Heterogeneity of Large Cell Carcinoma of the Lung

An Immunophenotypic and miRNA-Based Analysis

Mattia Barbareschi, MD,[1-3] Chiara Cantaloni, PhD,[1,3] Valerio Del Vescovo, PhD,[4] Alberto Cavazza, MD,[5] Valentina Monica, MD,[6] Rodolfo Carella, MD,[7] Giulio Rossi, MD,[8] Luca Morelli, MD,[1] Alberto Cucino,[1] Massimo Silvestri, MD,[9] Giuseppe Tirone, MD,[9] Giuseppe Pelosi, MD,[10] Paolo Graziano, MD,[11] Mauro Papotti, MD,[6] Paolo Dalla Palma, MD,[1] Claudio Doglioni, MD,[12] and Michela Alessandra Denti, PhD[4]

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Abstract

Large cell carcinomas (LCCs) of the lung are heterogeneous and may be of different cell lineages. We analyzed 56 surgically resected lung tumors classified as LCC on the basis of pure morphologic grounds, using a panel of immunophenotypic markers (adenocarcinoma [ADC]-specific, thyroid transcription factor-1, cytokeratin 7, and napsin A; squamous cell carcinoma [SQCC]–specific, p63, cytokeratin 5, desmocollin 3, and Δnp63) and the quantitative analysis of microRNA-205 (microRNA sample score [mRSS]). Based on immunoprofiles 19 (34%) of the cases were reclassified as ADC and 14 (25%) as SQCC; 23 (41%) of the cases were unclassifiable. Of these 23 cases, 18 were classified as ADC and 5 as SQCC according to the mRSS. Our data show that an extended panel of immunohistochemical markers can reclassify around 60% of LCCs as ADC or SQCC. However, a relevant percentage of LCCs may escape convincing immunohistochemical classification, and mRSS could be used for further typing, but its clinical relevance needs further confirmation.

Large cell carcinoma (LCC) of the lung is 1 of 4 major histopathologic tumor subtypes recognized by current classifications of lung tumors. However, although squamous cell carcinoma (SQCC), adenocarcinoma (ADC), and small cell carcinoma are well-defined entities with typical morphologic, immunophenotypic, and molecular features, LCCs, with the exception of the rare neuroendocrine, rhabdoid, basaloid, and lymphoepithelioma-like subtypes, are defined as poorly differentiated non–small cell tumors lacking features of ADC and SQCC. Therefore, the term LCC has frequently and improperly been used as a synonym of undifferentiated non–small cell lung carcinoma (NSCLC) and has been used as a "wastebasket" for tumors lacking a definite morphologic pattern.

Studies show that, by using ancillary techniques, a relevant percentage of LCCs could be reclassified as SQCC or ADC. Gene profiling shows that most LCCs have profiles quite similar to ADC or SQCC.[1-3] Similarly, by using appropriate immunohistochemical stains, almost two thirds of LCCs can be reclassified as poorly differentiated ADC or SQCC.[4,5] These studies have profound clinical relevance because rendering a diagnosis of LCC may represent a challenge for oncologists who need accurate subtyping of lung cancers to provide patients with optimal targeted chemotherapeutic agents, showing different efficacy with specific NSCLC categories (usually effective for ADC and not for others).[6,7]

Besides immunohistochemical studies and gene profiling, microRNA analysis seems to be a new promising diagnostic tool for lung cancer. Lebanony et al[8] and Bishop et al[9] reported that the relative quantification of microRNA-205, in comparison with 2 other small noncoding RNAs (the "oncomicroRNA" microRNA-21 and the housekeeping small

nuclear [sn]RNA U6, using the microRNA sample score [mRSS] method) correctly distinguishes lung SQCC from ADC with the same, if not higher, diagnostic power as expert histologic diagnosis. In a previous study of surgical samples of morphologically classifiable SQCC and ADC, Del Vescovo et al[10] confirmed the accuracy and robustness of the assay, which in our hands reached a sensitivity of 80.8% for ADC and 100% for SQCC.

The aims of the present study were to analyze a series of 56 surgically resected lung tumors that, on the basis of purely morphologic grounds, were classified as LCC, and to study their immunoprofiles by using a panel of immunophenotypic markers (ADC-specific, thyroid transcription factor [TTF]-1, cytokeratin [CK]7, and napsin A; SQCC-specific, p63, CK5, desmocollin [DSC]3, and Δnp63)[11-19] and the molecular test based on the quantitative analysis of microRNA-205.[8-10]

Materials and Methods

Histologic Material

A series of 56 consecutive LCCs of the lung, excluding neuroendocrine LCC,[20] resected between 1998 and 2005 were retrieved from the archives of the Units of Surgical Pathology of the S. Chiara Hospital, Trento, Italy; the Arcispedale S. Maria Nuova, Reggio Emilia, Italy; and San Maurizio Hospital, Bolzano, Italy. The patients included 9 women and 47 men; 36 were smokers, 3 were nonsmokers, and in 17 cases smoking habits were not known; 39 tumors were peripheral, 10 were central, and 7 had an unknown location. All cases were routinely formalin-fixed and paraffin-embedded; characteristics are described in detail in **Table 1**. Following complete anonymization of the samples, H&E-stained histologic slides from all cases were reviewed, and 1 representative tissue block was selected.

All tumors were immunostained for TTF-1, dilution 1:400 (clone SPT24, Leica Microsystems, Newcastle upon Tyne, England); napsin A, dilution 1:200 (clone TMU-Ad02, ARP American Research Products, Belmont, MA); p63, dilution 1:400 (clone 7JUL, Leica Microsystems); Δnp63, dilution 1:2,000 (p40 rabbit polyclonal, Calbiochem-Merck, Darmstadt, Germany); DSC3, dilution 1:30 (clone Dsc3-U114, PROGEN Biotechnik, Heidelberg, Germany); CK7, dilution 1:250 (clone OV-TL 12/30, Leica Microsystems); and CK5, dilution 1:600 (clone XM26, Leica Microsystems). Immunostaining was performed using the Bondmax automated incubation station (Leica Microsystems) as described.[21]

Immunohistochemical results were semiquantitatively evaluated as follows: 0, no reactivity; 1, fewer than 10% of faintly to moderately stained cells; 2, 10% to 50% of moderately to strongly reactive cells; and 3, more than 50% of moderately to strongly reactive cells. For statistical analysis, cases with 0 or 1 were considered as having low expression of the given marker and cases with 2 or 3 were considered as having high expression of the given marker (with the exception of TTF-1 and p40, for which a score of 1 was regarded as sufficient to consider the case as positive).

RNA Extraction

Four 10-μm sections were cut from the selected paraffin tissue blocks, placed in xylene, and heated at 50°C for 3 minutes. The tube was centrifuged at 12,000*g* for 2 minutes, and the xylene was decanted. Residual xylene was extracted by the addition of ethanol to the dewaxed tissue sections and centrifugation at 12,000*g* for 5 minutes. The ethanol was removed, and the process was repeated once. The samples were then air dried for 30 minutes at room temperature.

The RecoverAll kit (AM1975, Applied Biosystems [ABI], Foster City, CA) was used to extract total RNA from dried sections. This procedure involves DNase treatment, purification, and RNA elution. All samples were stored at –80°C until used for analysis. The concentration of each sample (ng/μL) along with the purity ratio (optical density, 260/280) was determined by using a NanoDrop 3300 spectrophotometer (Thermo Scientific, Wilmington, DE).

Reverse Transcription–PCR

Quantification of microRNA expression was carried out using TaqMan MicroRNA Assay kits according to the manufacturer's protocol (ABI). Prefabricated TaqMan MicroRNA assays (containing microRNA-specific forward and reverse polymerase chain reaction [PCR] primers and a microRNA-specific Taqman MGB probe) were used for the study of miR21 (ABI part No. 4373090) and miR205 (ABI part No. 4373093). We also quantified transcripts of U6 small RNA (ABI part No. 4373381) as an endogenous control for normalizing the levels of target microRNA. Complementary DNA was generated by using the TaqMan MicroRNA Reverse Transcription (RT) Kit (ABI part No. 4366596) according to the manufacturer's instructions. Reverse transcriptase reactions contained 10 ng of total RNA as the template, 5 μL of gene-specific stem-loop RT primer, 1.5 μL of the provided reverse transcription buffer, 0.15 μL of a 100-mmol/L concentration of deoxynucleoside triphosphates, 1 μL of MultiScribe reverse transcriptase, and 4.16 μL of nuclease-free water.

The 15-μL reactions were incubated on a GeneAmp PCR System (Bio-Rad, Hercules, CA) for 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C and then held at 4°C. Real-time quantitative PCR was carried out using the Rotor-Gene 6000 (Corbett Life Sciences, San Francisco, CA). The 20-μL PCR reactions contained 1.33

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Table 1
Comparison of Histologic, Immunohistochemical, and miRNA Findings for 56 Samples

	Immunohistochemical Findings*								miRNA Profile				
Case No./ Histologic Classification†	TTF-1	CK7	CK5	p63	p40	Napsin A	DSC3	Immunohistochemical Profile‡	miR21	miR205	Sample Score	Near Cutoff	Classification
1/LCC	3	2	0	0	0	1	0	ADC 1	15.77	22.91	3.51	Yes	ADC
2/LCC	3	2	0	0	0	2	0	ADC 1	17.36	27.47	7.17	No	ADC
3/LCC	3	2	0	0	0	0	0	ADC 1	17.45	26.69	5.825	No	ADC
4/LCC	2	2	0	0	0	0	0	ADC 1	17.2	30.85	11.415	No	ADC
5/LCC§	1	3	0	0	0	0	1	ADC 1	16.45	26.41	7.275	No	ADC
6/LCC§	2	3	0	1	0	3	0	ADC 1	17.08	22.75	2.695	Yes	ADC
7/LCC	2	3	0	0	0	3	0	ADC 1	18.6	26.93	6.225	No	ADC
8/LCC	2	3	0	0	0	2	0	ADC 1	18.14	22.31	1.35	Yes	SQCC
9/LCC	3	3	0	1	0	3	0	ADC 1	17.29	18.9	–1.535	No	SQCC
10/LCC	2	3	0	2	0	2	0	ADC 2	19.37	26.06	4.015	No	ADC
11/LCC	0	3	0	1	0	3	0	ADC 2	19.14	29.44	7.43	No	ADC
12/LCC	0	3	0	0	0	2	0	ADC 2	20.24	33.07	9.95	No	ADC
13/LCC	0	3	0	0	0	3	0	ADC 2	20.83	29.34	5.065	No	ADC
14/LCC	0	3	0	0	0	2	0	ADC 2	21.43	27.29	3.395	Yes	ADC
15/LCC	0	3	0	0	0	2	0	ADC 2	22.88	29.48	3.64	Yes	ADC
16/LCC	2	3	0	2	0	3	0	ADC 2	19.66	24.77	2.525	Yes	ADC
17/LCC	1	0	0	1	0	3	0	ADC 2	22.1	26.55	2.935	Yes	ADC
18/LCC	3	3	0	3	0	3	0	ADC 2	17.84	27.87	7.045	No	ADC
19/LCC	2	3	0	2	0	2	0	ADC 2	16.63	28.9	9.49	No	ADC
20/LCC	0	0	2	3	2	0	1	SQCC 1	16.68	18.51	–1.31	No	SQCC
21/LCC‖	0	0	3	2	2	1	0	SQCC 1	20.05	17.82	–3.655	No	SQCC
22/LCC	0	0	3	3	2	0	0	SQCC 1	30.09	30.21	–1.025	No	SQCC
23/LCC‖	0	0	3	3	2	0	2	SQCC 1	16.78	18.53	–1.155	No	SQCC
24/LCC‖	0	0	3	3	3	0	0	SQCC 1	17.77	18.15	–1.065	No	SQCC
25/LCC§	0	0	0	3	3	0	2	SQCC 2	17.37	32.05	12.65	No	ADC
26/LCC§	0	1	3	2	3	0	2	SQCC 2	16.43	19.15	–1.51	No	SQCC
27/LCC	0	1	3	3	3	0	2	SQCC 2	20.78	22.93	–0.175	No	SQCC
28/LCC	0	3	2	2	2	0	0	SQCC 2	19.27	23.22	1.38	Yes	SQCC
29/LCC‖	0	3	2	3	1	0	0	SQCC 2	15.39	20.4	2.15	Yes	SQCC
30/LCC‖	0	2	2	3	2	0	0	SQCC 2	19.96	20.9	–2.17	No	SQCC
31/LCC vs SP	0	3	2	3	1	1	0	SQCC 2	20.03	22.18	–0.85	No	SQCC
32/LCC	0	3	1	0	1	0	1	SQCC 2	17.25	21.06	0.31	No	SQCC
33/LCC	1	3	0	2	3	0	2	SQCC 2	21.44	21.82	–1.375	No	SQCC
34/LCC	0	2	0	0	0	0¶	0	NC	18.49	29.21	7.425	No	ADC
35/LCC	0	2	0	0	0	0	0	NC	19.98	31	7.945	No	ADC
36/LCC	0	3	0	1	0	0	0	NC	20.03	27.81	5.31	No	ADC
37/LCC#	0	2	0	0	0	0¶	0	NC	20.8	25.4	3.315	Yes	ADC
38/LCC	0	2	1	0	0	0	0	NC	17.27	25.77	5.995	No	ADC
39/LCC#	0	3	1	0	0	0	0	NC	16.44	26.98	7.735	No	ADC
40/LCC	0	2	2	0	0	0	0	NC	23.88	32.42	6.235	No	ADC
41/LCC	0	2	2	0	0	0	0	NC	17.83	23.13	3.55	Yes	ADC
42/LCC§	0	2	3	1	0	0	0	NC	17.74	30	9.345	No	ADC
43/LCC§	0	0	0	0	0	1	0	NC	15.6	25.28	6.07	No	ADC
44/LCC**	0	1	0	1	0	0¶	1	NC	16.87	24.07	4.98	No	ADC
45/LCC§	0	0	0	0	0	1	0	NC	15.6	25.28	6.07	No	ADC
46/LCC	0	3	0	2	2	2	0	NC	18.28	23.99	2.585	Yes	ADC
47/LCC	0	0	0	1	0	0	0	NC	22.56	31.37	6.995	No	ADC
48/LCC	0	0	0	2	0	0	0	NC	17.1	28.83	9.185	No	ADC
49/LCC	0	3	3	1	0	0	1	NC	18.42	24.78	4.035	No	ADC
50/LCC§,††	3	3	0	3	1	2	1	NC	17.61	26.72	6.05	No	ADC
51/LCC‖	0	3	1	2	0	0	1	NC	16.51	25.11	6.82	No	ADC
52/LCC††	2	3	2	2	1	3	0	NC	14.57	19.33	1.505	Yes	SQCC
53/LCC vs SP	0	0	2	1	2	2	0	NC	20.19	23.84	1.955	Yes	SQCC
54/LCC vs SP	0	3	3	0	0	0	2	NC	17.8	21.12	–0.86	No	SQCC
55/LCC vs SP	0	3	2	1	0	0	1	NC	19.08	21.79	–0.675	No	SQCC
56/LCC§	0	3	2	3	3	3	3	NC	19.35	23.3	1.425	Yes	SQCC

CK7, cytokeratin 7; CK5, cytokeratin 5; DSC3, desmocollin 3; NC, not classifiable.
* Interpretation of immunohistochemical findings is as follows: 0, no reactivity; 1, <10% of faintly to moderately stained cells; 2, 10%-50% of moderately to strongly reactive cells; 3, >50% of moderately to strongly reactive cells.
† LCC, large cell carcinoma; SP, sarcomatoid pleomorphic.
‡ ADC 1, typical adenocarcinoma immunoprofile (see text for description); ADC 2, less stringent adenocarcinoma immunoprofile (see text for description); SQCC 1, typical squamous cell carcinoma immunoprofile (see text for description); SQCC 2, less stringent squamous cell carcinoma immunoprofile (see text for description).
§ Clear cell.
‖ Basaloid.
¶ Rare positive cells.
# Lymphoepithelioma-like carcinoma.
** With rhabdoid features.
†† With adenosquamous features.

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μL of RT product, 10 μL of FastStart TaqMan Probe Master (product No. 04673417001, Roche Applied Science, Basel, Switzerland), 7.67 μL of nuclease-free water, and 1 μL of MicroRNA Assay. Reactions were incubated at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Auto increment settings, ramp rate setting, and data collection were accepted default values. The threshold cycle data (CT) and baselines were determined by using auto settings. The CT value was defined as the fractional cycle number at which the fluorescence passed the fixed threshold.

Sample Classification

The miR205, miR21, and U6 snRNA were measured by quantitative RT-PCR in triplicate. The average CT of the triplicates ($AvgCT_{miR205}$, $AvgCT_{miR21}$, and $AvgCT_{U6}$) was calculated, excluding outliers (replicates with CT differing by >1 cycle from the median), repeating the assay if no 2 replicates had a CT within 1 cycle. The normalized CT of miR205 and miR21 was calculated by subtracting the $AvgCT_{U6}$ from the $AvgCT_{miR205}$ or $AvgCT_{miR21}$, respectively; $CT_{205} = AvgCT_{miR205} - AvgCT_{U6}$ and $CT_{21} = AvgCT_{miR21} - AvgCT_{U6}$. The mRSS was then obtained by using the formula $mRSS \equiv AvgCT_{miR205} - [(AvgCT_{miR21} + AvgCT_{U6})/2] = CT_{205} - (CT_{21}/2)$. Lebanony et al[8] used an mRSS of 2.5 as the threshold for separating SQCCs from nonsquamous carcinomas.[7] Cancers with mRSSs below this threshold were classified as SQCCs, and those with scores above this threshold were classified as ADCs. Scores within 1.5 CT of the 2.5 cutoff (ie, between 1 and 4) were considered more prone to measurement error and, thus, were further qualified as "near cutoff."

Table 2
Relation Between Immunoprofiles, miR21, miR205, and mRSS Classification

Immunoprofile*	No. of Cases	miRNA 21†	miRNA 205†	mRSS ADC	mRSS SQCC
ADC 1	9	17.26	25.02	7	2
ADC 2	10	20.01	28.27	10	0
ADC any profile	19	18.70	26.73	17‡	2‡
SQCC 1	5	20.27	20.64	0	5
SQCC 2	9	18.65	22.63	1	8
SQCC any profile	14	19.23	21.92	1‡	13‡
Not classifiable	23	18.34	24.85	18	5

ADC, adenocarcinoma; miRNA, microRNA; mRSS, microRNA sample score; SQCC, squamous cell carcinoma.
* ADC 1, typical adenocarcinoma immunoprofile (see text for description); ADC 2, less stringent adenocarcinoma immunoprofile (see text for description); SQCC 1, typical squamous cell carcinoma immunoprofile (see text for description); SQCC 2, less stringent squamous cell carcinoma immunoprofile (see text for description).
† Arithmetic mean.
‡ $P < .000$ (χ^2 test).

Results

Immunohistochemical Results

The immunophenotypes and molecular results for all tumors are reported in detail in Table 1 and summarized in **Table 2**. The immunohistochemical markers applied in the present study have been subdivided into 2 groups: the "classical" panel, including TTF-1, p63, CK5, and CK7, and an additional panel, including napsin A, Δnp63, and DSC3. Tumor immunophenotypes were classified as perfectly concordant (type 1 immunoprofile) when TTF1, p63, CK5, and CK7 concordantly supported squamous (TTF-1–, p63+, CK5+, and CK7–) or adenocarcinomatous (TTF-1+, p63–, CK5–, and CK7+) differentiation. When the TTF-1/p63/CK5/CK7 immunoprofiles were nonconcordant (loss of expression of expected positive markers or aberrant or conflicting expression of markers), cases were considered ambiguous and were further classified on the basis of the results of the additional panel, which included napsin A, Δnp63, and DSC3. When these additional markers concordantly supported squamous (napsin A–, Δnp63+, and DSC3+) or adenocarcinomatous (napsin A+, Δnp63–, and DSC3–) differentiation, cases were classified as SQCC or ADC with the type 2, less stringent, immunoprofile. Cases were labeled as unclassifiable when even the second set of immunohistochemical markers provided conflicting results. Paradigmatic examples of different immunoprofiles are shown in **Image 1** and **Image 2**.

In detail, the results of the immunohistochemical study provided the following results: (1) Of the 56 tumors, 9 (16%) were TTF-1+, CK7+, p63–, and CK5–; these tumors were reclassified as ADC with a type 1 concordant immunoprofile. Within this group of tumors, 5 were immunopositive for napsin A and all were negative for Δnp63 and DSC3. (2) Of the 56 tumors, 5 (9%) were TTF-1–, CK7–, p63+, and CK5+; these tumors were reclassified as SQCC with a concordant type 1 immunoprofile; napsin A was negative in all cases, Δnp63 was positive in all cases, and DSC was positive in 2 cases. (3) Of the 56 tumors, 42 (75%) were considered ambiguous on the basis of the incompletely concordant immunophenotype, based on the expression patterns of TTF-1, CK7, p63, and CK5. Among these cases, 10 cases were further reclassified as ADC and 9 as SQCC with type 2 immunoprofiles.

By grouping the cases with type 1 and 2 immunoprofiles we could reclassify 19 (34%) cases as ADC and 14 (25%) as SQCC. The remaining 23 cases (41%) were regarded as nonclassifiable on the basis of our immunohistochemical markers.

microRNA Analysis and Relation With Immunohistochemical Results

RNA was successfully extracted from all formalin-fixed, paraffin-embedded samples. The levels of microRNA-21 and microRNA-205 were quantified in triplicate by RT-PCR and

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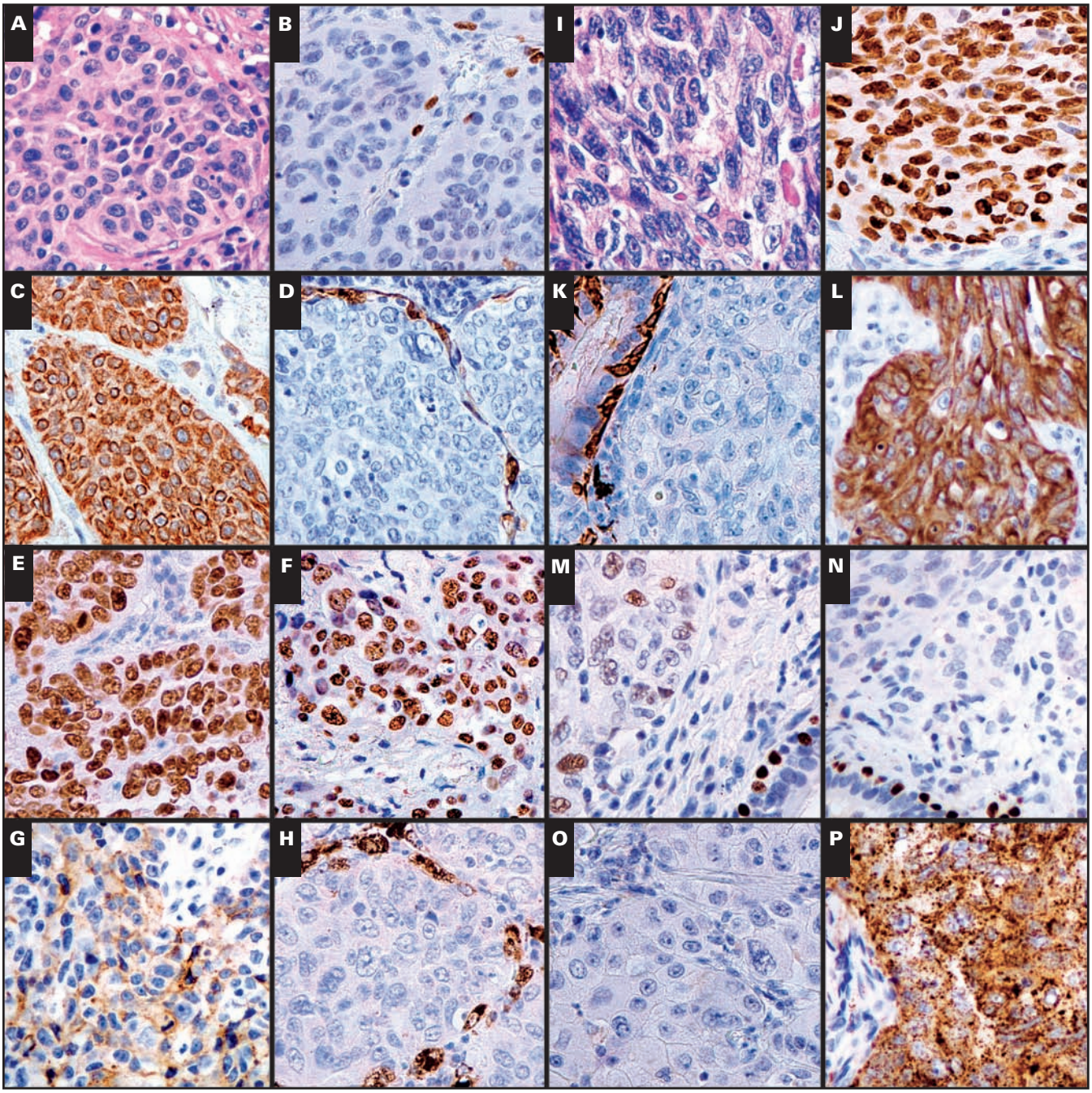

Image 1 Immunohistochemical phenotypes of large cell carcinoma (LCC) reclassified as squamous cell carcinoma (SQCC) and adenocarcinoma. **A**-**H**, A case of LCC showing the typical type 1 SQCC immunophenotype TTF-1–, CK5+, CK7–, p63+, p40+, DSC3+, napsin A– (**A**, H&E, ×400; **B**, TTF-1, ×400; **C**, CK5, ×400; **D**, CK7, ×400; **E**, p63, ×400; **F**, p40, ×400; **G**, DSC3, ×400; **H**, napsin A, ×400). **I**-**P**, A case of LCC showing a type 2 (less stringent—see text) adenocarcinoma immunophenotype: CK5–, CK7+, p63+ (note faint positivity of the cell nuclei), p40–, DSC3–, napsin A+ (**I**, H&E, ×400; **J**, TTF-1, ×400; **K**, CK5, ×400; **L**, CK7, ×400; **M**, p63, ×400; **N**, p40, ×400; **O**, DSC3, ×400; **P**, napsin A, ×400).

normalized to U6, with minimal variation between experiments, as described.[10] According to the mRSS, 36 cases were classified as ADC and 20 as SQCC. Table 2 shows the relation among the different immunoprofiles and microRNA expression levels and mRSS.

The mRSS classification showed a statistically significant relationship with the immunohistochemical classification (mRSS classification vs ADC or SQCC immunohistochemical classification with any profile, $P < .000$; χ^2; Table 2). All but 2 cases with an ADC immunohistochemical profile were

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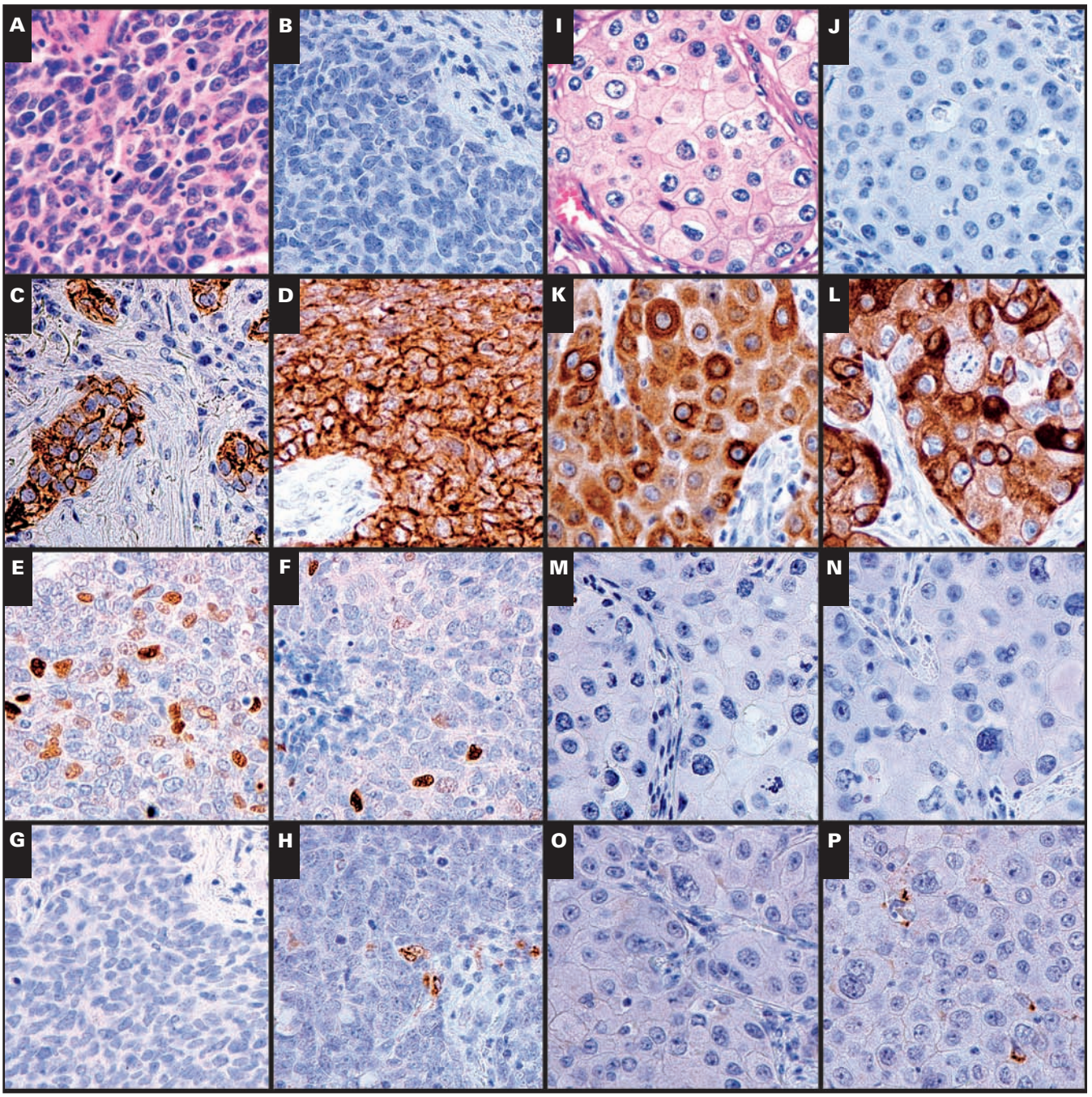

Image 2 Nondiagnostic immunohistochemical phenotypes of large cell carcinoma (LCC). **A**-**H**, A case of LCC showing basaloid features on H&E-stained slides and with the following immunophenotype: TTF-1–, CK5+, CK7+, p63+, p40+/–, DSC3–, napsin A– (**A**, H&E, ×400; **B**, TTF-1, ×400; **C**, CK5, ×400; **D**, CK7, ×400; **E**, p63, ×400; **F**, p40, ×400; **G**, DSC3, ×400; **H**, napsin A, ×400). **I**-**P**, A case of LCC with clear cell features and the following nondiagnostic immunophenotype: TTF-1–, CK5+, CK7+, p63–, p40–, DSC3–, napsin A– (**I**, H&E, ×400; **J**, TTF-1, ×400; **K**, CK5, ×400; **L**, CK7, ×400; **M**, p63, ×400; **N**, p40, ×400; **O**, DSC3, ×400; **P**, napsin A, ×400).

classified as such by mRSS. The 2 discrepant cases (cases 8 and 9) showed a classical type 1 ADC immunoprofile, with strong expression of TTF-1, CK7, and napsin and lack of CK5, Δnp63, and DSC (ie, clear-cut ADC immunoprofile): 1 showed mRSS values outside the near cutoff range (ie, definitive SQCC molecular profile), and 1 showed mRSS values within the near cutoff range (ie, nondefinitive SQCC molecular profile). All but 1 of the cases with an SQCC immunoprofile were classified as such by mRSS. The discrepant case had been classified as having a type 2 immunoprofile because of the lack

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of CK5 expression (case 25); its immunoprofile (strong p63, p40, and DSC3 reactivity associated with lack of TTF-1, CK7, and napsin) clearly identifies this case as an SQCC, although it showed the highest mRSS value of this series, clearly allowing its molecular classification as ADC. These discrepant cases have been reevaluated from immunohistochemical and molecular viewpoints, and repeated immunohistochemical stains and RT-PCR protocols have produced similar results.

Of the 23 cases with unclassifiable immunoprofiles, 18 were classified as ADC by mRSS (3 in the so-called near cutoff value range) and 5 as SQCC (3 in the so-called near cutoff value range). At histopathologic revision of this subset of cases, 3 of the 5 cases classified as SQCC by mRSS (cases 53, 54, and 55) showed features of sarcomatoid carcinoma, and 1 of the cases classified as ADC by mRSS (case 50) had a complex histologic pattern with clear cell and adenosquamous features and was reclassified as an adenosquamous carcinoma. The immunoprofiles of all cases are reported in detail in Table 1, and the relationships between the immunoprofiles and the mRSS values are shown in **Figure 1**.

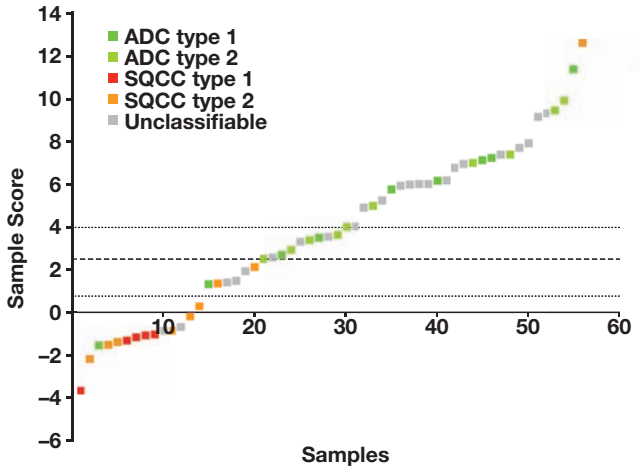

Figure 1 Scatter plot of all cases studied. The microRNA sample score was calculated as follows: Score ≡ $AvgCT_{miR205} - [(AvgCT_{miR21} + AvgCT_{U6})/2] = CT_{205} - (CT_{21}/2)$, according to Lebanony et al.[8] Samples are sorted by increasing score values. The dashed line at score 2.5 shows the cutoff for classification between squamous cell carcinoma (SQCC; score <2.5) and adenocarcinoma (ADC; score >2.5). The dotted lines at scores 1 and 4 show the cutoffs for high and low confidence. Each square represents a case; the colors refer to the different immunophenotypes. Immunophenotypes are as follows: ADC 1, typical adenocarcinoma immunoprofile (see text for description); ADC 2, less stringent adenocarcinoma immunoprofile (see text for description); SQCC 1, typical squamous cell carcinoma immunoprofile (see text for description); SQCC 2, less stringent squamous cell carcinoma immunoprofile (see text for description).

Discussion

LCCs of the lung are recognized as a diagnostic entity by the current World Health Organization classification of lung neoplasms. However, data suggest that LCCs represent a heterogeneous group of neoplasms. By using panels of immunohistochemical markers, around one half to two thirds of cases can be classified as ADC or SQCC.[4,5,22,23] The remaining cases, however, cannot be further classified because of incomplete, conflicting, or aberrant immunohistochemical data.

The present study further investigated the nature of LCC because we included, besides the traditional lung immunophenotypic markers (ie, TTF-1, p63, CK5, and CK7),[13,17,23-29] a panel of additional markers, such as Δnp63, napsin A, and DCS3[14,30-32] and the analysis of microRNA205 expression levels.[8-10] With our immunophenotypic approach, we could subdivide the series of LCCs into 3 groups, ie, 14 tumors (25%) with typical ADC (9 cases [16%]) or SQCC (5 cases [9%]) immunoprofiles, 19 tumors (34%) with less typical but still diagnostic ADC (10 cases [18%]) or SQCC (9 cases [16%]) immunoprofiles, and 23 tumors (41%) with nonclassifiable profiles. Almost all cases immunohistochemically classified as ADC or SQCC were concordantly classified by the molecular approach. This concordance strongly supports the value of our immunohistochemical approach, based on a 2-step procedure in which cases classified as having ambiguous phenotypes based on a first set of classical markers are further evaluated with a second panel of antibodies.

The cases that were considered nonclassifiable on the basis of our immunohistochemical approach could be further split into 2 groups according to the molecular approach, favoring ADC (18 cases) or SQCC (5 cases) differentiation. Although limited data still exist concerning miRNA-205 values in lung tumors of different histogenesis, our present data further support the hypothesis that most LCCs can, in fact, be reclassified as ADC or SQCC when multiple ancillary techniques are used.

Our immunohistochemical evaluation scheme of lung tumors using an extended panel of markers seems superior to the one suggested by some of us[13] and Terry et al.[24] In fact, by strictly applying these previous algorithms, almost 75% of cases in the present study would have been considered as "not otherwise specified" or with "ambiguous" immunophenotypes. Conversely, our present approach could reduce the number of nonclassifiable cases to 41% of cases. The robustness of our immunohistochemical approach is strongly supported by microRNA analysis, which seems a promising diagnostic tool for separating ADC from SQCC.[10] Conversely, the weakness of this multimarker approach is that it can be used

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only when abundant tumor material is available, which may not be the case with limited bioptic or cytologic specimens. In these cases, one should only use a very limited set of markers, such as TTF-1 and p63, or use double immunostains, possibly of nuclear and cytoplasmic markers, as recently suggested by Righi et al.[11]

In the present series of cases, 12 tumors showed CK7 and CK5 coexpression. In the literature, the diagnostic significance of CK7 and CK5 coexpression in lung tumors is unclear: some authors classify these cases according to their expression of TTF-1 and p63, whereas others consider these cases as unclassifiable.[13,24] In our series, 4 CK5+/CK7+ tumors were also positive for both p63 isoforms and negative for TTF-1; therefore, they were considered as SQCC with a type 2 immunoprofile. The very same cases were also classified as SQCC by mRSS. The remaining 8 CK5+/CK7+ cases were considered as nonclassifiable with our immunohistochemical approach; however, 4 of them were classified as SQCC by mRSS. Therefore, CK5/CK7 coexpression should not be considered as a criterion to exclude the diagnosis of SQCC, but these cases should be further studied with additional immunohistochemical or molecular markers. Further studies are needed to determine if these tumors belong to a specific histogenetic or molecular subtype of SQCC, such as the secretory type of SQCC identified by Wilkerson et al.[33]

In our series, a group of 7 cases showed concurrent TTF-1 and p63 expression. These cases were all positive for CK7 and all but 1 were negative for CK5. Four of these cases were negative for Δnp63 and DSC3 and positive for napsin A. We classified these 4 cases as poorly differentiated ADC with type 2 immunoprofiles, and all were classified as ADC by mRSS. Of the remaining 3 cases, all expressing Δnp63, 1 was classified on the basis of the immunohistochemical profile (very low expression of TTF-1, strong and diffuse expression of Δnp63 and DSC3, and absence of napsin A) as SQCC with a type 2 immunoprofile and also was classified as SQCC by mRSS. The other 2 cases were regarded as having an unclassifiable immunoprofile (low expression of Δnp63, strong expression of napsin A, absence of DSC3), and 1 each was classified as SQCC and as ADC by mRSS and may probably reflect an adenosquamous differentiation profile. These data provide further insight into the role of p63 expression in the lung.

Expression of p63 in ADC has been described by other authors[28,34] using the conventional p63 antibodies, ie, those that recognize all isoforms of the p63 protein.[35] Δnp63 is one of the 6 p63 known isoforms, which are defective in the transactivating N-domain. Δnp63 binds to p53 DNA sites, but because it lacks the transactivating domain, it does not produce p53-like effects on gene expression and, therefore, has an anti-p53 function, constraining p53-dependent apoptotic pathways. Owing to its anti-p53 effect, it is expressed in basal cell populations that require self-renewal and maintenance of a proliferative compartment. Δnp63 immunoreactivity has exquisite specificity for SQCC.[11] In the present series, Δnp63 was never expressed in tumors classified as ADC based on the immunohistochemical or molecular profile and was positive in all but 1 of the cases with an SQCC immunohistochemical or molecular profile. We therefore suggest that, in an algorithmic approach, cases with concurrent TTF-1 and p63 immunoreactivity with the usual antibody should not be classified as NSCLC, not otherwise specified, as suggested by Terry et al,[24] or as SQCC, as suggested by Conde et al.[22] These cases are almost always ADC,[12] and, if sufficient material is available, they should be submitted for additional immunostains with other markers or molecular analyses to ascertain their nature.

In the present study, 3 cases showed discrepant results in their immunohistochemical and molecular classification: 1 case with a squamous immunoprofile clustered within the adenocarcinomatous microRNA score range, and 2 cases with adenocarcinomatous immunoprofiles clustered within the squamous microRNA score range. These discrepancies, which have already been rarely reported in other series,[6] could be related to several factors, including technical artifacts and tissue preservation. Technical artifacts should not be relevant in our series because we repeated our molecular and immunohistochemical experiments twice, obtaining substantially identical results. The quality of the samples could be a source of discrepant results. Our samples were fixed in neutral buffered formalin, but the total time of fixation might have varied, influencing the quality of the biochemical components of the analyzed tissues and, hence, the results of a quantitative method such as real-time analysis of microRNA.

Our study supports the hypothesis that most LCCs represent poorly differentiated ADC or SQCC. Our data show that an integrated immunohistochemical approach, using an extended panel of markers, can provide accurate classification in a relevant percentage of cases diagnosed as LCC on purely morphologic grounds. The mRSS molecular diagnostic approach strongly correlates with our immunohistochemical approach, supporting the value of using additional markers (Δnp63, DSC3, and napsin A) with the markers most frequently used in clinical practice. However, a relevant percentage of LCCs may escape convincing immunohistochemical classification, and mRSS could therefore be used in clinical practice for this subset of cases. Further studies are needed to verify whether the proposed reclassification of LCCs on the basis of immunoprofiles or microRNA-205 quantification may influence the clinical and therapeutic management of patients with such tumors.

From the [1]Unit of Surgical Pathology, [2]Laboratory of Molecular Pathology, [3]Trentino Biobank, Unit of Surgical Pathology, and

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[9]Unit of Surgery, S. Chiara Hospital, Trento, Italy; [4]Centre for Integrative Biology, University of Trento, Trento; [5]Unit of Pathologic Anatomy, Arcispedale S. Maria Nuova, Reggio Emilia, Italy; [6]Unit of Pathologic Anatomy, San Luigi Hospital and University of Turin, Orbassano, Italy; [7]Unit of Surgical Pathology, S. Maurizio Hospital, Bolzano, Italy; [8]Section of Pathologic Anatomy, Azienda Ospedaliera-Universitaria Policlinico, Modena, Italy; [10]Unit of Pathology and Laboratory Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; [11]Unit of Pathologic Anatomy, Forlanini Hospital, Rome, Italy; and [12]Unit of Surgical Pathology, S. Raffaele Hospital, Milan, Italy.

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Address reprint requests to Dr Barbareschi: Unit of Surgical Pathology, Laboratory of Molecular Pathology, S. Chiara Hospital, Largo Medaglie Oro 9, 38122 Trento, Italy.

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