Abstract

Lung cancer (LC) is the leading cause of cancer-related mortality. Unfortunately, most patients of LC present at the advanced stage of the disease with a poor prognosis and 1-year survival of less than 20%. At the advanced stage of the disease, surgical resection cannot be possible, hence small biopsy or cytology specimens remain a choice for their correct diagnosis. The recognition of molecular drivers has revolutionized the treatment paradigm of non-small cell lung cancer (NSCLC) with introduction of tyrosine kinase inhibitors. Epidermal growth factor receptor (EGFR) gene mutations were identified, first, to be targeted in NSCLC followed by activating fusions in anaplastic lymphoma kinase (ALK) and rearrangements in c-ros oncogene 1 (ROS1) genes. In addition, the encouraging progress of immunotherapy in patients with NSCLC has been associated with predictive biomarker testing in the form of programmed death ligand-1 (PD-L1) immunohistochemistry assay. To test for these alterations, accurate biomarker testing is needed from biopsy or cytology specimens. In this brief review, testing of biomarkers is discussed using cytology specimens.

Keywords: Anaplastic lymphoma kinase, cytology, c-ros oncogene 1, Epidermal growth factor receptor, lung cancer, programmed death ligand-1

BACKGROUND

The diagnosis of lung cancer (LC) triggers a complex diagnostic workup in which cytology plays a key role in tissue acquisition for molecular testing. The latest guidelines from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology mandate testing for epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and c-ros oncogene 1 (ROS1) alterations in all patients with advanced non-small cell lung cancer (NSCLC) with an adenocarcinoma (ADC) component.[1] The National Comprehensive Cancer Network guidelines now recommend programmed death ligand-1 (PD-L1) testing on all tumor samples in patients diagnosed with advanced NSCLC.[2] The guidelines strongly advise for a broader molecular testing to identify rare mutations for which drugs may be available or for which the patient can be registered under a clinical trial. These include high-level mesenchymal–epithelial transition factor (MET) amplification or MET exon 14 skipping mutation, rearranged during transfection (RET) rearrangements, and ERBB2 (HER2/neu) mutations. In contrast to previous 2013 guidelines[3] where cell blocks (CBs) were preferred over smears for molecular testing, updated guidelines[1] recommend using either CBs or other cytologic preparations with adequate cellularity and preservation as suitable specimens for LC biomarker molecular testing. In this review, the use of cytology samples in molecular studies of LC is described, including mutational testing of EGFR and other genes, ALK and ROS1 fluorescence in situ hybridization (FISH), and PD-L1 immunohistochemistry (IHC) testing.

Molecular Alterations in LC

Activating EGFR mutations are usually found in lung ADCs, never smokers, females, and Asian patients.[4-6] These mutations are located in the tyrosine kinase domain (exons 18–21) of EGFR. Deletions in exon 19 and the point mutation L858R in exon 21 are the most common mutations which are tyrosine kinase inhibitor (TKI)-sensitive mutations. About 50% cases of acquired resistance to TKIs are related to a secondary T790M mutation in exon 20 of the EGFR gene.[7,8]

Contrarily, ALK gene rearrangements are due to inversion in chromosome 2 that juxtaposes the 5′-end of the echinoderm-microtubule-associated protein-like 4 (EML4)
gene with the 3’-end of the ALK gene, resulting in the novel fusion oncogene EML4-ALK. ALK testing is required to select patients for ALK-targeted TKI therapy. Other fusion oncogenes involve the ROS1 receptor tyrosine kinase, neuregulin 1 (NRG1), neurotrophic tyrosine kinase receptor type 1 (NTRK1), and rearranged during transfection (RET) gene. Due to similarity between the tyrosine kinase domains of ALK and ROS1, same inhibitors are used for ALK and ROS1 fusions. KRAS and BRAF function downstream of EGFR in the signaling pathway, and activating mutations are usually mutually exclusive in EGFR-mutated tumors. KRAS is mutated at codons 12, 13, and 61 in patients with lung ADC. BRAF, a serine/threonine kinase, is activated by somatic point mutations in exon 15, including V600E, in a minority of patients with LC. Other potentially targetable mutations include ERBB2, MET, and fibroblast growth factor receptors 1 and 2 (FGFR1 and FGFR2).

**SAMPLE PREPARATION**

The role of cytology has become more important by the increasing use of minimally invasive diagnostic approaches, such as endobronchial ultrasound-guided fine needle aspiration (FNA), which is able to provide material for the diagnosis and staging of LCs and for the application of ancillary techniques such as IHC and mutation testing. Current recommendations propose that the analysis of molecular targets be carried out through highly sensitive polymerase chain reaction (PCR)-based methods and/or FISH on lung cytology. In advanced stage of the LC, the diagnosis is based on the analysis of cytology samples, especially FNA or bronchial brushings/washings obtained through minimally invasive procedures. Although formalin-fixed samples give good results with molecular tests, formalin is also associated with some important drawbacks, including poor quality of nucleic acids due to structural damage and fragmentation in the process of DNA extraction. All cytology preparations are non-formalin-fixed, hence provide the best alternative source of well-preserved DNA. Unstained direct smears, alcohol-fixed Papanicolaou-stained, and air-dried Diff-Quik or Giemsa-stained smears can be used for molecular testing. DNA is readily extractable from all the different cytologic preparations and reasonably stable (from 6 months to 5 years). In both stained and unstained preparations, the number of intact nuclei that are suitable for FISH analysis is also higher. Second, cytology specimens allow for rapid on-site adequacy assessment (ROSE). ROSE maximizes cytology samples for molecular testing by on-site adequacy and diagnostic assessment. However, validation studies are essential with correct implementation of preanalytic factors for any molecular testing on cytologic samples.

**DNA EXTRACTION**

For DNA extraction, cells from stained cytology smears can be removed from glass slides by scraping and collected in microcentrifuge tubes without destaining of the smears. If needed, microdissection or macrodissection for tumor enrichment can easily be performed on cytology smears. Stained slides can be digitized for archival purposes if limited diagnostic slides are available in a particular case. Fresh frozen pellets can also be used for DNA extraction, which are prepared from effusion samples or Liquid based cytology (LBC) preparations. However, tumor content cannot be determined in pelleted preparations. The corresponding centrifuge or cytospin smears of exfoliative samples can be used for checking the presence of tumor.

**MUTATION DETECTION ASSAYS**

Usefulness of FNA and exfoliative specimens for mutation testing by Sanger sequencing and real-time PCR methods has been previously demonstrated. Commonly used mutation detection assays in LC cytology samples are Sanger sequencing, real-time PCR, high-resolution melting curve analysis, restriction fragment length polymorphism, and next-generation sequencing (NGS)-based techniques. Due to limited sensitivity of Sanger sequencing, it is no longer a method of choice for mutation detection in a small biopsy/cytology sample of LC specimen, although it has the ability to detect any mutation within the PCR amplification product without prior knowledge of the location or the type of mutation. On the other hand, real-time PCR-based methods offer much higher sensitivity than Sanger sequencing and are amenable to testing low tumor content samples. However, the disadvantage of these methods is related to analyzing only the mutations of interest and lack of the ability to detect additional novel mutations. NGS is a high-throughput method and has gained popularity because of the simultaneous screening of multiple genes. It requires a very low (as little as 10 ng of DNA) input of start DNA. There are targeted, multibiomarker NGS assays available which analyze amplifications and fusions in addition to mutations, including ALK and ROS1 gene fusions. NGS results from stained cytologic samples are similar to their matched frozen pellets indicating that preanalytical factors, such as fixation and staining of cytologic specimens, do not induce significant alterations in nucleic acid. Due to increased sensitivity and large gene panels used in NGS, it can detect alterations in a sample which was wild type by single-gene assays.

**FISH TESTING**

FISH is the gold standard method for identifying ALK and ROS1 rearrangements using dual-labeled, break-apart probes. A minimum number (at least 50) of evaluable tumor cells are required to perform the assay. Any cytology preparation can be used for FISH assay including CBs, Diff-Quik/Giemsa- and Papanicolaou-stained direct smears, and LBC smears. CBs have been used for ALK rearrangement analysis because the same protocols can be applied for formalin-fixed paraffin-embedded (FFPE) histology blocks. Advantages of direct smears include analysis of entire nucleus which eliminates signal loss from truncation artifacts, and good
nonoverlapped tumor areas can be selected for FISH analysis. The entire smear need not be subjected for FISH assay which is neither cost-effective nor necessary. Monolayered tumor areas with at least 100 tumor cells with the entire nuclei should be selected for FISH assay and for scoring the FISH signals. ALK or ROS1 FISH testing is considered positive if rearrangement is seen in at least 15% of cancer cells.

**Predictive Immunocytochemistry**

**ALK**

FISH is not a cost-effective technique to use in all cases of lung ADC given the high incidence of NSCLC and low frequency of ALK rearrangements. In contrast, ALK IHC is relatively cheap, easy to interpret, and can be incorporated into routine diagnostic laboratories. ALK IHC using either 5A4 or D5F3 clones shows high sensitivity and specificity for ALK rearrangements and is now approved for patient selection for TKIs. A majority of studies on ALK ICC have been performed on FFPE CBs, using 5A4 or D5F3 clones on various automated staining platforms. Alcohol-fixed smears, air-dried smears, cytospin smears, and LBC preparations have all been evaluated for ALK ICC. Over 50%–100% sensitivity has been reported for ALK rearrangement detection. A cut-off of 200 tumor cells for successful ICC testing on Pap-stained smears is suggested. Most studies used automated stainers. It is important to note that the performance of ALK IHC/ICC mainly depends on antibody clones, signal detection system, scoring system, and staining platform. ALK positivity is characterized by granular cytoplasmic staining in tumor cells. There are important pitfalls in interpretation of ALK IHC/ICC which include false positivity due to nonspecific stippling or staining of extracellular mucin and necrotic debris. On the other hand, false-negative results can occur due to usage of suboptimal antibody clones such as ALK1. Therefore, inclusion of a positive and negative control with each batch of cases is essential.

**ROS1**

ROS1 IHC using D4D6 clone is highly sensitive but is relatively less specific and has been recommended only as a screening tool and should be followed by confirmatory FISH testing. FFPE CBs and alcohol-fixed, Pap-stained, direct, and cytospin smears have all been reported to be suitable for ROS1 ICC. In contrast to ALK IHC, which is specific to tumor cells, ROS1 IHC shows false-positive staining in reactive pneumocytes and alveolar macrophages.

**PD-L1**

PD-L1 protein expression has been used as a predictive biomarker assay for anti-PD-1/PD-L1 therapies in NSCLC. However, it is fraught with numerous challenges related to the validation of five different predictive biomarker IHC assays on different staining platforms that tested five different drugs using variable cut-offs for positivity. Of these, the 22C3 pharmDx Dako assay and SP263 VENTANA assay are companion diagnostics and eligible for selection of patients for first-line and second-line pembrolizumab therapies, respectively. The use of cytology samples for assessing PD-L1 protein expression is not advocated because of intratumoral spatial heterogeneity in its expression, sampling error, and difficulty in differentiating tumor infiltrating immune cells or bystander lymphocytes from blood or native lymph nodes in case of metastatic LC. However, due to integral role of cytology samples in LC diagnosis, a number of studies have analyzed the concordance of PD-L1 expression in cytology and histology samples. PD-L1 IHC can be performed on CB, direct smears of aspiration, exfoliative samples, and LBC preparations. PD-L1 ICC can be performed in archived CBs up to 2 years old. Most of the studies used Dako 22C3 and 28-8 clones; however, due to unavailability of these clones in few parts of the world, SP263 assay has also been used with satisfactory results. A minimum of 100 viable tumor cells is the requisite for PD-L1 testing in histology, and similar criteria have been used in most cytology studies as well. PD-L1 staining is defined as complete circumferential or partial linear cell membrane staining of tumor cells at any intensity for Dako antibody clones, whereas SP263 shows cytoplasmic granular and membranous staining. There are certain challenges with PD-L1 staining interpretation both in cytology and histology which are related to nonspecific cytoplasmic staining of background macrophages and inflammatory cells. In effusion samples, it is even more difficult due to intricate admixture of tumor cells with inflammatory cells and mesothelial cells. PD-L1 immune cell scoring is not accurate on cytology samples due to admixture of immune cells derived from blood.

**Conclusion**

Adequate management of cytology specimens is essential for patients with NSCLC, especially when tissue biopsy is not an option. Rapid onsite evaluation from FNA procedures has emerged as an important tool in improving the adequacy and triage of cytology samples. Cytology specimens can be used for predictive molecular testing and other ancillary tests in the complete workup of LC diagnosis. There are challenges in using cytology specimens due to lack of standardization among laboratories for specimen collection, processing, and staining methodologies, hence validation studies are essential steps for any molecular testing on cytologic samples.

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**Conflicts of interest**

There are no conflicts of interest.

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