Biomarkers for ALK and ROS1 in Lung Cancer

Immunohistochemistry and Fluorescent In Situ Hybridization

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Context.—A small proportion of non–small cell lung cancers harbor rearrangements of ALK or ROS1 genes, and these tumors are sensitive to targeted tyrosine kinase inhibitors. It is crucial for pathologists to accurately identify tumors with these genetic alterations to enable patients to access optimal treatments and avoid unnecessary side effects of less effective agents. Although a number of different techniques can be used to identify ALK- and ROS1-rearranged lung cancers, immunohistochemistry and fluorescence in situ hybridization are the mainstays.

Objective.—To review the role of immunohistochemistry in assessment of ALK and ROS1 rearrangements in lung cancer, focusing on practical issues in comparison with other modalities such as fluorescence in situ hybridization.

Data Sources.—This manuscript reviews the current literature on ALK and ROS1 detection using immunohistochemistry and fluorescence in situ hybridization as well as current recommendations.

Conclusions.—Although fluorescence in situ hybridization remains the gold standard for detecting ALK and ROS1 rearrangement in non–small cell lung cancer, immunohistochemistry plays an important role and can be an effective screening method for detection of these genetic alterations, or a diagnostic test in the setting of ALK.

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lung cancer is the leading cause of cancer mortality in developed countries, and non–small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases. Unfortunately, most patients present with advanced disease at the time of diagnosis, and the prognosis remains grim, with a 1-year survival rate of just 15% to 19% for stage IV disease. This reflects the limited effectiveness of conventional chemotherapy and radiotherapy. The recognition of molecular heterogeneity in NSCLC and the development of tyrosine kinase inhibitors against specific genetic targets revolutionized the treatment of NSCLC with the introduction of personalized therapy. Activating epidermal growth factor receptor (EGFR) mutations were the first to be targeted by tyrosine kinase inhibitors, which improved survival over chemotherapy. More recently, activating fusions involving the anaplastic lymphoma kinase (ALK) gene have been identified in a small proportion of NSCLC patients who demonstrate significant response to ALK tyrosine kinase inhibitors such as crizotinib. Similarly, a subset of NSCLC patients harbor ROS1 rearrangements, and these also show a response to crizotinib. In order for these patient subsets to benefit from targeted therapy, accurate biomarker testing is needed to correctly identify the different genetic subtypes of lung cancer in a clinically appropriate time frame.

ALK IN LUNG CANCER

ALK encodes a receptor tyrosine kinase that is normally expressed in the central nervous system and plays a role in neural development. ALK rearrangement was initially described in anaplastic large cell lymphoma, where it is fused with the NPM gene. In NSCLC, the most common ALK rearrangement is fusion of its 3’ kinase domain with truncated portions of the (N-terminal) echinoderm microtubule-associated protein-like 4 (EML4) gene as a result of inversion within the short arm of chromosome 2. This results in constitutive activation of the kinase domain that is independent of ligand binding. Subsequently, there is activation of downstream signaling pathways, including the phosphoinositide 3-kinase–AKT, mitogen-activated protein kinase, and JAK-STAT pathways, promoting uncontrolled proliferation, survival, and migration. Indeed, forced expression of EML4-ALK fusion protein results in tumorgenesis in vitro and in mouse models, where tumor regression is seen following treatment with an ALK inhibitor. In addition to EML4, less common fusion partners have been described, including TRK-fused gene (TFG), kinesin light chain 1 (KLC1), and kinesin family member 5B (KIF5B),
although the clinical relevance of the different fusion partners is unknown.13

ALK rearrangement is found in approximately 3% to 5% of lung adenocarcinomas,13 and the patients tend to be young and nonsmokers, with no difference in ethnicity or sex. ALK-rearranged adenocarcinomas are almost always mutually exclusive with other driver mutations, such as those involving EGFR and KRAS.14 Distinctive histologic patterns including solid growth with focal signet ring cells and mucinous cribriform pattern associated with abundant extracellular mucin occur more frequently in ALK-rearranged lung adenocarcinomas.15

**METHODS OF ALK DETECTION**

Several assays are available to assess ALK status in tumors, including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), real-time polymerase chain reaction, and next-generation sequencing. Although real-time polymerase chain reaction and next-generation sequencing are highly sensitive and specific, it can be difficult to obtain sufficient quality RNA and DNA from formalin-fixed, paraffin-embedded tissues. Furthermore, these methods are not routinely available and are not currently recommended by the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology guidelines16 to select patients for ALK tyrosine kinase inhibitors.

Fluorescence in situ hybridization is the gold standard method for identifying ALK rearrangements, having been validated in most clinical trials, and this technique was the first to be Food and Drug Administration–approved. However, FISH is relatively expensive and labor intensive to perform and requires specific expertise as well as specialized equipment not routinely available in all laboratories. Furthermore, a minimum number (at least 50) of evaluable tumor cells are required to perform the assay. Given the high incidence of lung adenocarcinoma and low frequency of ALK rearrangements, testing using FISH is not a cost-effective technique to use in all cases. By contrast, ALK IHC is routinely available, rapid, relatively cheap, and

![Figure 1. ALK-rearranged lung adenocarcinoma can be identified by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). An ALK-rearranged lung adenocarcinoma visualized with (A) IHC (D5F3 clone) and (B) FISH using a break-apart probe showing a narrow, split signal pattern (×630). A lung adenocarcinoma without ALK rearrangement showing (C) negative ALK IHC and (D) no evidence of rearrangement with a break-apart FISH probe (original magnifications ×200 [A and C] and ×630 [B and D]).](image-url)
generally easy to interpret and can be readily incorporated into routine reporting. Furthermore, it is easier to identify tumor cells for assessment on IHC slides than by fluorescence microscopy, and testing can still be performed with fewer tumor cells, which is particularly useful in small biopsies with very limited material. Immunohistochemistry can also be attempted for evaluation of bone metastases where decalcification may preclude FISH. However, a negative result should be treated with caution in such instances, as a false-negative would be difficult to exclude.

A major challenge for ALK IHC in NSCLC is the low level of ALK protein expression in lung adenocarcinomas with ALK fusions compared with anaplastic large cell lymphoma.17 This may be due to weaker promoter activity of the EML4 gene in NSCLC compared with the NPM gene in anaplastic large cell lymphoma or to lower stability of the EML4-ALK protein compared with the NPM-ALK protein.18 Therefore, high-sensitivity visualization kits are required to amplify the signal.

ALK IHC positivity is characterized by granular cytoplasmic staining in tumor cells. There are several antibodies available for the detection of ALK expression in lung adenocarcinomas, including 5A4 (Novocastra NCL-ALK, Leica, Wetzlar, Germany), D5F3 (Ventana, Tucson, Arizona), ALK1 (Dako, Carpinteria, California), and 1A4 (OriGene, Rockville, Maryland). ALK IHC has high sensitivity and specificity for detecting ALK rearrangement compared with FISH when appropriate amplification systems are used to ensure cases with low epitope concentration are identified (Figure 1, A through D). The D5F3 antibody shows a sensitivity of 81% to 100% and a specificity of 75% to 100%17,19–22 and has been approved by the Food and Drug Administration as a companion diagnostic test for eligibility for ALK inhibitor treatment without the need for FISH confirmation. Similarly, the 5A4 antibody shows a sensitivity of 69% to 100% and a specificity of 86% to 100%.21–26 On the other hand, although ALK1 shows good specificity, it has lower sensitivity than 5A4 and D5F3 clones17,27,28 and is not recommended for lung cancer testing. The more recently developed 1A4 clone has been less extensively studied but is reported to have a sensitivity of 93.8% compared with FISH.29

PITFALLS OF ALK IHC AND FISH

In order for the interpretation for ALK IHC to be reliable, certain pitfalls must be avoided. False positives can result from overinterpretation of nonspecific stippling (Figure 2, A) or staining of extracellular mucin (Figure 2, B) as well as necrotic debris. On the other hand, false negatives can occur when using suboptimal antibodies such as ALK1 clone; therefore, inclusion of an appropriate positive control on the slide, ideally an ALK-positive NSCLC, is paramount. ALK cytoplasmic staining can also be masked by intracellular mucin (Figure 2, C), so care should be taken to closely assess the peripheral rim of cytoplasm in tumors with abundant intracellular mucin. It is also important to be aware that heterogeneous staining can occur in surgical resection

Figure 2. Pitfalls in ALK immunohistochemistry. A, False-positive staining can be seen with nonspecific cytoplasmic stippling much weaker than the diffuse granular cytoplasmic staining seen in the positive control (inset). B, False-positive staining can be seen in extracellular mucin. C, Positive cytoplasmic staining can be masked by large intracytoplasmic mucin droplets (original magnifications ×400 [A, A inset, and C] and ×200 [B]).
specimens in which the center of a large tumor may be subjected to delayed fixation.

Although there is generally very high correlation between ALK status as determined by IHC and FISH, a number of studies have reported some discrepancies. The most common scenario is when a tumor is IHC positive but FISH negative.\(^{19,20,24,26,30}\) False-negative FISH can arise from insufficient tumor cells within the sample for analysis, presence of reactive normal cells misinterpreted as cancer, or inappropriate fixation causing difficulty in interpreting the FISH. In borderline or atypical cases, the use of a different FISH probe, such as an assay including a probe for the most common ALK fusion partner, \(\text{EML4}\), may be beneficial.\(^{31}\) Negative FISH can also rarely occur because of complex rearrangements that generate an atypical negative FISH pattern in tumors still harboring a functional ALK fusion protein.\(^{32,33}\) Case reports suggest that these tumors are sensitive to tyrosine kinase inhibitor therapy. In addition, some tumors show positive heterogeneous IHC staining but the FISH shows high native ALK copy number instead of rearrangement.\(^{39}\) Although early evidence suggests ALK copy number status is not associated with sensitivity to crizotinib,\(^{34}\) these types of cases warrant further investigation.

In FISH-positive but IHC-negative cases, false-positive FISH can be due to atypical patterns. For example, cells can show normal fused signals with additional asymmetrically split 5′ centromeric signals that do not involve the ALK gene itself.\(^{24,35}\) In addition, IHC-negative cases can show borderline FISH positivity (15%–20% break-apart pattern). These tumors lack the usual \(\text{EML4}-\text{ALK}\) fusion transcripts, and patients show variable response to crizotinib.\(^{15}\) False-negative ALK IHC in ALK-rearranged lung cancers is also speculated to occur because of either different chimeric ALK proteins with lower stability, nontranslated ALK proteins, or technical artifacts related to fixation or preanalytical factors.\(^{26}\)

**ALK TESTING ALGORITHMS**

The methodology used for identification of ALK-rearranged lung adenocarcinomas in different centers depends on a combination of local resources and expertise as well as regulatory restrictions that dictate which methodology is required to determine access to treatment. ALK IHC alone using an approved assay may be appropriate, or, alternatively, ALK IHC can be used as an initial screening test, followed by FISH testing for confirmation\(^{23,36,37}\) (Figure 3).

In addition, Marchetti et al\(^{38}\) proposed that IHC-negative cases with clinicopathologic parameters frequently associated with ALK-positive tumors should still be tested with FISH. Whichever approach is used, it is important for pathologists to be aware of the pitfalls and limitations of each technique and to consider using an alternative test if results are equivocal.

**ROS1 in Lung Cancer**

The c-ros oncogene 1 (\(\text{ROS1}\)) is an oncogene that encodes a transmembrane receptor tyrosine kinase from the insulin receptor subfamily\(^{39}\) and shares 49% amino acid sequence homology with ALK in the kinase domain.\(^{40}\) The normal function of ROS1 has yet to be elucidated but is similar to that of ALK: ROS1 activates downstream signaling pathways, including the phosphoinositide 3-kinase–AKT, mitogen-activated protein kinase, and JAK-STAT pathways.\(^{31}\) The ROS1 fusion gene has been demonstrated to transform NIH3T3 fibroblasts in vitro and induce tumorigenesis in lung alveolar epithelial cells in vivo.\(^{42}\)

\(\text{ROS1}\) fusion can occur with partners that are intrachromosomal within the long arm of chromosome 6 as well as on other chromosomes. \(\text{CD74}\) is the most common fusion partner; however, at least 12 fusion variants have been identified with other partners, including tropomyosin 3 (\(\text{TPM3}\)), solute carrier family 34 member 2 (\(\text{SLC34-A2}\)), ezrin (\(\text{EZR}\)), syndecan 4 (\(\text{SDC4}\)), leucine rich repeats and
immunoglobulin-like domains 3 (LRIG3), KDEL endoplasmic reticulum protein retention receptor 2 (KDELR2), golgi-associated PDZ and coiled-coil motif containing (GOFPC), and coiled-coil domain containing 6 (CCDC6). ROS1 rearrangements have been identified in approximately 2% of lung adenocarcinomas, particularly, but not exclusively, in young never smokers. ROS1 rearrangements are mutually exclusive with EGFR, KRAS, BRAF, or ALK alterations. Similar to ALK, ROS1-rearranged adenocarcinomas often show solid growth with signet-ring cells or mucinous cribriform features. Clinical trials have shown that patients harboring ROS1-rearranged NSCLC respond to crizotinib, highlighting the need to identify this rare genetic subset of lung adenocarcinomas.

METHODS OF ROS1 DETECTION

Both IHC and FISH can be used to identify ROS1-rearranged lung cancers (Figure 4, A through D). Currently, only the D4D6 clone of ROS1 antibody (Cell Signaling Technology, Danvers, Massachusetts) is commercially available. A number of studies have compared ROS1 IHC with FISH, with reported IHC sensitivity of 94% to 100% and specificity of 76% to 100%. However, the results are highly dependent on the cutoff used to determine IHC positivity. By increasing the cutoff from 1+ to 3+, specificity can be increased from 76% to 100%. Although diffuse moderate to strong staining is more commonly associated with ROS1 rearrangement than focal or heterogenous staining, using a strictly 3+ cutoff significantly reduces the sensitivity of IHC. Indeed, assessment of staining intensity is subjective and prone to interobserver variability; therefore, we recommend FISH testing on all cases with any ROS1 IHC positivity. Furthermore, screening can be limited to adenocarcinomas that are wild type for EGFR, KRAS, and ALK (triple negative). Using this approach, IHC is highly sensitive with very high negative predictive value, and the
current data suggest that IHC is a cost-effective screening tool for ROS1 rearrangements followed by FISH confirmation. It has been reported that ROS1 IHC positivity regardless of ROS1 rearrangement is associated with longer survival. This suggests that the clinical significance of ROS1 expression extends beyond rearrangement alone. However, there is no evidence that IHC positivity alone is associated with response to ROS1 inhibitors.

PITFALLS OF ROS1 IHC AND FISH

In contrast to ALK IHC, which is specific to tumor cells, ROS1 IHC is more prone to false-positive staining. Tumors lacking ROS1 rearrangement, including tumors harboring ALK fusion or EGFR mutations, can still express ROS1 mRNA at levels comparable to those of tumors with ROS1 rearrangement. Although nonspecific expression tends to be weak and heterogenous, EGFR-mutant tumors can exhibit 3+ ROS1 IHC staining. A large proportion of ROS1- nonarranged mucinous adenocarcinomas also show reactivity, albeit with a distinct granular pattern. Furthermore, nonspecific staining can occur in reactive pneumocytes and alveolar macrophages, particularly giant cells.

Rarely, false-negative FISH can occur when ROS1 rearrangements involve genes that are closely located on the same chromosome. For instance, GOPC is only 134 kb upstream from ROS1 and the Abbott Molecular FISH 5’ telomeric probe (Abbott Molecular, Des Plaines, Illinois) covers both ROS1 and GOPC genes; therefore, a ROS1- GOPC fusion will not create a break-apart signal. On the other hand, the ZytoVision ROST fish probe (ZytoVision, Bremerhaven, Germany) can detect this. In addition, other genetic or epigenetic mechanisms, such as rearrangements occurring at a transcriptional level and alternative transcript initiation as seen in ALK, can lead to ROS1 overexpression not detectable by FISH.

CONCLUSIONS

ALK and ROS1 gene rearrangements are oncogenic drivers in a subset of lung adenocarcinomas that provide the option of targeted therapy. However, only a small proportion of NSCLCs harbor these genetic alterations, and accurate and cost-effective methods are required to select these patients for therapy. Both IHC and FISH play important roles in identification of lung adenocarcinomas harboring ALK and ROS1 fusions, and it is important that pathologists understand the strengths and limitations of different biomarker approaches in order to optimize diagnostic accuracy for each patient.

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