Molecular Testing of Non–Small Cell Lung Carcinoma Diagnosed by Endobronchial Ultrasound–Guided Transbronchial Fine-Needle Aspiration

The Cleveland Clinic Experience

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Context.—Given the increasing demand for molecular testing of non–small cell lung carcinoma specimens to guide therapeutic decision-making and the trend toward minimally invasive techniques for obtaining diagnostic tissue, cytopathology laboratories must devise strategies to maximize DNA yield for necessary molecular testing.

Objective.—To describe our experience at Cleveland Clinic with epidermal growth factor receptor (EGFR) mutation testing by next-generation sequencing and anaplastic lymphoma kinase (ALK) gene rearrangement testing by fluorescence in situ hybridization of non–small cell lung carcinomas diagnosed by cytology, with an emphasis on specimens obtained by endobronchial ultrasound–guided transbronchial fine-needle aspiration.

Data Sources.—Data sources include a review of the current literature, including published articles from our institution.

Conclusions.—At our institution, liquid-based cytology specimens are the primary resource used for molecular testing of non–small cell lung carcinomas; in most instances, adequate DNA can be extracted from the residual cell pellet for next-generation sequencing, and ThinPrep slides can be used reliably for fluorescence in situ hybridization testing for ALK gene rearrangements. In occasional cases where the cell pellet material is not adequate for molecular testing, cell blocks and/or surgical pathology specimens are secondary options. The cytopathologist’s role in specimen handling and triage is essential to ensure that molecular testing can be carried out successfully.

The treatment of advanced non–small cell lung carcinoma has changed tremendously in the past 10 to 15 years because of the discovery of molecularly defined subgroups of patients with non–small cell carcinoma who respond to targeted therapy. Activating mutations in the epidermal growth factor receptor (EGFR) gene in a subset of patients with lung adenocarcinoma were first identified in 2004, and subsequent clinical trials demonstrated improved progression-free survival and toxicity profiles in patients with EGFR mutations who received the tyrosine kinase inhibitor gefitinib compared with standard platinum-based chemotherapy. Shortly thereafter in 2007, a gene fusion between echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (EML4-ALK) was identified in a small subset of lung adenocarcinomas, leading to demonstration of a significant clinical response to the small-molecule ALK inhibitor crizotinib in patients harboring ALK gene rearrangements. The potential for treating patients with lung adenocarcinoma with targeted therapy has led to a shift in the approach to diagnosis and triage of specimens in order to ensure that patients with targetable molecular alterations are identified. The College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology recommend testing for EGFR mutations and ALK gene rearrangements in all patients with advanced-stage lung adenocarcinoma in order to guide patient selection for treatment with EGFR or ALK inhibitors.

Molecular testing of solid tumors has been traditionally performed on and standardized to formalin-fixed, paraffin-embedded surgical pathology tissue. However, clinicians are increasingly using minimally invasive methods to obtain diagnostic material, bringing cytopathology to the forefront of molecular testing. Cytology specimens provide high-quality DNA for molecular testing and are comparable to, if not superior to, formalin-fixed, paraffin-embedded surgical pathology specimens for the evaluation of clinically relevant mutations.

Although cell blocks are frequently used for molecular testing of non–small cell lung carcinomas, at our institution they were suboptimal for analysis in a high proportion of
cases. An alternate strategy for EGFR and ALK testing was therefore developed using material from the liquid-based cytology sample for EGFR mutation testing by next-generation sequencing (NGS) and the ThinPrep slide for fluorescence in situ hybridization (FISH) testing for ALK gene rearrangements.\textsuperscript{12,13} We briefly describe our experience with molecular testing of non–small cell lung carcinomas for EGFR mutations and ALK gene rearrangements on liquid-based cytopathology specimens at our institution, with an emphasis on material obtained from endobronchial ultrasound–guided transbronchial fine-needle aspiration (EBUS-TBNA) procedures.

**SPECIMEN TRIAGE AND PROCESSING**

At our institution, lung carcinomas are increasingly being diagnosed and staged by EBUS-TBNA; there were 765 EBUS-TBNA specimens submitted to our laboratory in 2016. Rapid on-site evaluation is provided in each case for determination of specimen adequacy and specimen triage (Figure 1). The specimen is obtained by an interventional pulmonologist. Two smears are made for each needle pass; 1 slide is immediately fixed in alcohol for Papanicolaou staining and the other is air-dried and stained with Diff-Quik for rapid on-site assessment by the cytopathologist. After dropping a small amount of specimen on the slide for smearing, the remainder of the specimen is rinsed into CytoLyt solution to ensure that enough material is collected for ancillary testing. The CytoLyt solution is centrifuged to generate a cell pellet, which is divided to make a ThinPrep slide and a cell block. The remainder of the cell pellet is resuspended and stored at 4°C for future molecular testing if needed (Figure 2). The cell pellet may be stored at this temperature for 1 to 2 months without compromising DNA yield.\textsuperscript{12}

![Figure 1. Specimen handling at the time of endobronchial ultrasound–guided transbronchial fine-needle aspiration. The cytopathologist is available for rapid on-site assessment to render a preliminary interpretation. A portion of the sample is smeared and then stained with Diff-Quik. If the slide is positive for non–small cell carcinoma, additional passes are requested and directly placed into CytoLyt solution to enrich the specimen for tumor DNA for downstream molecular analysis, if needed. Abbreviation: Pap, Papanicolaou.](image-url)
SELECTION OF CASES FOR MOLECULAR TESTING

We reflexively test all nonsquamous non–small cell lung carcinomas, including adenocarcinomas, combined adenocarcinomas and non–small cell carcinomas, or any other non–small cell carcinoma, not otherwise specified, for EGFR mutations and ALK gene rearrangements. If cytomorphologic features alone are not diagnostic of squamous cell carcinoma or adenocarcinoma, immunohistochemistry for thyroid transcription factor 1 (TTF-1), Napsin A, and p40 or p63 is routinely performed on Cellient cell block sections for further subtyping. All nonsquamous non–small cell carcinomas are then flagged for EGFR and ALK testing. Small cell carcinomas are tested for EGFR mutations if there is an adenocarcinoma component or in patients with known EGFR-mutated lung adenocarcinomas whose tumors have undergone small cell transformation.

ASSESSMENT OF SPECIMEN ADEQUACY FOR MOLECULAR TESTING

Because EGFR testing is performed on material derived from the cell pellet, adequacy for molecular testing is assessed on the corresponding ThinPrep slide, which is derived from the same sample. To avoid false-negative results, the specimen is deemed adequate for NGS testing if the tumor fraction is greater than 20%. We chose 20% as a cutoff value because that corresponds to an allele frequency of 10%, which is the lower limit of detection for NGS. Tumor fraction is estimated by assessing the proportion of tumor cells relative to all nucleated cells on the slide. In addition to assessing tumor fraction, we quantitate the number of groups of tumor cells to ensure that the specimen is adequately cellular; at least 20 groups of tumor cells are considered adequate cellularity. In cases with low tumor fraction and low tumor cellularity, testing is not performed, and an alternative sample is considered, if available.

EGFR MUTATION ANALYSIS

The use of the residual cell pellet material from liquid-based cytology specimens for EGFR mutational analysis was first validated in our laboratory using allele-specific polymerase chain reaction (PCR). In that study, even after using...
a portion of the cell pellet for cell block and ThinPrep slide creation, the quality of the remaining DNA obtained from the cell pellet was excellent; only 6 of the 228 cases (2.6%) tested in the clinical series failed analysis, and most of these because of insufficient DNA yield. This was a significant improvement compared with our prior experience of using cell block material for molecular testing, in which approximately a third of cases failed analysis.7

We switched to an NGS-based platform for evaluation of non-small cell lung cancer–related genes in 2015. With NGS sequencing technology, mutations in several clinically relevant genes, including EGFR, can be detected simultaneously using small amounts of DNA; the low input requirement of 10 ng of DNA is especially useful for analyzing cytology samples that may not yield larger amounts of DNA necessary for other testing platforms. Other groups have reported success with NGS on cytology specimens with material obtained from either smears or cell blocks.8,11,14

In our laboratory, the AmpliSeq Cancer Hotspot panel v2 (Thermo Fischer Scientific, Waltham, Massachusetts) is used with the Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, New York) to detect mutations in several hot spot regions in 50 oncogenes and tumor suppressor genes; currently, we only report mutations in EGFR, Kirsten rat sarcoma viral oncogene homolog (KRAS), and B-Raf proto oncogene (BRAF) on clinical samples (Figure 3). Next-generation sequencing testing on liquid-based cytologic samples was validated in our laboratory, and
the results were reported in a recent publication by Reynolds et al. In that study, EGFR mutation detection by NGS performed on the residual cell pellet of 20 EBUS-TBNA samples was successful and showed 100% concordance with results previously obtained by real-time PCR. Sequencing data were analyzed using NextGENe software (Soft Genetics, State College, Pennsylvania), and variants were confirmed using Torrent Suite software (version 4.2, Thermo Fischer Scientific). A total of 100 sequencing reads in genomic regions of interest was considered the minimum depth of coverage; mutations occurring at 5% to 10% allelic frequency could be identified. In the same study, 29 low-cellularity liquid-based cytology specimens yielding less than the recommended 10 ng of DNA were also tested by NGS; 18 of 29 (62%) were successfully sequenced with adequate depth of coverage in the non-small cell–related genes tested. Those that failed analysis were successfully tested by real-time PCR for EGFR mutations (Therascreen EGFR RGQ PCR kit, Qiagen, Hilden, Germany), thus providing a backup method for those liquid-based cytology specimens that do not achieve quality control criteria for NGS. If the cell pellet material from the liquid-based cytology specimen does not yield adequate DNA for mutational analysis, alternative available specimens are then tested; either the cell block or available surgical pathology material is used. This workflow model has been used successfully in our laboratory since 2015 (Figure 4). The results of NGS testing performed on our clinical non-squamous non–small cell lung cancer specimens in 2015 and 2016 are shown in Figure 5. Of a total of 1292 lung cancer specimens submitted for NGS, 1258 (97.4%) had sufficient quantity of DNA for successful DNA library preparation and sequencing. Most cases (686 of 1292; 53.1%) were EGFR, KRAS, and BRAF wild type, whereas 381 (29.5%) had KRAS mutations, 176 (13.6%) had EGFR mutations, and 15 (1.2%) had BRAF mutations. These results are similar to reported mutation rates in lung adenocarcinomas in Western patients.

**ALK GENE REARRANGEMENT ANALYSIS**

In our department, ALK FISH testing of liquid-based cytology specimens is directly performed on the existing...
ThinPrep slide using a dual-color ALK break-apart format FISH Probe Kit (Abbot Molecular Vysis, Des Plaines, Illinois). Areas with high tumor cellularity are marked on the reverse side of the slide before removal of the coverslip and application of 2 fluorescently labeled DNA FISH probes, which hybridize to the centromeric and telomeric segments flanking the ALK (2p23) gene. This method of specimen testing was validated in our laboratory; of 230 nonsquamous non–small cell lung carcinomas, ALK FISH testing on ThinPrep slides was successful in 228 (99.1%). Only 2 of the 230 cases (0.8%) tested showed no FISH signals, because of low specimen quantity/quality.13 In a subset of 154 cases with paired ThinPrep slides and adequately cellular cell blocks, ALK FISH performed on ThinPrep slides had a concordance of 98.7% compared with immunohistochemistry using the ultrasensitive ALK (D5F3) antibody (Ventana Medical Systems, Tucson, Arizona) performed on the corresponding cell block sections.13

In some cases, the ThinPrep slide has insufficient cellularity for ALK FISH testing. In such cases, we perform immunohistochemistry using the ultrasensitive ALK (D5F3) antibody either on the cell block material or on an available surgical pathology specimen. Positive immunohistochemical results are then confirmed by FISH (Figure 4).

**DISCUSSION**

Because there are no standardized guidelines for molecular testing on cytology specimens, cytopathologists must establish molecular testing strategies that work well for each individual laboratory. Given the diversity of cytopathologic specimen types and the different methods of fixation and processing available, options for procuring DNA from cytologic specimens for molecular analysis vary; material can be successfully obtained from cell blocks, smears, cytoswabs, and liquid-based preparations.7,9,11,12,14,19–23

Most commonly, cell blocks are used for molecular testing on cytopathologic specimens.21 The cell block closely approximates surgical pathology material; tissue fragments from cytopathologic specimens are embedded in paraffin and are sectioned akin to surgical specimens. Immunohistochemistry, PCR-based testing, and FISH can then be performed on the cell block material. However, cell blocks often suffer from low cellularity26,27 and may be inadequate for molecular testing, necessitating repeat biopsy procedures to obtain additional tissue. At our institution, cell block material was frequently paucicellular despite adequate cellularity on ThinPrep slides and smears, and failed analysis in a third of cases.7

Fortunately, cytology specimens often contain alternative sources of tumor DNA for molecular testing, such as direct smears or liquid-based preparations. The successful use of smeared slides to acquire DNA for EGFR testing has been described in several publications.9,11,28 Smears are decoverslipped, and tumor cells are removed from areas of the slide with a high tumor fraction by scraping with a scalpel or dissecting needle.31 Direct smears can also be used for ALK FISH testing.29 Although liquid-based cytology specimens are not commonly used for molecular testing of lung adenocarcinomas,21 a few groups have reported their utility.7,12,14,25

In our laboratory, we have found that the liquid-based cytology specimen is an excellent resource for EGFR mutation analysis and ALK FISH. We have implemented this method of molecular testing for all of our liquid-based cytology specimen types, including EBUS-TBNA, computed tomography–guided transthoracic fine-needle aspiration, peripheral fine-needle aspiration, and effusion cytology specimens. There are several advantages to using the residual cell pellet material for EGFR analysis compared with cell blocks or smears. First, cell blocks are not depleted, and smears are not sacrificed. The cell block material and smears can be saved with the archived specimen and retrieved if the specimen is needed for outside review or future molecular testing. Secondly, using the liquid-based specimen is less labor-intensive and therefore faster than obtaining material from smears or cell blocks, thus allowing for decreased turnaround time. In addition, the DNA quality is excellent and is often superior to formalin-fixed tissue derived from cell blocks. Because the liquid-based specimen can be stored without compromising DNA integrity, this method can be used in smaller cytopathology laboratories in which molecular testing may not be performed up front in all newly diagnosed non–small cell carcinoma patients. Advantages of using the ThinPrep slide for FISH testing include thin monolayer preparation with a clean background, allowing evaluation of whole nuclei without truncation artifacts that are often encountered from sectioning of paraffin-embedded cell block or surgical biopsy material.

Cytology specimens are ideally suited for NGS assays that require low-input DNA. However, in cases in which DNA quantity is not sufficient for NGS library preparation, backup testing methods should be employed to test for EGFR mutations. We have several backup options available, including attempting allele-specific PCR on residual cell pellet material or testing cell blocks and/or surgical pathology specimens. Often a transbronchial biopsy is obtained concurrently at the time of EBUS-TBNA for...
programmed death ligand-1 testing, and it can be used for EGFR and ALK testing if needed. If a surgical pathology specimen is not available, a follow-up procedure may be necessary to obtain material for molecular testing.

The success of molecular testing, regardless of the preparation type used, ultimately resides in the amount of tumor obtained at the time of the procedure. Although the minimum cellularity threshold differs depending on the sensitivity of the assay used, the specimen must be assessed for adequate cellularity before submission for EGFR or ALK testing. We have found that rapid on-site assessment by the cytopathologist has been invaluable in improving both quality and quantity of the specimen obtained. In addition to providing a preliminary interpretation, assessment of tumor viability and cellularity is helpful information to provide to the bronchoscopist. If a tumor is extensively necrotic, for example, sampling of a different site may help to increase the yield of viable tumor. Similarly, communicating the approximate cellularity to the clinician will provide useful information to guide further sampling. Rapid on-site assessment by the cytopathologist and effective communication between the cytopathologist and clinician are of utmost importance in ensuring that enough material is present for molecular testing.

CONCLUSIONS

Now more than ever, obtaining adequate material for molecular testing of non–small cell lung carcinomas is crucial. We have found that liquid-based cytology specimens are an excellent resource for high-quality DNA for EGFR mutation testing, by both allele-specific PCR and NGS, and that ALK FISH testing can be successfully done on ThinPrep slides prepared from the same sample. This testing strategy provides clinically useful information from material that may otherwise be discarded after preparation of a cell block, while preserving smears or cell blocks for future testing, and may be especially useful in institutions in which lung cancer is routinely diagnosed by cytology. The cytopathologist’s role in ensuring specimen adequacy for both diagnostic and ancillary testing at the time of specimen acquisition is critical.

References