrolizumab • Nivolumab + ipilimumab
PD-L1 ≥50%	Required	IHC	First-line therapy • Pembrolizumab • Carboplatin or cisplatin/ pemetrexed + pembrolizumab • Atezolizumab • Carboplatin + paclitaxel + bevacizumab + atezolizumab (nonsquamous) • Carboplatin + albumin-bound paclitaxel + atezolizumab (nonsquamous) • Nivolumab + ipilimumab + pemetrexed + carboplatin or cisplatin • Nivolumab + ipilimumab
NTRK rearrangement	Required[a]	NGS, preferably RNA based FISH for *NTRK 1/2/3* IHC	First-line therapy • Larotrectinib • Entrectinib
ERBB2 (HER2) mutation	Emerging[a]	NGS (as part of broad molecular profiling))	• Trastuzumab • Afatinib
KRAS mutation	Emerging[a]	PCR-based assays NGS (as part of broad molecular profiling)	• Sotorasib
High level *MET* amplification	Emerging	NGS IHC FISH	• Crizotinib
Tumor mutational burden (TMB)	Emerging[a]	NGS	• Nivolumab + ipilimumab • Nivolumab

Based on National Comprehensive Cancer Network Guidelines for non–small-cell lung cancer version 2.2021.
Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing; PCR, polymerase chain reaction; PD-L1, programmed death–ligand 1; RT, reverse transcriptase.
[a] Recommended as part of a broad molecular profiling panel.

patients.[13–15] Oncogenic driver mutations in *EGFR* localize to the tyrosine kinase domain, with approximately 85% of activating mutations seen as deletions in exon 19 and a point mutation (L858R) in exon 21. TKI therapy, including erlotinib, gefitinib, and afatinib, has shown efficacy in treating patients harboring sensitizing *EGFR* mutations.[16] Disease progression is frequently seen, most often secondary to the acquisition of a resistance mutation (T790M) in exon 20 that is treated with osimertinib, a third-generation *EGFR* TKI.[17] Although osimertinib has shown documented efficacy in both first-line and second-line settings, patients inevitably develop resistance, encompassing *EGFR*-dependent as well as *EGFR*-independent mechanisms including *MET* and *ERBB2* amplification, activation of the RAS-mitogen-activated protein kinase (MAPK) or RAS-phosphatidylinositol 3-kinase (PI3K) pathways, novel fusion events, and histologic transformation to small cell carcinoma.[18] Mutation testing for *EGFR* is recommended by polymerase chain reaction (PCR)-based sequencing techniques using assays that are able to detect mutations in samples with as low as 20% tumor content.[19]

ALK

In approximately 4% to 5% patients with NSCLC, *ALK* can undergo gene fusion, most frequently with *EML4*, leading to a constitutively active EML4-ALK fusion protein driving oncogenesis. Several approved oral TKIs, including crizotinib, alectinib, and ceritinib, have shown efficacy in treating patients whose tumors harbor an *ALK* gene rearrangement.[20,21] Conventionally, biomarker testing for *ALK* gene rearrangements have used FISH break-apart probes for detecting *ALK* rearrangements; however, IHC assays using ALK 5A4 and D5F3 monoclonal antibodies have been FDA approved and can be used an equivalent alternative to *ALK* FISH.[19] As with *EGFR*-mutated tumors, resistance mechanisms, either due to *ALK* kinase secondary mutations or *ALK*-independent mechanisms eventually develop in these patients requiring switching to second-generation or third-generation *ALK* TKIs, such as lorlatinib.

ROS1

ROS1 gene rearrangements are seen in 1% to 2% of patients with NSCLC. Although gene partners for *ROS1* can vary (most commonly *CD74, SLC34A2, CCDC6,* and *FIG*), the resulting constitutively active kinase signaling of the ROS1 fusion protein drives oncogenesis in these tumors and responds dramatically to crizotinib, currently approved by the FDA as first-line treatment in these tumors.[22] Testing for *ROS1* fusions are performed either by FISH break-apart probes, reverse transcriptase (RT)-PCR for known fusion partners of *ROS1*, or ROS1 IHC using the D4D6 antibody clone. However, a positive ROS1 result by IHC requires confirmation by a molecular/cytogenetic method.[19] Resistance to crizotinib eventually develops in patients with *ROS1*-rearranged tumors, and subsequent sequencing-based testing may be used to identify secondary resistance mutations that can be treated with other TKIs, such as lorlatinib.

BRAF

BRAF mutations are seen in 1% to 2% patients with NSCLC, with the V600E point mutation being the most commonly encountered alteration seen in these patients. Patients who harbor a *BRAF* V600E mutation are eligible for the FDA-approved dual dabrafenib (*BRAF* inhibitor) and trametinib (*MEK* inhibitor) therapy.[4] Although the CAP/IASLC/AMP testing guidelines do not recommend *BRAF* molecular testing as a routine stand-alone assay outside of testing it as part of a larger testing panel, the American Society of Clinical Oncology (ASCO) endorsement of these guidelines and the NCCN guidelines include *BRAF* in the current recommendations for advanced stage NSCLC biomarker testing.[4,23] *BRAF* testing is usually performed using PCR/sequencing based methods. BRAF IHC using the VE1 clone may be an alternative testing option, although the published literature on the use of BRAF VE1 IHC in lung cancer is limited.

MET

Genomic alterations in NSCLC for *MET* include gene amplification, activating point mutations, or splice mutations such as the exon 14 skipping mutation.[24–26] The FDA recently approved capmatinib therapy for patients with *MET* exon 14 skipping mutations and is currently included in the NCCN guidelines as a recommended biomarker for patients with NSCLC.[4,27] *MET* exon 14 testing is usually performed as part of an expanded NGS panel, due to complexity of exon 14 splice sites. *MET* amplification can be tested via FISH or IHC.

RET

RET gene fusions in NSCLC can involve multiple gene targets leading to a constitutive activation of the *RET* signaling pathways. The FDA has approved selpercatinib and pralsetinib for patients with RET fusion positive advanced stage

NSCLC.[25,28] Testing for *RET* rearrangements may be performed by FISH or RT-PCR; however, NGS-based testing as part of an expanded fusion panel is gaining popularity.

NTRK

NTRK fusions are seen in fewer than 1% of patients with NSCLC and multiple fusion partners have been identified.[29–32] The FDA has approved larotrectinib and entrectinib as first-line therapy in any patients with solid tumor *NTRK* fusions, and the NCCN guidelines include a section recommending evaluating *NTRK* fusions in their testing algorithm.[4,33] Testing methodologies may include FISH, IHC, and/or NGS-based assays.

PROGRAMMED DEATH–LIGAND 1

Immune checkpoint inhibitor therapy has emerged as a major therapeutic choice for tumors that do not harbor a targetable driver mutation. PD-L1 biomarker testing relies on the assessment of PD-L1 expression by IHC on tumor cells using a tumor proportion score (TPS) to determine which patients are most likely to respond to immune checkpoint inhibitor therapy.[34–36] Currently, there are a number of immune checkpoint inhibitor drugs for NSCLC that are FDA approved or in clinical trials, with a paired assay comprising a different antibody clone and an associated staining platform, with different clinical cutoff definitions of positivity that qualify for immune checkpoint inhibitor therapy. The NCCN guidelines recommend testing advanced stage NSCLC using IHC evaluation.[4]

ERBB2

Emerging biomarkers for NSCLC include *ERBB2* (*HER2*) mutations that may be susceptible to targeted therapy that are currently being evaluated in clinical trials. Clinical trials are ongoing for treating NSCLC with *ERBB2* mutations with targeted agents including trastuzumab and afatinib.[4] Currently *ERBB2* mutation testing in NSCLC is largely PCR/sequencing based, and is focused on sequence alterations, specifically insertions and duplications in exon 20.

KRAS

Mutations in the *KRAS* gene are much more common in patients with NSCLC and seen in approximately 15% to 25% of patients. Although there are currently no specific FDA-approved targetable therapies for *KRAS*-mutated NSCLCs, emerging data from clinical trials have shown promising results using sotorasib in *KRAS* G12C-mutated lung tumors.[37] Testing for *KRAS* mutations is generally done via PCR/NGS-based methods but testing is currently not recommended by NCCN guidelines.

EMERGING TARGETS

With the use of expanded platforms interrogating NSCLC tumors, the list of rare genomic alterations continues to grow.[5] Although the clinical significance of these alterations may not be entirely clear at this time, ongoing preclinical and clinical trials will continue to identify potential actionable targeted therapeutics for these tumors with rare alterations.

The reality of lung cancer molecular testing is the rapid pace of discovery of novel therapeutic targets and the complexity of providing this genomic information in a timely fashion that aligns with the clinical management of these patients. The clinical practice guidelines, such as the NCCN, recommend the use of expanded multiplexed panels for molecular profiling, thus encouraging a shift from single-gene assays to larger NGS panels to provide the most effective and efficient way to identify clinically relevant biomarkers from limited volume specimens. The judicious use of the tissue specimen for diagnostic workup and subsequent biomarker testing is critical for patients with NSCLC, as oncologic management relies heavily on the adequacy of the tissue for all clinically relevant and guideline recommended biomarker testing.[38] However, even with the increasing use of multiplexed genomic profiling that offers a more tissue-conserving approach, 10% to 20% of small specimens remain inadequate for comprehensive testing.[39,40] Although most molecular laboratories perform biomarker testing on traditional FFPE tissue blocks, mounting evidence shows that non-FFPE specimens such as cytology direct smears, liquid-based cytology, and even specimen supernatants can be used for NGS testing, if properly validated.[6,41–44] Including additional specimen substrates can increase the molecular adequacy of small specimens used for biomarker testing. For patients in whom tissue is inadequate for lung cancer biomarker testing, options include repeat biopsy/sampling in an attempt to collect sufficient tumor tissue. However, not all patients may be amenable for repeat biopsy, and therefore, the use of liquid biopsies that use NGS to evaluate circulating tumor DNA from plasma as a surrogate for tumoral genomic profiling is gaining popularity.[45,46] Plasma-based biomarker testing has limited sensitivity and current clinical practice guidelines

recommend using liquid biopsy as an alternative only when tissue-based testing is not feasible/available,[5] but with the clinical and technological advances in the field of biomarker testing, it is conceivable that the use of liquid biopsies will expand in the day-to-day clinical practice for NSCLC management.

SUMMARY

The identification of genomic alterations that play a role in lung cancer oncogenesis that can be treated with targeted therapeutics has been a paradigm shift in the management of these patients. Despite the clinical success of these targeted therapies in these patients, only a fraction of eligible patients with NSCLC worldwide currently have access to guideline-specified mandatory biomarker testing. Therefore, a multidisciplinary effort will be needed to optimize the adequate collection of tumor tissue for diagnosis and biomarker testing, to maximize the value of precision oncology for all patients who stand to benefit from these therapies.

CLINICS CARE POINTS

- The identification of genomic alterations in lung cancer targeted therapeutics has been a paradigm shift in the management of patients.
- While guideline recommendations have outlined the requirements for molecular testing, only a fraction of eligible patients with NSCLC worldwide currently have access to biomarker testing.
- A multidisciplinary effort to optimize the adequate collection of tumor tissue for diagnosis and biomarker testing is needed to treat all patients who stand to benefit from these therapies.

DISCLOSURE

The authors have nothing to disclose.

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