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ANATOMICAL PATHOLOGY

Agreement of CK5/6, p40, and p63 immunoreactivity in non-small cell lung cancer

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Summary

Histological subtyping of non-small cell lung cancer (NSCLC) is of utmost importance for therapy stratification. Common immunohistochemical markers to identify squamous lineage are CK5/6, p40, and p63. Although p40 is considered the gold standard by current guidelines, the agreement of all three markers is an important aspect for tumours more difficult to classify.

A total of 1244 NSCLC including 569 squamous cell carcinomas (SqCC) and 675 adenocarcinomas were assembled on a tissue microarray and stained with CK5/6, p40, p63, TTF-1, and Napsin-A. Sensitivity and specificity for squamous lineage markers as well as agreement of CK5/6, p40 and p63 were calculated.

Sensitivity of CK5/6, p40, and p63 for SqCC was 93%, 94%, and 94% and specificity was 98%, 97%, and 84%, respectively. Positivity for two of these markers was found in at least in 90% of SqCC. Highest agreement was observed for p40 and p63 (Cohen's kappa 0.80).

We report a similar sensitivity of CK5/6, p40, and p63, but a decreased specificity of p63 as compared to CK5/6 and p40 for the identification of squamous lineage. Our results support the use of either CK5/6 or p40 over p63 in the routine diagnostic setting.

Key words: NSCLC; lung cancer; CK5/6; p40; p63.

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INTRODUCTION

Approximately 6% of men and women will be diagnosed with lung cancer during their life which makes lung cancer the most common cancer in men and the second most common in women worldwide.[1] Despite recent advances in targeted therapy, the cancer related mortality remains high and only about 19% of patients survive 5 years or more after diagnosis.[1] Many patients (35–40%) are being diagnosed at an advanced, non-resectable clinical stage, and for most of these patients, therapy remains palliative. Biopsy or cytology material is often the only available option for precise tumour subtyping. In this regard, the use of a tissue microarray for immunohistochemical studies seems most appropriate to mimic the biopsy situation.[2]

Clinical management highly depends on the histological subtype as well as on the immunophenotype and genetic alterations.[3] Histologically, lung cancer is separated into non-small cell lung cancer (NSCLC, 85%) and small cell lung cancer (SCLC, 15%).[4] The main histological subtypes within the NSCLC group are squamous cell carcinoma (SqCC) and adenocarcinoma (ADC). At an advanced clinical stage, therapy highly depends on aberrations in genes such as *ALK*, *ROS1*, *EGFR*, *BRAF*, *RET*, *MET* or *HER2*.[5] In tumours without these alterations, immune checkpoint inhibitors are another promising option.[6,7]

As the histological subtype is also an important predictive factor,[8,9] reliable identification of glandular and squamous differentiation is of utmost importance. In tumours with typical morphological features such as lepidic, acinar, papillary or micropapillary growth, or evidence of keratinisation and/or intercellular bridges, the distinction between ADC and SqCC is often possible on morphology alone. In tumours with predominant solid growth and lack of specific morphological characteristics adjunct immunohistological staining may be necessary. In this regard, cytokeratin (CK) 5 or 5/6, p40, and p63 are common markers to identify squamous lineage. In order to save tissue for subsequent molecular analyses, the European Society for Medical Oncology (ESMO) currently recommended the use of only two markers to confirm the histological subtype.[10] The current World Health Organization (WHO) classification from 2015 encourages the application of thyroid transcription factor 1 (TTF-1) and p40 as the most reliable marker combination.[4] ESMO guidelines recommend the use of either p40 or p63.[11] p40 is favoured by some authors because of its higher specificity as compared to p63,[12] but there have also been reports that challenge this finding.[13] The WHO Classification

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of Tumours of the Lung, Pleura, Thymus and Heart 2015 also favours p40 over CK5/6 and p63.[14] To clarify the role of CK5/6, p40, and p63 in the identification of squamous differentiation, we compared the expression of all three immunohistochemical markers on 1244 patient samples, which is the largest respective dataset reported to date.

MATERIALS AND METHODS

Cohort characteristics

Formalin fixed and paraffin embedded NSCLC specimens resected from 2002 to 2010 in the Thoracic Hospital Heidelberg at Heidelberg University were extracted from the archive of the Institute of Pathology, Heidelberg University, with the support of the tissue bank of the National Center for Tumour Diseases (NCT, #180015 and #2591). Tissues were used in accordance with the ethical regulations of the NCT tissue bank defined by the local ethics committee. Diagnoses were made according to the recommendations of the 2015 WHO classification.[14] A cohort of 1244 NSCLC including ADC and SqCC, was selected. Tissue microarray construction was as described previously.[15–17] The results from the conventional NSCLC markers CK5/6, p63, Napsin-A, and TTF-1 were partially stained and published previously (cases from 2002 to 2008).[15] p40 was stained on the whole cohort for the first time and additional cases from 2008 to 2010 were stained by CK5/6, p63, Napsin-A, and TTF-1. A detailed description of the clinical characteristics of the NSCLC cohort is provided in Table 1.

Table 1 Patients' characteristics

Variable	Overall cohort	SqCC	ADC
Patients, *n*	1244	569	675
Gender, *n* (%)			
Male	869 (70)	477 (84)	392 (58)
Female	375 (30)	92 (16)	283 (42)
Age at first diagnosis, median (range)	64 (30–89)	65 (38–83)	63 (30–89)
TNM, *n* (%)			
pT1	243 (20)	107 (19)	136 (20)
pT2	765 (61)	345 (61)	420 (62)
pT3	198 (16)	97 (17)	101 (15)
pT4	38 (3)	20 (4)	18 (3)
pN0	612 (49)	275 (48)	337 (50)
pN1	287 (23)	184 (32)	103 (15)
pN2	305 (25)	100 (18)	205 (30)
pN3	7 (1)	1 (0.2)	5 (1)
pNX	34 (3)	9 (2)	25 (4)
pM1	38 (3)	8 (1)	30 (4)
pMX	1206 (97)	561 (99)	645 (96)
Clinical stage, *n* (%)			
I	466 (37)	196 (34)	270 (40)
II	366 (29)	220 (39)	146 (22)
III	374 (30)	145 (26)	229 (34)
IV	38 (3)	8 (1)	30 (4)

ADC, adenocarcinoma; SqCC, squamous cell carcinoma.

Immunohistochemistry

Immunohistochemical staining was performed as previously described.[18] In brief, slides were deparaffinised, pre-treated with an antigen retrieval buffer and stained using an automated device. Napsin-A and TTF-1 were stained on a Techmate 500plus (Dako, Germany) and CK5/6, p40, and p63 on a Ventana Benchmark Ultra (Roche, Switzerland). The antibody and staining characteristics are shown in Table 2. The evaluation was carried out by an experienced pathologist (MK). Any staining of tumour cells was evaluated as positive. Typical examples of positive and negative stainings of the three markers in SqCC are shown in Fig. 1.

Data analysis and software

All statistical analyses were performed using R-Statistical Software (www.r-project.org, v.3.4.2, Free Software Foundation), R-Studio (v.1.1.383, Affero General Public License, USA), or Excel 2017 (Microsoft, USA). Correlation of the immunohistochemical stains with clinicopathological characteristics was by unpaired t-test for equally distributed variables and Fisher-Freeman-Halton test for counted data. Agreement between markers was measured by Cohen's kappa which was calculated using the irr-package (v. 0.84) in R. Cohen's kappa: ≤0.1 no agreement, >0.1–≤0.4 low agreement, >0.4–≤0.6 medium agreement, >0.6–≤0.8 strong agreement, >0.8–≤1 full agreement. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using the EpiR package (v. 0.9–93). p values <0.05 were considered significant.

RESULTS

Patients' characteristics

Overall, 1244 NSCLC patient samples were analysed. Among the analysed patients 569 and 675 had a diagnosis of SqCC and ADC, respectively. There were 869 (70%) male patients and 375 (30%) female. The median age was 64 (range 30–89) years. Clinical stage I, II, and III disease was found in the majority of patients at first diagnosis and was distributed almost equally. Only 38 (3%) of patients had stage IV disease. Patients' characteristics are summarised in Table 1.

Expression of CK5/6, p40, and p63 in NSCLC

SqCC samples were positive for CK5/6, p40, and p63 in 532 (93%), 536 (94%), and 533 (94%) of 569 cases. Positivity for two of these markers was found at least in 90% of cases. CK5/6, p40, and p63 were expressed together in 509 (89%) of SqCC. Napsin-A and TTF-1 expression was observed in seven (1%) cases. Expression of these two markers was focal, present in the same cell population that showed reactivity for squamous markers and in <10% of tumour cells. Thus, these cases did not qualify for a diagnosis of adenosquamous carcinoma according to the 2015 WHO classification.[14]

ADCs were rarely focally positive for CK5/6 (15/675, 2%) or p40 (23/675, 3%). On the other hand, 106 (16%) ADC

Table 2 Antibodies used in this study and staining conditions

Antibody	Company	Clone	Pre-treatment	Buffer incubation time (min)	Antibody incubation time (min)	Dilution
CK5/6	Ventana	D5/16 B4	Tris/Borat/EDTA, pH 8.4	56	24	RTU
p40	Ventana	BC28	Tris/Borat/EDTA, pH 8.4	48	24	RTU
p63	Ventana	4A4	Tris/Borat/EDTA, pH 8.4	40	24	RTU
Napsin-A	Novocastra	IP64	Citrate, pH 6	10[a]	30	1:400
TTF-1	Novocastra	SPT24	EDTA, pH 9	10[a]	30	1:100

CK, cytokeratin, RTU, ready to use; TTF-1, thyroid transcription factor-1.
[a] Antigen retrieval under pressure for 10 minutes, then maintained in the same buffer for 30 minutes for cooling down.

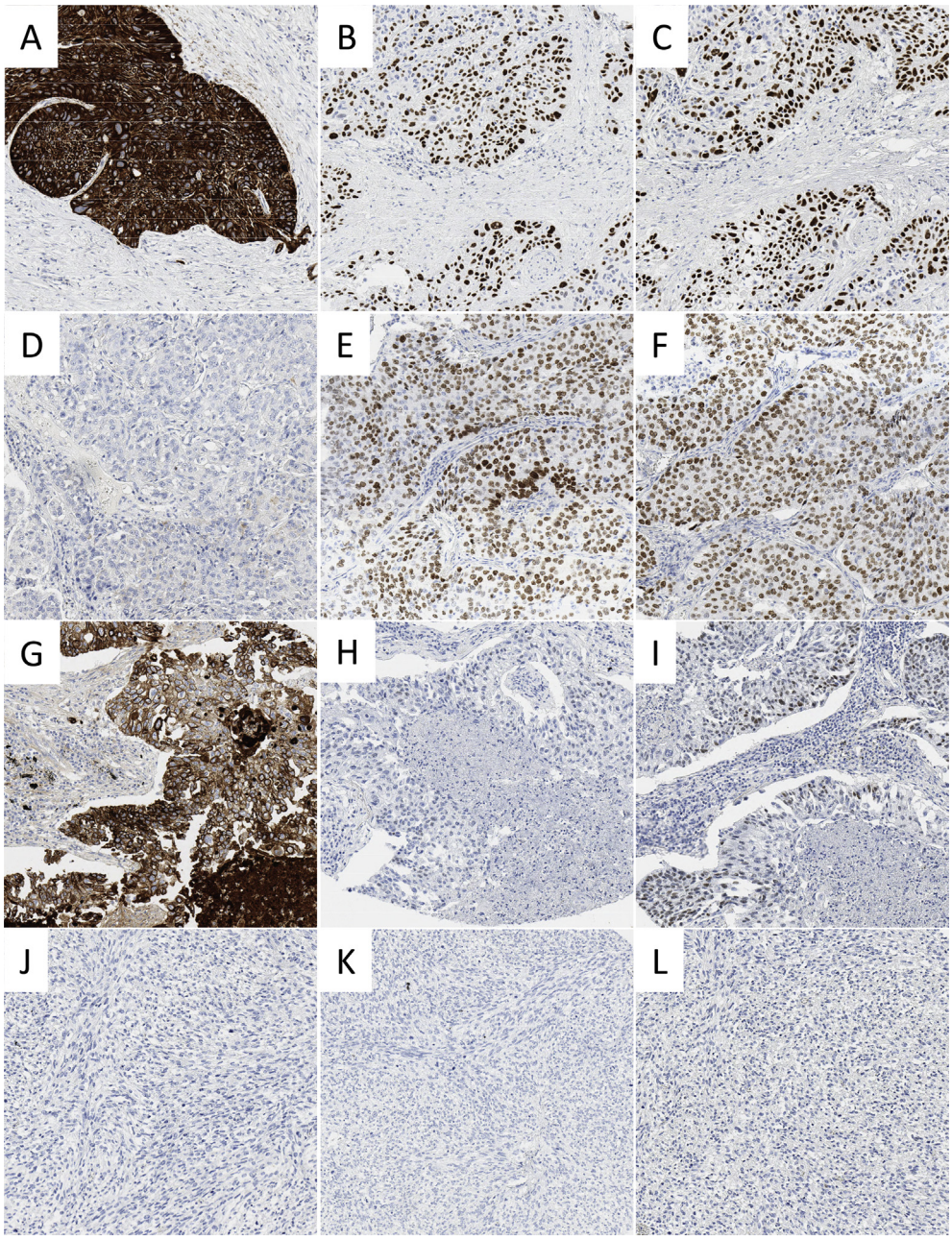

Fig. 1 Examples of expression patterns of SqCC. Most SqCC showed positivity for CK5/6 (A), p40 (B) and p63 (C). Rarely, either CK5/6 (D–F), or p40 (G–I) or p63 (not shown) were negative. Unique SqCC were triple negative (J–L) but exhibited keratinisation or intercellular bridges on the full slide.

cases showed positivity for p63. Cases positive for CK5/6 or p40 showed weak focal staining in <10% of tumour cells. Again, these cases did not qualify for a diagnosis of adenosquamous carcinoma according to the 2015 WHO classification.[14] Napsin-A (502, 74%) and TTF-1 (578, 86%) expression was found in a vast majority of ADC cases. ADC negative for Napsin-A and TTF-1 were also negative for CK5/6, p40 and p63.

Table 3 and Fig. 2 give an overview of the expression of the analysed markers in the considered entities.

Sensitivity, specificity, and agreement of CK5/6, p40 and p63 in SqCC

CK5/6, p40, and p63 were found to be positive in 532, 536, and 533 SqCC cases, therefore sensitivity for squamous differentiation was 93%, 94%, and 94%. As CK5/6, p40, and p63 were negative in all but 15, 23, and 106 of 675 analysed ADC cases, a specificity of 98%, 97%, and 84% was calculated for the three markers, respectively (Table 4). p40 showed the highest sensitivity/specificity combination for squamous differentiation compared to CK5/6 and p63. However, CK5/6 had similar sensitivity/specificity characteristics demonstrating just a 1% lower sensitivity compared to p40. Displaying comparable sensitivity of 98% for SqCC, p63 had a poor specificity.

Analysis of the expression of CK5/6, p40, and p63 in SqCC revealed that 509 of 569 (89%) cases were concordantly positive for all three markers. Forty-five (8%) SqCCs showed a discordant CK5/6, p40, and p63 expression with positivity of at least one marker. Fifteen (3%) SqCC cases showed negativity for all three markers. Among these triple-

Table 3 Staining characteristics

Entity	Cases, *n*	Positive[a] cases, *n* (%)								
		CK5/6	p40	p63	CK5/6, p40	CK5/6, p63	p40, p63	CK5/6, p40, p63	Napsin-A	TTF-1
Overall cohort	1244	547 (44)	559 (45)	639 (51)	525 (42)	522 (42)	551 (44)	518 (42)	509 (41)	685 (47)
SqCC	569	532 (93)	536 (94)	533 (94)	516 (91)	512 (90)	528 (93)	509 (89)	7 (1)	7 (1)
ADC	675	15 (2)	23 (3)	106 (16)	9 (1)	10 (1)	23 (3)	9 (1)	502 (74)	578 (86)

ADC, adenocarcinoma; SqCC, squamous cell carcinoma.
[a] Positivity was defined as any positivity.

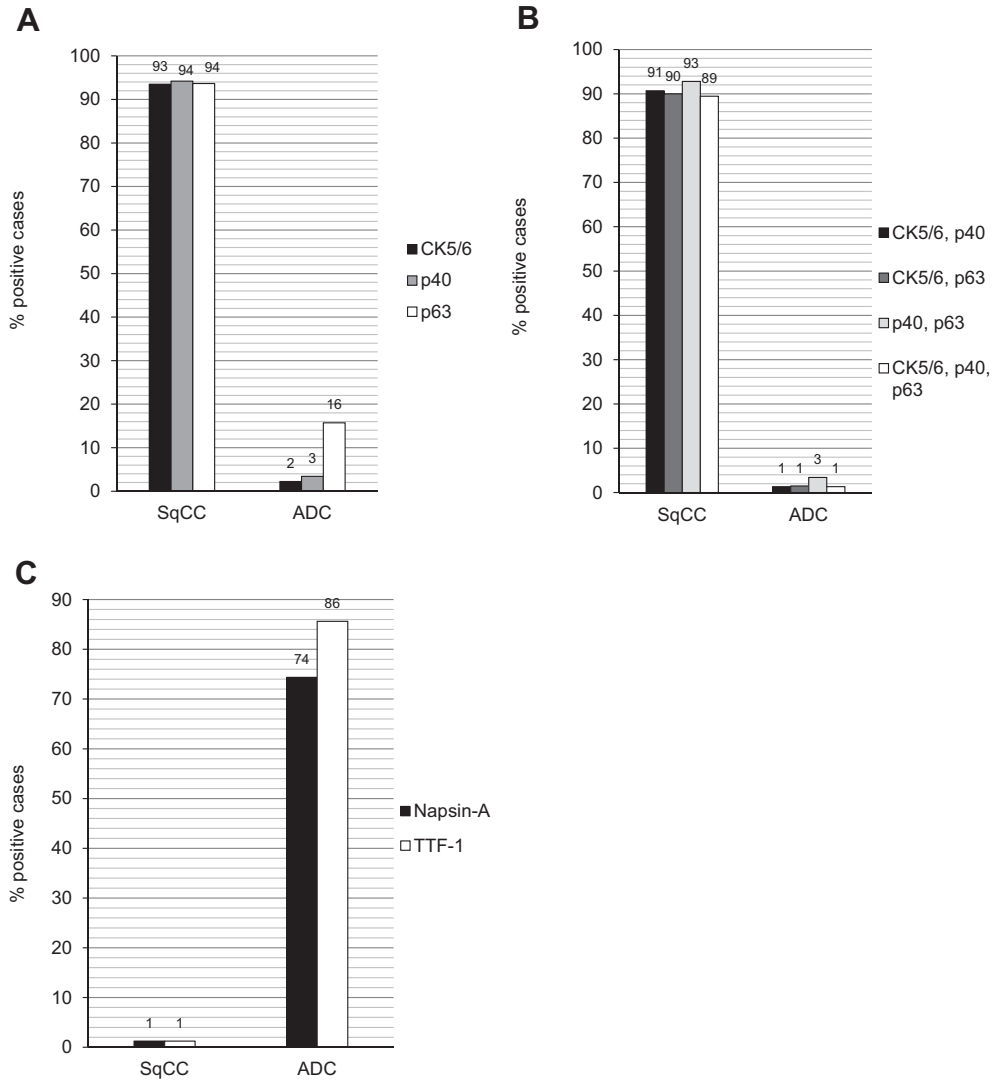

Fig. 2 Staining characteristics. Percentage of positive squamous cell carcinoma (SqCC) and adenocarcinoma (ADC) cases for (A) CK5/6, p40, p63, (B) the combination of these markers and (C) Napsin-A and TTF-1.

negative cases, all displayed negativity for Napsin-A and TTF-1. Moreover, all of these cases exhibited either keratinisation or intercellular bridges on whole slides and therefore qualified for a diagnosis of SqCC. It is not entirely clear why these tumours were negative for CK5/6, p40 and p63. One possibility is a sampling error as all of these markers might exhibit only focal immunoreactivity in some tumours. Absolute and relative numbers of concordant and discordant CK5/6, p40, and p63 expression in SqCC cases are summarised in Fig. 3. Among CK5/6, p40, and p63, p40 and p63 showed a strong inter-rater agreement (Cohen's kappa 0.80) in SqCC. The inter-rater agreement was medium (Cohen's kappa 0.45) between CK5/6 and p40 and low between CK5/6 and p63 (Cohen's kappa 0.40). The inter-rater agreement between all three markers was medium (Fleiss's kappa 0.55) in SqCC.

DISCUSSION

In the current study we investigated 1244 tissue specimens from patients with NSCLC including 569 SqCC to further

Table 4 Test quality criteria of CK5/6, p40 and p63 as markers for SqCC

Marker	IHC result	SqCC, *n*	ADC, *n*	Overall, *n*	Sensitivity	Specificity	PPV	NPV
CK5/6	Positive	532	15	547	93%	98%	97%	95%
	Negative	37	660	697				
	Overall	569	675	1244				
p40	Positive	536	23	559	94%	97%	96%	95%
	Negative	33	652	685				
	Overall	569	675	1244				
p63	Positive	533	106	639	94%	84%	78%	94%
	Negative	36	569	605				
	Overall	569	675	1244				

ADC, adenocarcinoma; IHC, immunohistochemistry, NPV, negative predictive value; PPV, positive predictive value; SqCC, squamous cell carcinoma.

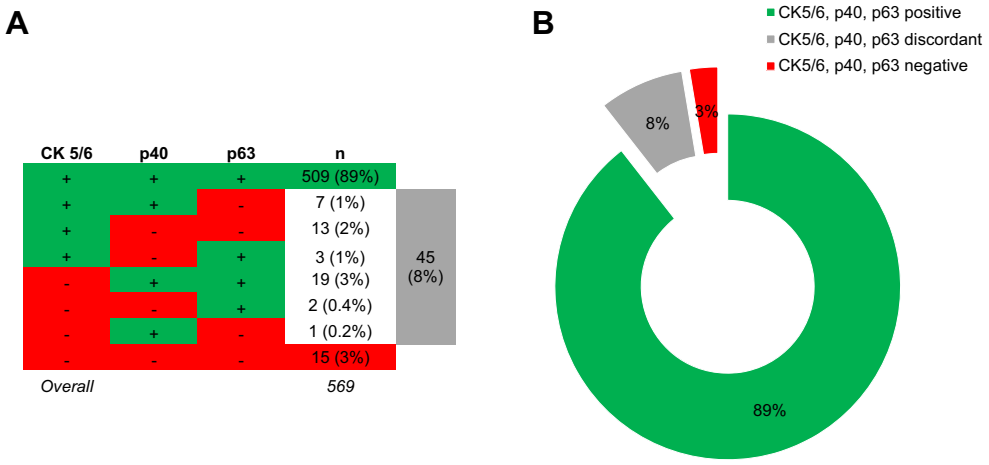

Fig. 3 Concordance and discordance of CK5/6, p40 and p63 positivity in SqCC. (A) CK5/6, p40 and p63 marker constellations with corresponding proportion of SqCC cases and (B) graphical visualisation.

clarify the role of CK5/6, p40, and p63 for the identification of squamous lineage. To date, this is by far the study with the largest number of NSCLC and SqCC investigated.

Our cohort seems representative for NSCLC in developed countries as the distribution of age, gender, TNM categories, and stage closely mimic the reported values.[1]

CK5/6 are intermediate-sized basic keratins with a molecular mass of 58 kDa.[19] p40 is the N-terminally truncated isoform of p63.[12] All three markers are commonly applied to identify squamous lineage in NSCLC. In lung cancer, the sensitivity for the identification of squamous lineage of CK5/6, p40, and p63 was similar in our study with 93, 94, and 94%, respectively, which is within the reported range of 90–97% for CK5/6, 81–100% for p40 and 80–100% for p63 (Table 5). The specificity was 98% and 97% for CK5/6 and p40 and considerably lower for p63 with 84%. Again, these values match the reported range of 80–99% for CK5/6, 85–100% for p40 and 60–98% for p63. Whereas some authors could not find a lower specificity of p63 as compared to p40,[13,20] our findings support the results from the majority of studies.[12,21] The lower specificity of p63 as compared to CK5/6 and p40 was mainly attributable to positive ADC (16% in our cohort) which has been recognised before.[21] These findings have also been confirmed in cytology

Table 5 Studies comparing p40 against other markers for pulmonary SqCC

Study	Year	Total samples, *n*	SqCC, *n*	CK5/6		p40		p63	
				Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Bishop *et al.*[12]	2012	470	81			100%	98%	100%	60%
Nonaka[25]	2012	200	50			100%	100%	100%	82%
Pelosi *et al.*[26]	2013	141	27			100%	97%	100%	78%
Ao *et al.*[27]	2014	154	77	90%	80%	81%	90%	94%	80%
Koh *et al.*[20]	2014	184	59	93%	94%	93%	98%	80%	98%
Tatsumori *et al.*[28]	2014	580	158	94%	93%	97%	97%	97%	73%
Kadota *et al.*[29]	2015	469	449			100%	85%	100%	60%
Tran *et al.*[21]	2016	557	167	95%	97%	94%	96%	95%	87%
Micke *et al.*[30]	2016	656	192	97%	99%	97%	98%	97%	74%

SqCC, squamous cell carcinoma.

specimens.[13,22–24] Interestingly, p40 and p63 show highest inter-rater agreement which might be due to the fact that both proteins have a common molecular structure.

Although CK5/6 and p40 show both a very high accuracy with regard to the identification of squamous differentiation, p40 might be easier to evaluate as a nuclear marker. As about 8% of SqCC show discordant results in our study, multiple squamous markers may be stained if any doubt.

In summary, we report a similar sensitivity of CK5/6, p40, and p63, but a decreased specificity of p63 as compared to CK5/6 and p40 for the identification of squamous lineage in the largest NSCLC cohort investigated to date. Our results support the use of either CK5/6 or p40 over p63 in the routine diagnostic setting.

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