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Original Research

CANCER

Classification of Non-small Cell Lung Carcinoma in Transthoracic Needle Specimens Using MicroRNA Expression Profiling

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Background: **Emerging targeted lung cancer therapies require the accurate morphologic subclassification of non-small cell lung cancer (NSCLC), even in scant and distorted specimens obtained by transthoracic needle aspiration (TTNA). MicroRNAs (miRNAs) are small noncoding genes recently reported as useful in differentiating squamous cell carcinoma (SCC) from adenocarcinoma (AD) in resected tumor specimens. We investigated their ability to do so in TTNA specimens.**
Methods: **Smears, immunocytochemistry slides, and corresponding cell blocks of 31 NSCLC TTNA specimens were retrieved and classified as AD or SCC based on their cytologic features and immunocytochemical profiles. Data on *EGFR* and *K-RAS* mutational status were available for all cases of AD. We quantified the *hsa-let-7* family and *hsa-miR-205* by quantitative reverse transcription-polymerase chain reaction and compared the miRNA expression levels in AD and SCC using Student *t* test.**
Results: **Eighteen cases were classified as AD and 13 as SCC by light microscopy and immunocytochemistry. miRNA expression profiles demonstrated considerable, statistically significant differences between AD and SCC, showing an upregulation of *hsa-let-7a*, *hsa-let-7b*, *hsa-let-7c*, *hsa-let-7f*, *hsa-let-7g*, *hsa-let-7i*, and *hsa-miR-98* and a downregulation of *hsa-miR-205* in AD specimens (all $P < .05$; *t* test).**
Conclusions: **Profiling the *hsa-let-7* family and *hsa-miR-205* is a promising method for differentiating AD from SCC, even in such small specimens as transthoracic aspirates. Subject to the validation of these findings in further, larger studies, this could prove to be a reliable, standardizable tool for the subclassification of NSCLC.** ***CHEST 2011; 140(5):1305–1311***

Abbreviations: AD = adenocarcinoma; CK = cytokeratin; EGFR = epidermal growth factor receptor; HRM = high-resolution melting; ICC = immunocytochemistry; miRNA = microRNA; NSCLC = non-small cell lung cancer; p63 = tumor protein 63; qRT-PCR = quantitative reverse transcription-polymerase chain reaction; SCC = squamous cell carcinoma; TTF-1 = thyroid transcription factor-1; TTNA = transthoracic needle aspiration

Lung cancer is the leading cause of cancer-related death worldwide.[1,2] Microscopically, lung cancer is classified as small cell or non-small cell lung cancer (NSCLC), the latter comprising a heterogeneous group that includes two of the most common subtypes (ie, adenocarcinoma [AD] and squamous cell carcinoma [SCC]).[3]

The emerging treatment revolution determined by new targeted therapies stresses the importance of accurate tumor subtyping as a mandatory step in the clinical workup of patients with lung cancer. For instance, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are effective in cases of lung

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cancer harboring an activating mutation of *EGFR*, which is more common in AD.[4-6]

Since a resection specimen cannot be obtained in most cases because of advanced and inoperable disease or patients having a low performance status, transthoracic needle aspiration (TTNA) under CT scan guidance and other such minimally invasive methods are widely used to obtain a definite pathologically confirmed diagnosis.[7-9]

On the other hand, the absence of the architectural structure of the tissue and the few malignant cells obtained in TTNA specimens do not always enable the required level of diagnostic accuracy to be obtained with light microscopy alone.[10] In addition, the analytical value of immunocytochemistry (ICC) is limited by intratumoral heterogeneity, inconsistent marker sensitivity and specificity, and variability in the interpretation of staining.[11-13]

MicroRNAs (miRNAs) are a class of short noncoding RNAs that modulate gene expression by targeting messenger RNAs and triggering either the repression of translation or RNA degradation.[14] miRNAs are involved in tissue differentiation during both normal development and carcinogenesis, and miRNA expression profiles have been seen as a promising new class of markers for tumor diagnosis and prognosis.[15-17] Recent studies have identified differences in miRNA expression between SCC and AD and have shown that miRNAs can be used to distinguish NSCLC subtypes reliably in histologic samples.[18-21]

In particular, *hsa-miR-205* expression was shown to be restricted to the squamous phenotype and was used to distinguish SCC from AD in a large series of resected NSCLC.[18-20] In their seminal work, Landi and colleagues[21] identified > 100 miRNAs differentially expressed in NSCLC subtypes and, after stratifying the results by stage, they found that the *hsa-let-7* family remained upregulated in AD by comparison with SCC. The aim of the present study was to investigate the role of the *hsa-let-7* family and *hsa-miR-205* in differentiating between the two major NSCLC subtypes in TTNA specimens, with a view to validating the reliability of these novel markers in routine diagnostic samples.

Materials and Methods

Patients and Specimens

Thirty-one cases (18 AD and 13 SCC) were retrieved from the files of the Surgical Pathology and Cytopathology Unit at Padova University Hospital. All patients had undergone CT scan-guided TTNA, improved by rapid on-site evaluation performed by an experienced cytopathologist (A. F.) at the Radiology Unit of Padova University Hospital between 2007 and 2010, as described elsewhere.[7] Overall, the male/female ratio was 25/6, and the patients' mean age was 65.3 ± 5.1 years (range = 47-83 years). For each patient, up to five cytologic smears were stained with Papanicolaou and/or Giemsa stains from the first TTNA aspirate. The second TTNA passage and the needle lavage were fixed in FineFix (Milestone Medical Technologies Inc; Kalamazoo, Michigan) for cell block preparation.[22] All cases were reviewed and classified by three pathologists (A. F., M. F., and R. C.) according to the World Health Organization classification.[3] A periodic acid-Schiff-diastase stain was performed on sections obtained from the cell blocks in cases of poorly differentiated carcinomas. Written informed consent to the study from the patients involved and the approval from the Padova University Hospital Ethical Committee were obtained.

Immunocytochemistry

ICC was performed on 3- to 4-μm paraffin sections obtained from cell blocks. Using the standard avidin-biotin-peroxidase complex method, staining was done automatically (BondmaX; Menarini; Florence, Italy), as described elsewhere,[7,23] for an accepted panel of ICC markers: (1) thyroid transcription factor-1 (TTF-1) (clone 8G7G3/1; DakoCytomation; Glostrup, Denmark; working dilution 1:100); (2) cytokeratin (CK) 5/6 (clone D5/16B4; DakoCytomation; working dilution 1:50); and (3) tumor protein 63 (p63) (clone 4A4; DakoCytomation; working dilution 1:100). Sections were then lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently for all the antisera applied. The expression of each ICC marker was jointly scored by two of the authors (A. F. and R. C.) and dichotomized as positive or negative.

High-Resolution Melting Analysis and DNA Sequencing

In AD samples, genomic DNA was extracted with the RecoverAll kit (Ambion; Austin, Texas) from five paraffin sections, each 5-μm thick, obtained from the cell blocks according to the manufacturer's instructions. The quality of the DNA was checked by gel electrophoresis, and its quantity was measured with the biophotometer (Eppendorf; Hamburg, Germany). High-resolution melting (HRM) was performed using specific primers for human *EGFR* and *K-RAS* genes, as described elsewhere.[7,8] Briefly, DNA was amplified by real-time polymerase chain reaction in the presence of a proprietary DNA-saturating dye contained in the LightCycler 480 High Resolution Melting Master (Roche Diagnostics; Indianapolis, Indiana). A melting curve was produced using high data-acquisition rates, and data were analyzed with the LightCycler 480 Gene Scanning Software Module to identify deletions and mutations. The reactions were performed on a gene scanning platform (LightCycler 480; Roche Diagnostics). After HRM assay, the reaction mixture was purified with the GenElute PCR Clean-Up kit (Sigma Aldrich; St. Louis, Missouri), according to the manufacturer's instructions, and the amplified products were sequenced. DNA sequencing was performed by BMR Genomics (Padova, Italy) on all the samples analyzed with the HRM assay. Sequence analysis was performed using Chromas 2.31 software (Technelysium Pty Ltd; Sydney, New South Wales, Australia).

RNA Extraction and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

All the residual cell clots were microdissected from the cell blocks previously fixed in FineFix and embedded in paraffin, then deparaffinized with xylene at 50°C for 3 min. Total RNA was extracted using the RecoverAll kit (Ambion), as reported elsewhere.[24-26] The NCode miRNA quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method (Invitrogen; Carlsbad, California) was used to detect and quantify *hsa-let-7a*, *hsa-let-7b*, *hsa-let-7c*, *hsa-let-7d*, *hsa-let-7e*, *hsa-let-7f*, *hsa-let-7g*,

Table 1—*Primers Used for the Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis*

Gene	Forward Primer (5′-3′)
Gene of interest	
hsa-let-7a	GCGGTGAGGTAGTAGGTTGTATAGTT
hsa-let-7b	CCTGAGGTAGTAGGTTGTGTGGTT
hsa-let-7c	CGCTGAGGTAGTAGGTTGTATGGTT
hsa-let-7d	GCAGAGGTAGTAGGTTGCATAGTT
hsa-let7e	GGCTGAGGTAGGAGGTTGTATAGTT
hsa-let-7f	GCCCCTGAGGTAGTAGATTGTATAGTT
hsa-let7g	GGCCTGAGGTAGTAGTTTGTACAGTT
hsa-let7i	GCTGAGGTAGTAGTTTGTGCTGTT
hsa-miR-98	GACGCTGAGGTAGTAAGTTGTATTGTT
hsa-miR-202	AGAGGTATAGGGCATGGGAA
hsa-miR-205	CTTCATTCCACCGGAGTCTG
Loading control	
RNU6B	ACGCAAATTCGTGAAGCGTT

hsa-let-7i, *hsa-miR-98*, *hsa-miR-202*, and *hsa-miR-205* (primer sequences in Table 1) on the LightCycler 480 Real-Time PCR System (Roche Diagnostics) according to the manufacturer's instructions. Normalization was done with the small nuclear RNA U6B (RNU6B; Invitrogen). qRT-PCRs were run in triplicate, including no-template controls.

Statistical Analysis

The data were analyzed using the STATA 8.1 software (STATA; College Station, Texas). Statistical significance was determined for qRT-PCR results using Student *t* test. A *P* value of $< .05$ was considered statistically significant.

Results

NSCLC TTNA Specimen Subclassification Using Conventional Pathologic Methods

Using standard methods (ie, integrating morphology with ICC), 18 cases were classified as AD and 13 as SCC (Table 2). The AD grades ranged from well (n = 5) to moderately (n = 9) to poorly differentiated (n = 4), and the SCC grades ranged from well (n = 3) to moderately (n = 7) to poorly differentiated (n = 3). Two AD specimens harbored a deletion in exon 19 of the *EGFR* gene, one had a missense mutation in exon 21 (affecting the activation loop of the [L858R] receptor) of the *EGFR* gene, and two had a point mutation in exon 2 of the *K-RAS* gene. At light microscopy, well- and moderately differentiated AD smears

Table 2—*Comparison of Cytologic, Immunocytochemical, and Mutational Findings for the Set of 31 Specimens*

	Cytology		Immunocytochemistry			
Case	Diagnosis	Grade	TTF-1	CK 5/6	p63	Mutation
1	AD	Well differentiated	+	−	−	None
2	AD	Moderately differentiated	+	−	−	None
3	AD	Well differentiated	+	−	−	None
4	AD	Moderately differentiated	+	−	−	None
5	AD	Poorly differentiated	+	−	−	*K-RAS*
6	AD	Well differentiated	+	−	−	None
7	AD	Well differentiated	+	−	−	*EGFR* (exon 19)
8	AD	Moderately differentiated	−	−	−	None
9	AD	Poorly differentiated	+	−	−	None
10	AD	Poorly differentiated	+	−	+	None
11	AD	Moderately differentiated	−	−	−	None
12	AD	Moderately differentiated	+	−	−	None
13	AD	Well differentiated	−	−	−	None
14	AD	Moderately differentiated	+	−	−	*EGFR* (exon 19)
15	AD	Moderately differentiated	+	−	−	*EGFR* (exon 21)
16	AD	Moderately differentiated	+	−	−	None
17	AD	Poorly differentiated	+	−	−	None
18	AD	Moderately differentiated	+	−	−	*K-RAS*
19	SCC	Moderately differentiated	−	−	+	Not performed
20	SCC	Moderately differentiated	−	−	+	Not performed
21	SCC	Well differentiated	−	+	+	Not performed
22	SCC	Moderately differentiated	−	+	+	Not performed
23	SCC	Poorly differentiated	−	−	+	Not performed
24	SCC	Moderately differentiated	−	+	+	Not performed
25	SCC	Poorly differentiated	−	+	+	Not performed
26	SCC	Moderately differentiated	−	+	+	Not performed
27	SCC	Well differentiated	−	+	+	Not performed
28	SCC	Poorly differentiated	−	−	+	Not performed
29	SCC	Moderately differentiated	−	+	+	Not performed
30	SCC	Moderately differentiated	−	+	+	Not performed
31	SCC	Well differentiated	−	+	+	Not performed

AD = adenocarcinoma; CK = cytokeratin; − = negative; p63 = tumor protein 63; + = positive; SCC = squamous cell carcinoma; TTF-1 = thyroid transcription factor-1.

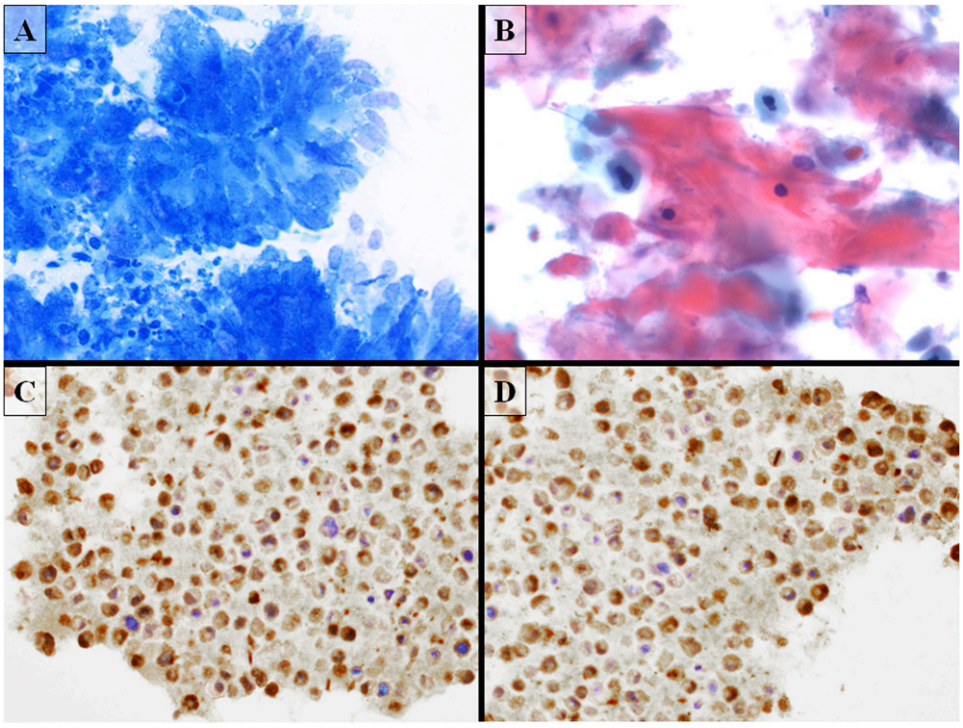

FIGURE 1. Smears and immunocytochemistry staining of non-small cell lung cancer transthoracic needle aspiration. A, Adenocarcinoma (AD) cells arranged in three-dimensional pseudo-papillary clusters, presenting delicate cytoplasm and peripheral nuclei (Papanicolaou, original magnification × 20). B, Squamous cell carcinoma (SCC) keratinized cells with abundant cytoplasm and irregular nuclei (Papanicolaou, original magnification × 40). C, AD cells exhibit strong and diffuse nuclear immunostaining for thyroid transcription factor-1 on cell block slide (original magnification × 20). D, SCC nuclei display broad tumor protein 63 labeling on cell block slide (original magnification × 20).

displayed tumor cell clusters arranged in morulae, acini, and pseudo-papillae, with clearly delineated margins and an abundant basophilic cytoplasm, with or without vacuoles (Fig 1A). The nuclei were eccentric, round to oval, with relatively smooth contours. Well- and moderately differentiated SCC smears usually showed isolated large tumor cells with central and abnormal nuclei, one or more small nucleoli, abundant cytoplasm, sometimes with keratin pearls against a background of necrosis and cell debris (Fig 1B). Cells were occasionally arranged in sheets, displaying spindle-shaped or elongated features. In the Papanicolaou-stained smears, all three well-differentiated SCC showed keratinized carcinoma cells with an orange cytoplasm. Seven specimens displayed a poorly differentiated morphology with few specific signs suggesting an AD or SCC differentiation, and their precise classification was possible through the support of both the periodic acid-Schiff-diastase stain and the ICC data.

TTF-1 is often expressed in AD derived from type 2 pneumocytes or nonciliated bronchiolar cells[13,27,28]; TTF-1 staining was detected in 15 AD specimens (83%) but none of the SCC specimens (Fig 1C). CK 5/6 are intermediate filaments found in stratified squamous epithelia and their labeling was detected in nine SCC (69%) and none of the AD specimens.[11,13,29] p63 is a protein in the p53 family that regulates stem cell commitment toward a squamous phenotype[11,13,30]; p63 immunostaining was seen in one AD specimen (6%), which was positive for TTF-1 and negative for CK 5/6, and in 13 (100%) of the SCC specimens (Fig 1D).

miRNA Expression Profiling

All 31 microdissected TTNA cell clots from cell blocks had valid amplifications as measured from the amounts of *U6B*. Analyzing the *hsa-let-7* family with qRT-PCR revealed that *hsa-let-7a* ($P = .030$), *hsa-let-7b* ($P = .048$), *hsa-let-7c* ($P = .031$), *hsa-let-7f* ($P = .032$), *hsa-let-7g* ($P = .014$), *hsa-let-7i* ($P = .045$), and *hsa-miR-98* ($P = .018$) levels were significantly higher in AD than in SCC (Fig 2), whereas the other members of the *hsa-let-7* family (*hsa-let-7d*, *hsa-let-7e*, and *hsa-miR-202*) showed no such differences between these NSCLC subtypes (Fig 2). Conversely, the amounts of the squamous marker *hsa-miR-205* were significantly higher in the SCC than in the AD specimens ($P < .001$) (Fig 2). No significant association was observed between miRNA expression and clinicopathologic variables.

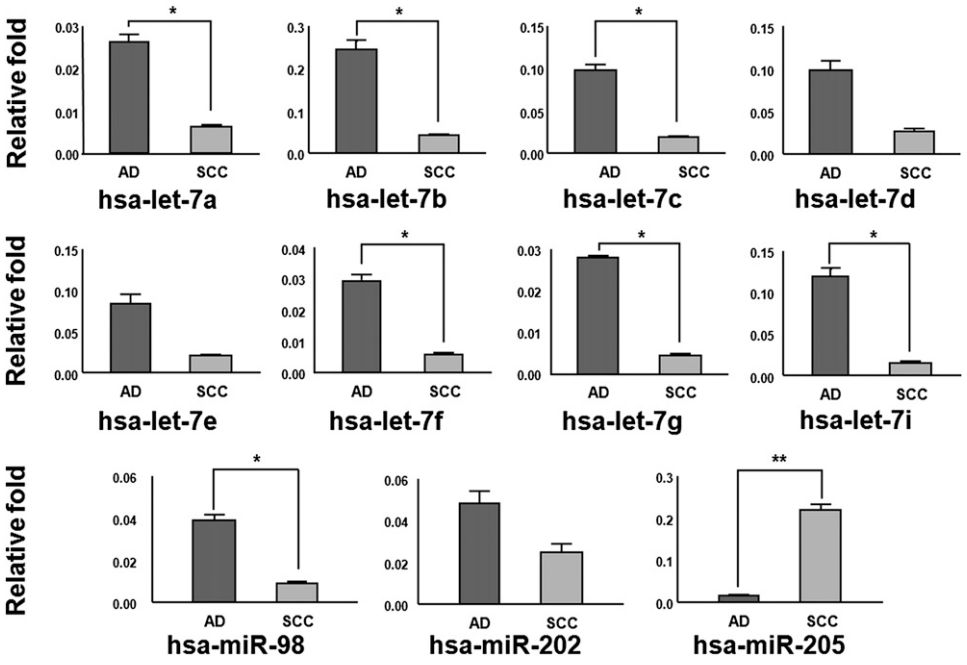

FIGURE 2. Relative fold of *hsa-let-7a*, *hsa-let-7b*, *hsa-let-7c*, *hsa-let-7d*, *hsa-let-7e*, *hsa-let-7f*, *hsa-let-7g*, *hsa-let-7i*, *hsa-miR-98*, *hsa-miR-202*, and *hsa-miR-205* levels in lung AD (dark gray) and SCC (light gray) transthoracic needle aspiration specimens. MicroRNA expression profiles markedly and significantly differed between AD and SCC, showing an upregulation of *hsa-let-7a* ($P = .030$), *hsa-let-7b* ($P = .048$), *hsa-let-7c* ($P = .031$), *hsa-let-7f* ($P = .032$), *hsa-let-7g* ($P = .014$), *hsa-let-7i* ($P = .045$), and *hsa-miR-98* ($P = .018$) and a downregulation of *hsa-miR-205* ($P < .001$) in AD by comparison with SCC specimens. Conversely, *hsa-let-7d* ($P = .228$), *hsa-let-7e* ($P = .273$), and *hsa-miR-202* ($P = .479$) did not show any difference between the two non-small cell lung cancer subtypes. Columns, representative images of reactions run in triplicate; bars, SD. $^{*}P < .05$; $^{**}P < .01$. See Figure 1 legend for expansion of abbreviations.

DISCUSSION

In routine practice, the majority of lung cancers are clinically identified in elderly patients with a poor performance status or when the disease is already in an advanced stage and consequently inoperable.[1,2] On the other hand, with the advances achieved in the new lung cancer targeted therapies, distinguishing between AD and SCC has now become crucially important, so minimally invasive methods are increasingly being used in such patients to arrive at a pathologically confirmed diagnosis. Indeed, CT-guided TTNA carries a low complication rate and enables the needle tip to be positioned precisely inside the lesion, and its diagnostic performance is considerably improved by combining it with rapid on-site evaluation, which can adequately ascertain the quality and quantity of tumor cells obtained with the aspirate.[7,9]

A precise oncological subtyping is not always possible on cytology, however, especially in the case of poorly differentiated tumor cells or limited, distorted specimens.[10] The use of an adequate panel of ICC stains could significantly improve diagnostic accuracy, although its effectiveness is influenced by the variable sensitivity and specificity of each marker, intratumoral heterogeneity, and the subjective interpretation of staining.[11-13] In this setting, the introduction of innovative, reliable markers of tumor differentiation is an impellent prerequisite for the achievement of an unequivocal diagnostic message and the adoption of histotype-specific therapeutic strategies.

In recent years, no biomarker has generated as much interest as miRNAs, which have been considered for a variety of purposes, including lung cancer differentiation. Indeed, the *hsa-let-7* family and *hsa-miR-205* expression are reportedly specific to AD and SCC, respectively,[18-21] and analysis of their expression has proved a reliable tool for subclassifying NSCLC, even in poorly differentiated tumor samples.[18-21]

Our findings confirm that miRNA expression profiles are a suitable tool for distinguishing between AD and SCC, with seven out of 10 members of the *hsa-let-7* family strongly overexpressed in AD by comparison with SCC, whereas *hsa-miR-205* is markedly up-regulated in SCC by comparison with AD. It is probably because of the limited number of our specimens that *hsa-let-7d*, *hsa-let-7e*, and *hsa-miR-202* did not appear to differ between the two NSCLC subtypes.

Although this study was not designed to compare miRNA and ICC methods, it did bring to light some noteworthy differences between the two techniques: The effectiveness of miRNA expression profiling is

not limited by any variability in subjective interpretation or in the sensitivity and specificity of each probe because the analysis is based on unambiguous scores. Moreover, none of the routinely used ICC markers alone could adequately diversify AD from SCC, confirming data previously reported by other authors.[11-13] For instance, TTF-1 was detected in none of the SCC specimens but also failed to react in 17% of the AD, whereas CK 5/6 labeling was absent in 31% of the SCC as well as in all of the AD. Last, although immunostaining for p63 was seen in all the cases of SCC, it was also detected in one AD, making it necessary to combine it with the use of other markers to obtain a reliable interpretation.

Conclusions

In conclusion, the present study strongly supports the value of the *hsa-let-7* family and *hsa-miR-205* expression profiling as an innovative method for differentiating AD from SCC in cytologic specimens obtained by means of such a minimally invasive and safe technique as CT-guided TTNA. If these findings are validated in further, larger studies, this approach could provide pathologists with a reliable and more standardizable method for subclassifying NSCLC, even in poorly differentiated tumor samples, to meet the needs of the new therapeutic standards.

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Dr Fassina: contributed to conceiving the hypotheses and planning the experiments, interpreting the data, critically revising the article, and approving the final submitted version.
Dr Cappellesso: contributed to conceiving the hypotheses, planning and carrying out the experiments, analyzing and interpreting the data, drafting the article, and approving the final submitted version.
Dr Fassan: contributed to conceiving the hypotheses, planning and carrying out the experiments, analyzing and interpreting the data, and approving the final submitted version.
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