

Use of dual-marker staining to differentiate between lung squamous cell carcinoma and adenocarcinoma

Rui Guo, Yi Tian, Na Zhang, Hong Huang, Ying Huang and Jun Yang 

Abstract

Objective: To assess the value of dual-marker immunostaining for detecting p40 and napsin A, and cytokeratin 5/6 (CK5/6) and thyroid transcription factor 1 (TTF1) in single sections of lung cancer tissue, for differentiating between lung squamous cell carcinoma and adenocarcinoma.

Methods: Lung cancer tissue sections from 58 patients were stained by dual-marker immunostaining using a mixture of anti-p40 and anti-napsin A, and anti-CK5/6 and anti-TTF1 primary antibodies. Sections stained with single markers were used as controls. Nuclear or cytoplasmic staining was considered as indicating positive p40 or napsin A expression, respectively, and cytoplasmic or nuclear staining was considered as indicating positive CK5/6 or TTF1 expression, respectively.

Results: p40/napsin A and CK5/6/TTF1 dual-marker staining showed high sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis of squamous cell carcinoma and adenocarcinoma respectively. There were no differences in marker expression between dual-marker and single-marker staining.

Conclusions: Dual-marker immunostaining is a relatively easy, time- and cost-conserving staining method for detecting two markers in a single section using one procedure and one chromogen. p40 and napsin A, and CK5/6 and TTF1 dual-marker staining were suitable for the differential diagnosis of lung squamous cell carcinoma and adenocarcinoma.

Keywords

Non-small cell lung carcinoma, immunohistochemistry, dual-marker immunostaining, squamous cell carcinoma, adenocarcinoma, differential diagnosis

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Department of Pathology, The Second Affiliated Hospital, Xi'an JiaoTong University, Xi'an, Shaan'xi Province, China

Corresponding author:

Jun Yang, Department of Pathology, Second Affiliated Hospital, Xi'an JiaoTong University; No. 157, The West 5th Road, Xi'an, Shaan'xi Province, China, 710004.
Email: yangjundr@163.com



Introduction

Lung cancer is one of the most frequently diagnosed malignant neoplasms.¹ Non-small cell lung carcinoma (NSCLC) accounts for approximately 80% to 85% of all lung cancers but is heterogeneous in nature, comprising many histological sub-types. For example, adenocarcinoma (ADC) accounts for approximately 50% to 70% and squamous cell carcinoma (SqCC) for approximately 20% to 30% of all NSCLC cases.²⁻⁴ Lung cancer treatments have recently changed from tumor stage-based approaches to histomorphological and genetic mutation-targeted therapies, which rely on the accurate histological sub-classification of NSCLC. This is particularly important for patients with advanced or metastatic NSCLC, given that the treatments and prognoses of these tumors differ.⁵⁻⁸ Hematoxylin and eosin (H&E) staining of sections is useful for classifying moderately differentiated NSCLC, but not for the classification of poorly differentiated NSCLC, when pathologists must differentiate between SqCC and ADC. Furthermore, it is difficult to obtain enough H&E sections from small biopsy specimens,⁹ making the histological differentiation of SqCC from ADC difficult due to the lack of sufficient tissue sections.

Immunohistochemistry (IHC) is routinely used to classify various types of cancers.^{10,11} Previous studies have reported that p40, cytokeratin 5/6 (CK5/6), thyroid transcription factor 1 (TTF1), and napsin A are reliable markers for the sub-classification of NSCLC.¹²⁻¹⁶ However, the lack of enough useful sections for immunostaining has always been an issue limiting the accurate classification NSCLC in small biopsy specimens. To circumvent this issue, many investigators have used a dual-immunostaining approach to stain two markers in one section; however, the existing approach requires two enzymes and two chromogenic substrates. Furthermore, this approach is time-

consuming and costly, and can generate high non-specific background staining.¹⁷⁻¹⁹

In the current study, we established a dual-marker immunostaining approach to simultaneously stain p40 and napsin A or CK5/6 and TTF1 in a single lung tumor section (China Patent Application No: 201911158385.3). The results suggest that this may provide a time-saving and economical approach for the simultaneous detection of two markers in sections from small biopsy specimens.

Materials and methods

Specimens

Surgical lung cancer specimens from 58 patients were retrieved from the Department of Pathology at the Second Hospital of Xi'an Jiaotong University from 2017 to 2019. The specimens included 26 SqCC and 32 cases of ADC. One additional case of adenosquamous carcinoma was employed as a control. The specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with H&E. The sections were then classified according to the 2015 WHO Classification of Lung Tumors by pathologists blinded to the patients' clinical data.^{3,4} All the lung tumors were well- or moderately differentiated. The use of human tissues was approved by the Second Hospital Review Board of Xi'an Jiaotong University. No ethical approval or patient consent was required because the study used surgically resected specimens solely for IHC experiments performed at the Second Affiliated Hospital, Xi'an JiaoTong University.

Reagents and equipment

Anti-p40 (clone ZR8), anti-TTF1 (clone MX011), anti-CK5/6 (clone D5/16B4), and anti-napsin A (clone MX015) antibodies and the antibody diluent (ABD-0030,

IVD) were all procured from Maixin Biotechnology Co., Ltd. (Fuzhou, China). The antibodies were used for IHC at dilutions of 1:100 in conjunction with an UltraView DAB Detection Kit and Ventana Benchmark XT Automated Stainer (Ventana Medical Systems, Shanghai, China).

Dual- and single-marker staining

Formalin-fixed, paraffin-embedded tissues were cut at a thickness of 4 to 5 μm . Dual- and single-marker staining were performed on sections according to a standard immunostaining protocol using a Ventana Benchmark XT Automated Stainer, following the manufacturer's instructions. Briefly, the sections were heated for 1 hour at 58°C, deparaffinized, and dehydrated in a graded alcohol series. Antigen retrieval was performed using Cell Conditioning 1 (Ventana) Tris/Borate/EDTA for 60 minutes with a Ventana Benchmark XT Automated Stainer followed by dual-marker immunostaining for p40/napsin A and CK5/6/TTF1, according to the manufacturer's instructions. The sections were blocked with hydrogen peroxide and normal goat serum and then incubated with a mixture of anti-p40 and anti-napsin A or anti-CK5/6 and anti-TTF1 antibodies at ratios of 1:1 for 30 minutes at 37°C. The antibody concentrations were about 1.0 $\mu\text{g}/\text{mL}$, respectively. The color was developed with 3,3'-diaminobenzidine (DAB), and the sections were then counterstained with hematoxylin. Positive controls were single-stained for p40, napsin A, CK5/6, or TTF-1. Single-marker immunostaining for p40, napsin A, CK5/6, and TTF1 was also performed synchronously using an UltraView DAB Detection Kit and Ventana Benchmark XT Automated Stainer, according to the manufacturer's instructions.

Interpretation of staining results

The sections were evaluated by two pathologists blinded to the clinical data.

The cutoff for positive staining for all four markers was according to the Best Practices Recommendations for Diagnostic Immunohistochemistry in Lung Cancer.²⁰ The staining of tumor cells, bronchial, and alveolar cells was scored separately. For the conventional single-marker staining approach, p40 or TTF1 nuclear staining was considered as positive for p40 or TTF1 expression, respectively, and CK5/6 or napsin A cytoplasmic staining was considered as positive for CK5/6 or napsin A expression, respectively. For dual p40 and napsin A staining, positive nuclear staining was considered as positive for p40 expression (p40(+)) and napsin A (-); diagnosis of SqCC), whereas positive cytoplasmic staining was considered as positive for napsin A expression (p40(-) and napsin A(+)); diagnosis of ADC). For dual CK5/6 and TTF1 staining, positive cytoplasmic staining was considered as positive for CK5/6 expression (CK5/6(+)) and TTF1 (-); diagnosis of SqCC), whereas positive nuclear staining was considered as positive for TTF1 expression (CK5/6(-) and TTF1 (+)); diagnosis of ADC) (Table 1).

Statistical analysis

Differences between the combined expression of p40 and napsin A or CK5/6 and TTF1 and the individual expression of p40, napsin A, CK5/6, and TTF1 were analyzed by Wilcoxon's test for paired samples. *P*-values <0.05 were considered significant. All analyses were carried out using IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY, USA).

Results

Clinical information

The median age of the patients was 58.2 years (range, 41–72 years). Among the 26 cases of SqCC, the male:female ratio was 15:11, there

Table 1. Dual-marker staining for the differential diagnosis of non-small cell lung cancer.

Dual markers	Staining pattern		Marker expression	Diagnosis
	Nuclear	Cytoplasmic		
p40 and napsin A	+	–	p40(+) and napsin A(–)	SqCC
	–	+	p40(–) and napsin A(+)	ADC
CK5/6 and TTF1	+	–	CK5/6(–) and TTF1(+)	ADC
	–	+	CK5/6(+) and TTF1(–)	SqCC

SqCC, squamous cell carcinoma; ADC, adenocarcinoma; CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor 1.

were 12 cases of pT1 and 14 cases of pT2 tumors, and the mean tumor size was 3.4 cm (range, 1.5–4.7 cm). Among the 32 cases of ADC, the male:female ratio was: 14:18, there were 15 cases of pT1, 16 cases of pT2, and one case of pT3 tumors, and the mean tumor size was 3.2 cm (range, 2.4–5.1 cm). The ADC were acinar (24 cases), solid (5 cases), papillary (1 cases), and invasive non-mucinous ADC (2 cases). One additional female patient with adenosquamous carcinoma was employed as a control (age, 48 years, tumor size, 2.6 cm, tumor stage, pT1).

Single-marker staining of p40, napsin A, CK5/6, and TTF1

There was no background staining in sections stained by the single-marker approach. In both bronchial basal and SqCC cells, nuclei stained positive for p40 and the cytoplasm stained positive for CK5/6. In both alveolar epithelial and ADC cells, the nuclei stained positive for TTF1 and the cytoplasm stained positive for napsin A. The nucleus stained positive for p40 and the cytoplasm stained positive for CK5/6 in all 26 (100%) cases diagnosed as SqCC, retrospectively. The nucleus stained positive for TTF1 in 30 of 32 (93.8%) and the cytoplasm stained positive for napsin A in 28 of 32 (87.5%) ADC cases (Figure 1 and Figure 2, Table 2). In the lung adenosquamous carcinoma sections, SqCC cells showed positive nuclear

staining for p40 and cytoplasmic staining for CK5/6, while ADC cells showed positive nuclear staining for TTF1 and cytoplasmic staining for napsin A.

Dual-marker staining of p40 and napsin A, and CK5/6 and TTF1

There was no background or cross-staining in sections stained by the dual-marker approach. In p40 and napsin A dual-stained sections, the nuclei and cytoplasm of bronchial basal cells stained positive and negative, respectively, whereas the nuclei and cytoplasm of alveolar epithelial cells of the paracarcinoma tissues stained negative and positive, respectively (Figure 1). p40 and napsin A dual-staining showed that the nuclei stained positive and the cytoplasm stained negative in all 26 (100%) SqCC cases, and the nuclei stained negative and the cytoplasm stained positive in 28 of 32 (87.5%) ADC cases (Table 2, Figure 1 and Figure 3).

In CK5/6 and TTF1 dual-stained sections, the nuclei and cytoplasm of bronchial basal cells stained negative and positive, respectively, whereas the nuclei and cytoplasm of alveolar epithelial cells stained positive and negative, respectively (Figure 2). CK5/6 and TTF1 dual-staining showed that the nuclei stained negative and the cytoplasm stained positive in all 26 SqCC cases (Table 2, Figure 3), and the nuclei stained positive

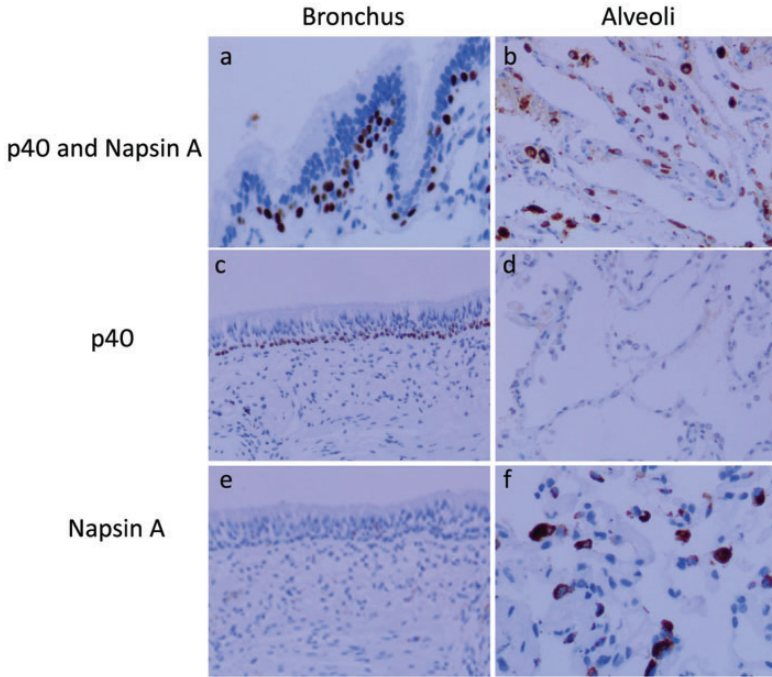


Figure 1. p40 and napsin A dual- and single-marker staining in lung tissue. (a) Bronchial basal cells showed positive p40 nuclear staining in p40/napsin A dual-staining. (b) Alveolar epithelial cells showed positive napsin A cytoplasmic staining in p40/napsin A dual-staining. (c) Bronchial basal cells showed positive p40 nuclear staining in p40 single-staining. (d) Alveolar epithelial cells showed negative p40 in single-staining. (e) Bronchial basal cells showed negative napsin A in single-staining. (f) Alveolar epithelial cells showed positive napsin A cytoplasmic staining in napsin A single-staining. a–e, 100 \times ; f, 200 \times .

and the cytoplasm stained negative in 30 of 32 (93.8%) ADC cases (Table 2, Figure 4).

In p40 and napsin A dual-stained sections of adenosquamous carcinoma, SqCC cells showed positive nuclear staining and ADC cells positive cytoplasmic staining. In CK5/6 and TTF1 dual-stained sections of adenosquamous carcinoma, SqCC cells showed positive cytoplasmic staining and ADC cells showed positive nuclear staining (Figure 5).

Similarities between dual- and single-marker staining

The expression of p40, napsin A, CK5/6, and TTF1 in 58 cases of lung carcinoma were completely consistent between dual- and single-marker staining (Table 2).

The results of dual-marker staining were therefore identical to those of conventional IHC single-marker staining for detecting p40, napsin A, CK5/6, and TTF1 in the 58 cases of lung carcinoma. All showed high sensitivity, specificity, positive predictive value, and negative predictive value for differentiating between SqCC and ADC (Table 3). Wilcoxon's test showed no difference in the expression of any of the markers detected by dual- compared with single-marker staining (Table 3, Figures 2 and 3).

Discussion

Cancer treatment relies on the differentiation of SqCC from ADC. Although well-differentiated SqCC can be easily

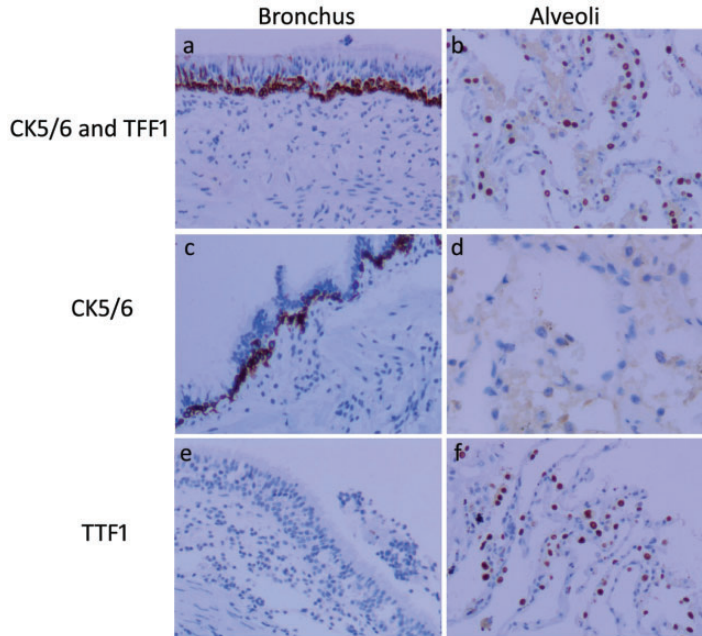


Figure 2. CK5/6 and TTF1 dual- and single-marker staining in lung tissue. (a) Bronchial basal cells showed positive CK5/6 cytoplasmic staining in CK5/6/TTF1 dual- staining; (b) Alveolar epithelial cells showed positive TTF1 nuclear staining in CK5/6/TTF1 dual-staining. (c) Bronchial basal cells showed positive CK5/6 cytoplasmic staining and (d) alveolar epithelial cells showed negative CK5/6 in single-staining. (e) Bronchial basal cells showed negative TTF1 and (f) alveolar epithelial cells showed positive TTF1 nuclear staining in single-staining. a–c, e, f, 100 \times ; d, 200 \times . CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor 1.

Table 2. Dual- and single-marker staining for differentiating between squamous cell carcinoma and adenocarcinoma in patients with non-small cell lung cancer.

Subtype of NSCLC	Cases (n)	Dual-marker staining				Single-marker staining			
		p40 and napsin A		CK5/6 and TTF1		p40	CK5/6	TTF1	napsin A
		p40	napsin A	CK5/6	TTF1	p40	CK5/6	TTF1	napsin A
SqCC	26	26/26	0/26	26/26	0/26	26/26	26/26	0/26	0/26
ADC	32	0/32	28/32	0/32	30/32	0/32	0/32	30/32	28/32

NSCLC: non-small cell lung cancer; SqCC: squamous cell carcinoma; ADC: adenocarcinoma; CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor 1.

distinguished from well-differentiated ADC, the classification of poorly differentiated cancers based on H&E sections is challenging.²¹ IHC is routinely used to classify various types of cancers, with approximately one-third of NSCLC cases requiring

IHC for accurate differentiation.^{22–24} The 2015 World Health Organization Classification Guidelines recommend the use of IHC with at least one SCC marker and one ADC marker.^{3–5,25} However, it is difficult to obtain sufficient sections from

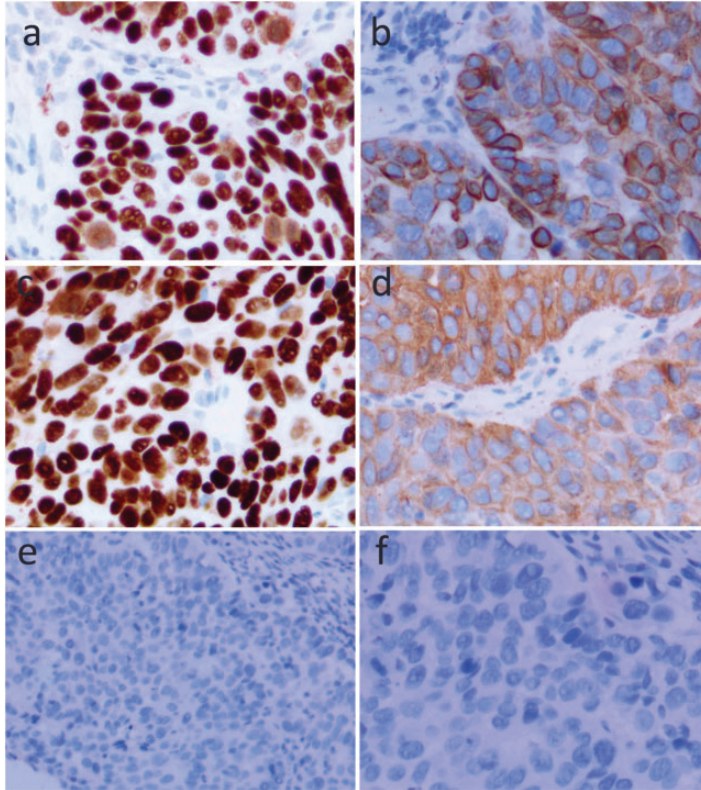


Figure 3. p40 and napsin A, and CK5/6 and TTF1 dual-marker staining in lung squamous cell carcinoma. (a) Tumor cells showed positive p40 nuclear staining and negative napsin A cytoplasmic staining in p40/napsin A dual-staining. (b) Tumor cells showed positive CK5/6 cytoplasmic staining and negative TTF1 nuclear staining in CK5/6/TTF1 dual-staining. (c) Tumor cells showed positive p40 nuclear staining in p40 single-staining, (d) positive CK5/6 cytoplasmic staining in CK5/6 single-staining, (e) negative napsin A staining in napsin A single-staining, and (f) negative TTF1 staining in TTF1 single staining. a-d,f, 400 \times ; e, 200 \times . CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor 1.

small biopsy specimens, which has impeded the diagnosis and treatment of patients and prompted us to explore other immunostaining approaches.

Many investigators have already established several highly sensitive and specific dual-staining protocols that yield reproducible and reliable staining results for lung cancer.^{26–29} Furthermore, various manufacturers offer dual-staining detection kits such as the EnVisionTM DuoFLEX Double Stain System from Dako Cytomation. An automated dual-staining system has also

recently been developed for visualizing both types of stains.³⁰ However, most dual-staining approaches require many sections and two antibodies from different species, usually rabbit and mouse, as well as two enzymes and two chromogenic substrates, to detect the expression of two markers in a single section. These products generally use horseradish peroxidase (HRP) and alkaline phosphatase reactions, involving DAB, alkaline phosphatase, Perma Blue, 3-amino-9-ethylcarbazole and/or Vulcan Fast Red. Petersen et al.³⁰

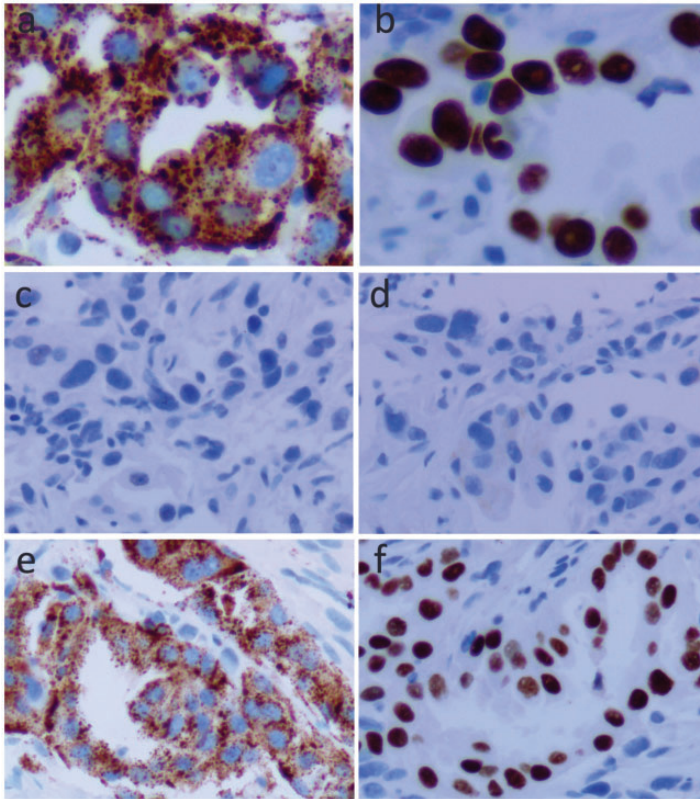


Figure 4. p40 and napsin A, and CK5/6 and TTF1 dual-marker staining in lung adenocarcinoma. (a) Tumor cells showed negative p40 nuclear staining and positive napsin A cytoplasmic staining in p40/napsin A dual-staining. (b) Tumor cells showed negative CK5/6 cytoplasmic staining and positive TTF1 nuclear staining in CK5/6/TTF1 dual-staining. (c) Tumor cells show negative p40 nuclear staining in p40 single-staining, (d) negative CK5/6 cytoplasmic staining in CK5/6 single-staining, (e) positive napsin A cytoplasmic staining in napsin A single-staining, and (f) positive TTF1 nuclear staining in TTF1 single-staining. a-d, 400 \times ; e-f, 200 \times . CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor I.

performed automated staining with a Dako Omnis system using the 'IHC Double Stain Template' with two HRP substrates as chromogens. They suggested that using antibodies from different species and using DAB as the first chromogen and HRP Magenta as the second chromogen could reduce the cross-reactivity in double staining protocols.³⁰ Although, traditional double staining can thus be used to detect two markers with the same or different expression patterns (subcellular

localizations) with different colors, this is a very tedious method.³¹⁻³³

In this study, we established a novel method to reduce the limitations of the dual-staining system. In theory, each IHC marker has its own subcellular localization and expression pattern, allowing investigators to detect the expression patterns of two markers with different subcellular localizations within a single immunostaining procedure. Based on this hypothesis, we developed a simple dual-marker approach

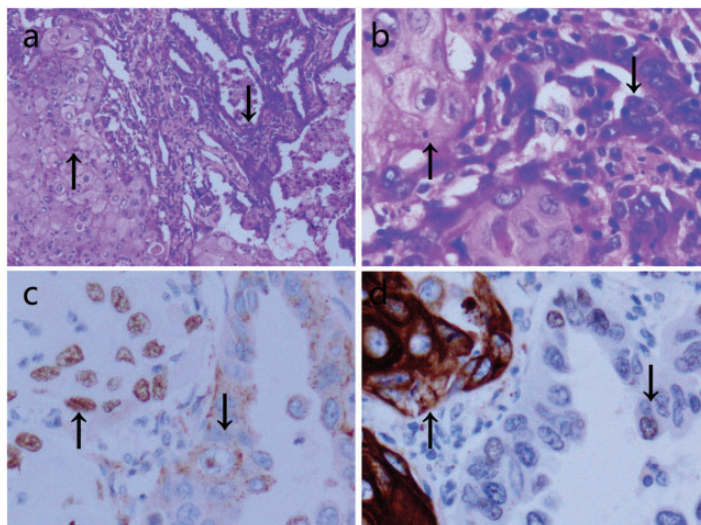


Figure 5. p40 and napsin A, and CK5/6 and TTF1 dual-marker staining in lung adenosquamous carcinoma (↑: SqCC cells; ↓: ADC cells). (a and b) Lung adenosquamous carcinoma with hematoxylin-eosin staining. (c) SqCC cells showed positively stained nuclei and ADC cells showed positively stained cytoplasm in p40/napsin A dual-stained cells. (d) SqCC cells showed positively stained cytoplasm and ADC cells showed positively stained nuclei in CK5/6/TTF1 dual-stained cells. a, 100×; b–d, 400×. SqCC, squamous cell carcinoma; ADC, adenocarcinoma; CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor I.

Table 3. Sensitivity, specificity, and positive and negative predictive values of dual- and single-marker staining for diagnosing squamous cell carcinoma and adenocarcinoma.

Dual- or single-marker staining	Sensitivity	Specificity	PPV	NPV	Accuracy	P-value
p40 and napsin A in SqCC	100%	100%	100%	100%	100%	<0.001
p40 in SqCC	100%	100%	100%	100%	100%	
CK5/6 and TTF1 in SqCC	100%	100%	100%	100%	100%	<0.001
CK5/6 in SqCC	100%	100%	100%	100%	100%	
p40 and napsin A in ADC	87.50%	100%	100%	86.67%	93.10%	<0.001
Napsin A in ADC	87.50%	100%	100%	86.67%	93.10%	
CK5/6 and TTF1 in ADC	94.12%	100%	100%	86.67%	93.10%	<0.001
TTF1 in ADC	94.12%	100%	100%	86.67%	93.10%	

PPV, positive predictive value; NPV, negative predictive value; SqCC: squamous cell carcinoma; ADC: adenocarcinoma; CK5/6, cytokeratin 5/6; TTF1, thyroid transcription factor I.

for detecting two markers in a single section using one staining procedure.

p40, napsin A, CK5/6, and TTF1 are reliable markers for the sub-classification of NSCLC.³⁴ p40 and CK5/6 are specific markers of lung SqCC, whereas TTF1 and

napsin A are specific markers of lung ADC. p40 and TTF1 are restricted to the nucleus, whereas CK5/6 and napsin A are restricted to the cytoplasm. Using two pairs of markers (p40 and napsin A, and CK5/6 and TTF1) allowed us to detect both

p40 and napsin A expression, as well as CK5/6 and TTF1 expression simultaneously, and thus to distinguish between SqCC and ADC.

In this study, we stained tissue sections from 58 cases of lung cancer by both dual- and single-marker immunostaining approaches using p40, CK5/6, TTF-1, and napsin A antibodies. Wilcoxon's test showed no difference in the expression of all markers detected by dual- and single-marker staining. Furthermore, there was no background or cross-staining of sections using the dual-staining method. Tumor cells occasionally showed heterogeneous expression in IHC but this did not affect the interpretation of the results and we therefore considered this to be acceptable. In addition, we reduced the influence of heterogeneity by using moderate staining as the cutoff for judging positive cells, and only cells with an IHC staining signal equal to or greater than moderate were defined as positive cells in the present study.

Although dual-marker staining can only be used to detect two molecular targets with different expression patterns, it has many advantages compared with the single-marker staining approach. The greatest advantage of the dual-staining approach is that only one procedure, as well as one enzymatic and one chromogenic reaction, is needed to detect the expression of two markers stained with the same color (i.e., brownish-red) in a single section, using a conventional IHC kit. Furthermore, the antibodies used can be from identical or different species (i.e., rabbit and/or mouse), the enzyme can be the same (i.e., HRP or alkaline phosphatase), and the chromogenic substrate can also be the same (i.e., DAB, Perma Blue, 3-amino-9-ethylcarbazole, or Vulcan Fast Red). This approach is thus both time-saving and economical. Another advantage of the dual-marker immunostaining approach is that the expression of p40 and napsin A, and of CK5/6 and

TTF-1 can be interpreted simultaneously based on the different localization patterns of the two markers. Compared with the single-marker staining approach, there was no background staining in sections stained for two markers. To the best of our knowledge, this is the first study to use dual-marker immunostaining to differentiate between sub-types of NSCLC.

This study had some limitations. Notably, this approach is not useful for two markers with similar localization patterns. Furthermore, this method relies on the high specificity of antibodies to avoid nonspecific or cross-reaction staining.

In this proof-of-concept study, we used p40 and napsin A, and CK5/6 and TTF1 antibodies to demonstrate the suitability of a novel approach for detecting two markers in single sections of well- or moderately differentiated tumors. In summary, the dual-marker staining approach is simple and economical and allows two markers to be stained simultaneously in a single section. This method is suitable for differentiating between SqCC and ADC in sections prepared from small biopsy specimens of NSCLC.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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ORCID iD

Jun Yang  <https://orcid.org/0000-0002-4965-2929>

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