Keratin 5 in Lung Cancer Specimens: Comparison of Four Antibody Clones and *KRT5* mRNA-ISH

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Abstract: Recent improvements in the medical treatment of non-small cell lung carcinoma have made the histopathological distinction between adenocarcinomas (ACs) and squamous cell carcinomas (SCCs) increasingly important. One immunohistochemical marker of squamous differentiation is Keratin 5 (K5). Several K5 antibody clones are commercially available, and data from external quality assessment (NordiQC) have shown large variations in their performance. However, comparing antibody performance characteristics of optimized K5 immunohistochemical assays in lung cancer specimens is needed. Tissue microarrays comprising 31 SCCs, 59 ACs, 17 large cell carcinomas, 8 large cell neuroendocrine carcinomas, 5 carcinosarcomas, and 10 small cell carcinomas were included. Serial sections from the tissue microarrays were stained using optimized assays based on the K5 mouse monoclonal antibodies D5/16 B4 and XM26, and the K5 rabbit monoclonal antibodies SP27 and EP1601Y, respectively. The staining reactions were assessed using H-score (0–300). In addition, p40 immunohistochemistry and *KRT5* mRNA-ISH analyses were conducted. Clone SP27 showed significantly higher analytical sensitivity than the other 3 clones. However, a distinct positive reaction was observed in 25% of the ACs using clone SP27 but not with the other clones. Clone D5/16 B4 displayed granular staining in 14 ACs, probably representing Mouse Ascites Golgi-reaction. A weak, scattered expression of *KRT5* mRNA was seen in 71% of the ACs. In conclusion, the K5 antibody clones D5/16 B4, EP1601Y, and XM26 showed equal sensitivity in lung cancer specimens, but D5/16 B4 also showed nonspecific Mouse Ascites Golgi-reaction. Clone SP27 demonstrated superior analytical sensitivity but lower clinical specificity in the differential diagnosis of SCC versus AC.

Key Words: Keratin 5, immunohistochemistry, lung cancer, mRNA *in situ* hybridization

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In recent years, new medical treatments for non-small cell lung carcinoma have posed a need for the distinction between squamous cell carcinomas (SCC) and adenocarcinomas (AC).[1] Often, this is possible based on morphology, but some cases do not display the features needed to distinguish these 2 main types of carcinomas reliably.[2] In such situations, an immunohistochemical (IHC) panel of squamous and adenomatous markers is necessary for the correct classification of the tumor, enabling the oncologist to provide the best medical treatment for the patient. Two frequently used markers of squamous differentiation are keratin (K) 5 and p40. Both markers have demonstrated high sensitivity and specificity in squamous cell lung cancer specimens, but with some variation, depending on antibody (Ab) clones, immunohistochemical protocols, and type of clinical samples (resection/biopsy, primary/metastasis).[3–6] The WHO Classification of Thoracic Tumors, fifth edition recommends the use of p40 over Keratin 5 (K5) as a first-line squamous marker for small samples.[7] However, recent studies have shown a comparable performance of p40 and K5, and K5 has been proposed as a supplementary marker in difficult cases.[5,8]

IHC evaluation of K5 expression in tissue specimens has been used in diagnostic pathology for decades, and many different K5 Ab clones are commercially available. Nordic immunohistochemical Quality Control (NordiQC), an external IHC proficiency testing organization, has assessed the performance of different K5 Ab clones several times. The results of these assessments have been described on www.nordiqc.org and summarized in a recent paper.[9] In the 2019 assessment of K5 (run 55) by NordiQC[10] with 263 participating laboratories, only 44% of the K5 IHC assays were considered of sufficient quality for diagnostic use. The majority of the less successful results were due to low analytical sensitivity or nonspecific reactions. Sufficient results could be obtained by almost all the assessed Ab clones, but great variations were observed with some clones, such as the mouse monoclonal (mm) Ab D5/16 B4, where only 23% of all assays based on this clone were able to produce a sufficient staining result. In contrast, the rabbit monoclonal (rm) Ab clone SP27 gave sufficient results with all assessed assays.[9] The NordiQC results give valuable information about the robustness and interlaboratory performance of the assessed Ab clones. However, to get more detailed information about possible differences in performance characteristics of the different

K5 Ab clones, a comparison based on IHC protocols optimized in a reference laboratory and tested on a large number of tissues is needed.

The aim of the present study was to compare the analytical sensitivity and specificity of the K5 Ab clones D5/16 B4, XM26, EP1601Y, and SP27 in a series of lung cancers and compare the K5 IHC results with *KRT5* mRNA in situ hybridization (ISH) results as well as with p40 IHC results.

MATERIALS AND METHODS

Tissue

Eight tissue microarrays (TMA) with tissue cores from anonymized lung cancer specimens, which had been constructed for the evaluation of several IHC biomarkers in 2010, were included in the study. The TMAs comprised in total 31 SCCs (including 2 adenosquamous carcinomas), 60 ACs, 16 large cell carcinomas (LCCs), 8 large cell neuroendocrine carcinomas, 5 carcinosarcomas (CSs), and 10 small cell carcinomas.

Immunohistochemistry

Serial 4 μm sections were cut from each TMA and mounted on coated slides (FLEX IHC slides K8020, Agilent). The sections were dried overnight at room temperature and stored at −20°C until staining. Before staining, the slides were dried at 60°C for 1 hour. The slides were then placed in a BenchMark Ultra instrument (Ventana). The slides were deparaffinized onboard and submitted to heat-induced epitope retrieval (HIER) in Cell Conditioning 1 for 48 minutes at 99°C. Following endogenous peroxidase blocking, the primary Abs for K5 and p40 (see Table 1) were applied for 32 minutes at 36°C. After a wash in the buffer the visualization system, OptiView DAB (HRP-labeled multimer, Ventana, 760–700) was applied. After a further wash in the buffer, the slides were finally developed with DAB (Ventana, 760–700) and counterstained with hematoxylin II (Ventana, 790–2208).

In situ Hybridization

Seven 5 μm consecutive sections were cut from each TMA and mounted on coated slides (Superfrost plus, Thermo Scientific). The sections were dried overnight at room temperature and dried at 60°C for 1 hour before staining. The fully automated RNAscope assay was performed on a Discovery Ultra (Ventana) according to the manufacturer's instructions[11] using the RNAscope VS Universal HRP reagent kit (Advanced Cell Diagnostics, Newark, CA). To determine which pretreatment conditions would yield the highest positive signal and the lowest negative control signal along with the best tissue morphology, every TMA was tested before running the assay with the target probe. Three sections from each TMA were tested with a positive RNAscope control probe targeting a common housekeeping gene, *PPIB,* with a heating time at respectively 16 minutes, 32 minutes, and 48 minutes in Cell Conditioning 1, all following protease treatment for 16 minutes. After evaluation, the optimized pretreatment conditions were used to run the assay with the target probe for *KRT5* mRNA (HS-KRT5-01, Product no: 547909, Advanced Cell Diagnostics, Newark, CA). In addition, sections from each TMA were tested using a negative control probe targeting a bacterial gene (*dapB*) with the same pretreatment settings to ensure specificity.

Sections were stained in the following order: HE, K5 IHC, p40, and mRNA-ISH.

TABLE 1. Antibodies Used in the Study

Epitope	Antibody Clone	Vendor	Product no.	Dilution*
K5/6	D5/16 B4	Agilent	M7237	1:50
K5	EP1601Y	Cell Marque	305R-14	1:25
K5	SP27	Thermo Scientific	MA5-16372	1:50
K5	XM26	Leica	NCL-L-CK5	1:50
P40	BC28	Biocare Medical	ACI3066C	1:20

*Dilution buffer: EnVision Flex Antibody Diluent, Agilent.

Scanning and Digital Microscopy

The IHC slides were scanned at ×20 magnification using a Hamamatsu Nanozoomer HT slide scanner. The mRNA-ISH slides were scanned at ×40 magnification using a Hamamatsu NanoZoomer S60 Digital slide scanner. All the digital slides were assessed using the software NDP.view2 (Hamamatsu).

Scoring of IHC

The IHC staining results were assessed by consensus of 2 of the authors (M.V. and L.B.-H.). The IHC stains were assessed by calculating an H-score that takes into account the percentage and intensity of staining. The percentage of positive tumor cells (0-100%) was multiplied by the intensity of the staining (0, negative; 1, weak; 2, moderate; and 3, strong intensity), thus creating an H-score ranging from 0 to 300. A positive cutoff level for H-scores of $\geq$ 10 was chosen, and slides containing less than 100 tumor cells were excluded.

Scoring of mRNA in situ Hybridization

The ISH slides were assessed by 2 of the authors (T.S. and C.T.). The ISH stains for K5 and p40 were scored using a system corresponding to the H-score, named ISH-score. Low expression was defined as 1 to 3 dots/cell, moderate expression as 4 to 15 dots/cell, and high expression as dot clusters or >15 dots/cell. Each entity (numbered 1, 2, and 3, respectively) was multiplied by their percentages to calculate the ISH-score (0–300). The preservation of mRNA in the tissue cores was considered sufficient if a moderate expression of the housekeeping gene, *PPIB*, was observed in almost all cells. Depending on an acceptable negative control (*dapB* ISH-score <1), tumor cores were considered *KRT5* mRNA positive if ISH-score $\geq$ 1.

Statistics

The H-scores of the different K5 Ab clones were compared using the Friedman test to investigate if there was a difference between any 2 Ab clones. Pairwise comparisons

using paired Wilcoxon signed-rank test were performed as a post hoc analysis to identify which Ab clones differed. P values <0.05 were considered significant. R-studio vers. 2022.02.0 build 443 with the packages *tidyverse* and *rstatix* was used for all statistics.

RESULTS

The results of each K5 Ab clone and p40 are summarized in Table 2, and the results of *KRT5*-ISH are summarized in Table 3. Clone SP27 gave significantly higher H-scores than the other clones, both overall and in the largest subgroups. Moreover, SP27 showed a distinct staining reaction in 15 of 59 ACs (25%), while no staining reaction in ACs was observed with any of the other clones (Fig. 1). Three of the K5-positive ACs with clone SP27 had an H-score ≥ 120. Two of them had corresponding *KRT5* mRNA-ISH of sufficient quality, with ISH scores of 0 and 7, respectively. The third core was considered insufficient due to poor preservation of mRNA, but despite that, scattered tumor cells were positive for *KRT5* mRNA (ISH-score 4). p40 was negative in all these 3 cores. In 14 ACs, a varying degree of granular, perinuclear cytoplasmic staining with mmAb clone D5/16 B4 was observed, probably false positive representing a Mouse Ascites Golgi (MAG) reaction (Fig. 2). When present, the nonneoplastic epithelium showed a similar reaction in these 14 AC tissue cores.

In general, all 4 K5 Ab clones provided high H-scores in the SCCs. However, in 2 of the tissue cores with SCC a large variation was observed (H-score core 1/core 2; SP27: 300/300; D5/16 B4: 180/105; XM26: 25/5; EP1601Y: 15/0). Both these tissue cores were strongly p40 positive (H-score: 180/280). Unfortunately, the corresponding slides for *KRT5* mRNA-ISH contained too few tumor cells in these 2 cores to be included in the analysis.

The LCCs comprised a heterogeneous group with both completely negative and diffusely positive tumor cores with all 4 Ab clones. There was a large variation both regarding the fraction of positive tumors (9 of 17 with SP27 vs. 4 of 17 with D5/16 B4) and the mean H-score, where SP27 demonstrated a significantly higher K5 expression. Two of the LCCs were p40 positive (H-score 110 and 270). These tumors might reflect misclassified, poorly differentiated squamous cell carcinomas. Two other LCCs showed positive reactions with SP27 (H-scores 30 and 140) while being negative with the other 3 clones. There was a low expression of *KRT5* mRNA (ISH-score 2) in those tumor cores.

Two of the 5 CSs were positive with all 4 clones, while 2 were negative. The remaining CS showed a positive reaction with SP27 (H-score 60) but was negative with the 3 other clones. This core had poor preservation of *PPIB* mRNA, but a few tumor cells showed weak expression of *KRT5* mRNA.

p40 Expression

The p40 staining reaction was sufficient in 127 of 130 tissue cores. The 3 insufficient cores were due to <100 tumor cells. In general, p40 was positive in the same or fewer number of tumor cores within each cancer subgroup, compared with the K5 Abs. Yet, one LCC was diffusely p40 positive (H-score 110) but negative with all 4 K5 Ab clones. A weak expression of *KRT5* mRNA (ISH-score 3) was observed in this case. In addition, one AC was weakly p40 positive (H-score 12) but negative with all K5 Ab clones and *KRT5* mRNA-ISH.

mRNA in situ Hybridization

In total, 81 of 130 tissue cores were sufficient for mRNA-ISH analysis. One of 8 TMAs (with neuroendocrine tumors: small cell carcinomas and large cell neuroendocrine carcinomas) containing 18 tissue cores was not available for mRNA-ISH.In addition, another 11 cores contained less than 100 tumor cells or were not representative of the tissue evaluated by IHC. The remaining 20 insufficient cores were due to low expression of the housekeeping gene, *PPIB*, indicating poor preservation of mRNA in these cores. All sufficient cores were completely negative for *dapB* (negative control).

All the sufficient cores (24 of 31) with SCCs, except 1 adenosquamous carcinoma, showed at least moderate expression of *KRT5* mRNA.

TABLE 2. Positivity-rates and H-scores in Lung Cancer Subtypes for Each Keratin 5 Antibody Clone and p40

Antibody	SP27		XM26		EP1601Y		D5/16 B4		p40	
Tumor type	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	Mean H-score	Pos. %	$P < 0.05$
SCC (n = 31, incl. 2 ASCs)	100	287	97	259	97	251	100	252	97	SP27 vs. all other
AC (n = 59)	25	13	0	0	0	0	0 (24*)	0	2	SP27 vs. all other
LCC (n = 17)	53	87	41	50	29	38	24	32	12	SP27 vs. all other
CS (n = 5)	60	132	40	120	40	120	40	120	40	—
LCNEC (n = 8)	50	91	38	69	25	61	25	62	25	—
SCLC (n = 10)	40	40	30	17	30	16	30	18	10	—
Overall (n = 130)	—	99	—	79	—	74	—	74	—	SP27 vs. XM26 vs. D5/16 B4 and EP1601Y

*Non-specific granular reaction, probably Mouse Ascites Golgi.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; CS, carcinosarcoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; Pos., H-score ≥ 10; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

TABLE 3. KRT5 mRNA-ISH Results for Each Lung Cancer Subtype

	mRNA in situ Hybridization		
Tumor type	Sufficient quality [%], (n)	Positive* [%], (n)	ISH-score [0–300], range (median)
SCC (n = 31, incl. 2 ASCs)	77 (24)	96 (23)	0–300 (270)
AC (n = 59)	68 (40)	71 (28)	0–10 (1)
LCC (n = 17)	81 (14)	100 (14)	1–270 (4)
CS (n = 5)	60 (3)	67 (2)	0–175 (3)
LCNEC (n = 8)	NA	NA	—
SCLC (n = 10)	NA	NA	—

*Positive: Sufficient quality and ISH-score ≥ 1.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; CS, carcinosarcoma; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

Among sufficient cores with AC (40 of 59), 71% showed weak and/or scattered expression of *KRT5* mRNA. Sixteen of these cores, including 1 with ISH-score 10, were negative with all 4 K5 Abs.

All the sufficient cores with LCCs (14 of 17) showed at least scattered tumor cells that were *KRT5* mRNA positive (ISH-score range: 1–270, median: 4). Five tumors had an ISH-score ≥ 55.

DISCUSSION

The K5 assay based on the rmAb SP27 showed significantly higher analytical sensitivity than the assays based on the other 3 examined K5 clones, both in general and in the larger groups of lung cancer subtypes, including a conspicuous positive reaction in 25% of the ACs. No significant difference in analytical sensitivity was observed between the mmAbs D5/16 B4 and XM26 and the rmAb EP1601Y. The observed difference between SP27 and the other clones could be the result of either superior analytical sensitivity or nonspecific reaction (inferior analytical specificity). In the lack of other methods than IHC to detect K5 *in situ, KRT5* (K5 encoding gene) mRNA-ISH was conducted as an indirect marker of K5 expression to evaluate the analytical specificity. We have previously used this indirect method to evaluate the specificity of IHC assays for estrogen receptors in breast cancers, where we found a nonlinear relationship between mRNA and protein expression.[12] In general, the level of protein in a cell population can be assumed to be dependent on the level of the corresponding mRNA under steady-state conditions.[13] To our knowledge, the relationship between K5 IHC and *KRT5* mRNA-ISH measurements in tumors has not previously been reported. In normal squamous epithelium, *KRT5* mRNA expression is restricted to the basal cell layer, whereas the K5 protein is retained in the more superficial layers of the epithelium.[14] In the SCCs, we observed *KRT5* mRNA in most tumor cells, indicating a more or less constant expression. However, transcription of genes to mRNA is either active or not and can be heterogeneous within a cell population.[13,15] It is therefore uncertain whether the scattered expression of *KRT5* mRNA in many ACs represents few cells with constant *KRT5* expression or a higher fraction of cells with only sporadic expression. However, our findings support at least a weak K5 expression in a large proportion of the non-squamous lung carcinomas. Thus, it cannot be concluded that the reaction pattern seen with SP27 is due to an unspecific reaction. Unfortunately, 20% of the cores that were available for mRNA-ISH showed poor preservation of mRNA and could not be included in the analysis. This is a limitation of the study, especially because these insufficient cores included some of those with the largest variation in K5 IHC. The low amounts of mRNA in some tissue cores may be a consequence of the relatively long archival time (10–17 years), as a time-dependent loss of mRNA has been described in previous studies.[16,17]

The increased analytical sensitivity of the SP27 assay may partially be related to a different dynamic range compared with the other K5 IHC assays. In addition to a higher ratio of positive cells, the SP27 assay also showed significantly more cells with high expression in all K5-positive tumors (data not shown). As the IHC protocol was identical to the other K5 assays, this must be related to a larger number of bound Ab molecules in the tissues.

IHC assays should be fit-for-purpose.[18] In the case of lung cancer diagnostics, the purpose of the K5 IHC assay is to discriminate between AC and SCC. Our findings support a superior analytical (and clinical) sensitivity of SP27, but the clinical/diagnostic specificity may be poorer compared with the other K5 Ab clones. There are several published studies on the use of D5/16 B4 and XM26 in lung cancer diagnostics, but to our knowledge, similar studies on SP27 and EP1601Y have not been published. This study was not designed to measure the clinical specificity, but the high ratio of SP27-positive ACs indicates a lower clinical specificity. Current lung cancer classification regarding K5 is based on older clones, such as D5/16 B4 and XM26. The introduction of a much more sensitive Ab clone could cause a shift in diagnostic cutoffs and requires diagnostic validation.[19] In lung cancer diagnostics, high sensitivity for K5 would enable the pathologist to correctly identify poorly differentiated SCCs, and encountering K5 reaction in an otherwise typical AC would probably not be problematic. However, K5 reaction in a poorly differentiated adenocarcinoma could cause troubles. Such issues may not be a problem in other diagnostic settings, for example, identification of basal cells in breast and prostate specimens, but it needs to be validated before use.[19]

In 14 of the ACs, a granular, perinuclear staining reaction with clone D5/16 B4 was observed, while the other clones were negative. This was probably a nonspecific MAG-reaction that can be seen in tissues from patients with blood group A with Abs of mouse ascites format.[20] Because of the characteristic staining pattern, which differed from true K5 reaction (eg, in basal cells), it was not included in the H-score, and the tumor cores were regarded as being negative. However, one needs to be aware of this issue with Ab clone D5/16 B4 in ascites format, as it might cause diagnostic challenges.

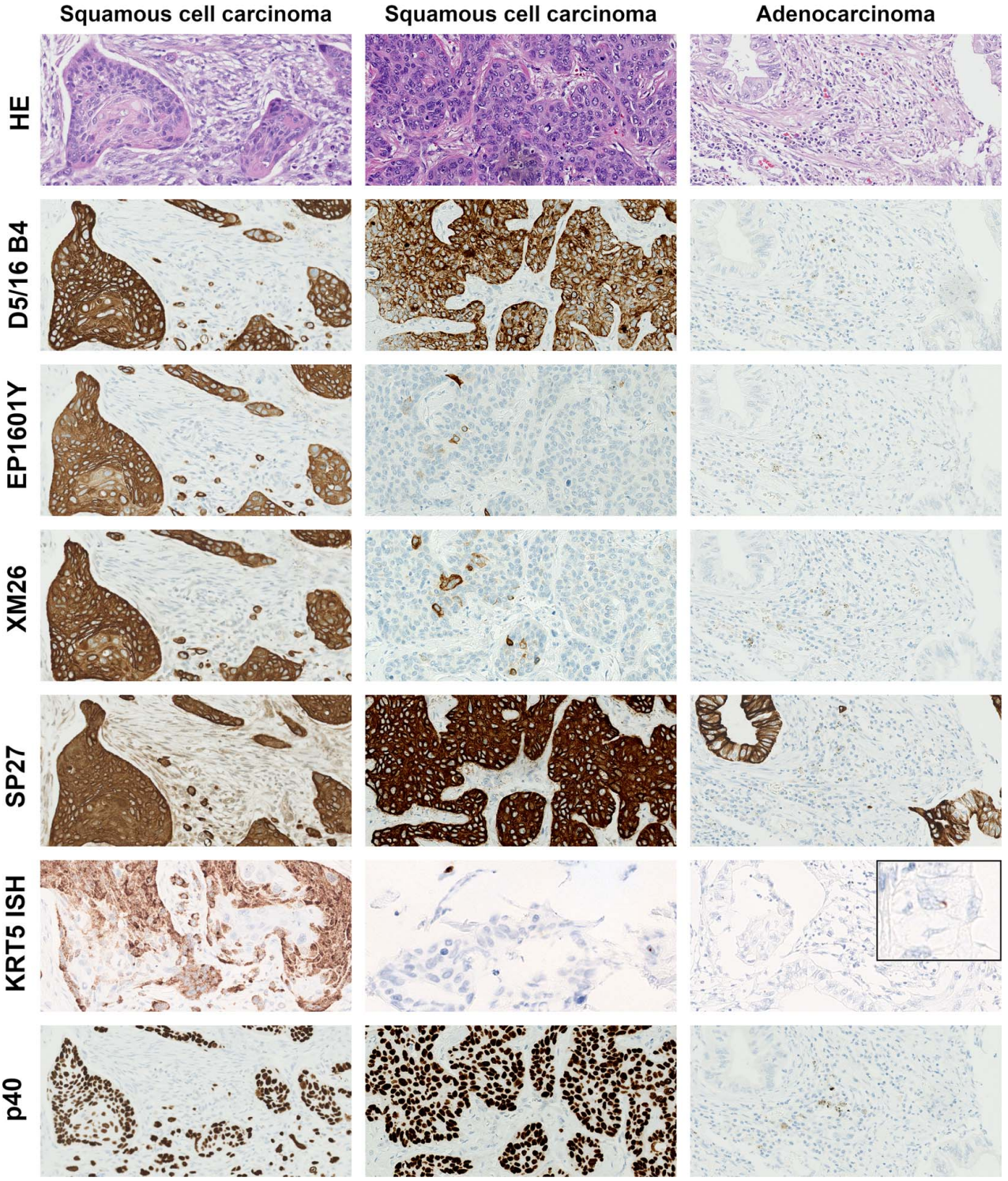

FIGURE 1. Examples of staining patterns for the 4 K5 Ab clones; D5/16 B4, EP1601Y, MX26, and SP27, as well as *KRT5* mRNA-ISH, and p40. Left column: Squamous cell carcinoma with high expression of K5, demonstrated by all 4 clones. Correspondingly, there is high expression of *KRT5* mRNA and p40. Middle column: Squamous cell carcinoma with divergent results by the 4 K5 Ab clones. <100 cells are represented in the *KRT5* mRNA-ISH, showing scattered cells with few dots, whereas p40 is strongly positive. Right column: Adenocarcinoma showing an intense staining with clone SP27, while the other 3 clones produce no staining reaction. mRNA-ISH shows very few cells with solitary dots. p40 shows negative staining reaction.

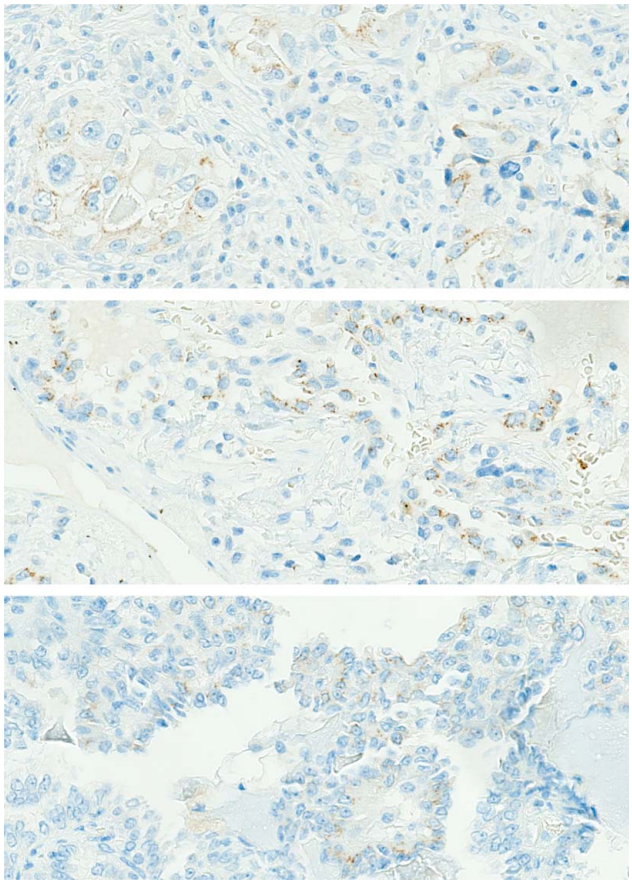

FIGURE 2. Three adenocarcinomas with a granular, mainly perinuclear staining reaction, that was only seen with the antibody clone D5/16 B4. It probably represents a nonspecific mouse ascites Golgi (MAG) reaction. full color online

To our knowledge, this is the first comparison of the performance of K5 Abs in lung cancer specimens. Bhargava et al compared the sensitivity of the Ab clones D5/16 B4 and XM26 in the identification of basal-like carcinomas of the breast.[21] They reported a significantly higher sensitivity of XM26 than D5/16 B4. Except for the LCCs, where XM26 was more sensitive, we did not observe a difference in the analytical sensitivity of these 2 clones. Conversely, XM26 failed to identify 1 SCC, while D5/16 B4 was positive in all SCC cases. The discrepancy of the findings in the 2 studies may be a consequence of different tumor types and different IHC protocols. However, in the NordiQC assessments of K5 IHC assays, optimal staining could be achieved with both Ab clones, but the likelihood of getting a successful result was much greater with XM26.[9] In a recent NordiQC assessment, EP1601Y showed an inferior performance compared with XM26 and SP27, the latter being the clone with the highest ratio of optimal results.[9] In our study, the performance of EP1601Y was similar to that of XM26 and D5/16 B4. The reason for this is probably differences in protocol settings compared with the participating laboratories in the NordiQC assessment.

p40 is another recommended marker of squamous differentiation.[5,8] In accordance, all SCCs (and 1 of 2 adenosquamous carcinomas) in this study were p40 positive. Interestingly, one AC was p40 positive, but K5 negative. Different expression profiles of K5 and p40 were also seen in the LCCs, with only 2 p40-positive tumors, while 4 of 17 were positive with all K5 Ab clones.

In conclusion, the expression profiles of K5 in lung cancer specimens are greatly dependent on the used Ab clone. SP27 has superior analytical sensitivity, but our results indicate a lower clinical specificity, which may limit its diagnostic utility in the differential diagnosis of SCC versus AC. The high sensitivity of SP27 may be preferred to identify poorly differentiated SCCs, but it has to be validated before use.[19] The Ab clones XM26, D5/16 B4, and EP1601Y provided overall equal analytical sensitivity, but D5/16 B4 also showed nonspecific MAG-reaction in many of the ACs. Based on experiences from NordiQC,[9] XM26 in general, provides the most robust performance among these three Ab clones.

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