Utilization of Ancillary Studies in the Cytologic Diagnosis of Respiratory Lesions: The Papanicolaou Society of Cytopathology Consensus Recommendations for Respiratory Cytology

[1]Lester J. Layfield, MD, [2]Sinchita Roy-Chowdhuri, MD, PhD, [3]Zubair Baloch, MD, PhD, [4]Hormoz Ehya, MD, [5]Kim Geisinger, MD, [6]Susan J. Hsiao, MD, PhD, [7]Oscar Lin, MD, PhD, [8]Neal I. Lindeman, MD, [9]Michael Roh, MD, PhD, [10]Fernando Schmitt, MD, PhD, [11]Nikoletta Sidiropoulos, MD

[1]Department of Pathology and Anatomical Sciences, University of Missouri, Columbia, MO; [2]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX; [3]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA; [4]Department of Pathology, Fox Chase Cancer Center, Temple University, Philadelphia, PA; [5]Department of Pathology, University of Mississippi, Jackson, MS; [6]Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY; [7]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY; [8]Department of Pathology, Harvard Medical School, Boston, MA; [9]Department of Pathology, University of Michigan School of Medicine, Ann Arbor, MI; [10]Laboratoire National de Sante, Luxembourg; [11]Department of Pathology and Laboratory Medicine, The University of Vermont, Burlington, VT

Address correspondence to:
Lester J. Layfield, MD
Professor and Chair
Department of Pathology and Anatomical Sciences
University of Missouri
One Hospital Drive, M263 Medical Sciences Building
Columbia, MO 65212 USA

Email: layfieldl@health.missouri.edu
Phone: (573) 882-1201
Fax: (573) 884-4612

Accepted Article

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version record. Please cite this article as doi:10.1002/dc.23549.

This article is protected by copyright. All rights reserved.

Running title: Consensus Recommendations for Ancillary Testing of Lung Specimens

Accepted Article

This article is protected by copyright. All rights reserved.

Abstract

The Papanicolaou Society of Cytopathology has developed a set of guidelines for respiratory cytology including indications for sputum examination, bronchial washings and brushings, CT-guided FNA and endobronchial ultrasound guided fine needle aspiration (EBUS-FNA), as well as recommendations for classification and criteria, ancillary testing and post-cytologic diagnosis management and follow-up. All recommendation documents are based on the expertise of committee members, an extensive literature review, and feedback from presentations at national and international conferences. The guideline documents selectively present the results of these discussions.

The present document summarizes recommendations for ancillary testing of cytologic samples. Ancillary testing including microbiologic, immunocytochemical, flow cytometric, and molecular testing, including next-generation sequencing are discussed.

Keywords: immunocytochemistry, flow cytometry, molecular diagnostics, next-generation sequencing, cytology

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Committee IV

Ancillary Testing for Respiratory Cytology

With the advent of targeted therapy for lung cancer, ancillary testing of specimens derived from the lower respiratory tract has obtained greater importance. Traditionally, ancillary testing was confined to culture techniques for microbiologic organisms, flow cytometry for lymphoid proliferations, and immunohistochemical and histochemical stains to aid in classification of pulmonary neoplasms. Targeted therapeutic options have expanded the need for ancillary testing and in particular molecular testing, to document the presence or absence of clinically relevant genetic alterations that include: single nucleotide variants, insertion/deletions, copy number variations, and structural variants that indicate a carcinoma's susceptibility to specific drug therapies (personalized medicine).

Some of the ancillary testing methods require dedicated transfer media or special preparation methods for optimal test performance. Rapid on-site evaluation (ROSE) allows intraprocedural cytologic evaluation with identification of the pathologic process and appropriate triage of material for specialized testing including microbiologic culture, flow cytometric evaluation, and molecular testing. Transport media requirements for microbiologic culture have been published.[1] Direct placement of aspirated material into RPMI (Roswell Park Memorial Institute) medium is a standard approach for the submission of a cytologic sample for flow cytometry testing.[2] While smears, liquid based preparations, and cell block preparations can all be used for immunohistochemistry and most molecular testing, formalin fixed and paraffin embedded (FFPE) cell block material is preferred by some laboratories.[3-8] FFPE cell block material is conventionally most convenient from a molecular pathology validation standpoint because this specimen type most closely approximates how small biopsies are processed and hence does not need its own validation process which can be cumbersome. The best preparation for immunocytochemistry (ICC) appears to be formalin-fixed, paraffin-embedded tissue (FFPE). A recent

This article is protected by copyright. All rights reserved.

study demonstrated problems with validation in nearly one half of antibodies tested using the Cellient cell block system (fixed in PreservCyt).[9] The antibodies had been previously validated for formalin-fixed., paraffin-embedded specimens. These findings bring into question the utility of methanol-fixed material for ICC and the probable need for revalidation of antibodies when methanol-fixed material is used. Other types of specimens should be individually validated and require libraries of controls for optimum validation.

Accepted Article

Microbiologic Culture

The lung is a favored site for localization of a number of infectious processes. These infectious agents can produce diffuse changes (lobar pneumonia) or more localized changes including abscesses and granuloma. A large number of infectious agents including viruses, fungi, bacteria, and mycobacteria may be responsible for pulmonary disease. Viral infections often have characteristic cytopathic effects facilitating their recognition. While viruses are rarely investigated by culture methods, other infectious agents are best definitively identified by a variety of culture techniques.

Culture of pulmonary specimens represents an important component of examination for many sputum, bronchoalveolar lavage and fine needle aspiration (FNA) specimens. Culture for acid fast bacilli, fungi and bacteria is often the most sensitive technique for establishing a specific diagnosis. Successful culture of a number of organisms requires specialized techniques and media. Optimal transport media have been identified for lymph node aspirates[1] and similar techniques and media can be used for pulmonary FNA specimens. ROSE examination may be helpful in selecting if and in what media material should be sent for culture.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Recommendation 1: Cytologic specimens from lesions suspected to be of infectious etiology should be sent for culture in appropriate transport media. Rapid on-site evaluation may be helpful in selecting the appropriate culture techniques.

Immunocytochemistry

Immunocytochemistry is an important diagnostic technique for the identification and classification of metastatic disease. The separation of primary adenocarcinoma from squamous cell carcinomas and the identification of small and large cell neuroendocrine carcinomas and carcinoid tumors may be facilitated by immunocytochemistry.

With the development of targeted therapy for pulmonary adenocarcinomas, the separation of adenocarcinomas from squamous cell carcinoma has become critically important.[10] Panels of antibodies have been found useful for this distinction. Immunohistochemistry is also of aid in determining the primary site of origin for metastatic disease. The use of differential cytokeratin staining and direction of differentiation markers can be particularly helpful in establishing the site of origin for a metastatic neoplasm. Finally, immunocytochemistry can be used for prediction of response to tyrosine kinase inhibitors and to identify targets for immunotherapy such as PD-L1.

Immunochemistry is important in the evaluation of neuroendocrine tumors. The World Health Organization guidelines require evaluation of Ki-67 index for classification of these neoplasms.[11] Enumeration of Ki-67 staining can be performed on cell block material.[12,13]

Antibodies Useful In Separating Pulmonary Adenocarcinomas from Squamous Cell Carcinomas

CK5/6, TTF-1, napsin A, p63, and p40 have been identified as helpful for separating pulmonary adenocarcinoma from squamous cell carcinoma.[10, 14-24] These antibodies can be used on smears, liquid

John Wiley & Sons
This article is protected by copyright. All rights reserved.

Accepted Article

based preparations, and formalin-fixed paraffin embedded cell block material. A recent study has questioned the reliability of the use of immunocytochemistry on methanol-fixed cell block material.[9]

TTF-1 reacts with cells differentiating towards follicular thyroid epithelium,[25] some gastric adenocarcinomas,[26] and some pulmonary adenocarcinomas and small cell carcinomas.[27,28] The sensitivity and specificity of TTF-1 for the separation of pulmonary adenocarcinoma from squamous cell carcinomas are approximately 85%[17,29] and 97%[29] respectively.

Napsin A has been shown to be reactive with pulmonary epithelium[20] and some neoplasms arising primarily within the ovary and other sites.[30] The sensitivity and specificity for napsin A in separating pulmonary adenocarcinomas from squamous cell carcinomas are 85% and 94% respectively.[17]

p63 is a marker helpful in distinguishing squamous cell carcinomas from adenocarcinoma.[29,31] While p63 is reactive in up to one third of pulmonary adenocarcinomas,[10] it remains useful for separating squamous carcinomas with a sensitivity and specificity of 100% and 85% respectively for the recognition of squamous cell carincoma.[31]

p40 appears more specific for squamous cell carcinomas than p63 and some authors have advocated its replacing p63.[10] p40 has a sensitivity of 94% and a specificity of 96% for squamous cell carincoma.[29] Other authors have also found p40 to be superior to p63 for the identification of squamous cell carcinomas of the lung.[32-34]

A number of authors have recommended panels of two to four immunohistochemical markers for the distinction of pulmonary adenocarcinoma from squamous cell carcinoma of the lung.[29,31-36] Potential panels include, (i) TTF-1, napsin A, p63, CK 5/6; (ii)TTF-1 and p40; (iii) napsin A and p40; or (iv)TTF-1 and p63. When performing immunohistochemistry for distinction of adenocarcinoma from squamous cell carcinoma it should be kept in mind that adequate material must be preserved for molecular testing. Core biopsy should be encouraged for back-up, especially when ROSE shows that the sample cellularity

This article is protected by copyright. All rights reserved.

Accepted Article

might be too low for analysis.[34] Kimbrell et al[37] addressed the issues concerning the utilization of immunocytochemistry in the sub-classification of non-small cell lung carcinomas.

Because diagnostic material must be saved for molecular testing, the use of double staining techniques should be considered when using immunocytochemistry for distinguishing squamous from adenocarcinomas. Johnson et al[38] has described a double staining method for TTF-1 and Napsin A.

Recommendation 2:

Pulmonary adenocarcinomas should be distinguished from squamous cell carcinomas. Use of immunohistochemical panels including some combination of TTF-1, napsin A, p63, p40 and CK 5/6 is recommended when significant cellular differentiation such as distinct keratinization is not seen. To preserve tissue for subsequent molecular testing, preferably one marker of adenocarcinoma and one of squamous cell carcinoma should be selected. Excessive immunostaining should be avoided to ensure preservation of cellular material for requested or anticipated molecular testing. The combination of TTF-1 and p40 appears optimal for separation of adenocarcinomas form squamous cell carcinoma.

Immunocytochemistry for Molecular Predictive Markers

While predictive testing for susceptibility to tyrosine kinase inhibitors is generally performed by molecular methods (FISH or PCR-based techniques), immunocytochemical methods exist for testing of some predictive markers. Immunocytochemical techniques have been used as initial tests which when positive, the specimen can be reflexed to a molecular laboratory for confirmatory testing. Antibodies directed against rearranged *ALK*,[39-41] *ROS1*, and mutated *EGFR*[42,43] are three such markers. However, the utility of this approach may be limited. Antibodies directed against mutated *EGFR* are limited to detecting specific mutations (15 base pair deletions in exon 19 and p.L858R mutation in exon 21) and fail

This article is protected by copyright. All rights reserved.

Accepted Article

to detect other *EGFR* mutations; while the *ALK* antibody may have false positive and false negative results.[43-46] Immunohistochemistry appears to be a useful screening technique for *ALK* rearrangements with subsequent reflex of positive results to FISH analysis. This approach using reflex testing reduces overall cost as the percentage of pulmonary adenocarcinomas with *ALK* alterations is low. Currently, the Food and Drug Administration (FDA) approved immunohistochemical method assessing *ALK* status uses the D5F3 antibody (Cell Signaling) with testing performed on the BenchMark XT instrument. This antibody detects expressed endogenous levels of total *ALK* protein (when present). Other antibody clones have been developed and are in clinical use.

Currently, immunohistochemistry may be a cost reduction technique for identification of molecular aberrations that occur at low frequency such as *ROS1*. A number of antibodies have been developed against *ROS1* and detect the inappropriately expressed endogenous protein. Such inappropriate expression of the endogenous protein has been shown to occur in approximately 1.6% of non-small cell carcinomas of the lung.[47] Antibodies raised against *ROS1* protein may represent a useful screening technique for *ROS1* rearrangements with subsequent reflex of positive results for FISH testing.[48]

Recommendation 3:

Immunocytochemical testing for mutated *EGFR* is not the preferred testing method for determination of tumor susceptibility to the associated tyrosine kinase inhibitors, but may be utilized in the setting of a limited volume sample when molecular testing cannot be performed.

Immunocytochemical testing for rearranged ALK may be used in place of FISH testing.

PD-L1 Immunocytochemical Testing

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Expression of Programmed Death Ligand-1 (PD-L1) is a predictive marker for anti-PD-1/PD-L1 therapies. PD-L1 is sometimes expressed in large amounts on cancer cells and allows their escape from immune surveillance and immune destruction.[49,50] A new class of drugs target PD-1 or PD-L1 and are reported to have activity against some malignancies including non-small cell lung cancers.[51,52] These drugs are useful for treatment of patients when standard chemotherapy has become ineffective. Nivolumab and pembrolizumab have been approved for treatment of non-small cell lung cancer, including both squamous cell carcinoma and adenocarcinoma. Section of the appropriate antibody clone for prediction of response to therapy depends on the drug selected. Testing protocols have been published.[52]

Recommendation 4:

Immunohistochemical testing for anti PD-1/PD-L1 therapy appears appropriate for some patients who have become refractory to standard chemotherapy regiments. Selection of the antibody used for testing depends on the specific anti PD-1/PD-L1 drug used. Immunocytochemical testing for PD-L1 in non-squamous, non-small cell pulmonary carcinomas may aid in the selection of targeted therapy. Detection of PD-L1 expressing carcinoma cells may indicate improved survival when patients are treated with Nivolumab therapy.[53] PD-L1 testing of cytology specimens has not undergone extensive validation in the published literature and specific recommendations fo its use for cytology material cannot be made at this time.

Accepted Article

John Wiley & Sons

This article is protected by copyright. All rights reserved.

PD-1/PD-L1 testing is performed at the discretion of the local oncology team and may be especially useful for patients non-responsive to tyrosine kinase inhibitor therapies. Treatment of squamous cell carcinoma with Nivolumab can be done without PD-L1 testing.

Immunocytochemical Testing for c-MET

Mesenchymal-epidermal transition (*MET*) receptor tyrosine kinase has been identified as a potential target for the treatment of non-small cell[54,55] and some drug resistant small cell lung cancers.[56] c-MET overexpression may be important in the development of resistance to EGFR-tyrosine kinase inhibitors[54] and some chemotherapeutic agents used for treatment of small cell lung cancer.[56] *MET* amplification occurs in up to 20% of non-small cell carcinomas refractory to *EGFR*-tyrosine kinase therapy and some studies have suggested utility for the combined use of drugs resulting in *MET* and *EGFR* co-inhibition.[54] The role of immunohistochemistry for the prediction of response of c-MET inhibitors has not yet been elucidated, but c-MET therapy based on immunohistochemical staining has shown promise.[57] MET testing may have potential value for predicting progression on targeted therapy (*EGFR*) but such testing is for MET amplification and is performed by FISH.

Recommendation 5:

Currently, MET testing cannot be recommended for routine use, but such testing can be performed at the discretion of the local oncology team.

Immunocytochemistry for the Identification and Classification of Metastatic Malignancies to the Lung

Accepted Article

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Metastatic disease is responsible for a significant percentage of lung nodules. While negative results of markers such as napsin A and TTF-1 may suggest a non-primary pulmonary adenocarcinoma, lack of staining for these markers does not exclude a lung origin, particularly in mucinous adenocarcinoma. A combination of cytomorphologic features and immunohistochemical reaction patterns is used to diagnose metastatic neoplasms. Differential cytokeratin staining patterns (CK7, CK20, and CK5/6) and specific lineage markers such as melan-A, HMB-45, prostatic specific antigen (PSA), leukocyte common antigen (LCA), p40, CDX-2, GATA-3, PAX-8, desmin, muscle specific actin (MSA), smooth muscle actin (SMA) and CD117 among others can be extremely helpful in the specific diagnosis of a metastatic neoplasm. Table I lists a selection of immunocytochemical markers helpful in the evaluation of metastatic neoplasms. Immunocytochemical testing of samples to determine tumor type or site of origin for metastases is a complex process with overlapping patterns of staining between pulmonary primaries and metastatic disease. For instance, some pulmonary adenocarcinomas may be CK20 positive (a finding often considered supporting a non-pulmonary origin). Similarly, CDX-2 a marker considered to support a gastrointestinal origin may be positive in some mucinous carcinomas of the lung. GATA-3, which usually supports a urothelial origin, may be expressed by some pulmonary squamous cell carcinomas. Thus, no single immunocytochemical staining result is definitively diagnostic for a specific type of neoplasm, but patterns of staining are helpful in assessing the organ of origin for metastatic disease.

Recommendation 6:

Selected panels of antibodies should be used to establish the origin and direction of differentiation in suspected metastatic disease to the lung. The precise panel of antibodies should be determined by

This article is protected by copyright. All rights reserved.

morphologic analysis of cytologic specimen and review of the patient's medical history and imaging findings.

Accepted Article

Molecular Techniques for Predictive Testing Associated with Targeted Therapy

The advent of a number of targeted therapies for lung cancer has placed new responsibilities on pathologists to perform molecular studies used to identify patients likely to respond to such therapies. Not only is the pathologist responsible for identifying which carcinomas should undergo testing, but he/she is responsible to ensure that adequate and representative material is present for ancillary testing. Presently, the majority of such targeted therapies are directed at adenocarcinomas (*EGFR*, *ALK,* and *ROS1*), but new therapies targeting squamous cell carcinomas are under development. The pathologist must make every attempt possible to obtain adequate tissue for molecular testing.[58,59] Proper tissue acquisition and management requires good communication between pulmonologist, radiologist and pathologist.[10] ROSE can be very helpful in assuring adequate tissue is collected when sampling is performed under endobronchial ultrasound (EBUS) or computerized tomography (CT) guidance. Since *EGFR, KRAS, ROS1,* and *ALK* testing can be reliably performed on formalin fixed paraffin embedded cell block material,[10,60-63] ROSE can be very useful in triaging material for processing to cell block.[63] The choice of the appropriate specimen type for molecular testing remains controversial. Studies have demonstrated that formalin fixed paraffin embedded cell block (CB) material is a satisfactory substrate for molecular testing[64] but cellularity of CBs remains a problem with up to 57% of CBs being acellular or of borderline cellularity for molecular testing.[65] Most studies have reported on the utility of formalin-sixed, paraffin-embedded surgical resection specimens or small biopsy specimens, but a number of studies have addressed the use of cytologic preparations.[66,67] These studies reviewed the use of CBs, direct smears, and liquid based preparations. Current data indicates that a variety of

This article is protected by copyright. All rights reserved.

cytologic preparations yield molecular testing results similar to those achieved with histologic samples.[67-70] In some cases, cytologic samples have been associated with detection rates for mutations higher than those obtained by histologic sampling methods.[70] Cell blocks have been shown to be comparable to core biopsies in some studies for detection of clinically important mutations.[71,72] A significant number of studies have shown that smears and CBs are equivalent for molecular testing.[68,73-75] In an analysis of 181 articles focusing on EGFR analysis in lung cancer, da Cunha Santos et al.[66] showed that cytologic techniques using an array of fixation and processing techniques yielded mutation detection rates similar if not superior to histologic material. Moreover, cell blocks, smears, and liquid based techniques were all useful for detection of clinically important mutations in non-small cell lung cancer.[66]

The majority of organizations and authors recommend molecular testing of pulmonary adenocarcinoma for *EGFR* and *ALK*.[75,76] In general, when tissue is limited, *EGFR* testing is prioritized and performed before *ALK*.[63,75] Additional markers that may be tested in pulmonary adenocarcinomas include *KRAS, ROS1, BRAF, RET*, *MET,* neurotrophic tyrosine kinase receptor type 1 (*NTRK1*) and v-erb-b2 erythroblastic leukemia viral oncogene homolog2 (*ERBB2*).[76,77] While no recommendations for prioritization of these markers have yet been published, some authorities recommend early performance of *KRAS* analysis. *KRAS* mutations appear to exclude mutations in *EGFR* and *EML4-ALK* and the *KRAS* gene product is present in the control pathway before *EGFR* and *EML4-ALK*, thus negating the impact of these latter two genes. With the recent FDA approval of crizotinib for treatment of *ROS1* rearranged tumors, immunocytochemical or FISH testing for *ROS1* rearrangements should now be performed following negative results for *EGFR* and *ALK* testing.

Molecular testing for pulmonary squamous cell carcinoma remains in its infancy with few markers yet having clinical utility. Potential target genes include: fibroblast growth factor receptor 1 (*FGFR1*) and the related amplification in *FGFR2, FGFR3*, and *FGFR4*.[78] Other potential targets are phosphatase and tensin

John Wiley & Sons
This article is protected by copyright. All rights reserved.

Accepted Article

homolog (*PTEN*), platelet-derived growth factor receptor alpha (*PDGFRA*) and discoidin domain-containing receptor 2 (*DDR2*).[76,78]

EGFR Testing For Pulmonary Adenocarcinoma

EGFR mutation testing should be performed at time of diagnosis for patients presenting with high stage disease (stage III and higher).[75] Patients should not be excluded from such testing based solely on clinical features. Lower stage patients may be tested at time of recurrence or progression. Retesting of EGFR should be considered when new recurrences or metastases are detected to look for new mutations. Patients stage I, II, or III may be tested at the discretion of the local oncology team. Results of testing should be available within 10 working days.[75]

EGFR testing can be performed on formalin-fixed paraffin-embedded (FFPE) material (cell block/core biopsy), fresh, frozen, air dried smears, or alcohol fixed specimens for polymerase chain reaction (PCR) based testing.[67-72] While FFPE material is one of the most common sources of material for molecular analysis in many laboratories,[75,79] recent studies have shown that material obtained from air-dried and alcohol-fixed cytologic smears can be reliably used.[62,63,76,77] Sample adequacy for analysis should be confirmed by a pathologist. While specimens with 20% or greater cancer cells are prefered,[63] many laboratories have reported successful results using significantly lower concentration of cancer cells, provided the sample meets the analytic sensitivity of the platform used and with appropriate validation studies. The testing method should be able to detect mutations in *EGFR* exons 18, 19, 20 and 21[75] as the two most commonly observed *EGFR* mutations in pulmonary adenocarcinomas are the p.L858R substitutions and small frame deletions in exon19.[76] Currently immunohistochemistry is not recommended for selection of EGFR based therapy.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

KRAS Testing For Pulmonary Adenocarcinoma

KRAS mutational analysis may be helpful in therapy selection, but it should not be the sole method for *EGFR* therapy selection. *KRAS* codon 12, 13, 61, and 146 mutations can be tested for by PCR-based techniques and when present are thought to exclude mutations in *EGFR* and *ALK* rearrangements. Moreover, patients with *KRAS* mutations may have poor responses to both *EGFR* based therapy and more traditional chemotherapy justifying to some oncologists, the need for early *KRAS* testing. The predictive value of *KRAS* testing for response to anti-*EGFR* based therapy remains controversial. Some recent data indicates that up to 3% of patients with *KRAS* mutations will respond to anti-*EGFR* therapy.[80] This finding brings into question *KRAS* testing as a guide for response to anti-*EGFR* therapy.

ALK Rearrangement Testing for Pulmonary Adenocarcinoma

ALK rearrangement testing should be performed on all high stage lung adenocarcinomas at time of diagnosis and on lower stage patients at time of recurrence or progression. Testing may be performed on FFPE material, but can also be performed on smears. In general, the slide should contain 50 cancer cells suitable for evaluation. When material is insufficient for both *EGFR* and *ALK* rearrangement testing *EGFR* testing has priority. The FISH method appears optimal for *ALK* rearrangement testing.[41] Screening for *ALK* rearrangements may be performed by immunocytochemistry with reflex to FISH for positive results.

Recommendation 7:

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

EGFR mutation testing should be performed at time of diagnosis for patients presenting with high stage disease. Testing of stage I, II and III patients may be performed at the discretion of the local oncology team. Reflex testing to *ALK* should be performed when *EGFR* mutational analysis is negative. Cell block and smears can be used for testing following appropriate validation of this specimen type.

Recommendation 8:

KRAS testing remains controversial and may be performed on pulmonary non-small-cell carcinomas at the discretion of the local oncology team.

ROS1 Testing for Primary Pulmonary Adenocarcinoma

ROS1 is a receptor tyrosine kinase phylogenetically related to *ALK*.[81,82] *ROS1* rearrangements are seen in 1 to 2.5% of non-small cell carincoma[83,84] and are mutually exclusive with other tyrosine kinase genetic abnormalities. Patients with *ROS1* rearrangments appear to benefit from crizotinib therapy.[85] Since *ROS1* rearranged pulmonary adenocarcinomas are rare, only patients lacking changes in *EGFR, KRAS*, and *ALK* should be tested for *ROS1*.[76]

Recommendation 9:

FISH testing for *ROS1* rearrangements may be performed at the discretion of the local oncology team in high stage pulmonary adenocarcinomas who have been shown to lack molecular/genetic changes in *EGFR, ALK,* and *KRAS*. Immunocytochemical testing for *ROS1* can be an acceptable alternative to FISH.

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Next-Generation Sequencing and Molecular Analysis of Pulmonary Carcinoma

In an era of precision medicine with new emerging biomarkers guiding therapeutic decisions in lung cancer, there is an increasing need for a more comprehensive approach to generate a complete molecular profile for cytologic specimens. Clinical next-generation sequencing (NGS) has allowed for simultaneous screening of multiple genes in a massively parallel manner from cytologic samples using very small amounts of DNA. Several recent studies have described the utility of NGS mutational analysis in pulmonary cytopathology utilizing both aspiration cytology (CT-guided FNA and EBUS-guided FNA)[86-90] as well as exfoliative cytology (body cavity fluids and bronchoalveolar lavage) specimens.[89,91,92] The high analytical sensitivity of NGS platforms (approximately 5-10%) permits evaluation of samples with relatively low tumor fraction.[87,91,93,94] One of the earlier studies utilizing NGS on cytologic specimens demonstrated the high sensitivity of NGS compared to conventional testing methods by using cells extracted from Papanicolaou stained smears of low tumor volume samples of bronchoalveolar lavage and pleural fluids.[91] NGS success rates do not vary significantly between aspiration and exfoliative cytology samples and NGS success rates of lung FNAs are similar to that of other sites.[89]

The most common causes of NGS failure in lung cytologic specimens are related to insufficient DNA yield, due to overall low cellularity, or insufficient tumor cellularity, in cases with high numbers of non-tumor background cells and/or where the tumor cells are heterogeneously distributed, frequently seen in EBUS FNA, samples obtained by brushing, or body cavity fluid samples.[88] Although aspiration cytology specimens usually provide inherently high tumor fraction due to lower numbers of background stromal components, they are frequently limited by the volume of tissue aspirated (overall cellularity) that dictates the quantitative DNA yield.[89,93] Implementing measures such as ROSE for adequacy assessment and additional needle passes may improve the overall cellularity to meet the input DNA requirement for

This article is protected by copyright. All rights reserved.

the NGS assay.[95] Also, tumor enrichment techniques by demarcating on the H&E stained cell block section or directly on the smear slides for macro/microdissection can improve the tumor fraction by eliminating background non-tumor cells, thereby reducing the chances of a false negative result.[96]

Cell block preparations as well as direct smears and liquid-based cytology (LBC) have been successfully employed for NGS.[86-90,92] The input DNA requirement for NGS analysis varies by testing platform but ranges between 10 ng for Ion Torrent PGM (Life Technologies, Carlsbad, CA) and 250 ng for Illumina (Illumina Inc, San Diego, CA) platforms.[97] Depending on the target capture method and the platform type, this roughly translates to approximately 100 to 1000 cells for Ion Torrent and 5000-15,000 cells for Illumina NGS.[98] There is no significant difference in NGS success rates between specimen preparations or cytological stains/fixatives.[88] The NGS sequencing performance metric (coverage depth, total number of reads, number of mapped reads and on-target reads, and variant calls) of cytologic smears and cell block preparations are comparable to that of FFPE histologic specimens.[88,90] In addition, NGS has been successfully performed using DNA extracted from residual LBC samples of FNA and body fluid samples with significantly higher DNA yield from the LBC rinse than the matched cell block sections.[92]

Molecular diagnostics in lung cytopathology has undergone a paradigm shift with the advent of NGS which allows for evaluation of multiple biomarkers in a single assay using minimal amounts of DNA.[97-99] In this changing landscape of precision medicine, the cytopathologist plays a critical role in triaging and selecting suitable material for successful implementation of NGS on lung cytologic specimens. However, one needs to keep in mind that NGS not only detects known driver mutations for specific targeted therapy, but will likely also identify passenger mutations and/or low level mutations in small subclonal populations, and variants of unknown significance (VUS), all of which need to be interpreted in the proper clinical context for subsequent clinical management. In the absence of “actionable” oncogenic mutations, these may have some relevance in the prospective enrollment of patients in pre-clinical or clinical trials for targeted therapy.[100,101]

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

John Wiley & Sons
This article is protected by copyright. All rights reserved.

REFERENCES

1. Layfield LJ, Glasgow BJ, DuPuis MH. Fine-needle aspiration of lymphadenopathy of suspected infectious etiology. Arch Pathol Lab Med. 1985 Sep;109(9):810-2.

2. Shetuni B, Lakey M, Kulesza P. Optimal specimen processing of fine needle aspirates of non-Hodgkin lymphoma. Diagn Cytopathol. 2012 Nov;40(11):984-6.

3. Rekhtman N, Brandt SM, Sigel CS, Friedlander MA, Riely GJ, Travis WD, Zakowski MF, Moreira AL. Suitability of thoracic cytology for new therapeutic paradigms in non-small cell lung carcinoma: high accuracy of tumor subtyping and feasibility of EGFR and KRAS molecular testing. J Thorac Oncol. 2011 Mar;6(3):451-8.

4. Navani N, Brown JM, Nankivell M, Woolhouse I, Harrison RN, Jeebun V, Munavvar M, Ng BJ, Rassl DM, Falzon M, Kocjan G, Rintoul RC, Nicholson AG, Janes SM. Suitability of endobronchial ultrasound-guided transbronchial needle aspiration specimens for subtyping and genotyping of non-small cell lung cancer: a multicenter study of 774 patients. Am J Respir Crit Care Med. 2012 Jun 15;185(12):1316-22.

5. Betz BL, Dixon CA, Weigelin HC, Knoepp SM, Roh MH. The use of stained cytologic direct smears for ALK gene rearrangement analysis of lung adenocarcinoma. Cancer Cytopathol. 2013 Sep;121(9):489-99.

6. Harada S, Agosto-Arroyo E, Levesque JA, Alston E, Janowski KM, Coshatt GM, Eltoum IA. Poor cell block adequacy rate for molecular testing improved with the addition of Diff-Quik-stained smears: Need for better cell block processing. Cancer Cytopathol. 2015 Aug;123(8):480-7.

7. van der Heijden EH, Casal RF, Trisolini R, Steinfort DP, Hwangbo B, Nakajima T, Guldhammer-Skov B, Rossi G, Ferretti M,Herth FF, Yung R, Krasnik M; World Association for Bronchology and Interventional Pulmonology, Task Force on Specimen Guidelines. Guideline for the acquisition and preparation of conventional and endobronchial ultrasound-guided transbronchial needle aspiration specimens for the diagnosis and molecular testing of patients with known or suspected lung cancer. Respiration. 2014;88(6):500-17.

8. Kossakowski CA, Morresi-Hauf A, Schnabel PA, Eberhardt R, Herth FJ, Warth A. Preparation of cell blocks for lung cancer diagnosis and prediction: protocol and experience of a high-volume center. Respiration. 2014;87(5):432-8.

9. Sauter JL, Grogg KL, Vrana JA, Law ME, Halvorson JL, Henry MR. Young investigator challenge: Validation and optimization of immunohistochemistry protocols for use on cellient cell block specimens. Cancer Cytopathol. 2016 Feb;124(2):89-100.

10. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, Ishikawa Y, Wistuba I, Flieder DB, Franklin W,Gazdar A, Hasleton PS, Henderson DW, Kerr KM, Petersen I, Roggli V, Thunnissen E, Tsao M. Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

Society/European Respiratory Society classification. Arch Pathol Lab Med. 2013 May;137(5):668-84.

11. Beasley MB, Brambilla E, Chiraeac LR, et al. Carcinoid Tumor. In: Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG, editors. WHO classification of tumours of the lung, pleura, thymus and heart. 4th ed. Lyon: International Agency for Research on Cancer: 2015. p 73-77.

12. Hasegawa T, Yamao K, Hijioka S, Bhatia V, Mizuno N, Hara K, Imaoka H, Niwa Y, Tajika M, Kondo S, Tanaka T, Shimizu Y, Kinoshita T, Kohsaki T, Nishimori I, Iwasaki S, Saibara T, Hosoda W, Yatabe Y. Evaluation of Ki-67 index in EUS-FNA specimens for the assessment of malignancy risk in pancreatic neuroendocrine tumors. Endoscopy. 2014 Jan;46(1):32-8.

13. Larghi A, Capurso G, Carnuccio A, Ricci R, Alfieri S, Galasso D, Lugli F, Bianchi A, Panzuto F, De Marinis L, Falconi M,Delle Fave G, Doglietto GB, Costamagna G, Rindi G. Ki-67 grading of nonfunctioning pancreatic neuroendocrine tumors on histologic samples obtained by EUS-guided fine-needle tissue acquisition: a prospective study. Gastrointest Endosc. 2012 Sep;76(3):570-7.

14. Rekhtman N, Ang DC, Sima CS, Travis WD, Moreira AL. Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. Mod Pathol. 2011 Oct;24(10):1348-59.

15. Yatabe Y, Mitsudomi T, Takahashi T. TTF-1 expression in pulmonary adenocarcinomas. Am J Surg Pathol. 2002 Jun;26(6):767-73.

16. Lau SK, Luthringer DJ, Eisen RN. Thyroid transcription factor-1: a review. Appl Immunohistochem Mol Morphol. 2002 Jun;10(2):97-102.

17. Ueno T, Linder S, Elmberger G. Aspartic proteinase napsin is a useful marker for diagnosis of primary lung adenocarcinoma. Br J Cancer. 2003 Apr 22;88(8):1229-33.

18. Nicholson AG, Gonzalez D, Shah P, Pynegar MJ, Deshmukh M, Rice A, Popat S. Refining the diagnosis and EGFR status of non-small cell lung carcinoma in biopsy and cytologic material, using a panel of mucin staining, TTF-1, cytokeratin 5/6, and P63, and EGFR mutation analysis. J Thorac Oncol. 2010 Apr;5(4):436-41.

19. Camilo R, Capelozzi VL, Siqueira SA, Del Carlo Bernardi F. Expression of p63, keratin 5/6, keratin 7, and surfactant-A in non-small cell lung carcinomas. Hum Pathol. 2006 May;37(5):542-6.

20. Chuman Y, Bergman A, Ueno T, Saito S, Sakaguchi K, Alaiya AA, Franzén B, Bergman T, Arnott D, Auer G, Appella E,Jörnvall H, Linder S. Napsin A, a member of the aspartic protease family, is abundantly expressed in normal lung and kidney tissue and is expressed in lung adenocarcinomas. FEBS Lett. 1999 Nov 26;462(1-2):129-34.

21. Bishop JA, Teruya-Feldstein J, Westra WH, Pelosi G, Travis WD, Rekhtman N. p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. Mod Pathol. 2012 Mar;25(3):405-15.

This article is protected by copyright. All rights reserved.

Accepted Article

22. Pelosi G, Fabbri A, Bianchi F, Maisonneuve P, Rossi G, Barbareschi M, Graziano P, Cavazza A, Rekhtman N, Pastorino U,Scanagatta P, Papotti M. ΔNp63 (p40) and thyroid transcription factor-1 immunoreactivity on small biopsies or cellblocks for typing non-small cell lung cancer: a novel two-hit, sparing-material approach. J Thorac Oncol. 2012 Feb;7(2):281-90.

23. Nonaka D. A study of ΔNp63 expression in lung non-small cell carcinomas. Am J Surg Pathol. 2012 Jun;36(6):895-9.

24. Klempner SJ, Cohen DW, Costa DB. ALK translocation in non-small cell lung cancer with adenocarcinoma and squamous cell carcinoma markers. J Thorac Oncol. 2011 Aug;6(8):1439-40.

25. Fabbro D, Di Loreto C, Beltrami CA, Belfiore A, Di Lauro R, Damante G. Expression of thyroid-specific transcription factors TTF-1 and PAX-8 in human thyroid neoplasms. Cancer Res. 1994 Sep 1;54(17):4744-9.

26. Choi SM, Furth EE, Zhang PJ. Unexpected TTF-1 Positivity in a Subset of Gastric Adenocarcinomas. Appl Immunohistochem Mol Morphol. 2015 Oct 13. [Epub ahead of print]

27. Zachara-Szczakowski S, Verdun T, Churg A. Accuracy of classifying poorly differentiated non-small cell lung carcinoma biopsies with commonly used lung carcinoma markers. Hum Pathol. 2015 May;46(5):776-82.

28. Miskovic J, Brekalo Z, Vukojevic K, Miskovic HR, Kraljevic D, Todorovic J, Soljic V. Co-expression of TTF-1 and neuroendocrine markers in the human fetal lung and pulmonary neuroendocrine tumors. Acta Histochem. 2015 May-Jun;117(4-5):451-9.

29. Tran L, Mattsson JS, Nodin B, Jönsson P, Planck M, Jirström K, Botling J, Micke P, Brunnström H. Various Antibody Clones of Napsin A, Thyroid Transcription Factor 1, and p40 and Comparisons With Cytokeratin 5 and p63 in Histopathologic Diagnostics of Non-Small Cell Lung Carcinoma. Appl Immunohistochem Mol Morphol. 2015 Oct 1. [Epub ahead of print]

30. Kandalaft PL, Gown AM, Isacson C. The lung-restricted marker napsin A is highly expressed in clear cell carcinomas of the ovary. Am J Clin Pathol. 2014 Dec;142(6):830-6.

31. Gurda GT, Zhang L, Wang Y, Chen L, Geddes S, Cho WC, Askin F, Gabrielson E, Li QK. Utility of five commonly used immunohistochemical markers TTF-1, Napsin A, CK7, CK5/6 and P63 in primary and metastatic adenocarcinoma and squamous cell carcinoma of the lung: a retrospective study of 246 fine needle aspiration cases. Clin Transl Med. 2015 Apr 21;4:16.

32. Tatsumori T, Tsuta K, Masai K, Kinno T, Taniyama T, Yoshida A, Suzuki K, Tsuda H. p40 is the best marker for diagnosing pulmonary squamous cell carcinoma: comparison with p63, cytokeratin 5/6, desmocollin-3, and sox2. Appl Immunohistochem Mol Morphol. 2014 May-Jun;22(5):377-82.

33. Vogt AP, Cohen C, Siddiqui MT. p40 (ΔNp63) is more specific than p63 and cytokeratin 5 in identifying squamous cell carcinoma of bronchopulmonary origin: a review and comparative analysis. Diagn Cytopathol. 2014 May;42(5):453-8.

Accepted Article

This article is protected by copyright. All rights reserved.

34. Bishop JA, Teruya-Feldstein J, Westra WH, Pelosi G, Travis WD, Rekhtman N. p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. Mod Pathol. 2012 Mar;25(3):405-15.

35. El-Maqsoud NM, Tawfiek ER, Abdelmeged A, Rahman MF, Moustafa AA. The diagnostic utility of the triple markers Napsin A, TTF-1, and PAX8 in differentiating between primary and metastatic lung carcinomas. Tumour Biol. 2015 Oct 1. [Epub ahead of print]

36. Collins BT, Wang JF, Bernadt CT. Utilization of p40 (ΔNp63) with p63 and cytokeratin 5/6 immunohistochemistry in non-small cell lung carcinoma fine-needle aspiration biopsy. Acta Cytol. 2013;57(6):619-24.

37. Kimbrell HZ, Gustafson KS, Huang M, Ehya H. Subclassification of non-small cell lung cancer by cytologic sampling: a logical approach with selective use of immunocytochemistry. Acta Cytol. 2012;56(4):419-24.

38. Johnson H, Cohen C, Fatima N, Duncan D, Siddiqui MT. Thyroid transcription factor 1 and Napsin A double stain: utilizing different vendor antibodies for diagnosing lung adenocarcinoma. Acta Cytol. 2012;56(6):596-602.

39. Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya H, Motoi N, Mun MY, Sakao Y, Okumura S, Nakagawa K, Soda M,Choi YL, Mano H, Ishikawa Y. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol. 2009 Apr;22(4):508-15.

40. Jokoji R, Yamasaki T, Minami S, Komuta K, Sakamaki Y, Takeuchi K, Tsujimoto M. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. J Clin Pathol. 2010 Dec;63(12):1066-70.

41. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ. Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: implications for optimal clinical testing. Arch Pathol Lab Med. 2012 Jul;136(7):796-803.

42. Kitamura A, Hosoda W, Sasaki E, Mitsudomi T, Yatabe Y. Immunohistochemical detection of EGFR mutation using mutation-specific antibodies in lung cancer. Clin Cancer Res. 2010 Jul 1;16(13):3349-55.

43. Atkins D, Reiffen KA, Tegtmeier CL, Winther H, Bonato MS, Störkel S. Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. J Histochem Cytochem. 2004 Jul;52(7):893-901.

44. Brevet M, Arcila M, Ladanyi M. Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. J Mol Diagn. 2010 Mar;12(2):169-76.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

45. Selinger CI, Rogers TM, Russell PA, O'Toole S, Yip P, Wright GM, Wainer Z, Horvath LG, Boyer M, McCaughan B, Kohonen-Corish MR, Fox S, Cooper WA, Solomon B. Testing for ALK rearrangement in lung adenocarcinoma: a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. Mod Pathol. 2013 Dec;26(12):1545-53.

46. Sholl LM, Weremowicz S, Gray SW, Wong KK, Chirieac LR, Lindeman NI, Hornick JL. Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. J Thorac Oncol. 2013 Mar;8(3):322-8.

47. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP,Gu TL, Polakiewicz RD, Rush J, Comb MJ. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell. 2007 Dec 14;131(6):1190-203.

48. Stumpfova M, Jänne PA. Zeroing in on ROS1 rearrangements in non-small cell lung cancer. Clin Cancer Res. 2012 Aug 15;18(16):4222-4.

49. Omori S, Kenmotsu H, Abe M, Watanabe R, Sugino T, et al. Changes in PD-L1 expression in non-small cell lung cancer by immunohistochemical analysis. J Clin Oncol 33, 2015 (suppl; abstr e22118).

50. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012 Mar 22;12(4):252-64.

51. Ji M, Liu Y, Li Q, Li XD, Zhao WQ, Zhang H, Zhang X, Jiang JT, Wu CP. PD-1/PD-L1 pathway in non-small-cell lung cancer and its relation with EGFR mutation. J Transl Med. 2015 Jan 16;13:5.

52. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, Carcereny E,Ahn MJ, Felip E, Lee JS, Hellmann MD, Hamid O, Goldman JW, Soria JC, Dolled-Filhart M, Rutledge RZ, Zhang J,Lunceford JK, Rangwala R, Lubiniecki GM, Roach C, Emancipator K, Gandhi L; KEYNOTE-001 Investigators. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med. 2015 May 21;372(21):2018-28.

53. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol. 2012 Apr;24(2):207-12.

54. Landi L, Minuti G, D'Incecco A, Cappuzzo F. Targeting c-MET in the battle against advanced nonsmall-cell lung cancer. Curr Opin Oncol. 2013 Mar;25(2):130-6.

55. Sierra JR, Tsao MS. c-MET as a potential therapeutic target and biomarker in cancer. Ther Adv Med Oncol. 2011 Nov;3(1 Suppl):S21-35.

56. Ozasa H, Oguri T, Maeno K, Takakuwa O, Kunii E, Yagi Y, Uemura T, Kasai D, Miyazaki M, Niimi A. Significance of c-MET overexpression in cytotoxic anticancer drug-resistant small-cell lung cancer cells. Cancer Sci. 2014 Aug;105(8):1032-9.

This article is protected by copyright. All rights reserved.

57. Korpanty GJ, Graham DM, Vincent MD, Leighl NB. Biomarkers That Currently Affect Clinical Practice in Lung Cancer: EGFR, ALK, MET, ROS-1, and KRAS. Front Oncol. 2014 Aug 11;4:204.

58. Suh J, Rekhtman N, Ladanyi M, Riely GJ, Travis WD. Testing of new IASLC/ATS/ERS criteria for diagnosis of lung adenocarcinoma (AD) in small biopsies: minimize immunohistochemistry (IHC) to maximize tissue for molecular studies. Mod Pathol 2011;24(15):424A.

59. Travis WD, Rekhtman N. Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing. Semin Respir Crit Care Med. 2011 Feb;32(1):22-31.

60. Savic S, Tapia C, Grilli B, Rufle A, Bihl MP, de Vito Barascud A, Herzog M, Terracciano L, Baty F, Bubendorf L. Comprehensive epidermal growth factor receptor gene analysis from cytological specimens of non-small-cell lung cancers. Br J Cancer. 2008 Jan 15;98(1):154-60.

61. Otani H, Toyooka S, Soh J, Yamamoto H, Suehisa H, Kobayashi N, Gobara H, Mimura H, Kiura K, Sano Y, Kanazawa S,Date H. Detection of EGFR gene mutations using the wash fluid of CT-guided biopsy needle in NSCLC patients. J Thorac Oncol. 2008 May;3(5):472-6.

62. Rafael OC, Aziz M, Raftopoulos H, Vele OE, Xu W, Sugrue C. Molecular testing in lung cancer: fine-needle aspiration specimen adequacy and test prioritization prior to the CAP/IASLC/AMP Molecular Testing Guideline publication. Cancer Cytopathol. 2014 Jun;122(6):454-8.

63. Pusztaszeri M, Soccal PM, Mach N, Pache JC and Mc Kee T. Cytopathological diagnosis of non-small cell lung cancer: recent advances including rapid on-site evaluation, novel endoscopic techniques and molecular tests. J Pulmonar Respirat Med 2011;S5:002.

64. Harada S, Agosto-Arroyo E, Levesque JA, Alston E, Janowski KM, Coshatt GM, Eltoum IA. Poor cell block adequacy rate for molecular testing improved with the addition of Diff-Quik-stained smears: Need for better cell block processing. Cancer Cytopathol. 2015 Aug;123(8):480-7.

65. Knoepp SM, Roh MH. Ancillary techniques on direct-smear aspirate slides: a significant evolution for cytopathology techniques. Cancer Cytopathol. 2013 Mar;121(3):120-8.

66. da Cunha Santos G, Saieg MA. Preanalytic parameters in epidermal growth factor receptor mutation testing for non-small cell lung carcinoma: A review of cytologic series. Cancer Cytopathol. 2015 Nov;123(11):633-43.

67. da Cunha Santos G, Saieg MA, Geddie W, Leighl N. EGFR gene status in cytological samples of nonsmall cell lung carcinoma: controversies and opportunities. Cancer Cytopathol. 2011 Apr 25;119(2):80-91.

68. Billah S, Stewart J, Staerkel G, Chen S, Gong Y, Guo M. EGFR and KRAS mutations in lung carcinoma: molecular testing by using cytology specimens. Cancer Cytopathol. 2011 Apr 25;119(2):111-7.

69. Malapelle U, Bellevicine C, De Luca C, Salatiello M, De Stefano A, Rocco D, de Rosa N, Vitiello F, Russo S, Pepe F,Iaccarino A, Micheli P, Illiano A, Carlomagno C, Piantedosi FV, Troncone G. EGFR mutations detected on cytology samples by a centralized laboratory reliably predict

This article is protected by copyright. All rights reserved.

Accepted Article

response to gefitinib in non-small cell lung carcinoma patients. Cancer Cytopathol. 2013 Oct;121(10):552-60.

70. Shiau CJ, Babwah JP, da Cunha Santos G, Sykes JR, Boerner SL, Geddie WR, Leighl NB, Wei C, Kamel-Reid S, Hwang DM, Tsao MS. Sample features associated with success rates in population-based EGFR mutation testing. J Thorac Oncol. 2014 Jul;9(7):947-56.

71. Stigt JA, 'tHart NA, Knol AJ, Uil SM, Groen HJ. Pyrosequencing analysis of EGFR and KRAS mutations in EUS and EBUS-derived cytologic samples of adenocarcinomas of the lung. J Thorac Oncol. 2013 Aug;8(8):1012-8.

72. Rekhtman N, Brandt SM, Sigel CS, Friedlander MA, Riely GJ, Travis WD, Zakowski MF, Moreira AL. Suitability of thoracic cytology for new therapeutic paradigms in non-small cell lung carcinoma: high accuracy of tumor subtyping and feasibility of EGFR and KRAS molecular testing. J Thorac Oncol. 2011 Mar;6(3):451-8.

73. Lozano MD, Zulueta JJ, Echeveste JI, Gúrpide A, Seijo LM, Martín-Algarra S, Del Barrio A, Pio R, Idoate MA, Labiano T,Perez-Gracia JL. Assessment of epidermal growth factor receptor and K-ras mutation status in cytological stained smears of non-small cell lung cancer patients: correlation with clinical outcomes. Oncologist. 2011;16(6):877-85.

74. Pang B, Dettmer M, Ong CW, Dhewar AN, Gupta S, Lim GL, Nga ME, Seet JE, Qasim A, Chin TM, Soo R, Soong R, Salto-Tellez M. The positive impact of cytological specimens for EGFR mutation testing in non-small cell lung cancer: a single South East Asian laboratory's analysis of 670 cases. Cytopathology. 2012 Aug;23(4):229-36.

75. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, Jenkins RB, Kwiatkowski DJ, Saldivar JS, Squire J,Thunnissen E, Ladanyi M. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Thorac Oncol. 2013 Jul;8(7):823-59.

76. VanderLaan PA. Molecular markers: Implications for cytopathology and specimen collection. Cancer Cytopathol. 2015 Aug;123(8):454-60.

77. Sholl LM. Biomarkers in lung adenocarcinoma: a decade of progress. Arch Pathol Lab Med. 2015 Apr;139(4):469-80.

78. Kim HS, Mitsudomi T, Soo RA, Cho BC. Personalized therapy on the horizon for squamous cell carcinoma of the lung. Lung Cancer. 2013 Jun;80(3):249-55.

79. da Cunha Santos G, Saieg MA, Geddie W, Leighl N. EGFR gene status in cytological samples of non-small cell lung carcinoma: controversies and opportunities. Cancer Cytopathol. 2011 Apr 25;119(2):80-91.

80. Jackman DM, Miller VA, Cioffredi LA, Yeap BY, Jänne PA, Riely GJ, Ruiz MG, Giaccone G, Sequist LV, Johnson BE. Impact of epidermal growth factor receptor and KRAS mutations on clinical

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

outcomes in previously untreated non-small cell lung cancer patients: results of an online tumor registry of clinical trials. Clin Cancer Res. 2009 Aug 15;15(16):5267-73.

81. Birchmeier C, Sharma S, Wigler M. Expression and rearrangement of the ROS1 gene in human glioblastoma cells. Proc Natl Acad Sci U S A. 1987 Dec;84(24):9270-4.

82. Acquaviva J, Wong R, Charest A. The multifaceted roles of the receptor tyrosine kinase ROS in development and cancer. Biochim Biophys Acta. 2009 Jan;1795(1):37-52.

83. Bergethon K, Shaw AT, Ou SH, Katayama R, Lovly CM, McDonald NT, Massion PP, Siwak-Tapp C, Gonzalez A, Fang R,Mark EJ, Batten JM, Chen H, Wilner KD, Kwak EL, Clark JW, Carbone DP, Ji H, Engelman JA, Mino-Kenudson M, Pao W,Iafrate AJ. ROS1 rearrangements define a unique molecular class of lung cancers. J Clin Oncol. 2012 Mar 10;30(8):863-70.

84. Kim HR, Lim SM, Kim HJ, Hwang SK, Park JK, Shin E, Bae MK, Ou SH, Wang J, Jewell SS, Kang DR, Soo RA, Haack H,Kim JH, Shim HS, Cho BC. The frequency and impact of ROS1 rearrangement on clinical outcomes in never smokers with lung adenocarcinoma. Ann Oncol. 2013 Sep;24(9):2364-70.

85. Shaw AT, Camidge DR, Engelman JA, Solomon BJ, Kwak EL, Clark JW, et al. Clinical activity of crizotinib in advanced non-small cell lung cancer (NSCLC) harboring ROS1 gene rearrangement. 2012 ASCO Meeting. Ab J Clin Oncol 30, 2012 (suppl; abstr 7508).

86. Young, G. *et al.* Clinical next-generation sequencing successfully applied to fine-needle aspirations of pulmonary and pancreatic neoplasms. *Cancer cytopathology* 121, 688-694, doi:10.1002/cncy.21338 (2013).

87. Scarpa, A. *et al.* Molecular typing of lung adenocarcinoma on cytological samples using a multigene next generation sequencing panel. *PloS one* 8, e80478, doi:10.1371/journal.pone.0080478 (2013).

88. Kanagal-Shamanna, R. *et al.* Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics. *Mod Pathol* 27, 314-327, doi:10.1038/modpathol.2013.122 (2014).

89. Roy-Chowdhuri, S. *et al.* Factors affecting the success of next-generation sequencing in cytology specimens. *Cancer cytopathology* 123, 659-668, doi:10.1002/cncy.21597 (2015).

90. Karnes, H. E., Duncavage, E. J. & Bernadt, C. T. Targeted next-generation sequencing using fine-needle aspirates from adenocarcinomas of the lung. *Cancer cytopathology* 122, 104-113, doi:10.1002/cncy.21361 (2014).

91. Buttitta, F. *et al.* Effective assessment of egfr mutation status in bronchoalveolar lavage and pleural fluids by next-generation sequencing. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19, 691-698, doi:10.1158/1078-0432.CCR-12-1958 (2013).

John Wiley & Sons

This article is protected by copyright. All rights reserved.

Accepted Article

92. Wei, S. *et al.* Using "residual" FNA rinse and body fluid specimens for next-generation sequencing: An institutional experience. *Cancer cytopathology*, doi:10.1002/cncy.21666 (2015).

93. Gailey, M. P., Stence, A. A., Jensen, C. S. & Ma, D. Multiplatform comparison of molecular oncology tests performed on cytology specimens and formalin-fixed, paraffin-embedded tissue. *Cancer cytopathology* 123, 30-39, doi:10.1002/cncy.21476 (2015).

94. Chen, H., Luthra, R., Goswami, R. S., Singh, R. R. & Roy-Chowdhuri, S. Analysis of Pre-Analytic Factors Affecting the Success of Clinical Next-Generation Sequencing of Solid Organ Malignancies. *Cancers* 7, 1699-1715, doi:10.3390/cancers7030859 (2015).

95. Monaco, S. E. Cytopathology of Lung Cancer: Moving from Morphology to Molecular. *Diagnostic Histopathology* 18, 313-320 (2012).

96. Roh, M. H. The Utilization of Cytologic Fine-Needle Aspirates of Lung Cancer for Molecular Diagnostic Testing. *Journal of pathology and translational medicine* 49, 300-309, doi:10.4132/jptm.2015.06.16 (2015).

97. Dumur, C. I. & Kraft, A. O. Next-generation sequencing and the cytopathologist. *Cancer cytopathology* 123, 69-70, doi:10.1002/cncy.21515 (2015).

98. Vigliar, E., Malapelle, U., de Luca, C., Bellevicine, C. & Troncone, G. Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective. *Cytopathology : official journal of the British Society for Clinical Cytology* 26, 271-283, doi:10.1111/cyt.12265 (2015).

99. Bellevicine, C., Malapelle, U., de Luca, C., Iaccarino, A. & Troncone, G. EGFR analysis: current evidence and future directions. *Diagn Cytopathol* 42, 984-992, doi:10.1002/dc.23142 (2014).

100. Tripathy, D., Harnden, K., Blackwell, K. & Robson, M. Next generation sequencing and tumor mutation profiling: are we ready for routine use in the oncology clinic? *BMC medicine* 12, 140, doi:10.1186/s12916-014-0140-3 (2014).

101. Redig, A. J. & Janne, P. A. Basket trials and the evolution of clinical trial design in an era of genomic medicine. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 33, 975-977, doi:10.1200/JCO.2014.59.8433 (2015).

Accepted Article

This article is protected by copyright. All rights reserved.

Table I. Useful Immunohistochemical Markers for Identification of Unknown Primaries

Organ	**Immunohistochemical Marker**
Adrenocortical	Inhibin, MelanA, Ad4BP
Adrenomedullary	NSE, synaptophysin, chromogranin, PGP9.5
Biliary	CK7, CDX-2, CK19, CEA, MOC-31
Breast	ER, PR, GATA-3, CK7, CEA
Cervix (adenocarcinoma)	CEA, CK7, p16
Cervix (squamous cell)	CK7, p16
Colon	CK20, villin
Esophagus (adenocarcinoma)	MUC5AC, MUC4, CA19-9, CEA, EMA
Esophagus (squamous cell)	AE1/3, CK5/6, p63, CK19
Endometirum	CK7, vimentin, ER, p53, inhibin, EMA
Gastric (adenocarcinoma)	EMA, MUC5AC, MUC4, BerEP4, CA19-9, CEA
Germ Cell Tumor	CD30, hCG, Oct4, CK, AFP, Glypican 3, CD117, PLAP, WT-1, CD10, EMA
Hepatocellular	EMA, Hep Par-1, CEA
Kidney	EMA, CD10, vimentin, RCC
Lung (adenocarcinoma)	CK7, TTF-1, Napsin, villin
Lung (squamous cell)	p63, p40
Lung (small cell)	CK7, chromogranin, synaptophysin
Ovary (mucinous)	CK7, CK20
Ovary (serous)	CK7, WT-1
Pancreas (ductal)	CK7, CK20
Pancreas (neuroendocrine)	CK7, CK20, chromogranin, synaptophysin
Prostate	PSA, PAP
Salivary Gland	GFAP, s-100 protein, c-kit, Bcl-2, CK7
Thymus	CD5 (Thymic carcinoma)
Thyroid	TTF-1, Thyroglobulin, CK19 (papillary), HBME-1 (papillary), galectin-3 (papillary), calcitonin (medullary)
Urothelium (bladder)	CK7, CK20, p63, Thrombomodulin, GATA-3
Mesothelioma	Mesothelin, CK7, WT-1, calretinin, CK5/6, D2-40

Accepted Article

This article is protected by copyright. All rights reserved.