Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Accurate Classification of Non–Small Cell Lung Carcinoma Using a Novel MicroRNA-Based Approach

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Abstract

Purpose: Advances in targeted lung cancer therapy now demand accurate classification of non–small cell lung cancer (NSCLC). MicroRNAs (miRNA) are recently discovered short, noncoding genes that play essential roles in tissue differentiation during normal development and tumorigenesis. For example, hsa-miR-205 is a miRNA that is highly expressed in lung squamous cell carcinomas (SqCC) but not in lung adenocarcinomas. The differential expression of miRNAs could be exploited to distinguish these tumor types.

Experimental Design: One hundred and two resected NSCLCs were classified as SqCC or adenocarcinoma based on their histologic features and immunohistochemical profiles. Corresponding preoperative biopsies/aspirates that had been originally diagnosed as poorly differentiated NSCLCs were available for 21 cases. A quantitative reverse transcription-PCR diagnostic assay that measures the expression level of *hsa-miR-205* was used to classify the carcinomas as SqCC or adenocarcinoma based solely on expression levels. The two sets of diagnoses were compared.

Results: Using standard pathologic methods of classification (i.e., microscopy and immunohistochemistry), 52 resected lung carcinomas were classified as SqCCs and 50 as adenocarcinomas. There was 100% concordance between the diagnoses established by conventional and miRNA-based methods. MiRNA profiling also correctly classified 20 of the 21 preoperative biopsy specimens.

Conclusions: MiRNA profiling is a highly reliable strategy for classifying NSCLCs. Indeed, classification is consistently accurate even in small biopsies/aspirates of poorly differentiated tumors. Confirmation of its reliability across the full range of tumor grades and specimen types represents an important step toward broad application. *Clin Cancer Res; 16(2); 610–9. ©2010 AACR.*

Lung cancer is the leading cause of cancer-related death in both men and women worldwide. In the United States, there are over 215,000 new cases of lung cancer each year resulting in over 160,000 deaths annually (1). Eighty-five percent of these lung cancers are non–small cell lung cancers (NSCLC), a heterogeneous group comprised of mostly squamous cell carcinomas (SqCC) and adenocarcinomas. In the past, the only diagnostic branch point in the classification of lung cancers that carried any relevance was the distinction between small cell carcinoma and NSCLC. Further subclassification of the NSCLCs has not been important from a therapeutic or prognostic standpoint. The recent emergence of targeted lung cancer therapies directed against specific cellular alterations, however, now demands the most precise classification possible for NSCLCs. For example, lung adenocarcinomas are more likely to harbor activating mutations of epidermal growth factor receptor and, in turn, are more likely to respond to epidermal growth factor receptor tyrosine kinase inhibitors (2). Similarly, certain angiogenesis inhibitors such as bevacizumab are more effective in the treatment of adenocarcinomas. Not only is bevacizumab less effective in treating squamous cell lung carcinoma, but the squamous phenotype is associated with higher rates of life-threatening pulmonary hemorrhage (3, 4). As other targeted drugs enter the clinical arena, the precise subclassification of NSCLCs will become increasingly important to better assess differential side effects and efficacy profiles.

Routine histopathology is the current standard of lung cancer classification, but this time-honored method is unreliable (5–7). In one study addressing the reproducibility of histopathologic classification of resected lung cancers, the central expert pathologist disagreed with the regional pathologists in one third of the cases (5). The difficulty is vastly inflated in the preoperative setting where attempts at tumor classification in small diagnostic samples (e.g., fine-needle aspirates) are further handicapped by the paucity of tumor cells and the absence of tissue architecture

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Translational Relevance

Recent advances in targeted lung cancer therapy now demand accurate classification of NSCLC. Whereas the distinction between squamous cell carcinoma and adenocarcinoma used to be of no clinical relevance, accurate subclassification is becoming increasingly important. This recent emphasis on accurate subclassification has exposed weaknesses in traditional microscopy and has prompted the expansion of adjunct diagnostic techniques including immunohistochemistry, notwithstanding persistent problems with immunohistochemical staining relating to suboptimal sensitivity/specificity, absence of quantification standards, and staining deviation reflecting technical inconsistencies. MicroRNAs (miRNA) are recently discovered short, noncoding genes that play essential roles in tissue differentiation during normal development and tumorigenesis. Certain miRNAs are differentially expressed in lung squamous cell carcinomas and adenocarcinomas. Retention of distinguishing miRNA profiles across all histologic grades and specimen types could provide a reliable and standardized method for the classification of NSCLC, even preoperative biopsies/fine-needle aspirates of poorly differentiated cancers.

(6, 8, 9). The inadequacy of traditional light microscopy alone has prompted the pursuit of adjunctive assays such as immunohistochemistry. Although immunohistochemistry has improved the precision and accuracy of lung cancer classification to some degree (8–10), its effectiveness is limited by the variable sensitivity and specificity of each individual marker, capricious staining owing to technical inconsistencies and tumor heterogeneity, and the lack of standardization in staining interpretation (11, 12).

MicroRNAs (miRNA) are a recently discovered group of short (21-23 nucleotides in length), noncoding control genes that play an important role in regulating gene expression (13). The mature miRNA is incorporated into a large protein complex-the RNA-induced silencing complex-where it binds its corresponding mRNA and where it inhibits protein translation. MiRNAs are believed to play essential roles in tissue differentiation during normal development and oncogenesis (14). A subset of these miRNAs shows considerable tissue specificity (15). For example, *hsa-miR-205* is believed to be a marker of stratified squamous epithelium (16), and its expression is retained in SqCCs of the upper and lower respiratory tracts (17, 18). Indeed, the differential expression of hsa-miR-205 in lung SqCCs but not in adenocarcinomas provides a rationale for the classification of NSCLC based on miRNA expression profiling. Quantification of *hsa-miR-205* expression has recently been optimized for formalin-fixed and paraffin-embedded clinical samples such that levels of expression can now be used to classify NSCLCs with a high degree of sensitivity and specificity (19). The purpose of this study was to: (*a*) expand that experience to a large group of NSCLCs across a full spectrum of histologic grades; (*b*) establish the overall sensitivity and specificity of miRNA profiling based on the current benchmark of NSCLC classification, namely integration of microscopy with a panel of differentiation-specific immunohistochemical probes; and (*c*) apply miRNA profiling to the most common and problematic area of lung cancer classification-the limited pretreatment sampling of poorly differentiated NSCLCs.

Materials and Methods

Cases. Study approval was obtained from the Johns Hopkins Medical Institutions Internal Review Board. One hundred patients who had undergone lung resections for primary NSCLC between 2005 and 2007 were identified from a search of the archival surgical pathology files of The Johns Hopkins Hospital. Cases were included only if they had been originally diagnosed as adenocarcinoma or SqCC based on histologic evidence of glandular differentiation or squamous differentiation, respectively. A second set of 26 resected primary lung carcinomas was selected based on the availability of a preoperative biopsy diagnosed noncommittally as "poorly differentiated non–small cell carcinoma." After initial patient identification, all original histologic slides were reviewed, and an appropriate block containing >50% tumor was retrieved for RNA extraction and immunohistochemical analysis. We have found that the specificity and sensitivity of tumor classification by miRNA profiling is diminished when the majority of the test sample is contaminated by nonneoplastic cells (19). For those cases with a corresponding preoperative biopsy specimen, blocks from both the biopsies and subsequent resections were obtained for analysis.

Immunohistochemistry. All tumors were evaluated immunohistochemically for the expression of various markers that have been promoted for the classification of NSCLCs. Thyroid transcription factor-1 (TTF-1) is expressed in alveolar type II cells and in bronchiolar cells, and its expression is often retained in adenocarcinomas derived from these cell types (20, 21). Napsin-A is an aspartic proteinase that is involved in the maturation of surfactant protein B (22). Like TTF-1, Napsin-A is specifically expressed in lung adenocarcinomas (23, 24). P63 is a protein of the p53 family that plays an important role in the regulation of stem cell commitment toward a squamous phenotype. Accordingly, p63 immunostaining has been advocated as a means of discerning squamous differentiation in NSCLC (10, 11, 25).

Imunohistochemistry was done on a Leica Bondmax autostainer (Leica Microsystem) for Napsin A (monoclonal mouse anti-human antibody, clone IP64; 1:800 dilution; Novocastra), and on a Ventana XT autostainer (Ventana Medical Systems) for TTF-1 (monoclonal mouse anti-human antibody, clone 8G7G3/1; 1:500 dilution;

Cell Marque) and p63 (monoclonal mouse anti-human antibody, clone 4A4; prediluted; Cell Marque). For p63 and TTF-1, only distinct nuclear staining was regarded as positive expression, and for Napsin-A, only granular cytoplasmic staining was regarded as positive. Bronchial reserve cells served as positive internal controls for p63, and pneumocytes lining the alveolar septa served as positive internal controls for TTF-1 and Napsin-A.

RNA extraction. Total RNA was extracted as described previously (26). Briefly, sections from formalin-fixed and paraffin-embedded tissues were deparaffinized with xylene, washed in ethanol, and digested with Proteinase K. The RNA was extracted by acid phenol/chloroform followed by ethanol precipitation and DNase digestion. Following a second acid phenol/chloroform extraction, the pellet was resuspended in DDW and analyzed using spectrophotometer (Nanodrop1000).

Quantitative reverse transcription-PCR. MiRNA amounts were quantified using a quantitative reverse transcription-PCR method as recently described in ref. (27). RNA was incubated in the presence of poly(A) polymerase (Ambion, AM2030), $MnCl_2$, and ATP for 1 h at 37°C. Using an oligodT primer harboring a consensus sequence, reverse transcription was then performed on total RNA using SuperScript II RT (Invitrogen). An amount of cDNA equivalent to 0.5 to 10 ng of the total RNA extracted from the resected tumor or an equivalent dilution of the RNA extracted from the FNA samples (RNA quantity was below measurable threshold) was amplified by reverse transcription-PCR. The reaction contains a miRNA-specific forward primer, a universal reverse primer complementary to the consensus 3′ sequence of the oligodT tail, and a Taqman probe complementary to the 3′ of the specific miRNA sequence as well as to part of the polyA adaptor sequence. The cycle threshold (C_T, the PCR cycle at which probe signal reaches the threshold) was determined for each well.

A known SqCC with a defined C_T score was used as a positive control. A no-RNA sample was used as a negative control to detect potential contamination.

Score calculation and interpretation. The miRNAs *hsa-miR-205*, *hsa-miR-21*, and *U6 snRNA* were measured by quantitative reverse transcription-PCR in triplicate. The average C_T of the triplicates ($AvgCt_{miR205}$, $AvgCt_{mir21}$, and $AvgCt_{U6}$) was calculated, excluding outliers (replicates with C_T differing by more than one cycle from the median). Assays were repeated when no two replicates had a C_T within one cycle and when the $AvgCt_{U6}$ was not between 20 and 32 cycles. If the sample failed to meet these criteria a second time, it was classified as an assay failure. Sample score was then obtained using the formula Score = $AvgCt_{mir205} - [(AvgCt_{mir21} + AvgCt_{U6})/2]$.

Based on thresholds established in a previous study (19), a sample score of 2.5 was used as the threshold for separating SqCCs from nonsquamous carcinomas. Cancers with sample scores below this threshold were classified as SqCCs, and those with scores above this threshold were classified as adenocarcinomas. Scores within 1.5 C_T of the 2.5 cutoff (i.e., between 1 and 4) were considered to be more prone to measurement error and thus were further qualified as "near cutoff."

Results

Twenty-four of the lung cancer resections were eliminated from further analysis because the tissue blocks did not contain >50% tumor cells. Of the 26 preoperative biopsies, 5 tumor samples were depleted during the sectioning of the block for immunohistochemical analysis, and these too were eliminated from further analysis. The remaining 102 lung cancer resections and 21 preoperative biopsies formed the basis of this study, and the study results are shown in Table 1. Based on the integration of the morphologic and immunohistochemical findings, 52 of the tumors were SqCCs with histologic grades ranging from well (n = 2) to moderate (n = 35) to poor (n = 15), and 50 were adenocarcinomas with grades ranging from well (n = 9) to moderate (n = 24) to poor (n = 17).

Immunohistochemical staining. TTF-1 labeling was detected in 39 of the 50 (78%) resected adenocarcinomas and in none of the 52 resected SqCCs (Fig. 1). Staining for Napsin-A closely reflected TTF-1 staining with a concordance rate of over 99%. In effect, the addition of Napsin-A did not appreciably enhance the sensitivity or diminish the specificity of TTF-1 alone. P63 immunolabeling was detected in 46% of the adenocarcinomas and in 100% of the SqCCs (Fig. 1). The extent of p63 labeling (i.e., percent of tumor cells staining) was much greater in the SqCCs (range, 40-100%; average, 89%) than in adenocarcinomas (range, 0-80%; average, 9%). Six (12%) of the adenocarcinomas showed p63 staining in 40% or more of the tumor cells, but all six of these tumors were also positive for TTF-1 and Napsin-A. In effect, the sensitivity and specificity of p63 labeling for diagnosing SqCC was highly dependent on the quantitative thresholds and on the correlative analysis of TTF-1/Napsin-A expression. For example, the positive predictive value of nonquantified p63 staining alone for diagnosing SqCC was only 53%, but it was 100% when positive staining is defined as labeling of at least 40% of tumor cells in the context of negative TTF-1/Napsin-A staining.

Nine of the resected adenocarcinomas and 12 of the SqCCs were preceded by a biopsy or aspirate diagnosed as poorly differentiated NSCLC. In all cases, the inability to further classify these specimens as adenocarcinoma or SqCC reflected the combined effects of limited number of tumor cells, poorly differentiated morphology, and obscuring background inflammation. TTF-1 and Napsin-A staining was detected in 7 (78%) and 8 (89%) of the adenocarcinomas respectively, but in none of the SqCCs. P63 staining was present in 2 (22%) of the adenocarcinomas and 12 (100%) of the SqCCs. Although p63 labeling was high in the two adenocarcinomas (40% and 80%), the coexpression of TTF-1 and Napsin-A in these tumors did not support squamous differentiation.

MiRNA profiling. All 102 lung cancer resections and the 21 paired preoperative biopsies had valid amplifications

Table 1. Comparison of histologic, immunohistochemical, and miRNA findings for NSCLCs

Case	Histologic findings				Immunohistochemical findings						MiRNA findings					
	Diagnosis		Grade		TTF-1		Napsin		P63		Score		Classification		Near cutoff	
	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx
1	AC	—	Poor	—	100	—	100	—	20	—	9.33	—	AC	—	No	—
2	AC	—	Well	—	80	—	100	—	40	—	6.37	—	AC	—	No	—
3	AC	—	Mod	—	70	—	100	—	0	—	8.62	—	AC	—	No	—
4	AC	—	Mod	—	80	—	100	—	0	—	5.84	—	AC	—	No	—
5	AC	—	Mod	—	100	—	100	—	0	—	5.29	—	AC	—	No	—
6	AC	—	Well	—	100	—	100	—	60	—	5.94	—	AC	—	No	—
7	AC	—	Mod	—	100	—	100	—	70	—	5.08	—	AC	—	No	—
8	AC	—	Mod	—	80	—	100	—	1	—	3.89	—	AC	—	Yes	—
9	AC	—	Mod	—	0	—	0	—	0	—	9.19	—	AC	—	No	—
10	AC	—	Mod	—	100	—	100	—	0	—	7.53	—	AC	—	No	—
11	AC	—	Mod	—	60	—	100	—	5	—	7.23	—	AC	—	No	—
12	AC	—	Well	—	0	—	0	—	0	—	6.70	—	AC	—	No	—
13	AC	—	Mod	—	100	—	100	—	30	—	6.30	—	AC	—	No	—
14	AC	—	Well	—	100	—	100	—	20	—	6.34	—	AC	—	No	—
15	AC	—	Well	—	100	—	100	—	0	—	7.89	—	AC	—	No	—
16	AC	—	Poor	—	0	—	0	—	0	—	4.05	—	AC	—	No	—
17	AC	—	Poor	—	20	—	40	—	5	—	4.75	—	AC	—	No	—
18	AC	—	Mod	—	100	—	100	—	2	—	6.22	—	AC	—	No	—
19	AC	—	Mod	—	90	—	100	—	2	—	7.03	—	AC	—	No	—
20	AC	—	Mod	—	100	—	100	—	0	—	7.97	—	AC	—	No	—
21	AC	—	Well	—	0	—	0	—	0	—	6.88	—	AC	—	No	—
22	AC	—	Poor	—	100	—	100	—	2	—	8.68	—	AC	—	No	—
23	AC	—	Poor	—	100	—	100	—	0	—	8.44	—	AC	—	No	—
24	AC	—	Poor	—	100	—	100	—	30	—	8.10	—	AC	—	No	—
25	AC	—	Mod	—	80	—	100	—	0	—	8.18	—	AC	—	No	—
26	AC	—	Mod	—	80	—	100	—	5	—	5.09	—	AC	—	No	—
27	AC	—	Mod	—	80	—	50	—	0	—	5.01	—	AC	—	No	—
28	AC	—	Well	—	0	—	0	—	0	—	7.20	—	AC	—	No	—
29	AC	—	Poor	—	0	—	0	—	2	—	2.56	—	AC	—	Yes	—
30	AC	—	Well	—	100	—	100	—	0	—	11.22	—	AC	—	No	—
31	AC	—	Mod	—	100	—	100	—	2	—	5.97	—	AC	—	No	—
32	AC	—	Poor	—	0	—	0	—	0	—	9.86	—	AC	—	No	—
33	AC	—	Poor	—	0	—	0	—	2	—	3.69	—	AC	—	Yes	—
34	AC	—	Poor	—	100	—	100	—	0	—	5.62	—	AC	—	No	—
35	AC	—	Mod	—	100	—	100	—	3	—	7.86	—	AC	—	No	—
36	AC	—	Mod	—	100	—	100	—	50	—	4.55	—	AC	—	No	—
37	AC	—	Mod	—	100	—	100	—	0	—	5.70	—	AC	—	No	—
38	AC	—	Poor	—	10	—	30	—	0	—	7.22	—	AC	—	No	—
39	AC	—	Mod	—	100	—	100	—	0	—	5.34	—	AC	—	No	—
40	AC	—	Poor	—	80	—	100	—	5	—	6.24	—	AC	—	No	—
41	AC	—	Poor	—	0	—	0	—	5	—	7.98	—	AC	—	No	—
42	SqCC	—	Poor	—	0	—	0	—	80	—	0.51	—	SQCC	—	No	—
43	SqCC	—	Poor	—	0	—	0	—	100	—	0.09	—	SqCC	—	No	—
44	SqCC	—	Mod	—	0	—	0	—	70	—	2.17	—	SqCC	—	Yes	—
45	SqCC	—	Mod	—	0	—	0	—	90	—	0.62	—	SqCC	—	No	—
46	SqCC	—	Mod	—	0	—	0	—	100	—	0.48	—	SqCC	—	No	—
47	SqCC	—	Mod	—	0	—	0	—	80	—	0.82	—	SqCC	—	No	—
48	SqCC	—	Mod	—	0	—	0	—	90	—	–0.17	—	SqCC	—	No	—

(*Continued on the following page*)

Table 1. Comparison of histologic, immunohistochemical, and miRNA findings for NSCLCs (Cont'd)

Case	Histologic findings				Immunohistochemical findings						MiRNA findings					
	Diagnosis		Grade		TTF-1		Napsin		P63		Score		Classification		Near cutoff	
	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx
49	SqCC	—	Mod	—	0	—	0	—	100	—	0.60	—	SqCC	—	No	—
50	SqCC	—	Mod	—	0	—	0	—	100	—	0.63	—	SqCC	—	No	—
51	SqCC	—	Mod	—	0	—	0	—	100	—	0.04	—	SqCC	—	No	—
52	SqCC	—	Poor	—	0	—	0	—	40	—	−1.34	—	SqCC	—	No	—
53	SqCC	—	Mod	—	0	—	0	—	90	—	0.79	—	SqCC	—	No	—
54	SqCC	—	Mod	—	0	—	0	—	100	—	−0.29	—	SqCC	—	No	—
55	SqCC	—	Mod	—	0	—	0	—	100	—	0.52	—	SqCC	—	No	—
56	SqCC	—	Mod	—	0	—	0	—	100	—	0.40	—	SqCC	—	No	—
57	SqCC	—	Mod	—	0	—	0	—	100	—	0.07	—	SqCC	—	No	—
58	SqCC	—	Poor	—	0	—	0	—	100	—	0.76	—	SqCC	—	No	—
59	SqCC	—	Mod	—	0	—	0	—	100	—	0.65	—	SqCC	—	No	—
60	SqCC	—	Mod	—	0	—	0	—	100	—	0.12	—	SqCC	—	No	—
61	SqCC	—	Poor	—	0	—	0	—	100	—	0.27	—	SqCC	—	No	—
62	SqCC	—	Mod	—	0	—	0	—	100	—	2.36	—	SqCC	—	Yes	—
63	SqCC	—	Mod	—	0	—	0	—	100	—	1.83	—	SqCC	—	Yes	—
64	SqCC	—	Poor	—	0	—	0	—	90	—	1.32	—	SqCC	—	Yes	—
65	SqCC	—	Mod	—	0	—	0	—	90	—	0.94	—	SqCC	—	No	—
66	SqCC	—	Mod	—	0	—	0	—	100	—	0.93	—	SqCC	—	No	—
67	SqCC	—	Mod	—	0	—	0	—	100	—	1.63	—	SqCC	—	Yes	—
68	SqCC	—	Mod	—	0	—	0	—	100	—	0.41	—	SqCC	—	No	—
69	SqCC	—	Mod	—	0	—	0	—	80	—	1.67	—	SqCC	—	Yes	—
70	SqCC	—	Poor	—	0	—	0	—	100	—	2.11	—	SqCC	—	Yes	—
71	SqCC	—	Mod	—	0	—	0	—	100	—	1.43	—	SqCC	—	Yes	—
72	SqCC	—	Mod	—	0	—	0	—	100	—	0.46	—	SqCC	—	No	—
73	SqCC	—	Poor	—	0	—	0	—	100	—	0.68	—	SqCC	—	No	—
74	SqCC	—	Mod	—	0	—	0	—	100	—	−1.06	—	SqCC	—	No	—
75	SqCC	—	Well	—	0	—	0	—	100	—	1.58	—	SqCC	—	Yes	—
76	SqCC	—	Well	—	0	—	0	—	100	—	1.58	—	SqCC	—	Yes	—
77	SqCC	—	Mod	—	0	—	0	—	100	—	0.79	—	SqCC	—	No	—
78	SqCC	—	Mod	—	0	—	0	—	100	—	0.63	—	SqCC	—	No	—
79	SqCC	—	Poor	—	0	—	0	—	100	—	−0.04	—	SqCC	—	No	—
80	SqCC	—	Mod	—	0	—	0	—	80	—	−0.75	—	SqCC	—	No	—
81	SqCC	—	Mod	—	0	—	0	—	100	—	1.83	—	SqCC	—	Yes	—
82	AC	NSCC	Poor	Poor	0	20	5	90	0	5	3.95	5.74	AC	AC	No	No
83	AC	NSCC	Poor	Poor	70	0	90	80	0	0	7.15	9.21	AC	AC	No	No
84	AC	NSCC	Poor	Poor	0	0	0	0	0	0	10.91	11.79	AC	AC	No	No
85	AC	NSCC	Mod	Poor	90	100	100	100	0	0	3.89	4.75	AC	AC	Yes	No
86	AC	NSCC	Well	Poor	90	10	100	100	0	0	8.54	8.23	AC	AC	No	No
87	AC	NSCC	Mod	Poor	60	80	100	90	80	80	7.75	6.00	AC	AC	No	No
88	AC	NSCC	Poor	Poor	90	90	100	100	0	5	8.86	10.51	AC	AC	No	No
89	AC	NSCC	Mod	Poor	100	90	100	100	40	0	7.28	6.04	AC	AC	No	No
90	AC	NSCC	Mod	Poor	80	100	100	100	0	5	8.48	7.73	AC	AC	No	No
91	SqCC	NSCC	Poor	Poor	0	0	0	0	100	100	0.26	−1.46	SqCC	SqCC	No	No
92	SqCC	NSCC	Mod	Poor	0	0	0	0	100	100	−0.55	1.23	SqCC	SqCC	No	Yes
93	SqCC	NSCC	Mod	Poor	0	0	0	0	90	90	1.46	0.87	SqCC	SqCC	Yes	No
94	SqCC	NSCC	Mod	Poor	0	0	0	0	100	100	2.34	0.88	SqCC	SqCC	Yes	No
95	SqCC	NSCC	Mod	Poor	0	0	0	0	100	100	−0.78	0.67	SqCC	SqCC	No	No
96	SqCC	NSCC	Poor	Poor	0	0	0	0	100	10	2.33	1.77	SqCC	SqCC	Yes	Yes

(*Continued on the following page*)

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	Diagnosis		Grade		TTF-1		Napsin		P63		Score		Classification		Near cutoff	
	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx	Res	Bx
97	SqCC	NSCC	Mod	Poor	0	0	0	0	100	100	1.10	1.05	SqCC	SqCC	Yes	Yes
98	SqCC	NSCC	Poor	Poor	0	0	0	0	100	80	1.76	1.60	SqCC	SqCC	Yes	Yes
99	SqCC	NSCC	Poor	Poor	0	0	0	0	100	100	1.90	0.49	SqCC	SqCC	Yes	No
100	SqCC	NSCC	Mod	Poor	0	0	0	0	100	100	1.00	−2.34	SqCC	SqCC	Yes	No
101	SqCC	NSCC	Poor	Poor	0	0	0	0	100	100	1.14	2.15	SqCC	SqCC	Yes	Yes
102	SqCC	NSCC	Poor	Poor	0	0	0	0	80	90	0.64	3.51	SqCC	AC	No	Yes

as measured by *U6* levels. Using the previously established threshold of 2.5 for separating between squamous and nonsquamous differentiation, the sensitivity of identifying SqCC in the resection specimens was 100% (52 of 52), and the specificity for classifying tumors as nonsquamous was 100% (50 of 50; Fig. 2). The miRNA-based classification was outside the near cutoff region in 79 of 102 (77%) cases. There was no statistically significant difference

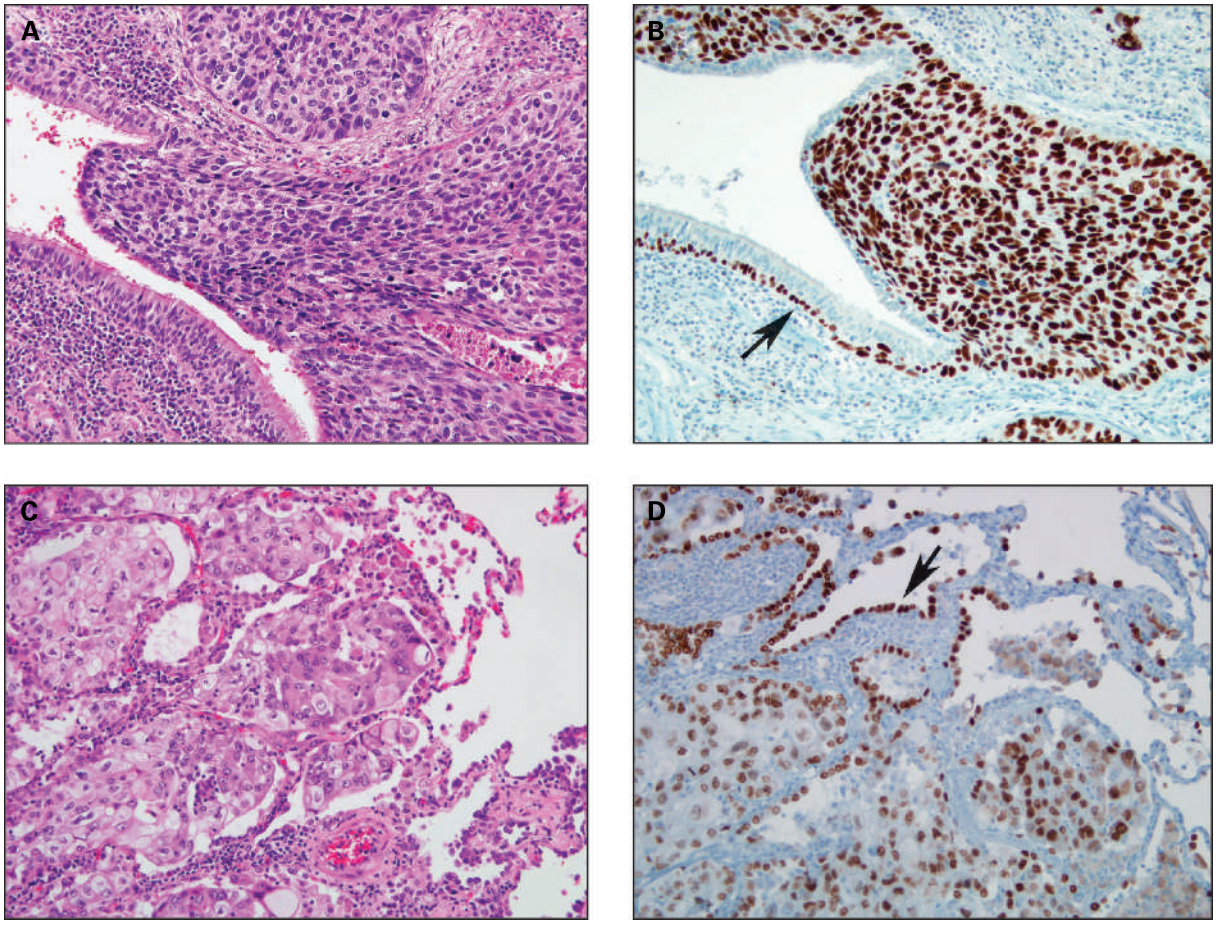

Fig. 1. Immunohistochemical staining of NSCLCs. A SqCC arising from the lining of an airway (*A*) exhibits strong and diffuse nuclear staining for p63 (*B*). The basal cell layer of the nonneoplastic airway epithelium serves as a positive internal control. An adenocarcinoma (*C*) exhibits nuclear staining for TTF-1 (*D*). The nonneoplastic pneumocytes serve as a positive internal control.

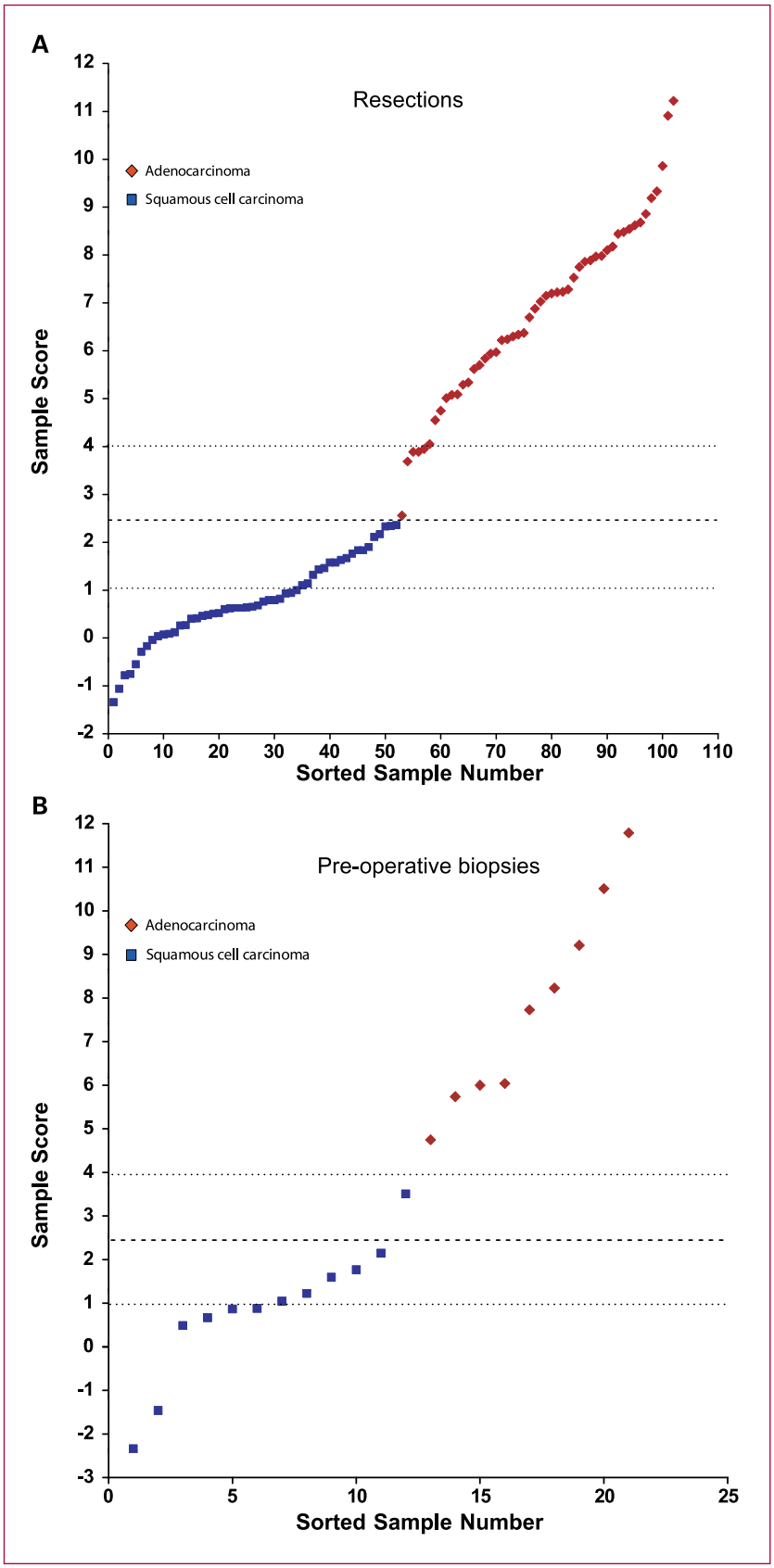

Fig. 2. Lung cancer classification by miRNA score values. The samples are sorted by increasing values of score. For the lung cancer resections (*A*), the reference score of 2.5 (*dashed line*) was found to have 100% sensitivity and 100% specificity in differentiating SqCCs (*blue boxes*) and adenocarcinomas (*red diamonds*). The dotted lines at scores 1 and 4 indicate the boundaries of the near cutoff region. For the preoperative biopsies and aspirates (*B*), one of the 21 lung cancers was misclassified using the same reference score—a SqCC with a score (3.51) in the near cutoff region for adenocarcinoma.

between poorly differentiated carcinomas and well- or moderately differentiated carcinomas in terms of the proportion of cases near the cutoff (25% versus 21%; P = 0.80). Classification remained highly accurate even for the 23 cases with scores near the 2.5 threshold (i.e., $1 \leq$ score ≤ 4). Thus, we did not consider values within the near cutoff range differently. MiRNA profiling correctly classified 20 of the 21 (95%) preoperative biopsies (Fig. 3). The single discordant case (case 102) involved a SqCC that was classified as adenocarcinoma by miRNA profiling with a score near the cutoff (score, 3.51). In 15 of the 21 (71.4%) biopsies, miRNA classification was made outside the near cutoff region.

Discussion

MiRNAs are short noncoding single-stranded RNAs that downregulate gene expression and, in turn, modulate cell growth and differentiation (13). As a promising new class of biomarkers, the detection of miRNAs in clinical samples may potentially affect all aspects of clinical care from early cancer detection (28, 29), to disease prognosis (17, 30, 31), to discernment of site of origin in those patients presenting with metastatic spread (15, 26). MiRNA profiling may also be useful in discriminating specific differentiation in tumors that are poorly differentiated and where accurate pathologic classification guide therapy. The subclassification of NSCLCs is an area of exceptional need and opportunity. For most advanced lung cancers, small specimens obtained by biopsy or fine-needle aspiration are the only available diagnostic material. In these scant and distorted specimens, accurate tumor classification is usually difficult if not impossible using conventional diagnostic techniques (6, 8, 9). Even in resection specimens, classification by routine light microscopy is subjective and vulnerable to interpathologist variability (5–7). Attempts

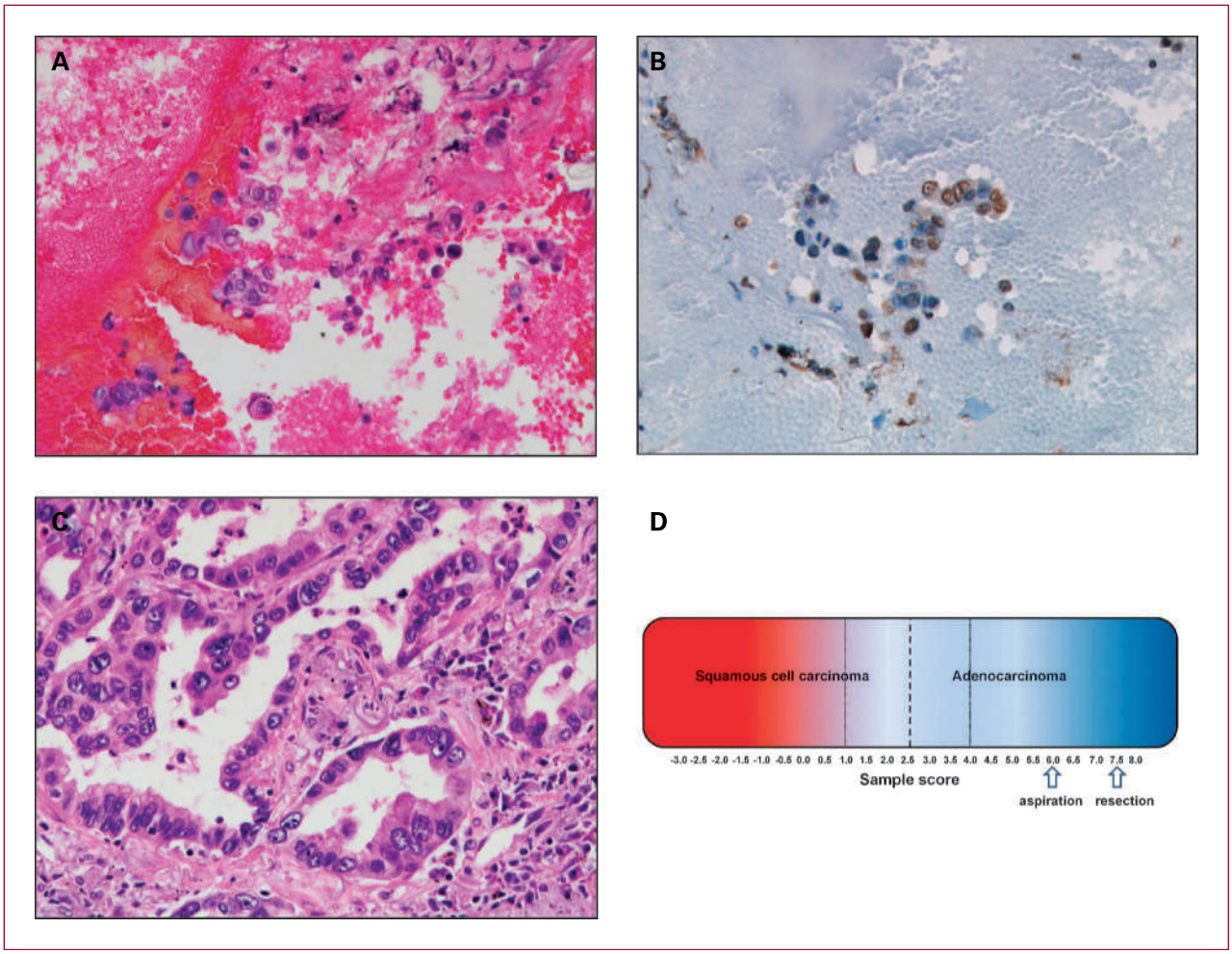

Fig. 3. This lung carcinoma (case 87) was diagnosed as a poorly differentiated non–small cell carcinoma based on a preoperative fine-needle aspiration specimen showing scattered discohesive and degenerating atypical cells in a background of blood (*A*). Although the presence of nuclear p63 immunolabeling (*B*) suggests a SqCC, the subsequent lung resection shows an adenocarcinoma with clear-cut glandular differentiation (*C*). Both the preoperative aspirate and resection samples had miRNA scores that were diagnostic of adenocarcinoma outside the near cutoff region (*D*).

to offset the deficiency of traditional light microscopy with the liberal use of adjunctive immunohistochemical stains are constrained by the sensitivities, specificities, and variabilities of the probes individually and as extended panels. Notwithstanding these difficulties, novel targeted therapies now demand consistently accurate classification of NSCLCs where treatment success and complications correlate with tumor classification.

Previously, it was shown that the expression of *hsa-miR-205* was restricted to the squamous phenotype, and that the measurement of *hsa-miR-205* expression was a highly reliable method of distinguishing lung SqCCs from adenocarcinomas (19). In this study, miRNA profiling inerrantly classified all 102 resected lung carcinomas as either SqCC or adenocarcinoma. Whereas the absence of clear-cut microscopic features of cellular differentiation (e.g., gland formation, keratinization) presents a formidable obstacle for traditional methods of classification, tumor grade did not impact on the accuracy of miRNA analysis. Poorly differentiated carcinomas were as reliably characterized as well-differentiated tumors, and with the same proportion of near cutoff scores. Likewise, limited tumor sampling did not significantly diminish the discriminating power of this approach. Twenty (95%) of the 21 preoperative fine-needle aspirates and core biopsies were correctly classified, although the specimens were scant and lacked any microscopic evidence of differentiation (Fig. 3). For lung cancer, the ability to apply a diagnostic test to small samples is compulsory. The majority of lung cancers are detected as advanced and inoperable tumors where resection specimens are simply unavailable. In these cases, treatment decisions must necessarily be guided by information gleaned from scant tissue and cytologic samples.

In this study, NSCLCs were classified as either SqCC or adenocarcinoma based on the current "gold standard" practice of integrating the microscopic and immunohistochemical analysis. Although the study does not serve as a direct comparison of immunohistochemical and miRNA approaches, it does highlight some notable differences between these methods. Unlike miRNA analysis, none of the immunohistochemical markers alone were entirely sensitive and specific in their ability to differentiate SqCC and adenocarcinoma. Consistent with other studies, staining for Napsin-A and TTF-1 were highly specific for adenocarcinoma, but staining was absent in 12% of the tumors showing microscopic evidence of glandular differentiation (23, 24, 32). Conversely, p63 staining was noted in all of the SqCCs, but it was also observed in 46% of the adenocarcinomas. Unlike miRNA analysis where sample scores unambiguously reside on one side or the other of a standardized threshold, immunohistochemical labeling is, at best, a "semi"quantitative assay that does not lend itself to objective quantitative analysis. Reproducibility of immunohistochemical analysis is highly vulnerable to staining variation as a function of tissue preservation and processing, to disparities in assay protocol and reagents, and to the interpretation bias inherent with observer-based assessment devoid of quantitative scoring systems (33). P63 labeling, for example, is touted as a marker of squamous differentiation (8, 9, 25, 34), but it is far from lineage specific when interpreted without quantitative thresholds (10, 12). Past experience with other immunohistochemical markers of therapeutic and prognostic importance such as estrogen receptor, progesterone receptor, and Her2 have underscored the difficulty in achieving diagnostic uniformity when it comes to staining interpretation (33, 35, 36).

As for the reproducibility of miRNA analysis, this study used a threshold established in a previous study using an independent set of lung cancer cases (19). The fidelity of this reference standard across independent sets of study cases represents an important step toward broad application. Quantitative separation of squamous and nonsquamous NSCLCs may not only benefit patients individually, but will promote enhanced objectivity among those trials correlating clinical end points with defined pathologic variables. Based on traditional approaches of lung cancer classification, recent studies of novel targeted therapies have underscored a compelling need to distinguish between SqCCs and adenocarcinomas. These subtypes are associated with their own differential side effects and efficacy profiles. Incorporation of miRNA analysis into future clinical trials may help further clarify the relationship of lineage differentiation and critical clinical end points in ways that help individualize oncologic therapy.

Disclosure of Potential Conflicts of Interest

H. Benjamin, A. Chajut, employees and/or equity holders, Rosetta Genomics; W. Westra, consultant, Prometheus Laboratories. The other authors disclosed no potential conflicts of interest.

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