Cytology Workshop #3

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Disclosure information

“The speaker has no relationship that represents a possible conflict of interest with respect to the content of this presentation.”
Background
Lung carcinoma is the leading cause of cancer mortality for both genders in the United States and throughout the world. Cytologic techniques are usually part of the initial workup of patients with suspected pulmonary malignancy and are commonly used for both diagnosis and staging. Opportunistic infections, specific inflammatory processes and some benign neoplasms may also be diagnosed cytologically and often mimic malignancy clinically. Advances in bronchoscopic techniques together with improved diagnostic methods have resulted in the ability to do more with progressively smaller specimens. Endobronchial ultrasound-guided FNA (EBUS) is a relatively new procedure that relies heavily on cytology to maximize diagnostic results. Cytologists play an important role in on-site assessment of specimen adequacy, sample triage and final diagnosis of the case including appropriate therapy-related molecular testing. Ensuring a cellular, adequate sample, and using a combination of cytomorphologic features and results of ancillary studies including immunohistochemistry and flow cytometry make a definitive, specific diagnosis possible in the majority of cases.

Endobronchial Ultrasound-Guided (EBUS) FNA
Accurate staging of lung cancer is critical, as stage of disease dictates choice of therapy and prognosis. Currently, more than 75% of new lung cancer patients present with local or distant metastases. Mediastinal lymph nodes are the most common site of involvement. Surgery is most appropriate for patients with disease confined to the lung and hilar lymph nodes. Patients with metastases in mediastinal lymph nodes are generally treated with chemotherapy, radiation therapy, or both. A subset of these patients may also benefit from surgery at some point in their course. Clinical staging of patients may differ significantly from pathologic staging. Staging with radiologic methods alone, using CT or PET is associated with high rates of false positive and false negative diagnoses, respectively. Currently, combination PET/CT is the most sensitive and specific method, with reported sensitivity of 60-85%, and specificity of 84-94%. The American College of Chest Physicians recommends invasive staging with tissue confirmation of suspected metastatic mediastinal lymph nodes. Mediastinoscopy is the current gold standard for evaluation of mediastinal lymph nodes. Recently, less invasive methods with reduced morbidity and complications have emerged as promising potential alternatives.

Perhaps the most promising non-invasive staging technique is endobronchial ultrasound-guided (EBUS) FNA. This method is increasingly used to stage patients with non-small cell lung cancer. Other uses include sampling central lung tumors and sampling lymph nodes in patients with mediastinal adenopathy. It can be performed in a bronchoscopy suite or operating room with patients under conscious sedation or general anesthesia.
Anesthetic choice does not appear to alter sensitivity or accuracy of the staging results. In this technique, a bronchoscope has an ultrasound transducer at the tip and a video processor, allowing real time optical and ultrasound images. As in conventional bronchoscopy, airway anatomy and associated abnormalities are evaluated using the fiberoptic component. On ultrasound, using a linear scanning probe, wedge-shaped images of structures in the mediastinum adjacent the major airways may be seen. Integrated color Doppler allows identification of blood vessels in the vicinity. These real time images guide the biopsy procedure. The needle is directly observed in the lymph node or mass to be aspirated. Fine needle aspiration of the target is performed through the working channel of the bronchoscope with a 22 gauge needle. Multiple nodal stations in the hilum and mediastinum may be sampled. In general, 3-4 passes per lymph node are obtained unless the earlier passes are diagnostic of malignancy. Diagnostic yield appears to plateau after the third pass.

EBUS FNA is more sensitive than traditional transbronchial FNA (TBNA) and allows sampling of nodal stations not accessible by TBNA. Transbronchial FNA is performed using pre-procedural imaging studies to determine the likely location of the node or mass to be sampled. Compared to EBUS-FNA, it is a relatively “blind” procedure. Sensitivity of TBNA depends on size and location of the targets. Several studies have compared the sensitivity and specificity of TBNA and EBUS-FNA and have shown superior results with EBUS-FNA. Stoll and colleagues report a sensitivity of 54.5% and 85.2% for TBNA and EBUS-FNA, respectively in lymph node aspirates with 100% specificity for both. Herth and colleagues studied a large series of EBUS cases and reported 94% sensitivity and 100% specificity with a low risk of complications. Endobronchial bleeding is the most common complication, which can usually be controlled with suctioning. Another advantage of the procedure is the cost. EBUS is estimated to be approximately one third of the cost of mediastinoscopy. There are few contraindications to the biopsy procedure including coagulopathy, respiratory failure and uncontrollable coughing. Thus, EBUS is useful in many patients who are not candidates for surgery.

**Rapid On-Site Evaluation of Specimen Adequacy (ROSE)**

Rapid on-site evaluation (ROSE) of specimen adequacy by cytologists increases the value of the EBUS procedure for patients. It allows additional passes or needle redirection in the event of a non-diagnostic sample. Conversely, ROSE provides an opportunity to stop sampling when the diagnostic goal is achieved. A preliminary diagnosis may be rendered to guide urgent therapy when indicated. Another advantage of ROSE is the ability to immediately triage the aspirate sample for ancillary testing. Additional material for flow cytometry, microbiologic cultures or cell blocks for immunohistochemistry and/or molecular testing may be obtained which often results in a more accurate and meaningful final diagnosis. At our institution, two smears are prepared from each FNA pass and the needle is rinsed in Cytolyte® or, if a lymphoproliferative disorder is suspected, RPMI. One of the paired smears is stained with a Diff-Quik® stain for ROSE. The second smear is fixed in 95% ethanol for subsequent Papanicolaou staining. Material for cell blocks is routinely procured. Cell blocks are useful in providing material for microbiologic stains and immunohistochemistry. They are also helpful in the
setting of non-diagnostic sample by ROSE due to excess blood. The cell block may provide the diagnostic material in these cases.

The EBUS procedure is not without challenges. Early in the EBUS experience, there is a learning curve for clinicians and pathologists. Communication is essential and the two groups need to work together to develop an integrated process. Depending on experience, cytologists may have to adjust to a new stain or to the rapid pace of the procedure. False positive diagnoses may occur, especially early on. Over-interpretaion of bronchial cell contamination, reactive bronchial cells, granulomas or smear preparation artifact may cause a false positive result. False negative interpretations may be due to cytologically bland neoplasms, such as carcinoid neoplasms, low grade lymphoproliferative disorders, or sampling error. Adequacy criteria for EBUS samples are not well defined and a non-diagnostic test should not be considered negative. The presence of lymphoid cells improves the negative predictive value of a benign EBUS-FNA. Numeric criteria for an adequate lymphoid sample have been proposed but are difficult to apply. It is generally agreed upon that the presence of at least moderate numbers of lymphocytes and/or pigmented macrophages is required to conclude that a lymph node has been sampled. A description of the lymphoid component should be included in the final report of lymph node aspirates obtained by EBUS that are not otherwise specifically diagnostic, keeping in mind that even a lymphoid rich aspirate may be subject to sampling error.

The ROSE procedure is time consuming for cytopathology with inadequate remuneration for time spent. The time for the procedure ranges between 12 and 22 minutes per site sampled. The average cytology time per procedure at our institution is between 45 and 60 minutes. Since initiating ROSE, procedural time has increased with sampling of additional lymph node stations and use of general anesthesia. Intra-procedural adequacy assessments are insufficiently compensated at this time by Medicare compensation schedules (CPT code 88172). Pathology costs for this evaluation exceed reimbursement. However, the cost of the EBUS procedure with ROSE remains far below the cost of mediastinoscopy.

The success of the EBUS-FNA procedure requires close communication between the cytology team and the bronchoscopy team. The cytopathologist must have access to clinical information and imaging results. The goal of the cytopathologist is to obtain a representative cellular sample from the target lesion to allow ancillary testing when needed in order to arrive at an accurate and complete final diagnosis that will direct patient management and prognosis.

**Importance of Definitive Classification in Lung Carcinoma**
Transbronchial fine needle aspiration and EBUS-FNA are accurate and sensitive procedures for the diagnosis and staging of lung carcinoma. While the cytologic material available from the samples may be limited when compared to surgical transbronchial biopsies, maximizing this material for definitive diagnosis is important in regard to patient treatment and prognosis. Prior to 2004, there was a two-pronged approach to the treatment of lung carcinoma, with small cell carcinoma patients receiving chemotherapy and non-small cell carcinoma patients receiving surgery and/or
chemotherapy, dependent upon stage. However, in 2004, new targeted therapies were approved for specific subtypes of non-small cell lung carcinoma which now necessitate a more refined cytologic diagnosis.

Use of Stains to Distinguish Types of Lung Carcinoma
Both special stains and immunostains can assist in the definitive classification of lung carcinomas. By morphology alone, it may be difficult to distinguish between certain types of lung carcinomas, such as basaloid squamous cell carcinomas and small cell carcinomas, while treatments options vary greatly. Likewise, the distinction between poorly-differentiated non-small cell carcinomas may be difficult solely by cytomorphology. Discussed below are examples of both special stains and immunostains that can assist in further classifying lung carcinomas.

A diastase resistant periodic acid Schiff (D-PAS) stain can be used to identify neutral mucin in non-small cell lung carcinomas (NSCLC) in cell block material. A positive mucin stain would help to confirm adenocarcinoma and exclude squamous cell carcinoma. While a special stain for mucin is quite specific, it is not a very sensitive marker for mucin-producing cells. In addition, both benign bronchial epithelial cells and adenocarcinomas can be positive for mucin, thus necessitating a definitive malignant cytologic diagnosis prior to staining.

Immunocytochemical stains can also assist in definitively distinguishing the various types of lung carcinoma. These stains include TTF-1, p63, 4A4 (anit-p63) and 34ßE12. Thyroid transcription factor – 1 (TTF-1) is a 38kDa nuclear protein that regulates gene transcription in the thyroid gland, lung, and diencephalon. It stains normal and neoplastic thyroid follicular epithelial cells as well as pulmonary epithelium. Pathologists use antibodies to TTF-1 most frequently in the evaluation of epithelial lesions of lung or thyroid origin. As a nuclear stain, it is positive in approximately 80% pulmonary adenocarcinomas and the majority of small cell carcinomas. In contrast, pulmonary squamous cell carcinomas are only rarely positive for TTF-1.

p63 is a nuclear transcription factor that regulates cell growth and development. As a member of the p53 oncogene family, it shares high structural homology with p53. Nuclear staining for p63 supports squamous differentiation in pulmonary non-small cell carcinomas as it positive in the vast majority of squamous carcinomas, while less than one-third of adenocarcinomas are positive. 4A4 (anit-p63) is monoclonal antibody and also a nuclear stain which may have less background staining. The 34ßE12 antibody consists of a subset of high molecular weight cytokeratins (CK’s 1,5,10, and 14) that reacts within the cytoplasm of squamous, ductal and other complex epithelia. While squamous cell carcinomas are almost always positive with this marker, about a third of adenocarcinomas are also positive. In contrast, CK 7 can be positive in all lung carcinomas, including small cell carcinoma, and will not help to distinguish between adenocarcinoma and squamous cell carcinoma. The particular combination of immunostains needed to adequately classify a lung carcinoma should be ascertained following the differential diagnosis based upon cytomorphologic features.
Importance of Molecular Diagnostics in Non-Small Cell Lung Carcinoma

As we continue to learn more about the molecular genetic basis for certain lung carcinoma types as well as the implications of tumor molecular heterogeneity, there is a movement to treat patients with personalized therapy that targets the molecular biology of these tumors\textsuperscript{20}. Molecular diagnostic testing is being increasingly requested in patients with certain types of non-small cell lung carcinoma, in particular testing for epidermal growth factor receptor (EGFR)\textsuperscript{25} and for the transforming echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (EML4-ALK) fusion gene.

EGFR is a tyrosine kinase receptor of the ErbB family that is overexpressed in many human carcinomas, including NSCLC. EGFR has extracellular, transmembrane and intracellular kinase domains. In 2003 and 2004, the FDA approved two EGFR directed-tyrosine kinase inhibitors (TKI) in clinical trials of advanced, chemo-refractory NSCLC, gefitinib (Iressa) and erlotinib (Tarveca). These are non-chemotherapy agents and are small molecules that inhibit the tyrosine kinase activity of EGFR by blocking cell proliferation, inducing apoptosis and reducing angiogenesis. Both gefitinib and erlotinib have demonstrated the greatest response in those patients who have tumors harboring somatic EGFR mutations\textsuperscript{28,30}. In fact, a clinical trial of first-line gefitinib treatment in patients with advanced NSCLC containing somatic EGFR mutations showed a response rate of 55% with good tolerance for the therapy\textsuperscript{24}.

EGFR mutations are present in 10-40\% of pulmonary adenocarcinomas and tumors of this subtype are the most sensitive to the EGFR directed-tyrosine kinase inhibitors\textsuperscript{19}. The two most common mutations are present on chromosome 7 with an in-frame deletion at exon 19 or the L858R point mutation at exon 21 (leucine substitutes for arginine at codon 858). These two mutations comprise approximately 90\% of all EGFR mutations in NSCLC\textsuperscript{18}. The clinical correlates of the tumors harboring EGFR mutations are East Asian female never smokers with lung adenocarcinomas, particularly those with at least some bronchioloalveolar morphology\textsuperscript{20}.

Several studies have shown that screening for these somatic EGFR mutations in adenocarcinomas can be performed by EGFR sequencing and accurately reported in cytologic samples\textsuperscript{23,26}. Therefore, it is important to appropriately triage samples based upon the cytologic diagnosis for further EGFR molecular testing.

The EML4-ALK fusion transcript has been identified in a small subset of non-small cell lung carcinomas, reported in between 3 and 7\% of patients\textsuperscript{16,27}. The fusion occurs on chromosome 2p, where one of the two genes can become inverted and fuse with the other gene. EML4-ALK mRNA can be detected by reverse transcription-polymerase chain reaction (RT-PCR) and the protein product of the fused gene has also been confirmed immunohistochemically within the tumor cell cytoplasm. Interestingly, the NSCLC patients with the EML4-ALK fusion gene were not the same as those harboring EGFR mutations and comprise a discrete subset of patients\textsuperscript{16,27}. Histomorphology of the adenocarcinomas has also been statistically linked to the particular variant gene fusions, with EML4-ALK variant 1 demonstrating mixed type adenocarcinomas with papillary and bronchioloalveolar components and the variant 2 types being acinar.
adenocarcinomas. These investigators studying the detection of the EML4-ALK fusion gene in NSCLC have the ultimate goal of providing additional targeted treatment approaches based on the individualized molecular features of a NSCLC tumor type.

In summary, the pathologist needs to create a clear and concise final cytopathology report in patients with pulmonary carcinomas. The distinction between non-small cell lung carcinoma and small cell carcinoma is no longer enough information for the treating physician. It is increasingly important to classify non-small cell carcinomas as either adenocarcinomas or squamous cell carcinomas whenever possible, to help direct further diagnostic testing or provide the physician with an opportunity to direct treatment options. Cytomorphologic features can be used first to classify cases; however, stains may help to further clarify origin if cytologic distinction is difficult. Finally, patients with adenocarcinomas can be further triaged to EGFR testing or EML4-ALK testing of their samples to help predict a therapeutic response.

References: EBUS and ROSE


References: Cytochemistry, Immunocytochemistry and Molecular Diagnostics


