Biomarker Testing in Lung Carcinoma Cytology Specimens

A Perspective From Members of the Pulmonary Pathology Society

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- The advent of targeted therapy in lung cancer has heralded a paradigm shift in the practice of cytopathology with the need for accurately subtyping lung carcinoma, as well as providing adequate material for molecular studies, to help guide clinical and therapeutic decisions. The variety and versatility of cytologic-specimen preparations offer significant advantages to molecular testing; however, they frequently remain underused. Therefore, evaluating the utility and adequacy of cytologic specimens is critical, not only from a lung cancer diagnosis standpoint but also for the myriad ancillary studies that are necessary to provide appropriate clinical management. A large fraction of lung cancers are diagnosed by aspiration or exfoliative cytology specimens, and thus, optimizing strategies to triage and best use the tissue for diagnosis and biomarker studies forms a critical component of lung cancer management. This review focuses on the opportunities and challenges of using cytologic specimens for molecular diagnosis of lung cancer and the role of cytopathology in the molecular era.


In an era of personalized medicine, with an increasing need for molecular testing, cytologic specimens comprise a crucial component in providing prognostic and predictive information for clinical management of patients with lung cancer.1 Because, currently, these critical decisions are based on the morphologic, immunocytochemical, and molecular features of the lung carcinoma sample, which is often obtained using minimally invasive approaches, optimizing the methods for tissue collection, processing, and triage for molecular testing is needed to adequately use lung cytologic samples for the myriad ancillary studies necessary for clinical management.

The 2013 College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology (CAP/IASLC/AMP) clinical practice guidelines for molecular testing of lung cancer include subtyping non–small cell lung carcinoma into adenocarcinoma and squamous cell carcinoma by morphology, by limited immunoperoxidase staining, or by both methods, as well as providing adequate material for EGFR mutational analysis and detection of ALK rearrangements.2 Many patients with lung cancer are diagnosed with advanced-stage disease, which precludes them from being surgical...
candidates.3,4 The diagnosis is frequently made using
minimally invasive procedures, including fine-needle aspi-
ration (FNA) or body-cavity fluid collection. Although the
variety and versatility of specimen preparations offered by
cytology for ancillary studies provide some distinct advan-
tages over their histologic counterpart, they also pose
unique challenges in validation, partly because of the lack
of standardization among laboratories for specimen collec-
tion, processing, and staining methodologies.3,6 Several
recent articles have discussed the preanalytic variables in
cytologic specimens and the need for standardization to
validate and evaluate molecular testing in lung cancer using
a variety of cytologic preparations.7–9

In patients with lung cancer, the most common
sampling methods for obtaining cytologic specimens
include computed tomography–guided FNAs of the lung
(frequently coupled with core needle biopsies), endobron-
chial ultrasound–guided FNAs of lymph nodes, body
cavity fluids/effusions, bronchial brushings, bronchial
washings, bronchoalveolar lavages, and sputum, along
with other minimally invasive aspirations of distant, deep-
seated or superficial metastatic lesions.3 Samples from
aspirations are typically (1) smeared directly onto glass
slides, air-dried, or fixed in alcohol and stained using Diff-
Quik or Papanicolaou stain; (2) processed as liquid-based
cytology (LBC), analogous to the processing of cervical
cytology specimens, performed by collecting the needle
rinse in an alcohol-based fixative, such as CytoLyt
(Hologic, Bedford, Massachusetts) solution or CytoRich
Red (Fisher Scientific UK Ltd, Loughborough, Leicesters-
shire, England) fixatives and preparing a cell monolayer
slide; or (3) both processes. The remaining LBC fixative
fluid or the residual needle rinse from the aspiration
(collected in RPMI medium, formalin, or other media) can
be used for a paraffin-embedded, cell-block preparation.
Body-cavity fluids, bronchial brushings, bronchial wash-
ings, bronchoalveolar lavages, and sputum samples can be
processed as cytospin preparations or as LBC, with or
without cell block preparations. Multiple studies have
evaluated ancillary testing using the various cytologic-
specimen preparations with the following salient points
pertaining to lung cancer samples.

Cell block preparations are the most commonly used
cytologic-specimen preparation employed for ancillary
testing in lung cancer.5 This is not only because most
laboratories have validated most ancillary studies on
paraffin-embedded sections but also because of the
relative ease of acquiring multiple serial sections to
perform immunocytochemistry and molecular diagnostic
assays.1 However, the adequacy assessment of cell-block
preparations are largely based on the rapid, onsite
evaluation performed on the corresponding direct smears,
which may or may not be entirely representative of the cell
block cellularity. In addition, rapid, onsite evaluation may
not be available in all practice settings. Performing
dedicated needle passes for the cell block at the time of
aspiration may improve the cellular yield but does not
always guarantee specimen adequacy for performing all
the necessary ancillary studies. There is also extreme
variability in cell-block preparation techniques across
institutions, with many laboratories reporting dissatis-
faction with their cell-block preparation methods.5,10 As
a consequence, cell-block preparations can sometimes be
paucicellular and inadequate for molecular testing, espe-
cially for assays that require relatively large amounts of
dNA.3,6,7,11,12 In addition, the standard 4- to 5-μm cell-
block sections do not represent the entire nuclei from the
cell and are likely to have lower nucleic acid yields for
molecular testing per cell than the whole cells obtained
from other nonformalin-fixed cytologic substrates. Finally,
similar to histologic sections, the nucleic acid extracted
from cell blocks subject to formalin fixation is prone to
sequencing artifacts, thus, leading to possible false-
negative and false-positive results in downstream molec-
ular assays.5,13,14 Despite all the issues associated with
the use of cell-block preparations, the 2013 CAP/IASLC/
AMP clinical practice guidelines for molecular testing
of lung cancer recommended the use of cell-block sections
over smears for all molecular testing.2 However, an update
of these guidelines is expected in the near future, and it
will be interesting to see if this topic is revisited. A recent
article reviewing the current literature on EGFR mutation
analysis on cytologic samples from non–small cell lung
carcinoma reported that cell blocks were the most
commonly used cytology preparation, even though failure
rates on cell-block preparations were comparable to that
of direct smears.13 This may reflect the concern of the
cytopathologist for preserving diagnostic material on the
smear/cytospin preparations and the general reluctance to
sacrifice the slides for molecular testing, given the
regulatory requirement for archival slide retention. Re-
cently updated CAP guidelines, however, have made an
exemption to allow the sacrifice of cytology smears for
molecular testing, if that benefits the patient.

Direct smear/cytospin preparations have been employed
by several groups for immunocytochemistry, as well as for
molecular studies in lung cancer.11,16–19 Unlike cell blocks,
smears and cytopsin preparations are not formalin-fixed preparations
and typically provide higher-quality nucleic acids for
molecular testing than do formalin-fixed, paraffin-embed-
ded (FFPE) sections.12 In addition, they offer the advantage
of adequacy assessment during rapid, onsite evaluation
and better triaging of the sample for diagnosis and ancillary
studies.1 However, the additional rigorous validation for
each individual assay performed on smear and cytopsin
preparations for clinical reporting poses the biggest
challenge in using these specimen preparations for
ancillary studies. In addition, certain clones of antibodies
that have been optimized for immunoperoxidase assays on
FFPE material may not work well in alcohol-fixed
material.3 In cases in which all or most of the diagnostic
material is on a single smear/cytospin preparation that will
be used for molecular testing, the slide can be digitally
scanned or photographed to maintain diagnostic material
for the archives to mitigate the medicolegal constraints.12
However, digital imaging of smears poses significant
challenges because of the 3-dimensional nature and
variable thickness of the material requiring z-axis scan-
ing, which, unlike whole slide imaging of histology slides,
is time consuming and results in relatively large digital-
image files.

Liquid-based cytology provides the ease of specimen
collection, especially in settings in which there is no
technical support to prepare smears or when there is no
on-site cytopathologist or cyto technologist available to
perform an assessment of specimen adequacy.3,12 The
specimen is typically expelled in its entirety into a cell-
preservative solution to be processed subsequently in the
cytology laboratory as a cell–monolayer slide that can
confirm the presence of tumor and assess tumor fraction,
with or without a cell block. The advantages of LBC specimens are the optimal preservation of cells, the ease of specimen transportation because of the stability of cells at room temperature, and the minimal amount of background debris and blood on the slide. Although most commercially available LBC fixatives are designed to optimize cell preservation, the preservative in which the needle rinse is collected, which is frequently alcohol-based, can affect certain assays, such as immunoperoxidase staining. Several studies have described using LBC specimens for molecular analysis, either by scraping off cells from the LBC slides for DNA extraction or extracting directly from the rinse solution, and as mentioned above, many cytology laboratories prepare cell blocks directly from those solutions. The properties of the different preservative solutions used in LBC may affect downstream molecular analysis. Some studies have indicated that cells preserved in CytoLyt solution provide higher DNA yields than do cells preserved in CytoRich Red fluid. One study comparing cellularity and DNA yield between ThinPrep slides (CytoLyt LBC) and direct smears reported greater cellularity and significantly higher average DNA yields in the latter, whereas a more recent study reported issues with long-term DNA stability and more-accelerated DNA degradation in LBC samples when compared with conventional smears.

The use of fresh/frozen cells or cells collected in Whatman FTA cards (GE Healthcare Life Sciences, Buckinghamshire, England) for molecular testing, although not very common, has been described in patients with lung cancer. The inability to assess for the presence of tumor and to quantify tumor fraction makes them less-than-ideal specimen preparations for routine molecular testing. However, similar to LBC, an aliquot of the sample can be processed by conventional methods for cytomorphicologic assessment and estimation of tumor fraction, while the remaining specimen is centrifuged into a cell pellet that can be used for immediate DNA extraction or frozen at −20°C or placed on an FTA card until needed for molecular testing. The advantage of using fresh/frozen/FTA-collected cells is the high quality of nucleic acid retrieved from the specimen; however, negative results need to be interpreted with significant caution because false-negatives can commonly occur if the sample tested does not have an adequate tumor fraction. Some recent studies have explored the option of using cell-free plasma DNA in a subset of cases in which limited tissue precludes molecular testing for EGFR. Although highly specific, these methods are limited by their sensitivity for detection of EGFR mutations and their utility in the context of a negative result remains limited.

The 2013 CAP/IASLC/AMP guidelines for molecular testing of lung cancer recommend testing for EGFR mutations and ALK rearrangements to select patients for tyrosine kinase inhibitor therapy in all lung tumors with an adenocarcinoma component. However, our understanding of the genomic landscape of lung cancer is constantly evolving. Crizotinib (Pfizer, New York, New York) was recently granted breakthrough status and priority review by the US Food and Drug Administration (FDA) for treatment of patients with rearrangements in a related biomarker, ROS1. Several other emerging target molecules, including mutations in BRAF and ERBB2, amplifications in MET, and rearrangements in RET, are currently under investigation for other targeted therapeutics that could be included in future biomarker-testing guidelines. Likewise, in squamous cell carcinomas, molecular markers like DDR2 mutations and amplifications in FGFR1, PDGFRα, and PIK3CA are currently under investigation. The wide repertoire of emerging molecular markers in lung cancer, therefore, underscores the need to judiciously triage limited-volume samples to ensure sufficient material is conserved for additional molecular studies.

Molecular testing for EGFR mutations in cytologic specimens have been described using a variety of polymerase chain reaction (PCR)–based techniques, including direct sequencing, real-time PCR, pyrosequencing, fragment–length analyses, high-resolution melting analyses, amplification-refractory mutation system, peptide nucleic acid–locked nucleic acid, coamplification at lower denaturation temperature, and next-generation sequencing (NGS). Although the choice of platform used for the detection of EGFR mutations remains a decision of the individual molecular laboratories performing the assay, the CAP/IASLC/AMP guidelines recommend that the technique employed detect mutations in specimens with at least 50% tumor fraction, although more-sensitive platforms that are able to detect mutations in specimens with as little as 10% tumor are strongly encouraged. In addition, it is recommended that the platform employed for clinical EGFR mutation testing should be able to detect all EGFR mutations that have been reported in the literature with a frequency of at least 1%. Aspiration cytology samples, therefore, provide a potential advantage over corresponding core needle biopsies for clinical mutational analysis because they frequently have a high overall tumor fraction because of the presence of fewer nontumor stromal cells. This is of even greater relevance in patients undergoing rebiopsy following initial therapy in which the tissue shows extensive therapy-related fibrosis. Similar to histologic samples, tumor-fraction enrichment in cytologic specimens can be achieved through macrodissection and microdissection, by demarcating tumor-rich areas directly on the slide. For cell-block sections, the circled hematoxylin-eosin–stained slide can then be used to guide tissue extraction from unstained sections, whereas for smear/cytospin/LBC preparations, the deroversipped slide is etched on the bottom using a diamond-tip pen for tissue extraction from designated tumor-rich areas.

EGFR copy number analysis or immunoperoxidase staining for total EGFR is not recommended for selecting patients for tyrosine kinase inhibitor therapy, although some recent studies suggest that there may be a revised role for these approaches for consideration of monoclonal anti-EGFR therapy. Some studies have reported using EGFR mutation-specific monoclonal antibodies for immunoperoxidase staining on cytologic cell-block preparations for detection of a subset of EGFR-mutated lung tumors. However, because of the relatively low, negative predictive value, the utility of this approach may be limited to situations in which the paucity of tumor cells precludes PCR-based molecular testing for EGFR mutations, and a positive result with the antibodies can prevent the need to rebiopsy the patient for a more-comprehensive molecular study.

For detection of ALK rearrangements the CAP/IASLC/AMP guidelines recommend a fluorescence in situ hybridization assay, using dual-labeled, break-apart probes as the preferred technique for selecting patients for ALK tyrosine kinase inhibitor therapy.
kinase inhibitor therapy. Although the Vysis ALK break-apart probe (Abbott Molecular, Abbott Park, IL) has been approved by the FDA specifically for use in FFPE tissue fragments, the extent to which this approval extends to cell-block preparations is not clear. This potential regulatory limitation notwithstanding, the use of the dual break-apart probe for ALK fluorescence in situ hybridization on FFPE cell-block sections has become a commonly deployed approach to allow for testing on specimens without corresponding adequate histologic sections. 

Beyond the cell-block preparation, several groups have reported the feasibility and utility of the probe in nonformalin cytology specimen preparations, including Diff-Quik and Papanicolaou-stained smears, as well as LBC ThinPrep slides, with some groups reporting better performance than seen with cell-block sections. The advantage to using smears or LBC is that entire cell nuclei are analyzed, eliminating signal loss from truncation artifacts, as seen in FFPE sections. However, the challenge for using smears and LBC for fluorescence in situ hybridization–based assays is the need for independent standardization and validation for each specimen type because the thresholds for positive and negative cutoffs have been set using FFPE histology material. Immunoperoxidase staining for antibodies directed against the ALK protein has been described in cytologic specimens as a quick and relatively inexpensive alternative to the fluorescence in situ hybridization assay, with the Ventana (Ventana Medical Systems, Tucson, Arizona) ALK D5F3 (Cell Signaling Technology, Danvers, Massachusetts) antibody receiving FDA approval in 2015, as a companion diagnostic for ALK-directed therapies. Although the use of reverse-transcription PCR has not been recommended because it may bias detection only for previously well-described fusion partners, in limited cytology specimens, the use of this approach may be preferable to not testing, and laboratories may elect to use this method.

With the new and emerging biomarkers guiding therapeutic decisions in lung cancer, the need for a multigene diagnostic approach using small amounts of nucleic acid extracted from limited volume samples is important. The clinical implementation of NGS allows for the use of a single platform to evaluate for single-nucleotide variations, insertions and deletions, gene amplifications, and gene rearrangements across multiple genes in a massively parallel manner. The NGS design can be modified to enhance detection of a particular alteration type, thus allowing laboratories to customize the assay to best suit their clinical needs, albeit with some trade-offs in cost and clinical sensitivity. Several recent studies have outlined the feasibility and utility of performing NGS on cytologic samples using smears, cell blocks, and LBC samples from patients with lung cancer. Cytologic samples including computed tomography–guided FNA and endobronchial ultrasound–guided FNA, as well as body cavity fluids and bronchoalveolar lavage, have been used successfully for NGS analysis. A recent study comparing NGS success rates in cytologic specimens did not find any significant difference between specimen types, including computed tomography–guided FNA, endobronchial ultrasound–FNA, and body cavity fluids. In addition, aspirates from the lung showed NGS success rates comparable to those from other sites. One study that evaluated lung FNA and pleural fluid samples showed successful sequencing from both smears and cell blocks with no significant difference in NGS success rates between specimen preparations or cytologic stains and fixatives. Another study showed comparable sequencing performance metrics (coverage depth, total number of reads, number of mapped and on-target reads, and variant calls) for smears and FFPE sections of corresponding surgical resections. As with most molecular assays, the most common causes of failure with cytologic specimens on NGS are likely related to limited volume and insufficient DNA yield (because of overall low cellularity). The minimum amount of input DNA required for NGS depends on the target-capture method and specific NGS platform used for analysis, with manufacturer recommended input DNA ranging from 10 ng for the Ion Torrent (Thermo Fisher Scientific, Waltham, Massachusetts) to 250 ng for Illumina (Illumina Inc, San Diego, California) platforms. However, given the high quality of nucleic acid retrieved from nonformalin-fixed cytologic samples, this can be optimized in a clinical setting for significantly lower DNA input than the manufacturer recommended amounts.

In this era of personalized medicine, cytologic specimens have a crucial role in the diagnosis and therapeutic management of patients with lung cancer by providing a variety of specimen preparations for ancillary studies. Aspiration cytology samples are often more enriched in tumor than their histologic counterparts, and nonformalin-fixed cytologic specimen preparations have been shown to provide better quality nucleic acid for molecular testing. However, cytologic specimens continue to be excluded from most biomarker-driven clinical trials, primarily because of the failure to exploit the variety and versatility of different specimen preparations; the lack of validation for different assays, which is labor intensive and time consuming; and the potpourri of information in the literature. The key limitation of cytology samples lies in the typically low tissue volume obtained in aspirates and the lack of standardization of specimen processing across laboratories. Therefore, implementing strategies to optimize and standardize procedures for specimen acquisition, processing, and tissue extraction are critical to maximize use of cytologic samples for ancillary studies and to provide relevant information for inclusion in clinical trial design. It is clear that, although approaches to increase standardization of processes would be valuable, given the diversity of practices globally, this is unlikely to be achieved. Therefore, strategies to maximize tissue preservation for molecular studies need to be implemented at an institutional level, depending on the available resources for cytologic-specimen collection and processing and the molecular laboratory testing approach. Thus, a critical component of continued patient access to critical testing and inclusion in clinical trials, which often can be performed on cytology specimens, is continued laboratory flexibility to design, implement, adopt, and update assays in which cytology specimens are the substrate. Numerous regulatory structures have been proposed as mechanisms for oversight of laboratory testing, and it is critical that the flexibility of laboratories to continue to apply innovative approaches for use of these critical samples is preserved. In the molecular era of lung cancer management, the integration of cytopathology and molecular diagnostics has changed the practice of cytopathology and how we evaluate, assess, and triage cytologic specimens for diagnosis as well as ancillary studies. As we continue to evolve in this growing landscape of molecular diagnostics,
cytopathology has to rise to the challenge of doing more with less tissue to provide prognostic and predictive information for the clinical management of lung cancer.

References


