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*

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Introduction: The two essential requirements for pathologic specimens in the era of personalized therapies for non-small cell lung carcinoma (NSCLC) are accurate subtyping as adenocarcinoma (ADC) versus squamous cell carcinoma (SqCC) and suitability for *EGFR* and *KRAS* molecular testing. The aim of this study was to comprehensively review the performance of cytologic specimens for the above two goals in a high-volume clinical practice.

Methods: Subtyping of primary lung carcinomas by preoperative cytology was correlated with subsequent resection diagnoses during a 1-year period ($n = 192$). The contribution of various clinicopathologic parameters to subtyping accuracy and utilization of immunohistochemistry (IHC) for NSCLC subtyping were analyzed. In addition, the performance of cytologic specimens submitted for *EGFR*/*KRAS* molecular testing during a 1-year period ($n = 128$) was reviewed.

Results: Of the 192 preoperative cytology diagnoses, tumor subtype was definitive versus favored versus unclassified in 169 (88%) versus 15 (8%) versus 8 (4%) cases, respectively. Overall accuracy of cytologic tumor subtyping (concordance with histology) was 93% and accuracy of definitive diagnoses 96%. For a group of patients with ADC and SqCC ($n = 165$), the rate of unclassified cytologic diagnoses was 3% and overall accuracy 96%. IHC was used for subtyping of 9% of those cases, yielding 100% accuracy. The strongest predictors of difficulty in subtyping of ADC and SqCC were poor differentiation ($p = 0.0004$), low specimen cellularity ($p = 0.019$), and squamous histology ($p = 0.003$). Of 128 cytologic specimens submitted for molecular testing, 126 (98%) were suitable for analysis, revealing *EGFR* and *KRAS* mutations in 31 (25%) and 25 (20%) cases, respectively.

Conclusions: Cytologic subtyping of NSCLC is feasible and accurate, particularly when morphologic assessment is combined with IHC. Furthermore, routine cytologic specimens can be successfully used for *EGFR/KRAS* mutation analysis. Our data strongly support the suitability of cytologic specimens for the new therapeutic paradigms in NSCLC.

Key Words: Non-small cell carcinoma, Adenocarcinoma, Squamous cell carcinoma, Cytology, *EGFR*, *KRAS*.

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Non-small cell lung carcinoma (NSCLC) comprises adenocarcinoma (ADC) and squamous cell carcinoma (SqCC) as the two major subtypes and several rare tumors, including adenosquamous carcinoma, large cell carcinoma (LCC), and pleomorphic/sarcomatoid carcinoma. Until recently, subtyping of NSCLC has received little attention in pathology because of a similar treatment strategy for all histologic subtypes. Three major advances in thoracic medical oncology have led to a large paradigm shift in NSCLC diagnosis, resulting in a new emphasis on accurate NSCLC subtyping.[1] First was the discovery that epidermal growth factor receptor (*EGFR*) and *KRAS* mutations, which are largely confined to ADC, are predictive of responsiveness and resistance, respectively, to *EGFR* tyrosine kinase inhibitors, erlotinib and gefitinib, and accurate subtyping as ADC versus SqCC became important for the selection of patients for molecular testing.[2] More recently, two other agents, bevacizumab and pemetrexed, were found to have differential toxicity and activity, respectively, in patients with SqCC versus non-SqCC, and these agents are currently approved by the U.S. Food and Drug Administration only for patients with advanced non-SqCC and are excluded from use in patients with SqCC.[3–7]

Approximately 60% of patients with NSCLC present with unresectable stage IIIB or IV disease,[8] where the only pathologic material guiding systemic therapy may be small biopsy or cytology specimens. A major advantage of cytology is that procurement of these specimens can be accom-

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plished by minimally invasive procedures, and immediate on-site assessment can provide real-time feedback on specimen adequacy and triage. It is widely recognized that cytology has exceptional accuracy for distinguishing small cell lung carcinoma (SCLC) from NSCLC.[9,10] However, the performance characteristics of cytology in NSCLC subtyping and predictive marker testing are not well established.

The key morphologic criteria for ADC versus SqCC are glandular architecture versus keratinization, respectively. Although in the majority of cases a line of differentiation can be readily identified by morphology, the difficulty arises in a subset of cases, which are poorly differentiated, scant, or poorly preserved, where distinguishing morphologic features are not apparent. Another difficulty is presented by tumors with mixed histology, although true adenosquamous carcinomas are quite rare.[11] These potential morphologic limitations, combined with the lack of clinical impact, were the reason that a noncommittal diagnosis of NSCLC-not otherwise specified (NSCLC-NOS) was widely advocated until recently in small specimens.[12,13]

In parallel with the advances in thoracic oncology, which have made the pathologic distinction of ADC and SqCC important, in recent years, there has been growing evidence in pathology that immunohistochemistry (IHC) is a powerful tool for revealing a line of differentiation as ADC versus SqCC in morphologically unclassifiable cases.[14–17] Therefore, IHC is increasingly incorporated in routine diagnostic practice for NSCLC subtyping, but the actual impact of this new approach to NSCLC diagnosis on unclassified rate and accuracy has not been reviewed.

The feasibility of NSCLC subtyping in cytologic specimens, particularly before the advances in IHC, has been controversial. A common perception is that cell dispersal and the loss of tissue architecture prevent accurate tumor subtyping. Despite the lack of conventional architecture, there are rigorous cytology-specific criteria. In fact, cytology provides several notable advantages over surgical specimens for the diagnosis of NSCLC. First, the Papanicolaou (Pap) stain, a routine stain in cytology, was originally developed to detect SqCC in cervical smears, and it has exquisite sensitivity for even minimal keratinization aiding in the distinction of SqCC from ADC (in contrast, hematoxylin and eosin [H&E]-stained histologic sections do not have this capability). Second, the morphologic patterns, which emerge in tumor smears, provide a clue to a tumor subtype, which may not even be apparent in surgical specimens. Third, cytology smears do not have formalin-fixation artifact that can limit interpretation of some surgical specimens; therefore, cytology frequently provides greater nuclear and cytoplasmic resolution than histology.

Another common concern is low cellularity, which is sometimes viewed as an a priori limitation for using cytologic specimens for ancillary studies, such as IHC or molecular testing. Although some cytologic specimens (sputum and bronchial brush/wash/lavage) are indeed typically scant, the cellularity of fine needle aspirates (FNAs) can be similar to or even exceed that of a small biopsy.[18] Importantly, for any cytologic specimen with cellular material in suspension (such as FNA needle rinse), a paraffin-embedded cell pellet (cell block) can be prepared, which can be used for IHC or molecular studies analogous to a surgical specimen.

With the emerging clinical evidence for importance of NSCLC subtyping and growing evidence for effectiveness of IHC in distinguishing ADC and SqCC, in the last few years, we have modified our cytology practice to attempt to subtype NSCLC whenever possible by morphology and by IHC in morphologically equivocal cases. This study represents a comprehensive review of accuracy of this clinical practice. In addition, we review the performance of cytologic specimens submitted for *EGFR*/*KRAS* molecular testing.

MATERIALS AND METHODS

Cytologic/Histologic Correlation

The pathology department electronic medical record was searched to identify all thoracic cytology specimens that had subsequent resection for primary lung epithelial neoplasms (carcinomas and neuroendocrine tumors) during a 1-year period (September 1, 2006, to August 31, 2007). Metastatic tumors were excluded. Cytologic/histologic correlation was performed for same-site cytology and resection only. To prevent sampling issues, only resected tumors (wedge, lobectomy, and pneumonectomy) were included. Because the focus of this study was accuracy of tumor subtyping rather than sensitivity for tumor detection, only cases diagnosed as positive or suspicious for epithelial neoplasm on cytology were included.

One hundred ninety-two paired cytology and resection specimens were identified and formed the basis of this study. For this study, the categories of positive and suspicious (nearly diagnostic for malignancy but limited by scant cellularity or poor cell preservation) were considered together as positive (suspicious diagnoses were infrequent; $n = 6$). If a patient had more than one preoperative cytologic specimen (such as bronchial brush, wash, and lavage), only the specimen with the most specific diagnosis was included for analysis. For a small subset of cases where cytology was accompanied by a biopsy ($n = 13$, 7%), cytology diagnosis was usually rendered without the knowledge of the biopsy diagnosis because (1) in our practice cytology and biopsy are reviewed by different pathologists and (2) cytology is reviewed a day earlier due to shorter processing time.

On-site specimen adequacy assessment was provided by cytotechnologists for all imaging-guided fine needle aspirations. Slides were prepared and stained by standard methodology. Routine cytologic preparations included (1) Diff Quick (DQ)-stained air-dried smears, (2) H&E and Pap-stained alcohol-fixed smears, (3) Pap-stained Thin Prep slides monolayer prepared by transfer of cells in suspension, such as FNA needle rinse or effusion fluid, from CytoLyt fixative onto a slide by an automated processor ThinPrep 2000 and (4) H&E-stained cell blocks (paraffin-embedded cell pellets prepared by contrifugation of CytoLyt fluid after the addition of HistoGel). The subtyping of NSCLC and the diagnosis of other tumors followed the standard cytomorphologic criteria.[19] Diagnoses for cytologic specimens were rendered by pathologists with subspecialty expertise in cytopathology.

Resected tumors were classified and graded according to the 2004 World Health Organization (WHO) Classification of Tumors by pathologists with subspecialty expertise in thoracic pathology.[20]

Utilization of IHC in routine clinical practice was reviewed. During the period of this study, IHC was routinely performed for all NSCLC for which a definite subtype could not be determined by cytomorphology alone and which were sufficiently cellular. Typical IHC panel included TTF-1 (Novocastra; Newcastle Upon Tyne, UK; dilution 1:50), p63/4A4 (Dako; dilution 1:5000), and high-molecular-weight cytokeratins (HMWCK)—34βE12/CK903 (Dako; Carpinteria, CA; dilution 1:800) and CK5/6 (Dako; dilution 1:200). Exact marker panels for various cases were selected at the discretion of individual pathologist but at the minimum included TTF-1 and p63. Interpretation was based on the following algorithm: TTF-1 negative/p63-positive/HMWCK-positive profile was interpreted as supportive of SqCC, whereas expression of TTF-1 was interpreted as supportive of ADC. On the basis of other studies, we allowed p63 and HMWCK coexpression with TTF-1 for classification as ADC.[21]

If cytomorphology was equivocal for NSCLC subtype, but cellularity was insufficient for IHC—a case was diagnosed as "NSCLC-NOS." For cases where cytomorphology was suggestive but not diagnostic of a NSCLC subtype, but material was insufficient for IHC confirmation—the case was given a "favored" diagnosis.

Data Analysis

For accuracy analysis, both definitive and favored diagnoses were counted as diagnostic of a tumor type. A match for at least one component (such as cytologic diagnosis of "ADC" for histologic diagnosis of "adenosquamous carcinoma" or "pleomorphic carcinoma with a component of ADC") was counted as correct.

For analysis of binary tumor categories (such as SqCC versus non-SqCC), cytologic diagnoses were designated as true positive (TP)—correct cytologic diagnosis of SqCC; true negatives (TN)—correct cytologic diagnosis of non-SqCC; false positives (FP)—incorrect cytologic diagnosis of SqCC; and false negatives (FN)—incorrect cytologic diagnosis of non-SqCC. The accuracy parameters were calculated as follows: sensitivity = TP/(TP + FN), specificity = TN/(FP + TN), positive predictive value = TP/(TP + FP), negative predictive value = TN/(FN + TN), and accuracy (TP + TN)/total number of cases.

Significance of associations was analyzed by two-tailed Fisher's exact test tests for categorical variables and unpaired *t* test for continuous variables. *p* values of ≤ 0.05 were considered statistically significant.

Review of *EGFR*/*KRAS* Molecular Testing on Cytologic Specimens

For review of molecular testing on cytologic specimens, we performed a separate search of the pathology department medical records to identify all cytologic specimens submitted to the Diagnostic Molecular Pathology Laboratory at Memorial Sloan-Kettering Cancer Center (New York City, NY) for *EGFR*/*KRAS* mutation testing during a 1-year period (January 1, 2009, to December 31, 2009). Typically, DNA was extracted from 5-μm-thick sections cut from the paraffin-embedded cell blocks (median number of sections was 14). Only cell blocks subjectively judged by a pathologist as "adequate" (easily identifiable tumor cells by light microscopy, with tumor cells representing at least 25% of overall cellularity[22]) were submitted for testing, although in rare cases a lower proportion of tumor cells was accepted. Testing for *EGFR* exon 19 deletion mutations, *EGFR* exon 21 L858R point mutations, and *KRAS* codon 12 and 13 point mutations was performed as described previously.[23,24]

Approval

This study was performed with the approval of the Institutional Review Board of Memorial Sloan-Kettering Cancer Center.

RESULTS

Specimen and Patient Characteristics

Cytologic specimens ($n = 192$) included transthoracic FNAs ($n = 181$), exfoliative specimens (bronchial brush/wash/lavage) ($n = 10$), and transbronchial FNA ($n = 1$). The underrepresentation of bronchial specimens and the lack of pleural effusion and sputum specimens in this series can be explained by selection for cases with subsequent resection and, therefore, limited representation of patients with advanced disease. Concurrent biopsy was obtained in addition to cytology for 13 (7%) patients. Resected specimens included wedge resections ($n = 64$), lobectomies ($n = 120$), and pneumonectomies ($n = 8$). Patient demographics were M:F ratio: 1:1.8, average age: 68 years, and age range: 42–86 years.

Feasibility and Accuracy of Tumor Subtyping in Cytology

Of 192 cytologic specimens, definitive versus favored versus unclassified carcinoma subtypes were diagnosed for 169 (88%) versus 15 (8%) versus 8 (4%) cases, respectively. The rate of unclassified diagnoses for patients with ADC and SqCC as a group was 3% (5/165 cases). Patients with ADC were more likely to have definitive subtyping by cytology than patients with SqCC (93% versus 74%, respectively; $p = 0.008$) (Table 1).

Cytologic/histologic correlation for patients with a specific tumor subtype diagnosed by both cytology and histology ($n = 183$) (Table 2) revealed that 171 cytologic diagnoses were concordant with histology (overall accuracy 93%). Discrepant diagnoses ($n = 12$) included three cytologic diagnoses of "ADC," which were reclassified as SqCC on resection, three "SqCC" reclassified as ADC, and one "carcinoid tumor" reclassified as ADC (a rare but well-known pitfall for carcinoid tumors with glandular-like architecture).[25] All remaining discrepancies were in the large cell histologic category, including three LCCs and two large cell neuroendocrine carcinomas, all of which were subtyped as ADC on cytology based on evidence of glandular features in cytologic preparations (see Discussion section). The eight cases with "unclassified carcinoma" or "NSCLC-NOS" diagnoses in cytol-

TABLE 1. Frequency of Definitive vs. Favored vs. Unclassified Tumor Subtypes in Cytology

	Histology			
Cytology	**All Tumors Combined (n = 192)**	**Adenocarcinoma (n = 142)**	**Squamous Cell Carcinoma (n = 23)**	**Adenocarcinoma + Squamous Cell Carcinoma Combined (n = 165)**
Definitive subtype	169 (88%)	133 (93%)	17 (74%)	150 (91%)
Favored subtype	15 (8%)	5 (4%)	5 (22%)	10 (6%)
Unclassified	8 (4%)	4 (3%)	1 (4%)	5 (3%)

TABLE 2. Correlation of Cytologic (Preoperative) vs. Histologic (Postoperative) Diagnoses

	Histology									
Cytology	**ADC**	**SqCC**	**Ad-Sq[a]**	**PC[b]**	**LCC**	**LCNEC**	**Carcinoid**	**SCLC**	**Unclassified Carcinoma[c]**	**Total**
ADC	132	1	1	2	2	2	1		1	142
NSCLC, favor ADC	3	2			1					6
SqCC	1	16	1							18
NSCLC, favor SqCC	2	3								5
SCLC or NE neo							7	2		9
Favor SCLC or NE neo							3	1		4
Unclassified carcinoma[d]	4	1		2					1	8
Total	142	23	2	4	3	2	11	3	2	192

Green shading—concordant cytology/histology; red shading—discordant cytology/histology; and gray shading—unclassified by cytology and/or histology.

[a] A predominant component of Ad-Sq carcinoma was sampled by cytology specimen and was correctly identified.

[b] Both pleomorphic carcinomas had a component of ADC, which was correctly identified by cytology.

[c] "Unclassified" histologic category includes two carcinomas with extensive necrosis and only rare viable tumor cells limiting further classification. One case had cytologic features diagnostic of ADC, but only minimal viable tumor remained in resected specimen after neoadjuvant therapy, preventing histologic classification.

[d] "Unclassified" cytologic category includes diagnoses of "NSCLC-not otherwise specified (NSCLC-NOS)" or "Carcinoma-not otherwise specified."

ADC, adenocarcinoma; SqCC, squamous cell carcinoma; Ad-Sq, adenosquamous carcinoma; PC, pleomorphic carcinoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; NE neo, neuroendocrine neoplasm (included tumors diagnosed as carcinoid tumor, NE carcinoma, NE neoplasm, and carcinoma with NE features); NOS, not otherwise specified.

TABLE 3. Sensitivity and Specificity of Cytologic Tumor Subtyping

	Sensitivity, % (95% CI)	**Specificity, % (95% CI)**	**PPV,[a] % (95% CI)**	**NPV, % (95% CI)**	**Accuracy, % (95% CI)**
SCLC vs. non-SCLC	100 (29.2–100)	100 (97.9–100)	100 (29.2–100)	100 (97.9–100)	100 (98.1–100)
Squamous vs. nonsquamous	87 (66.4–97.2)	98 (94.6–99.6)	87 (66.4–97.2)	98 (94.6–99.6)	97 (93.2–98.6)
Adenocarcinoma vs. nonadenocarcinoma	98 (93.9–99.6)	79 (63.2–89.7)	94 (88.7–97.2)	92 (77.5–98.3)	93 (89.1–96.3)

Analysis performed for all tumors with at least favored tumor subtype diagnosed by cytology and histology (n = 183). See Materials and Methods section for details.

[a] PPVs for definitive cytologic diagnoses of squamous cell carcinoma and adenocarcinoma were 94% and 96%, respectively.

CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; SCLC, small cell lung carcinoma.

ogy included pleomorphic carcinomas (n = 2), necrotic carcinoma, which remained unclassified on resection (n = 1), and cytologic specimens with insufficient cellularity for further characterization by IHC (n = 5).

While overall accuracy (concordance with histologic diagnosis) of cytologic tumor subtyping was 93%, the accuracy of definitive diagnoses was 96%. For a group of patients with histologic diagnosis of ADC and SqCC (n = 165), overall accuracy was 96%, and accuracy of definitive diagnoses was 99%. Definitive cytologic diagnoses of ADC or SqCC had positive predictive values for correct tumor type of 96% and 94%, respectively. For analysis of binary categories (Table 3), accuracy of cytology for the distinction of SCLC versus NSCLC was 100%, SqCC versus non-SqCC 97%, and ADC versus non-ADC 93%. Diagnosis of SqCC had lower sensitivity than ADC (87% versus 98%, respectively; p = 0.037) but higher specificity (98% versus 79%, respectively; p = 0.0001).

Clinicopathologic Factors Contributing to the Difficulty of NSCLC Subtyping in Cytology

For a group of patients with resection diagnosis of ADC and SqCC, clinicopathologic factors associated with difficulty in assigning correct definitive subtype by preoperative cytology are summarized in Table 4. They included low specimen cellularity (p = 0.019) and exfoliative (bronchial brush/wash/lavage) rather than FNA specimen type (p = 0.037), consistent with lower

TABLE 4. Clinicopathologic Factors Contributing to Difficulty of Cytologic Subtyping of Adenocarcinoma and Squamous Cell Carcinoma

	Correct Definitive Subtyping (n = 148)	Difficulty in Subtyping[a] (n = 17)	p[b]
Age: mean (range)	68 (42–86)	66 (53–86)	0.756
Gender			
Male	43 (86%)	7 (14%)	
Female	105 (91%)	10 (9%)	0.402
Specimen type			
FNA	143 (91%)	14 (9%)	0.037[c]
Exfoliative	5 (62%)	3 (38%)	
Specimen cellularity[d]			
Low	51 (82%)	11 (18%)	0.019[c]
High	97 (94%)	6 (6%)	
Differentiation[e]			
Well-moderately differentiated	128 (87%)	8 (47%)	0.0004[c]
Poorly differentiated	20 (13%)	9 (53%)	
Histologic diagnosis			
Adenocarcinoma	132 (93%)	10 (7%)	0.003[c]
Squamous cell carcinoma	16 (70%)	7 (30%)	

Analysis performed for cases with final (resection) diagnosis of adenocarcinoma and squamous cell carcinoma (n = 165).

[a] "Difficulty in subtyping" category includes cases that were incorrectly classified (n = 6), unclassified (n = 5), or underclassified/subtype favored (n = 6) by cytology.

[b] Fisher's exact test tests or unpaired t test.

[c] Considered significant ($p \leq 0.05$).

[d] Cellularity was defined by the presence (high) or absence (low) of cell block material.

[e] Grade of differentiation was based on resected specimens.

FNA, fine needle aspirates.

TABLE 5. Frequency and Accuracy of Morphologic vs. Immunohistochemistry-Aided Diagnoses of Adenocarcinoma and Squamous Cell Carcinoma in Cytology

	Frequency	Accuracy
Diagnosis based on morphology	146 (88%)	96%
Diagnosis based on IHC	14 (9%)	100%
Diagnosis that needs IHC but cellularity insufficient (diagnosis: NSCLC-NOS)	5 (3%)	na

Analysis performed for cases with final (resection) diagnosis of adenocarcinoma and squamous cell carcinoma (n = 165).

IHC, immunohistochemistry; na, nonapplicable; NSCLC, non-small cell lung carcinoma; NOS, not otherwise specified.

cellularity of the former specimens. The strongest predictor for difficulty in NSCLC subtyping was poor differentiation (p = 0.0004), as determined by the grade of the resected tumor. This analysis shows a greater difficulty with correct definitive identification of SqCC compared with ADC (70% versus 93%, respectively; p = 0.003), as also reflected by fewer definitive diagnoses (Table 1) and lower sensitivity (Table 3) for SqCC compared with ADC.

Accuracy of Subtyping Aided by IHC

As summarized in Table 5, for a group of patients with ADC and SqCC, morphologic features alone were sufficient to identify a tumor subtype (as definitive or favored) in the majority of cytologic specimens (146/165; 88%). Fourteen of 165 cases (9%) could not be subtyped by morphology, but after analysis with IHC, a tumor subtype was identified (based on the algorithm described in the Materials and Methods section). Histologic correlation revealed that 100% of IHC-aided diagnoses were correct, whereas morphologic diagnoses were 96% correct. Five of 165 cases (3%) could not be subtyped by morphology, but the cellularity was insufficient for IHC, and the diagnoses remained unclassified (NSCLC-NOS).

TABLE 6. Suitability of Cytologic Specimens for *EGFR* and *KRAS* Mutational Analysis: Review of Specimens Submitted for Molecular Testing during 1-yr Period (n = 128)

	N (%)
Types of cytology specimens tested	
Fine needle aspirates, transthoracic and transbronchial	67 (52)
Fine needle aspirates, extrathoracic	29 (23)
Pleural effusions	29 (23)
Bronchial brush/wash/lavage	3 (2)
Suitability for analysis	
Suitable	126 (98)
Unsuitable	2 (2)
Frequency of mutations	
EGFR mutant	31 (25)
KRAS mutant	25 (20)
No *EGFR*/*KRAS* mutations	70 (55)

Feasibility of *EGFR*/*KRAS* Molecular Testing on Cytologic Specimens

Because molecular testing on cytology is typically requested for patients with advanced disease, this analysis was performed on a different cohort of patients than the one used for the above cytologic/histologic correlation. As summarized in Table 6, cytologic specimens submitted for molecular testing during the study period (n = 128) included transthoracic and transbronchial FNAs (n = 67), extrathoracic FNAs (n = 29), pleural effusions (n = 29), and bronchial brush/wash specimens (n = 3). Of these, 126 (98%) were suitable for *EGFR*/*KRAS* testing, whereas two cases were not analyzable due to PCR failure (both failures were from poorly preserved specimens submitted from outside institutions). Of 126 analyzable samples, *EGFR* mutations were identified in 31 (25%) and *KRAS* mutations in 25 (20%) cases.

DISCUSSION

We confirm prior observations that cytology has exceptional accuracy for the distinction of SCLC versus NSCLC (100%). We also find that cytologic subtyping of ADC versus SqCC in a practice with routine utilization of IHC is highly feasible (3% unclassified rate) and accurate (96% concordance with resection diagnosis). In this series, IHC was used to subtype 9% of NSCLC that would otherwise be diagnosed as NSCLC-NOS by morphology, reflecting a significant reduction in this category as a result of IHC. Although there were rare morphologic misclassifications, the diagnoses confirmed by IHC had 100% accuracy. Of note, only a small

subset (13%) of cytologic specimens in this series was accompanied by a concurrent small biopsy; therefore, a separate study will be needed to make a direct comparison of efficacy and accuracy of NSCLC subtyping between cytology and small biopsy.

We find that the diagnosis of SqCC has a very high specificity (98%), indicating that false-positive diagnoses are rare, but the sensitivity for SqCC is lower than that of ADC (87% versus 98%, respectively), indicating that SqCC is underdiagnosed compared with ADC. Interestingly, several earlier studies, which contained predominantly bronchoscopic specimens, reported lower sensitivity for ADC rather than SqCC.[26,27] This difference may be due to the predominance of peripheral SqCC (sampled by transthoracic FNAs) in this series over central SqCC (sampled by bronchoscopic techniques) in prior studies: peripheral SqCC are generally less keratinizing than central SqCC and, therefore, more difficult to classify correctly by morphology.[28–30] Another potential contributing factor is the changing epidemiology of NSCLC, where SqCC is becoming an uncommon tumor type in North America (SqCC represented 12% of specimens in this study), and this may contribute to underrecognition of SqCC. Despite the lower sensitivity, the overall accuracy of SqCC versus non-SqCC diagnoses is high (97%), supporting that cytology is suitable for guiding the therapeutic decisions based on these binary categories.

In contrast to SqCC, we find that the issue with cytologic diagnosis of ADC is with lower specificity (79%). Rather than misdiagnoses of SqCC as ADC, the lack of specificity is in large part due to tumors that are classified as ADC in cytology and LCC in resection; and as discussed next, we favor that these do not represent true misdiagnoses. LCC is defined in the 2004 WHO Classification of Tumors as NSCLC lacking clear evidence of glandular or squamous differentiation by light microscopy after the entire tumor has been examined[20] (therefore, this definition is not applicable to cytology or small biopsy, where unclassified NSCLC is designated as NSCLC-NOS rather than LCC[20,31]). Although the WHO definition of LCC is based on light microscopy, it is long known that by electron microscopy[32,33] and more recently by IHC[34,35] a line of differentiation as ADC versus SqCC can be readily revealed in the majority of LCC. All LCC ($n = 3$) in this study were diagnosed cytologically as ADC based on evidence of glandular differentiation, which was inapparent in histology. Even though histologic diagnosis is considered a gold standard in cytologic/histologic correlation studies, in this instance, rather than representing an "incorrect" diagnosis, discordance was due to cytology allowing a more specific diagnosis because cytologic preparations preserved more identifiable features of differentiation than histology.

Only 3% of NSCLC in this study were unclassified by cytology, whereas a wide-range NSCLC-NOS frequency (8–37%) has been previously reported.[12,36,37] Similarly, compared with ADC/SqCC subtyping accuracy of 96% in this study, a wide range of typing accuracy (64–98%) has been reported.[27,29,30,38–43] There are three main factors that may explain the variability in the reported feasibility and accuracy of NSCLC subtyping.

1. Utilization of IHC: This is becoming routine for NSCLC subtyping in clinical practice, whereas most prior studies that reported higher rates of unclassified NSCLC and lower accuracy were based largely on morphology alone. Recent studies have demonstrated that IHC can effectively reveal the line of differentiation in small specimens originally diagnosed as NSCLC-NOS[16,17,44] and that IHC-aided diagnoses show a greater interobserver agreement than morphologic diagnoses.[17] In this study, we show the impact of IHC in actual clinical practice—IHC reduced the rate of unclassified NSCLC by 9% and increased accuracy over morphologic diagnoses.
2. Cytologic specimen type/diagnostic procedure: We show that accuracy of NSCLC subtyping was higher for FNAs than exfoliative specimens, reflecting higher cellularity of the former specimens. Therefore, studies dominated by exfoliative cytology (or sputums) can be expected to have lower accuracy of NSCLC subtyping than this study, in which the majority of specimens are transthoracic FNAs. In addition, because this is a surgical series, consisting of lower stage tumors, studies of advanced NSCLC can be anticipated to have a higher rate of difficult-to-classify tumors because poorly differentiated tumors (which are the main culprit for unclassified cytology) are overrepresented in advanced disease.[45]
3. Utilization of on-site immediate adequacy assessment: This practice is not universal, but at our institution, it is performed for every imaging-guided FNA. This practice has been previously shown to significantly increase adequacy, diagnostic accuracy, and cost-effectiveness of thoracic cytology.[46,47] On-site assessment ensures optimal cellularity, which is essential for accurate NSCLC subtyping (and is a critical consideration for ensuring sufficiency for predictive marker testing.)

In a recent review of the California Cancer Registry, it was reported that NSCLC-NOS represents 22% of pathologic (32% of cytologic and 19% of histologic) diagnoses of NSCLC and that there has been a substantial increase in this diagnosis between 1989 and 2006.[37] The aforementioned parameters (utilization of IHC, specimen type/diagnostic procedure, and on-site assessment) were not reported, precluding a direct comparison with the results of this study. We suggest that at least in part the high rate of NSCLC-NOS in recent years reflects a "practice trend," in which a generic diagnosis of NSCLC-NOS was widely encouraged in pathology,[13] and IHC was not yet widely used to classify difficult cases. Similar to our findings, there have been several studies before the emergence of the "NSCLC-NOS trend," which showed high feasibility and accuracy of cytologic subtyping of NSCLC, even in pre-IHC era.[38,40,42,48] We predict that in combination with morphologic criteria, utilization of IHC for difficult cases and greater awareness of clinical impact will lead to a substantial decline in the rate of NSCLC-NOS in the coming years. Nevertheless, this study

represents a single-institution experience, where cytology is practiced in a subspecialty setting, and the detailed data from other institutions will be needed to assess the experience with NSCLC subtyping in different practice settings at the time of the "paradigm shift" in the approach to NSCLC diagnosis.

An important consideration is a potential impact of the new approach to NSCLC diagnosis on the clinical outcomes data, particularly the treatment-by-histology interaction, which was established based on specimens diagnosed largely by light microscopy alone.[1] As discussed earlier, this and several recent studies show that IHC substantially reduces the rate of unclassified NSCLC,[16,17] while increasing the accuracy and interobserver agreement between pathologists.[17] How the more specific, accurate, and reproducible subtyping of advanced NSCLC will affect the clinical correlation studies will require empirical clinical data. Standardized criteria for classification of NSCLC in small biopsies and cytology specimens, which incorporates the utilization of IHC, are being proposed for the first time in a new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification.[31] This should help standardize terminology and criteria and hopefully will be incorporated into future clinical trials.

The optimal IHC algorithm for NSCLC subtyping is not firmly established. We find that our routine panel (which includes TTF-1, p63, and HMWCK) performed well in specimens with sufficient cellularity. Nevertheless, what constitutes a minimal panel with greatest accuracy needs to be assessed in focused studies. Several newer markers, including Desmocollin-3[35] and micro-RNA (hsa-miR-205)[44,49] have been suggested to distinguish SqCC from ADC, but their utility compared with standard markers needs to be evaluated further.

Finally, we find that in a large screen of various cytologic specimens (FNAs, effusions, and exfoliative specimens) submitted for *EGFR*/*KRAS* testing, 98% of samples were suitable for analysis. Because the specimens were submitted for testing only if an adequate cell block was available (as determined by a pathologist's triage), the actual rate of adequacy for molecular testing in consecutive specimens is not reflected by this analysis and needs further study. Based on a recent study of patients enrolled in a rebiopsy protocol at our institution, 79% of FNAs and 89% of core biopsies were suitable for molecular testing,[50] but further studies are needed to define the criteria for minimal cellularity requirements and to establish optimal parameters for specimen procurement. We can conclude that testing is feasible and that specimens subjectively interpreted as "adequate" by pathologists yield sufficient DNA for mutational analysis, with only rare exceptions, which is in agreement with several other studies.[22,50–52] The rate of *EGFR* and *KRAS* mutations detected in cytologic specimens is comparable with the rate detected in surgical specimens at our institution.[53]

CONCLUSIONS

To our knowledge, this is the largest cytologic/histologic correlation study with a focus on accuracy of NSCLC subtyping in modern pathology practice, where IHC is routinely used for subtyping of morphologically unclassifiable NSCLC. In addition, this is the largest review of utilization of cytologic specimens for *EGFR*/*KRAS* molecular analysis. We find that cytologic subtyping of ADC and SqCC is highly feasible (unclassified rate 3%) and accurate (overall accuracy 96% and accuracy for IHC-aided diagnoses 100%). Furthermore, various cytologic specimens are suitable for *EGFR*/*KRAS* molecular testing, although precise guidelines for minimal cellularity requirements need further study. We suggest that the approach to NSCLC diagnosis in the era of histology and predictive marker-based therapeutic decisions need not shift to more invasive surgical procedures as has been suggested[54] and that cytologic specimens, when sufficiently cellular, can provide not only accurate diagnosis but also adequate material for molecular testing.

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