Non-small cell lung cancer is a different disease from what it was a decade ago. The last 10 years were based on remarkable advances in the understanding of key genetic alterations that function as oncogenic drivers and serve as therapeutic targets, thereby defining new molecular subsets. These changes have had an impact on clinical care, patient outcomes, and pathologic diagnosis and present new challenges in the approach of the cytopathologist to this still deadly disease.

To meet these new challenges and appropriately train the next generation of cytopathologists, the complex molecular background underlying this disease and the implications that cytologic and histologic diagnoses have on treatment must be understood. Herein, the author reviews the background leading to this new approach and explains how, why, and what cytologists need to know to successfully contribute to the care of the patient with lung cancer.

KEY WORDS: genetic alterations; molecular subsets; non-small cell lung cancer; oncogenic drivers; therapeutic targets.

INTRODUCTION

Few oncologists have been as dependent on cytopathologists for the diagnosis and care of patients as those who treat lung cancer. Still the major cancer killer in the Western world, lung cancer has been diagnosed using cytologic methods for decades, if not centuries.1 Physicians started with sputum and then large needles and basic stains to sample palpable lymph nodes, and advanced to using fluoroscopy for locating intrathoracic lesions (Fig. 1). Later, with computed axial tomography and dedicated interventional radiologists and pulmonologists armed with more sophisticated bronchoscopic techniques, a larger amount of tumor material was made more easily available. Pathologists helped to direct the care of the patient with lung cancer by separating those with small cell lung cancer from those with non-small cell lung cancer (NSCLC). This was of utmost importance, because although both had dismal prognoses due to late presentation and surgical unresectability, patients with these 2 cancers were treated differently.

Today, the majority of patients with lung cancer still present too late for potentially curative surgical treatment. Late presentation is why cytology became so vital in the diagnosis of lung cancer: cytology provides a non-invasive means for the diagnosis and initiation of appropriate treatment without the morbidity and mortality of surgical procedures in very sick patients.

Although some improvements in the treatment of lung cancer were made, such as doublet chemotherapy instead of single-agent regimens and better surgical procedures, the state of the art of lung cancer oncology remained virtually unchanged until the early 2000s. In 2004, several articles appeared nearly simultaneously identifying activating mutations in NSCLC that rendered these tumors treatable by so-called “targeted therapy.”2–4 These new agents, usually in the form of pills taken daily for sometimes years, were game changers for the lung...
cancer community. The type of NSCLC that responded to the targeted therapy soon was identified as almost exclusively adenocarcinoma (ACA).

In the initial articles, the response to agents such as gefitinib and erlotinib were clearly linked to mutations in epidermal growth factor receptor (EGFR) exons 19 and 21. Rapidly following this breakthrough in understanding of the molecular changes in the development of lung cancer, smoking notwithstanding, were the discoveries of other mutations and translocations involving ERBB2, ROS1, and MET. To my knowledge, not much work has been done to date in identifying ROS1, RET, and MET alterations in cytology samples, although it recently has been shown that they can be detected in such samples using fluorescence in situ hybridization (FISH) assays. In patients with ROS1-rearranged NSCLC, clinical studies have shown a high response rate to crizotinib.\(^5\)\(^6\) MET amplification is rare in patients with untreated NSCLC, but can be found in up to 20% of patients with EGFR-mutated tumors as a mechanism of acquired resistance and MET-amplified tumors respond to crizotinib. The 3 most common targetable alterations currently are EGFR, ALK, and MET. Driver mutations now have been identified in nearly 75% of all lung adenocarcinomas (Fig. 2),\(^8\) and although the pipeline is rapidly expanding, not all driver mutations (KRAS among them) have yet been matched with effective targeted drugs.\(^9\)

Treatment with these new targeted agents improved the response of patients, controlled symptoms, extended disease-free and overall survival, and led to more development and/or approval of drugs for each newly identified mutation. In fact, it was this class of drugs that initiated a new rapid approval program from the US Food and Drug Administration (FDA) for the quicker release of new agents. Although to my knowledge none of these drugs has yet provided a durable cure for the majority of patients, the era of identifying small cell and NSCLC as the major diagnostic role of the lung cancer cytologist is over.

It now is essential that ACA be separated from the NSCLC category, and it is becoming obvious that squamous cell carcinoma (SCC) also is a separate category in terms of mutations and treatment.\(^10\) The importance of recognizing SCC as distinct from ACA has led to the clinical use of the term “nonsquamous carcinoma.” At first resisted by the pathology community, this term currently is becoming more accepted and it is understood that nonsquamous tumors are associated with different types of analysis and different drug therapies, both indicated and contraindicated. With that knowledge, it becomes easier to understand the need for this term in conveying information to the treating physicians.
In 2015, the WHO [World Health Organization] Classification of Tumors of the Lung, Pleura, Thymus and Heart was updated and provided “standardized terminology for lung cancer diagnosis in small biopsies and cytology.”\textsuperscript{11} This classification grouped small biopsies and cytology specimens together and did not distinguish between different types of cytology specimens such as touch preparations, fine-needle aspirations (FNAs), monolayer preparations, and cell blocks. Although some of the recommendations as applied to cytology are good practice (eg, subclassifying NSCLC), others appear to be unnecessarily prescriptive (eg, it should be clarified whether a diagnosis was made using light microscopy or if special stains were required and this information should be used to stratify cases for future research and clinical trials). It also should be cautioned that the classification has proven difficult to reproduce in actual practice.\textsuperscript{12} Nevertheless, the inclusion of cytology and small biopsies in the WHO system is a positive step toward the recognition of the important role these specimens play in lung cancer diagnosis.

It is essential that new cytology trainees learn the intricacies of lung cancer diagnosis, from FNA adequacy determinations to the incorporation of new classification systems and terminology to choosing appropriate therapies. The use of rapid on-site evaluation (ROSE) of cytology specimens often is considered essential in good practice, but in actuality the literature is rife with confusion regarding this technique and where and when it is best used.\textsuperscript{13}

Advanced disease at the time of presentation still is frequently encountered and early detection methods for lung cancer are not fully developed.\textsuperscript{14} Cytologists still are called on to make these distinctions based on limited amounts of material. These cytologic samples must be handled appropriately to supply both morphologic diagnoses and material for molecular analysis.

Several developments have aided cytologists in this: pathologists now are relearning the morphologic criteria to distinguish ACA from SCC in cytology specimens,\textsuperscript{15} immunohistochemistry (IHC) has provided new and effective stains for ACA (eg, thyroid transcription factor 1 [TTF-1] and napsin A) and SCC (p63 and p40), and cyto-technologists and trainees have aided in the on-site triage of diagnostic material to ensure sufficiency for additional studies. The morphologic features that are useful in distinguishing ACA from SCC are most obvious in well-differentiated tumors: gland formation in ACA and keratinization in SCC. Other identifying features include intercellular bridges in SCC and glandular architecture such as mucin vacuoles, acini, and papillae in ACA. However, making these distinctions can be difficult in poorly differentiated tumors, in which these features may be overlapping or absent. In those cases, IHC stains are very useful. I do not find it absolute that IHC stains must be used when clear-cut features of ACA or SCC are present. The use of IHC traditionally has been to support a morphologic diagnosis and stating that the results of the staining panels favor ACA or SCC is good practice. Metastatic tumors to the lung should be ruled out using additional IHC panels when the morphology and/or primary panel of IHC stains are not determinative.

As techniques to identify lung lesions at an earlier stage and therefore a smaller size improve and even less material is obtained, decisions must be made regarding what tests to perform and in what order to perform them. Care should be taken to maximize the amount of tissue available for additional studies; these techniques may vary from laboratory to laboratory and with specimen type. In my practice, I tend to avoid trimming the cell blocks to preserve as much material as possible. Another approach includes obtaining multiple passes during FNA procedures and setting one pass aside for molecular analysis.

Although the literature points to solid success in differentiating NSCLC by tumor type and retrieving molecular profiles from cytology material,\textsuperscript{16,17} difficulties remain in the ability to do this and in understanding the correct sequence of studies, particularly outside of major cancer centers.

The lung cancer molecular testing guidelines jointly written and published by the College of American Pathologists,\textsuperscript{18} the International Association for the Study of Lung Cancer,\textsuperscript{19} and the Association for Molecular Pathology\textsuperscript{20} and widely adopted have wisely included cytologic material. These guidelines already are being updated. For what to my knowledge is the first time, recognition of the role of cytology has been extended to classification systems for lung cancer, emphasizing clinical impact.\textsuperscript{21} This classification system, although flawed, serves to highlight the importance of cytology to the general surgical pathologist and the treating oncologist.

Considering the scant number of cells retrieved in FNA specimens compared with surgical resection specimens, the development of mutation-specific IHC stains applicable to cytology material, such as for the detection of mutant forms of EGFR and for ALK (Figs. 3 and 4),\textsuperscript{22,23}
have helped in identifying these critical targetable alterations in single tumor cells, present for example in cerebrospinal fluid.

Newer immunotherapy agents in lung cancer deserve some critical attention. These agents include checkpoint inhibitors that require the assessment of programmed death-ligand 1 (PD-L1) expression by IHC stains for patient selection. These checkpoint inhibitors work by interrupting PD-L1 binding with its receptor programmed cell death protein (PD-1) on the surface of cytotoxic T cells. Many tumor cells are able to upregulate the expression of PD-L1 as a mechanism with which to evade the body’s natural immune response. Activated T cells recognize the PD-L1 marker on the tumor cell and this renders the T cell inactive. The tumor cell escapes the immune response, avoids detection, and proliferates. Anti-PD-1 therapy enhances the immune response against tumors. The lung tumors treated by these agents include both ACA and SCC in advanced stages, for which other therapies have failed. Anti-PD-1 therapy appears to be more effective in tumors with high mutation burdens such as melanomas and smoking-associated lung cancers. Determining which patients are most likely to respond to this therapy is done by the pathologist using IHC to assess the percentage of tumor cells with membranous staining using the monoclonal anti-PD-L1 antibody. The FDA has approved the use of pembrolizumab in the treatment of patients with advanced NSCLC when staining using a specific assay is performed. This is the concept of companion diagnostics such as the FDA-directed use of the Vysis FISH assay (Abbott Laboratories, Abbott Park, Ill) for the assessment of ALK status in patients with lung cancer before crizotinib can be administered.

There are at least 4 different biomarker assays, each associated with one of the anti-PD-1 or anti-PD-L1 agents available (nivolumab, pembrolizumab, atezolizumab, and avelumab). This has added to the complexity of laboratory biomarker testing for the selection of cancer therapeutics.

A minimum of 100 tumor cells are needed to assess the positivity of the PD-L1 IHC stain. Although these stains have not become routine or even common in cytopathology, they have been applied successfully to patients with malignant mesothelioma in pleural effusions. The paucicellularity of cell blocks can make the use of this stain problematic in cytology. The complexity of PD-L1 staining assessment is another area in which cytopathologist expertise will be needed to select the appropriate treatment modality.

**Figure 3.** Mutation-specific stain for EGFR exon 19 applied to cerebrospinal fluid.

**Figure 4.** Identification of echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) fusion using cytology cell block material. Ab indicates antibody; ALK, anaplastic lymphoma kinase; EML4-ALK, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; H & E, hematoxylin and eosin; IHC, immunohistochemistry.
The FDA directives indicating how tests must be performed before therapeutic agents can be prescribed can have a significant impact on the cytology laboratory. The decision to validate these tests on cytology samples and ensure adequate cellularity represents an investment of time, money, and personnel for cytology laboratories. It also impacts on a laboratory’s decision to develop in-house versus outsource testing for lung markers. The choice between establishing testing for key predictive markers of lung cancer in house versus sending samples out to a reference laboratory is, as in other clinical laboratory settings, driven primarily by departmental and institutional resources and clinical priorities. If a reference laboratory is used, the overall turnaround times (including shipping time) for results should be within recommended guidelines.\(^1\) However, the process of selecting slides or blocks, the necessity of scanning slides that may be exhausted in the testing, gathering consents, recording information in the pathology record, and conveying that information to the clinician may slow the process considerably. Outside testing using pathology departments as a conduit should not be so burdensome that it encourages interventionists and surgeons to bypass pathology and send materials directly to reference laboratories. In considering in-house testing, pathologists must decide on the feasibility of introducing companion diagnostic assays that may require the purchase of not only specifically manufactured antibodies but particular staining platforms as well.\(^2\)\(^9\)\(^3\)

The increasing numbers of tests requested for lung cancer specimens and the expanding use of next-generation sequencing (NGS) have proven exciting but somewhat frustrating for the cytologist. Low-dose helical scans have identified smaller lung lesions at earlier stages. Not only does this confront the cytopathologist with the need to accurately identify malignancy on limited material and at an in situ stage of tumor growth, it also means that the specimens obtained during FNA are smaller and decisions regarding how to best use that limited sample may be subject to competing interests. It also requires the cytopathologist to become familiar with the morphologic appearance of these very early lesions (Fig. 5), which is quite different from diagnosing advanced and clinically suspected carcinoma, and becoming more adept at judging whether the percentage and absolute number of cancer cells in the sample are likely to be sufficient for current NGS-based testing. Different NGS platforms have different DNA input requirements. NGS assays based on amplicon sequencing require very low amounts of input DNA; the parameters of successfully sequenced cytology samples recently were studied in detail.\(^3\)\(^1\) In contrast, NGS based on broad hybrid capture requires a higher number of tumor cells (up to several thousand) or at least 100 ng of DNA, but can interrogate larger panels of genes for mutations and also provides more accurate gene copy number data; practical approaches to optimizing the use of cytology samples for this assay type recently were described.\(^3\)\(^2\) It is difficult to obtain enough material to confidently make a diagnosis while providing clinicians with all the laboratory information needed to select therapy. Because there are mounting data that FNA cytology samples perform better in NGS-based testing than concurrent core needle biopsies,\(^3\)\(^3\) cytopathologists will have to assume a greater amount of clinical participation and acquisition of molecular and therapeutic knowledge to provide optimal patient care, which is quite different from diagnosing advanced and clinically suspected carcinoma. As these new roles become incorporated into cytology training programs, the value of a combined cytology-molecular pathology fellowship is becoming obvious.

Cytology has been and continues to be crucial in the diagnosis and treatment of lung cancer. The proficiency required of today’s cytologist includes recognizing the differences in cytology specimens between ACA and SCC, facilitating the testing of small samples of tumor for mutations such as \textit{EGFR} and \textit{KRAS} and \textit{ALK} gene rearrangements, using mutation-specific antibodies and FISH techniques in cytology, and estimating tumor staining in semiquantitative methods for anti-PD-1 therapy. In

\textbf{Figure 5.} Typical scant cellularity in cytology specimens of subcentimeter lesions of adenocarcinoma in situ of the lung (Papanicolaou stain, original magnification ×60).
addition, the recognition of the pitfalls in the default to clinical ordering of core needle biopsies to obtain material for protocols and trials has led to clinicians being instructed regarding how to best approach diagnosing the patient with lung cancer. It is interesting to note that lung cancer has caused cytologists to assume a more clinical role as they provide the information that determines the choice of therapeutic agents, and thus they must be familiar with current and emerging drugs and the indications for their use. Drug companies now are targeting advertisements toward the pathology community. This is a long way from diagnosing advanced small cell carcinoma on FNA.

Cytology has experienced rapid growth in the field of thoracic oncology. The usefulness of cytology in molecular diagnostics is likely to expand to other areas of cancer care, perhaps in monitoring early recurrences or defining metastatic disease, especially in cases in which resistance mutations are involved. Much has been learned regarding patient testing and treatment from the appropriate handling of cytologic material and the incorporation of cytology specimens into molecular analysis. This has led to improvements in the use of resources and in diminishing risk and inconvenience to patients. Molecular pathology is a training subspecialty that must incorporate cytology into its curriculum, just as cytology needs to incorporate molecular methods into its fellowship training. Knowledge and experience in both these fields will provide the pathologist with better tools for disease detection, treatment, and cure.

FUNDING SUPPORT
No specific funding was disclosed.

CONFLICT OF INTEREST DISCLOSURES
The author made no disclosures.

REFERENCES
1. Hajdu SI, Ehya H. Foundation of diagnostic cytology. Ann Clin Lab Sci. 2008;38:296-299.
2. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA. 2004;101:13306-13311.
3. Lynch TJ, Bell DW, Sordella R. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129-2139.
4. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004;304:1497-1500.
5. Savic S, Bubendorf L. Common fluorescence in situ hybridization applications in cytology. Arch Pathol Lab Med. 2016;140:1323-1330.
6. Shaw AT, Ou SH, Bang YJ, et al. Crizotinib in ROSI-rearranged non-small-cell lung cancer. N Engl J Med. 2014;371:1963-1971.
7. Mazieres J, Zalcman G, Crino L, et al. Crizotinib therapy for advanced lung adenocarcinoma and a ROSI rearrangement: results from the EUROS1 cohort. J Clin Oncol. 2015;33:992-999.
8. Jordan EJ, Kim HR, Arcila ME, et al. Prospective comprehensive molecular characterization of lung adenocarcinomas for efficient patient matching to approved and emerging therapies [published online ahead of print March 23, 2017]. Cancer Discov. DOI: 10.1158/2159-8290.CD-16-1337.
9. Wood K, Hensing T, Malik R, Salgia R. Prognostic and predictive value in KRAS in non-small-cell lung cancer: a review. JAMA Oncol. 2016;2:805-812.
10. Rekhtman N, Paik PK, Arcila ME, et al. Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: lack of EGFR/KRAS and presence of PIK3CA/ AKT1 mutations. Clin Cancer Res. 2012;18:1167-1176.
11. Travis W, Brambilla, E, Burke A, et al, eds. WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. Lyon, France: IARC Press; 2015.
12. Zakowski MF. Cytology nomenclature and 2015 World Health Organization classification of lung cancer. Cancer Cytopathol. 2016;124:81-88.
13. Zakowski MF. "...That which we call a rose...": a critical analysis of rapid on-site evaluation. Cancer Cytopathol. 2016;124:857-861.
14. Veronesi G, Novellis P, Voulaz E, Alloisio M. Early detection and early treatment of lung cancer: risks and benefits. J Thorac Dis. 2016;8:E1060-E1062.
15. Zakowski MF, Rekhtman N, Auger M, et al. Morphologic accuracy in differentiating primary lung adenocarcinoma from squamous cell carcinoma in cytology specimens. Arch Pathol Lab Med. 2016;140:1116-1120.
16. Sigel CS, Moreira AL, Travis WD, et al. Subtyping of non-small cell lung carcinoma: a comparison of small biopsy and cytology specimens. J Thorac Oncol. 2011;6:1849-1856.
17. Rekhtman N, Brandi SM, Sigel CS, et 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;6:451-458.
18. Lindeman NI, Cagle PT, Beasley MB, et al. 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. Arch Pathol Lab Med. 2013;137:828-860.
19. Lindeman NI, Cagle PT, Beasley MB, et al. 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. 2015;8:823-859.
20. Lindeman NI, Cagle PT, Beasley MB, et al: College of American Pathologists International Association for the Study of Lung Cancer and Association for Molecular Pathology. 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 Mol Diagn. 2013;15:415-453.
21. Travis WD, Brambilla E, Riel GJ. New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. J Clin Oncol. 2013;31:992-1001.
22. Nakamura H, Koizumi H, Kimura H, Marushima H, Saji H, Takagi M. Epidermal growth factor receptor mutations in adenocarcinoma in situ and minimally invasive adenocarcinoma detected using mutation-specific monoclonal antibodies. Lung Cancer. 2016;99:143-147.
23. Rosenblum F, Hutchinson LM, Garver J, Woda B, Cosar E, Kurian EM. Cytology specimens offer an effective alternative to formalin-fixed tissue as demonstrated by novel automated detection for ALK break-apart FISH testing and immunohistochemistry in lung adenocarcinoma. *Cancer Cytopathol*. 2014;122:810-821.

24. Hirsch FR, McEllinney A, Stanforth D, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *J Thorac Oncol*. 2017;12:208-222.

25. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015;348:124-128.

26. Dako North America Inc. PD-L1 IHC 22C3pharmDX Interpretation Manual: US Version. Carpinteria, CA: Dako North America Inc; 2015.

27. Kerr KM, Hirsch FR. Programmed death ligand-1 immunohistochemistry: friend or foe? *Arch Pathol Lab Med*. 2016;140:326-331.

28. Khanna S, Thomas A, Abate-Daga D, et al. Malignant mesothelioma effusions are infiltrated by CD3+T cells highly expressing PD-L1 and the PD-L1+ tumor cells within these effusions are susceptible to ADCC by the anti-PD-L1 antibody avelumab. *J Thorac Oncol*. 2016;11:1993-2005.

29. Sholl LM, Aisner DL, Allen TC, et al; Members of Pulmonary Pathology Society. Programmed death ligand-1 immunohistochemistry–a new challenge for pathologists: a perspective from members of the Pulmonary Pathology Society. *Arch Pathol Lab Med*. 2016;140:341-344.

30. Vignar E, Malapelle U, Bellevicine C, de Luca C, Troncone G. Outsourcing cytological samples to a referral laboratory for EGFR testing in non-small cell cancer: does theory meet practice? *Cytotechnology*. 2015;26:312-317.

31. Roy-Chowdhuri S, Gowami RS, Chen H, et al. Factors affecting the success of next-generation sequencing in cytology specimens. *Cancer Cytopathol*. 2015;123:659-668.

32. Tian SK, Killian JK, Rekhtman N, et al. Optimizing workflows and processing of cytologic samples for comprehensive analysis by next-generation sequencing: Memorial Sloan Kettering Cancer Center experience. *Arch Pathol Lab Med*. 2016;140:1200-1205.

33. Roy-Chowdhuri S, Chen H, Singh RR, et al. Concurrent fine needle aspirations and core needle biopsies: a comparative study of substrates for next-generation sequencing in solid organ malignancies. *Mod Pathol*. 2017;30:499-508.

34. Rekhtman N, Kazi S, Yao J, et al. Depletion of core needle biopsy cellularity and DNA content as a result of vigorous touch preparations. *Arch Pathol Lab Med*. 2015;139:907-912.