Reproducibility of Histopathological Diagnosis in Poorly Differentiated NSCLC

An International Multiobserver Study

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Introduction: The 2004 World Health Organization classification of lung cancer contained three major forms of non–small-cell lung cancer: squamous cell carcinoma (SqCC), adenocarcinoma (AdC), and large cell carcinoma. The goal of this study was first, to assess the reproducibility of a set of histopathological features for SqCC in relation to other poorly differentiated non–small-cell lung cancers and second, to assess the value of immunohistochemistry in improving the diagnosis.

Methods: Resection specimens (n = 37) with SqCC, large cell carcinoma, basaloid carcinoma, sarcomatoid carcinoma, lymphoepithelial-like carcinoma, and solid AdC, were contributed by the participating pathologists. Hematoxylin and eosin (H&E) stained slides were digitized. The diagnoses were evaluated in two ways. First, the histological criteria were evaluated and the (differential) diagnosis on H&E alone was scored. Second, the added value of additional stains to make an integrated diagnosis was examined.

Results: The histologic criteria defining SqCC were consistently used, but in poorly differentiated cases they were infrequently present, rendering the diagnosis more difficult. Kappa scores on H&E alone were for SqCC 0.46, large cell carcinoma 0.25, basaloid carcinoma 0.27, sarcomatoid carcinoma 0.52, lymphoepithelial-like carcinoma 0.56, and solid AdC 0.21. The κ score improved with the use of additional stains for SqCC (combined with basaloid carcinoma) to 0.57, for solid AdC to 0.63.

Conclusion: The histologic criteria that may be used in the differential diagnosis of poorly differentiated lung cancer were more precisely refined. Furthermore, additional stains improved the reproducibility of histological diagnosis of SqCC and AdC, uncovering information that was not present in routine H&E stained slides.

Key Words: Non–small-cell lung cancer, Pathology, Reproducibility.

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The 2004 World Health Organization (WHO) classification of lung cancer contained three major forms of non–small-cell lung cancer (NSCLC): squamous cell carcinoma (SqCC), adenocarcinoma (AdC), and large cell carcinoma.[1] With an update to the classification of AdC, being published by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society containing additional

changes to terminology make the classification more relevant to clinical management and the molecular biology of AdC.[2] Following this, the IASLC pathology committee published a reproducibility study supporting its usage in a routine diagnostic setting[3] and the accuracy in distinguishing better differentiated SqCCs from AdCs has been repeatedly demonstrated elsewhere.[4–7]

However, there are a few data on the reproducibility between pathologists in relation to more poorly differentiated tumors, especially in small biopsies.[8] Specifically, in this setting, the differential diagnosis often lies between a poorly differentiated SqCC and large cell carcinoma, basaloid carcinoma, sarcomatoid or pleomorphic carcinoma, lymphoepithelial-like carcinoma (LELC), and solid AdC. Until recently, there was no clinical imperative for further differentiation, with cases being classified as NSCLC-not otherwise specified (NOS). However, with advances in chemotherapy, there is an increasing need for accurate subdivision, even in these more poorly differentiated neoplasms.[9] Current WHO criteria are purely morphologic for SqCC, these being the presence of keratinization and/or the presence of intercellular bridges, the latter criterion being especially relevant in more poorly differentiated tumors.

Therefore, the goal of this study was first, to assess the reproducibility of a set of histopathological features for SqCC in relation to other poorly differentiated NSCLCs, using a panel of pulmonary pathologists from three continents. Second, we assessed the value of immunohistochemistry (IHC) in improving the diagnosis as an ancillary tool.

MATERIALS AND METHODS

Resection specimens ($n = 80$) with SqCC (well and poorly differentiated), and other cases with diagnosis according to the WHO classification 2004[1] (large cell carcinoma, basaloid carcinoma, sarcomatoid or pleomorphic carcinoma, LELC, and AdC with a solid pattern), were contributed by 10 participating pathologists. Hematoxylin and eosin (H&E)–stained slides were sent to the Tsukuba Critical Path Research and Education Integrated Learning Center at the University of Tsukuba. NanoZoomer 2.0-HT:C9600-13 system was used to scan the slides, Hamamatsu Photonics, Hamamatsu, Japan. The digitized cases were made available on the Internet for reading by the participants.

In this pilot study, 19 histological criteria were considered by the IASLC pathology committee as of possible value in the discrimination of poorly differentiated tumors and, in 80 cases, were scored for their presence, as described in Table 1, with a preferred diagnosis made by 12 pathologists, revealing a low κ score (data not shown).

After this pilot study, the histological criteria were re-evaluated. Six of the criteria were discarded as they were not informative (their presence or absence was not discriminating for any diagnostic category) for the cases, leaving **12 criteria** to be used for further evaluation (printed in **bold** in Table 1). Detailed images were taken from the digitally scanned slides, placed in a Powerpoint file as examples, and consensus definitions of individual histological features were agreed upon (Table 2).

To facilitate the application of the WHO criteria, a flow chart was made, see Figure 1.

TABLE 1. The Criteria Evaluated in the First Round Are Shown and Scored for Each Case as One of the Three Choices

Criterion			
1 Keratinization	Yes	No	Uncertain
2 Pearl formation	Yes	No	Uncertain
3 Intercellular bridges	Yes	No	Uncertain
4 Peripheral palisading of nuclei	Yes	No	Uncertain
5 Intercellular gaps	Yes	No	Uncertain
6 Sheets of polygonal cells	Yes	No	Uncertain
7 Spindle cells	Yes	No	Uncertain
8 Giant cells	Yes	No	Uncertain
9 Pleomorphic cells	Yes	No	Uncertain
10 Cell borders	Rarely seen	In between	Sharp
11 Nuclei	Monomorphic	In between	Pleomorphic
12 Nuclear moulding	Yes	No	Uncertain
13 Chromatin	Finely granular	In between	Vesicular
14 Nucleoli	Inconspicuous	In between	Prominent
15 Glassy eosinophilic cytoplasm	Yes	No	Uncertain
16 Intracytoplasmic vacuoles	Yes	No	Uncertain
17 Mitoses	Low <1/10 HPF = ~0.5 mm	In between	Many >1–2/HPF = ~0.5 mm
18 Mitoses	Absent	In between	Prominent
19 Lymphocytic infiltrate	Absent		
	Present in stroma	Between tumor cells	In both compartments
Comment: [max500 characters]			

In the second round only the bold criteria were scored.
HPF, high power field.

TABLE 2. Definitions for Individual Criteria Formulated after First Ring Study

Intercellular bridges	The gaps that have real bridges are often relatively narrow, are always bordered by cytoplasm, usually plenty of eosinophilic, glassy stuff, and show a constant width. Intercellular bridges are typically seen between elongated cells and are best confirmed at 40× microscope objective (not 20×). The intercellular bridges show several (at least three) connections across the intercellular gap and have a regular quality, similar to the spokes of a wheel or the parallel rungs of a ladder, see Figure 1. In very well-fixed specimens, one may see a tiny dot in the center of the strand between the two adjacent cells, which represents the macula adherens (desmosome), but this is uncommonly seen. Intercellular bridges are a defining criterion for squamous differentiation. They are also prone to overinterpretation, especially when the gaps are not tight and parallel.
Keratin/keratinization	A squamous pearl or a maturing sheet or ball of stratified (± palisading!) epithelium is an easily recognized feature of SqCC. Usually the cytoplasm is glassy, pink, and intercellular bridges are present. The individual cell with keratinization has an intact nucleus with eosinophilic ring around the nucleus and correlate with perinuclear tonofibrillar bundles ultrastructurally. This may be tricky and prone to over interpretation, i.e., pyknotic nuclei with dense eosinophilic cytoplasm cannot be used for identification of individual cell keratinization. Individual cell death (apoptosis) can be falsely interpreted as keratinization. Individual cell keratinization is beside pearl formation and intercellular bridges also defining for squamous differentiation.
Intercellular gaps	Intercellular gaps are quite a common finding in many lung cancers, which is mostly an artifact, representing fixation and processing changes. It is a change which is often misinterpreted as indicating squamous differentiation, probably because gaps are needed to see bridges and so, pathologists can "see"—maybe actually imagine—bridges when gaps are present. Image 2 show lots of gaps where there are no intercellular bridges and the impression is that the gaps without bridges are wide, uneven, and variable. Sometimes there is little or no cytoplasm apparent between the nucleus and the gap. Intercellular gaps are not diagnostic for squamous cell or other carcinomas.
Intracytoplasmic vacuoles	Vacuoles are according to Stedman's Medical Dictionary, defined as a "clear space in the substance of a cell, sometimes degenerative in nature." Vacuoles may hint at the possibility of an adenocarcinoma but never define or confirm the diagnosis—and may be seen in all sorts of tumors as degenerative changes. Vacuoles might trigger a mucin stain—the mucin stain may confirm adenocarcinoma differentiation. For the purpose of this study with intracytoplasmic vacuoles those vacuoles are meant, which may have a high chance to be positive in the mucin stain, see Figure 3. Vacuoles can be round, oval or odd shapes and their location in the cell is not of much help. Mucin stain is less likely to be positive if there were multiple vacuoles and just beneath the cell membrane and more likely to be positive if single, big and look as if they had something in them. In essence vacuoles are frequently seen in any carcinoma, but those which are more frequently associated with mucin positivity open the possibility of adenocarcinoma (solid type).
Palisading	Palisading is defined as layer of relatively long cells with nuclei arranged loosely perpendicular to a surface and parallel to each other. Palisading is not a defining feature of SqCC and is seen in other tumors (i.e., basaloid carcinoma, adenocarcinomas and neuroendocrine carcinomas). However, in the correct context, it might be useful to raise the possibility of SqCC, assuming other features are present. There was no consensus on what was exactly meant with this feature, Figure 4.
Lymphocytic infiltrate	Lymphocytes occur regularly in stroma of NSCLC. In LELC, an appreciable number of intratumoral lymphocytes are present: i.e., in the nest between tumor cells, Figure 5. Small numbers get ignored. In Eastern countries, EBV is usually positive, but in Western countries usually negative. The LELC may become defined by the presence of EBV. For the purpose of this study, the presence and location of the lymphocytes were recorded.
Sheets of polygonal cells	Sheets or nests of polygonal cells are a characteristic of epithelial architecture, see Figure 1. In case of a malignant tumor, it is pointing toward a carcinoma instead of sarcoma or lymphoma. "Sheets of polygonal cells" is thus not restricted to SqCC. In larger areas of sarcomatoid carcinoma, sheets are usually lacking.
Spindle cells	According to the WHO 2004, a spindle cell carcinoma "consists of only spindle-shaped cells," architecturally nests/fascicles with overtly malignant nuclear features (Fig. 6). As for calling, a case sarcomatoid carcinoma unanimous consensus was present in case of diffuse mesenchymal spindle appearance of the tumor, this pattern was used for designation of "spindle." Whereas with some spindle-like cells in more nested tumors (mixed with epitheloid nuclei), the variation was much larger.
Glassy eosinophilic cytoplasm	Is an eosinophilic cytoplasm, which is not unique to SqCC carcinoma, but is frequently present when defining criteria for SqCC were also present. Eosinophilic cytoplasm may also occur in adenocarcinomas, especially in invasive areas.
Giant cells	No consensus
Nuclear moulding	Nuclear moulding is a frequent finding in small-cell lung carcinoma, but a rare feature in SqCCs. However, it may occasionally be present in small-cell type SqCC.

SqCC, squamous cell carcinoma; NSCLC, non–small-cell lung cancer; LELC, lymphoepithelial-like carcinoma; WHO, World Health Organization.

To assess the reproducibility of diagnosis in poorly differentiated tumors, a ring study was performed in which 37 of the initial 80 digitized cases were read in a second ring study by 16 pathologists. The reduction to 37 cases was based on the information from the pilot study that κ scores did not essentially change by reading more cases. The interval between the pilot study and this ring study was 12 months.

In contrast to the pilot study, two levels of diagnosis were evaluated. First, the (differential) diagnosis on H&E alone was scored. Subsequently, the immunohistochemical information (i.e., the result from the submitting pathologist regarding TTF1, mucin, and p63/p40) was provided, allowing the pathologist to make a "second level" integrated diagnosis based on H&E plus additional stains, should they wish.

Statistics

The distribution of the readers' diagnoses was compared with the original submitting pathologist's diagnosis. The κ scores were calculated in two different ways: (1) between all diagnostic categories for all possible combinations of pathologists and diagnostic categories and (2) for each individual diagnostic category versus all the other diagnostic categories combined. For each pathologist, the sensitivity and specificity of the score, for

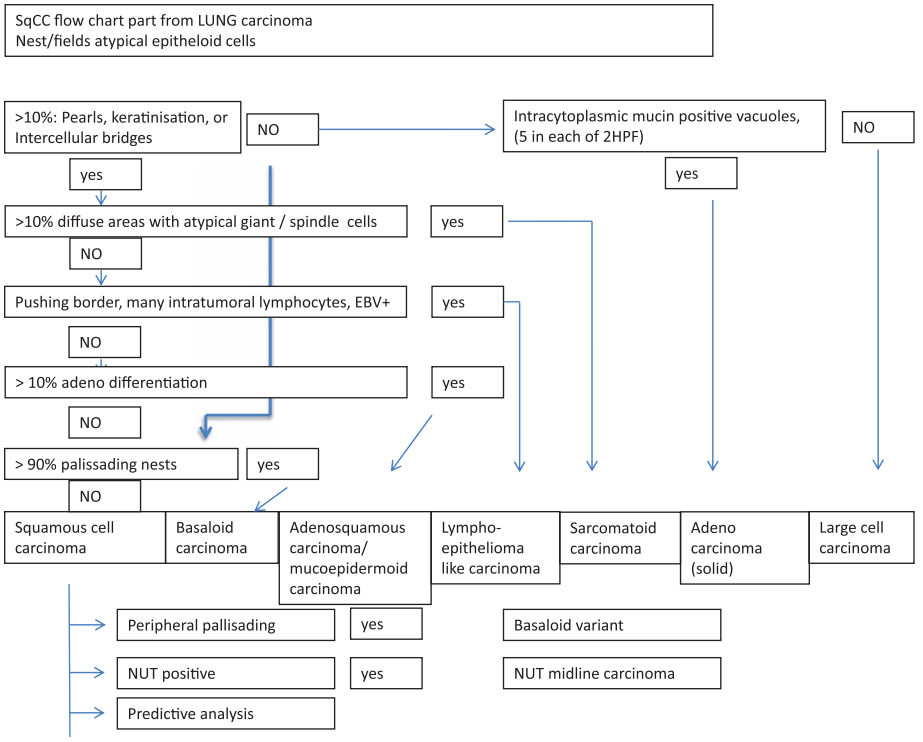

FIGURE 1. A flow chart for the application of histological criteria in poorly differentiated SqCC based on the WHO classification 2004.[1] HPF, high power field; NUT, *NUT* midline carcinoma, family member 1 (*NUTM1*); SqCC, squamous cell carcinoma; WHO, World Health Organization.

example, SqCC (criteria 1–3 in Table 1 with at least one "yes") and for p63/40 positivity was computed, as well as Youden's J statistic (the sum of the sensitivity and specificity minus 1). The same was undertaken for the sensitivity, specificity, and Youden's J statistic for a positive score for intracytoplasmic vacuoles ("yes") and for thyroid transcription factor/mucin positivity. Associations between the two categorical variables were tested by the χ^2 test.

For statistical analysis, the SPSS software package version 20.0 (IBM Corp., Armonk, NY) was used.

RESULTS

Descriptions of Histological Criteria

Detailed descriptions for the interpretation of histological criteria were made (Table 2 and Figs. 2–7). To assess the reproducibility of poorly differentiated NSCLC, 16 pathologists read 37 cases. The original contributors' diagnoses were SqCC (n = 13), basaloid carcinoma (n = 5), AdC solid type (n = 4), large cell carcinoma (n = 6), sarcomatoid carcinoma (n = 5), and LELC (n = 4).

In nine cases, the presence of "keratinization" was scored by a majority of pathologists (mean, 83%; range, 62–94%), while in the other 28 cases, on an average, one pathologist (mean, 4%; range, 0–25%) scored "keratinization" as being present. In six of these first nine cases, "pearl formation" was also scored as being present by the majority of the pathologists (mean, 63%; range, 25–88%). "Intercellular bridges" were also seen in seven of these nine cases by the majority (mean, 74%; range, 25–94%), while these features were rarely scored in the other 26 cases. Thus, only a minority of the 37 cases consisted of SqCC according to the readers. "Intercellular gaps" were scored as being present slightly more often in the cases with keratinization, as compared to the others, suggesting that this feature is not specific for this differential diagnosis.

To establish consistency in the use of the histological criteria, the relation between scored histological criteria and specific diagnosis provided by the same observer is shown in Table 3. In general, the three squamous cell criteria (keratinization, squamous pearls, and intercellular bridges) were scored present in 17 to 25% of all the scores (n = 592: 16 pathologists × 37 cases). These three criteria were highly correlated (r > 0.50, data not shown) and mainly distributed over SqCC and sarcomatoid carcinoma. In these sarcomatoid carcinomas, an area of SqCC was present according to the original diagnosis.

"Sheets of polygonal cells" and "intercellular gaps" were scored as present in 85% and 58%, respectively, scored in all diagnostic categories, but mainly distributed over SqCC and large cell carcinoma. "Peripheral palisading of the nuclei," "glassy eosinophilic cytoplasm," "spindle cells," and "giant cells" were scored in SqCC and large cell carcinoma. "Glassy eosinophilic cytoplasm" and "spindle cells" were also scored in sarcomatoid carcinoma. "Giant cells" were frequently scored in sarcomatoid carcinoma. "Nuclear moulding" was rarely scored (12%), but when present, was seen in SqCC, basaloid, and large cell carcinoma. Lymphocytes in stroma were present in 80% of the scores, and lymphocytes admixed amongst the tumor cells in 35%. The score for "intracytoplasmic vacuoles" was 22%, distributed over large cell carcinoma, SqCC, and AdC.

Additional Stains and Criteria

In cases with submitted diagnosis of SqCC, additional stains for AdC differentiation (TTF1/mucin) and p63/40 were

TABLE 3. The Histologic Criteria Scored to Be Present (*n*/% of Positive Scores) for the Diagnosis of the Same Observer Is Shown, Plus Sum of These Scores and % of Total Scores (Total = 592)

Diagnosis	SqCC	Basaloid	AdC	Ad.SqC	LCC	Sarc.	LELC	Total	% Total
Keratinization	109	2	0	1	5	21	0	138	23%
	79%	1%	0%	1%	4%	15%	0%		
Pearl formation	74	2	0	1	4	18	0	99	17%
	75%	2%	0%	1%	4%	18%	0%		
Intercellular bridges	122	1	0	0	5	20	0	148	25%
	82%	1%	0%	0%	3%	14%	0%		
Intercellular gaps	140	14	16	1	130	34	6	341	58%
	41%	4%	5%	0%	38%	10%	2%		
Peripheral palisading nuclei	67	24	6	0	51	6	0	154	26%
	44%	16%	4%	0%	33%	4%	0%		
LI between tumor cells	3	0	3	0	13	3	3	25	4%
	12%	0%	12%	0%	52%	12%	12%		
LI in stroma	116	21	14	1	127	15	0	294	49%
	39%	7%	5%	1%	43%	5%			
LI in both compartments	40	3	12	1	93	14	22	185	31%
	22%	2%	6%	1%	50%	8%	12%		
Sheets polygonal cells	152	22	27	2	244	39	19	505	85%
	30%	4%	5%	0%	48%	8%	4%		
Spindle cells	35	3	6	1	39	43	0	127	21%
	28%	2%	5%	1%	31%	34%	0%		
Giant cells	32	0	4	0	30	23	0	89	15%
	36%	0%	4%	0%	34%	26%	0%		
Glassy eosinophilic cytoplasm	112	3	7	2	83	27	2	236	40%
	47%	1%	3%	1%	35%	11%	1%		
Intracytoplasmic vacuoles	23	0	18	1	75	10	1	128	22%
	18%	0%	14%	1%	59%	8%	1%		
Nuclear moulding	10	16	2	1	40	2	0	71	12%
	14%	23%	3%	1%	56%	3%	0%		

SqCC, squamous cell carcinoma; Basaloid, basaloid carcinoma; AdC, adenocarcinoma; Ad.SqC, adenosquamous carcinoma; LCC, large cell carcinoma; Sarc, sarcomatoid carcinoma; LELC, lymphoepithelial-like carcinoma; LI, lymphocytes.

requested in 73% and 78% of the cases, respectively. For the other diagnoses, this fraction was higher with 91 and 92%, respectively, indicating the relatively undifferentiated morphology of these latter cases, as well as, the wish for immunohistochemical information in these cases.

In Table 4, the relation between the scored histological criteria and additional stains for AdC (TTF1 and mucin) and SqCC is shown. In general, the squamous cell criteria are usually scored as present in cases positive for p63/p40, and not scored as present when AdC stains were positive (for all $p < 0.001$). A similar finding was shown for palisading of the nuclei. Intracytoplasmic vacuoles scored as present were more frequently associated with positive AdC differentiation stains and less frequently with positive p63/p40. The remaining criteria did not show a significant relation with these additional stains.

Observer's Diagnosis Compared to Diagnosis of Submitting Pathologist

The distribution of original diagnosis from submitting pathologist compared to diagnosis of 16 pathologists is shown for two different levels in Table 5. The first level is based on hematoxylin and eosin (H&E) only and the second level on H&E combined with information from the additional stains: p63/p40, TTF1, and/or mucin. The overall κ score (95% confidence interval [CI]) at the first level was 0.31 (eight categories, 95% CI, 0.23–0.40), and increased to 0.45 (seven categories, 95% CI, 0.37–0.53) at the second level, showing an essential improvement with the use of additional stains.

Reproducibility of Diagnostic Categories

In Table 6, the κ scores for each individual diagnostic category versus all other categories combined are shown. For most individual categories, the κ score improved (or remained above 0.46) with the use of additional stains, in the case of SqCC, solid AdC, sarcomatoid carcinoma, and LELC. For basaloid carcinoma, adenosquamous carcinoma, and large cell carcinoma, the κ score remained below 0.30 after the use of additional stains.

Interobserver Variation Criteria Related to Additional Stains

For each pathologist, the relation between the presence of H&E features of squamous differentiation and p63/40 was

TABLE 4. Relation between a Score of "Yes" for Individual Criteria (*n* and %) and TTF1 (Total Scores *n* = 576) and p63/p40 (Total Scores = 560)

	TTF1 and/or Mucin					p63/p40				
	Negative		Positive			Negative		Positive		
Criterion	***n***	**%**	***n***	**%**	***p* Value**	***n***	**%**	***n***	**%**	***p* Value**
Keratinization	132	29%	6	5%	<0.001	2	2%	134	31%	<0.001
Pearl formation	94	21%	5	4%	<0.001	1	1%	97	22%	<0.001
Intercellular bridges	139	31%	9	7%	<0.001	7	5%	139	32%	<0.001
Intercellular gaps	284	63%	61	48%	0.001	81	63%	254	59%	0.36
Peripheral palisading of nuclei	136	30%	21	16%	0.002	20	16%	136	31%	<0.001
Sheets polygonal cells	392	88%	110	86%	0.64	109	85%	379	88%	0.44
Spindle cells	95	21%	32	25%	0.36	32	25%	80	19%	0.11
Giant cells	74	17%	16	13%	0.27	23	18%	60	14%	0.25
Glassy eosinophilic cytoplasm	185	41%	58	45%	0.42	59	46%	180	42%	0.37
Intracytoplasmic vacuoles	78	17%	42	33%	<0.001	36	28%	79	18%	0.02
Nuclear moulding	63	14%	9	7%	0.03	16	13%	55	13%	0.94

TABLE 5. The Original Diagnosis of Submitting Pathologist Is Compared to Diagnosis (in %) of 16 Pathologists Based on H&E only and Including Information Additional Stains (p63/p40, TTF1, Mucin)

		Original Diagnosis					
		SqCC	**Basal**	**Adeno**	**LCC**	**Sarcom.**	**LELC**
	Diagnosis	**%**	**%**	**%**	**%**	**%**	**%**
H&E only	SqCC	59%	11%	9%	11%	20%	9%
	Basaloid	2%	24%	0%	7%	0%	0%
	AdC	0%	1%	33%	6%	5%	3%
	AdenoSqmC	0%	1%	2%	0%	0%	0%
	LCC	33%	55%	52%	65%	28%	56%
	Sarcom.	2%	1%	0%	5%	48%	0%
	LELC	2%	0%	2%	2%	0%	28%
	DD SqCC-AdC	2%	6%	3%	3%	0%	3%
	Total	100%	100%	100%	100%	100%	100%
H&E plus stains	SqCC	76%	31%	3%	19%	13%	14%
	Basaloid	4%	33%	0%	14%	0%	0%
	AdC	0%	1%	70%	8%	10%	8%
	AdenoSqmC	0%	6%	2%	1%	0%	2%
	LCC-Sqcc	10%	19%	3%	19%	1%	14%
	LCC-AdC	0%	1%	11%	7%	9%	5%
	LCC-AdenoSqmC	0%	3%	8%	2%	3%	0%
	LCC-undiff	4%	6%	2%	22%	5%	28%
	Sarcom.	2%	0%	0%	6%	60%	0%
	LELC	3%	0%	2%	2%	0%	30%
	Total	100%	100%	100%	100%	100%	100%

SqCC, squamous cell carcinoma; Basaloid, basaloid carcinoma; AdC, adenocarcinoma; AdenoSqmC, adenosquamous carcinoma; LCC, large cell carcinoma; Sarcom., sarcomatoid carcinoma; LELC, lymphoepithelial-like carcinoma; DD SqCC-AdC, combination of SqCC and adenocarcinomas in the differential diagnosis; LCC-SqCC, large cell carcinoma favor SqCC; LCC-AdC, large cell carcinoma favor adenocarcinoma, LCC-Adenosquameus carcinoma; LCC-undiff, large cell carcinoma, additional stains negative.

similar: the mean and SD of the observers' sum of sensitivity and specificity were 133±13%. The ideal maximum is the sum of 100% sensitivity and 100% specificity = 200%. For the presence of intracytoplasmic vacuoles and TTF1/mucin positivity, these values were 115±17%. These data emphasize that more information about differentiation is obtained in undifferentiated or poorly differentiated tumors with the use of additional stains, than is recognized by light microscopic criteria alone in H&E-stained slides.

TABLE 6. Kappa Score (Two Categories: Specific Category vs. the Others; 95% Confidence Interval in Brackets) for Diagnostic Categories Based on (1) H&E Diagnosis Alone and on (2) Stains: Diagnosis Including Information Additional Stains (p63/p40, TTf1, Mucin)

	SqCC	Basaloid	AdC	AdenoSqmC	LCC	Sarcom.	LELC
H&E	0.46 (0.33–0.59)	0.27 (0.19–0.36)	0.21 (0.10–0.33)	0.05 (0.0–0.19)	0.25 (0.15–0.36)	0.52 (0.35–0.69)	0.56 (0.36–0.76)
H&E + stains	0.46 (0.33–0.59)	0.25 (0.16–0.34)	0.53 (0.33–0.73)	0.09 (0.0–0.19)	Range[a] (0.12–0.21)	0.52 (0.34–0.69)	0.47 (0.34–0.60)
Combining categories							
H&E	0.36 (0.25–0.48)		0.21 (0.10–0.33)	0.05 (0.0–0.10)	0.25 (0.15–0.36)	0.52 (0.35–0.69)	0.56 (0.36–0.76)
H&E + stains	0.57 (0.45–0.70)		0.63 (0.46–0.81)	0.20 (0.10–0.29)	0.21 (0.04–0.38)	0.52 (0.34–0.69)	0.47 (0.34–0.60)

After combining two diagnostic categories κ scores were recalculated. For this SqCC and basaloid was defined as one category; large cell carcinoma favoring SqCC was combined with SqCC; large cell carcinoma, favoring AdC with adenocarcinoma; large cell carcinoma, favoring AdenoSqmC to AdenoSqCC. Large cell carcinoma, unclassified after stains remained the large cell category.

SqCC, squamous cell carcinoma; Basaloid, basaloid carcinoma; AdC, adenocarcinoma; AdenoSqmC, adenosquamous carcinoma; LCC, large cell carcinoma; Sarcom., sarcomatoid carcinoma; LELC, lymphoepithelial like carcinoma.

[a]Range for the following categories: large cell carcinoma, favoring SqCC; large cell carcinoma, favoring AdenoC; large cell carcinoma, favoring AdenoSqCC; large cell carcinoma, unclassified.

DISCUSSION

Histologic criteria are consistently used by pathologists according to WHO criteria[1], but in poorly differentiated cases of NCSLC, many may not be present and the definitions are subject to individual interpretation, rendering consistent diagnosis more difficult and raising the possibility of a range of differential diagnoses based on cytological pleomorphism rather than aspects of differentiation. Also, this study demonstrates that, with the use of ancillary stains in poorly differentiated NSCLC, improved reproducibility can be obtained for the histopathological categories SqCC, solid AdC, sarcomatoid carcinoma, and LELC. Furthermore, it highlights the need for more precise definitions of individual histologic criteria, something evidenced in earlier studies relating to the definition of invasion.[3]

In relation to ancillary stains, the reproducibility between pathologists in poorly differentiated lung cancer has been shown to be poor in the past,[10,11] indicating that in H&E stained sections, diagnostic criteria are harder to find and may increase the likelihood of diagnosis of large cell carcinoma in resection specimens. There are numerous publications showing that the addition of IHC reduces the NOS rate in biopsy and also may potentially reclassify the number of cases termed LCC,[12–17] but our data additionally suggest that applying the current criteria and practice would lead to higher κ values in daily practice than on H&E alone, especially in these more poorly differentiated tumors.[7]

However, it is important to realize that these additional staining criteria do not have a defining capacity by themselves, as these stains are neither 100% specific nor sensitive. p63 may stain some AdCs, and TTF1 has a sensitivity of 70 to 80% in AdCs, plus both antibodies may stain tumors from other sites. Nevertheless, in the context of tumors where classification comes down to the differential of more poorly differentiated NSCLCs, these stains provide ancillary data that improve the accuracy of pathological diagnosis.

The consistent use of histological criteria is essential for histopathological diagnosis. In our study, the first phases of the study uncovered variations in interpretation of the individual criteria and we, therefore, made slightly stricter definitions (Table 2) even though some of the defining SqCC criteria (keratinization, squamous pearls, and intercellular bridges) originated 5 decades ago.[18] Again, this highlights the importance of ensuring that the written definitions are open to as little variation in interpretation as possible.

Even with stricter definitions, some criteria were found to be problematic in relation to the distinction of SqCCs from other subgroups. These included the term "intercellular gaps," which some pathologists were misinterpreting as the "intercellular bridges" of true squamous differentiation. Furthermore, "glassy eosinophilic cytoplasm" may also be recognized in AdCs and large cell carcinomas with mucin or TTF1 positivity and may reflect what some have termed "pseudo-squamoid" morphology.[19] Neither of these criteria appear to be of value in histologic distinction on H&E staining. Furthermore, the architectural terms "sheets of cells" may have a limited descriptive value in the distinction of epithelioid tumors from sarcomatoid tumors, but otherwise has little diagnostic value in the subtyping of NSCLCs.

Conversely, cytological criteria such as "spindle cells" in the context of a diffuse pattern with fascicles reached consensus and were of value in the classification of sarcomatoid carcinomas, although they were described in cases classified by some as SqCC. This, therefore, highlights the importance in documenting evidence of both squamoid and AdC differentiation in these more poorly differentiated epithelial tumors that are showing increased cellular plasticity. Moreover, the presence of sarcomatoid areas in greater than10% renders the diagnosis of sarcomatoid carcinoma and trumps squamous cell or AdC (Fig. 1).[1]

Despite some histological criteria being shown to have diagnostic value in more poorly differentiated tumors, our study shows that there is considerable overlap in these features that are helpful, for recognition of certain subtypes and are also discerned in the others. The examples are: features such as "intracytoplasmic vacuoles" which were scored as being present in cases finally classified as both SqCC and large cell carcinoma. Some of these may reflect a lack of mucin stains. Recently, the presence of two mucin droplets, instead of five in two high power fields was shown to be sufficient for diagnosis of AdC.[8] Moreover, recognition of these features is also dependent on having a good quality, well stained section of the correct thickness to examine.

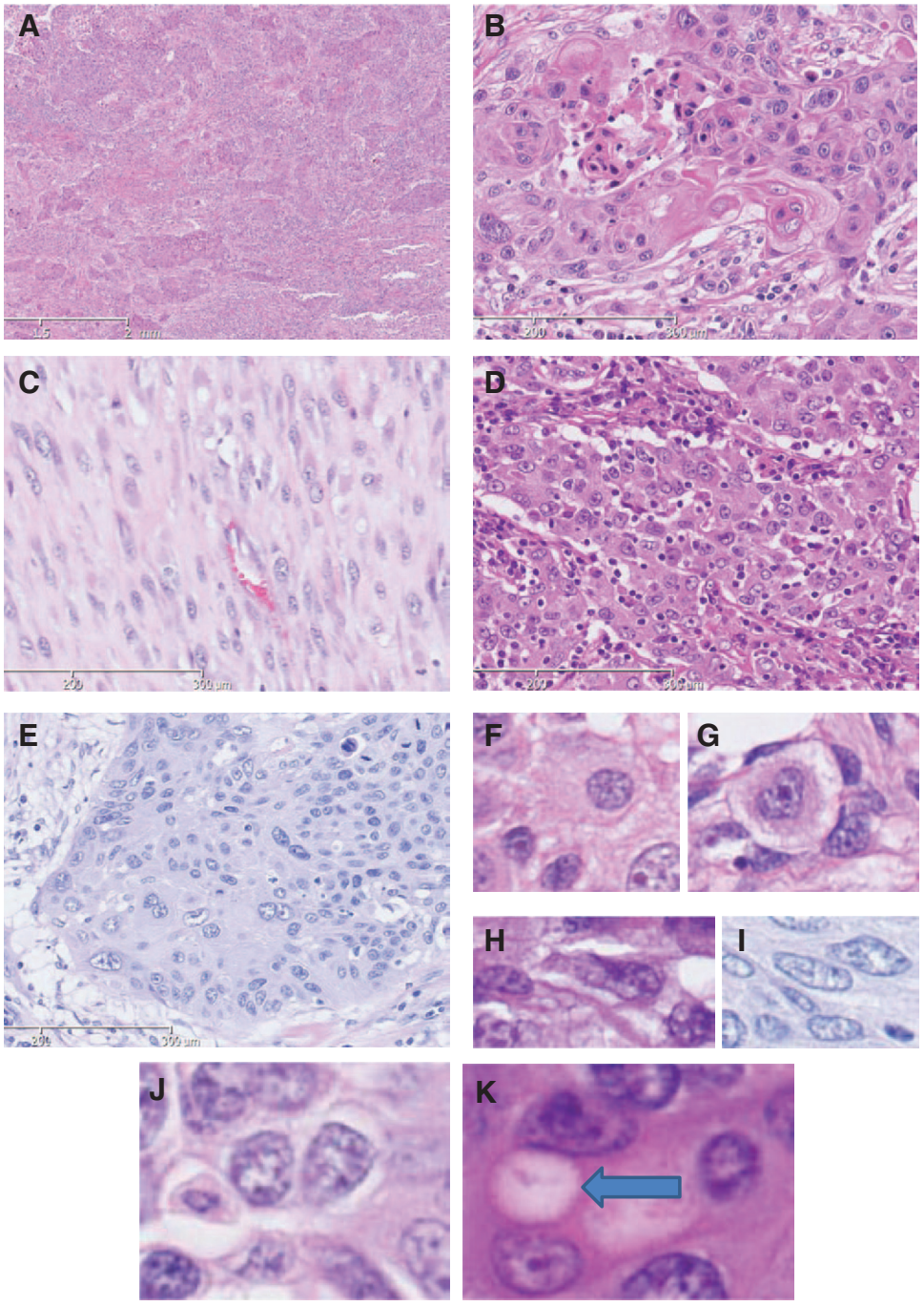

FIGURE 2. Example of consensus squamous cell carcinoma. *A*, Overview, ×20 (*B*), example of spindle cells in diffuse architecture (no nests or fields) (*C*), consensus score for lymphocytic infiltrate between the tumor cells (*D*), For palisading, no consensus was obtained: 25% of the pathologists called this palisading (*E*), detailed images with around the centered cell in at least part of the perimeter features of intercellular bridges (*F*, *G*), detailed images with three examples of intercellular gaps (called by most pathologists) (*H–J*), example of intracytoplasmic vacuole (arrow, consensus by all the pathologists) (*K*).

Basaloid carcinoma was described in 1992. The cardinal histopathologic features distinguishing this tumor from the other NSCLCs are a lobular growth pattern of small cells with moderately hyperchromatic nuclei without prominent nucleoli, and with scant cytoplasm, a high mitotic rate, and peripheral palisading.[20] This pattern could be present in a pure form or mixed with SqCC and was associated with a poor prognosis in stage I cases when compared to other NSCLCs.[21] When the prognosis of basaloid carcinomas is compared to poorly differentiated SqCCs, however, no differences were found, although the case numbers in these studies were relatively small.[22,23] In many instances, the differential diagnosis includes SCLC small-cell lung cancer and large cell neuroendocrine carcinoma (LCNEC).[24–27] Additional staining showing positive 34βE12 and p63 may suggest a diagnosis of basaloid SqCC. Taking the additional information from this study into account, arguments exist to consider basaloid carcinoma as a variant of SqCC. In this instance, IHC is superimposable with this interpretation.

Limitations of this study are that (1) the reader had only one section in images to examine, whereas the submission diagnosis was based on all the sections on glass slides

available; (2) IHC outcome was used and not the reproducibility of IHC interpretation; (3) images were scanned at ×20 magnification, making interpretation of subtle morphologic features at ×40 less optimal.

In conclusion, the histologic criteria that may be used in the differential diagnosis of poorly differentiated lung cancer need to be more precisely refined. Furthermore, additional stains improve the reproducibility of histological diagnosis of SqCC and AdC, uncovering information that is not present in regular H&E-stained slides.

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