

RESEARCH ARTICLE

Roles of Immunohistochemical Staining in Diagnosing Pulmonary Squamous Cell Carcinoma

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Abstract

Background: Differentiating morphologic features based on hematoxylin-eosin (HE) staining is the most common method to classify pathological subtypes of non-small-cell lung cancer (NSCLC). However, its accuracy and inter-observer reproducibility in pathological diagnosis of poorly differentiated NSCLC remained to be improved. **Materials and Methods:** We attempted to explore the role of immunohistochemistry (IHC) staining in diagnosing pulmonary squamous cell carcinoma (SQCC) with poorly differentiated features by HE staining or with elevated serum adenocarcinoma-specific tumor markers (AD-TMs). We also compared the difference of epidermal growth factor receptor (EGFR) mutation rate between patients with confirmed SQCC and those with revised pathological subtype. Logistic regression analyses were used to test the association between different factors and diagnostic accuracy. **Results:** A total of 132 patients who met the eligible criteria and had adequate specimens for IHC confirmation were included. Pathological revised cases in poor differentiated subgroup, biopsy samples and high-level AD-TMs cases were more than those with high/moderate differentiation, surgical specimens and normal-level AD-TMs. Moreover, biopsy sample was a significant factor decreasing diagnostic accuracy of pathological subtype (OR, 4.037; 95% CI 1.446-11.267, $p=0.008$). Additionally, EGFR mutation rate was higher in patients with pathological diagnostic changes than those with confirmed SQCC (16.7% vs 4.4%, $p=0.157$). **Conclusions:** Diagnosis based on HE staining only might cause pathological misinterpretation in NSCLC patients with poor differentiation or high-level AD-TMs, especially those with biopsy samples. HE staining and IHC should be combined as pathological diagnostic standard. The occurrence of EGFR mutations in pulmonary SQCC might be overestimated.

Keywords: Non-small-cell lung cancer - pulmonary squamous cell carcinoma - epidermal growth factor receptor

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Introduction

Lung cancer is the leading cause of cancer death with the rapidest growth of morbidity and mortality all over the world during the past 50 years (Ferlay et al., 2007; Ferlay et al., 2010). In China, lung cancer also tops the list of mortality in all kinds of cancer among both male and female patients (Guo et al., 2012). The World Health Organization (WHO) divides lung cancer into two broad categories based on its biology, therapy, and prognosis: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which account for 15%~25% and 70%~85% of patients respectively (Beasley et al., 2005). Histologically, NSCLC includes adenocarcinoma (ADC), squamous cell carcinoma (SQCC), adenosquamous carcinoma (ADSQC), large-cell carcinoma (LCC) and other cell types. ADC and SQCC are major types of

NSCLC occurring in approximately 50% and 30% of cases (Ginsberg et al., 2007; Ettinger et al., 2010).

Previously, the histologic subtyping of advanced NSCLC was not clinically or therapeutically important because of a lack of differential treatment options for NSCLC subtypes (Goldstraw et al., 1993; Thomas et al., 1993). However, the differential activity or limited indications of newer agents called for a distinction between SQCC and ADC of the lung. For instance, several clinical trials showed superior efficacy of pemetrexed in nonsquamous NSCLC, while SQCC obtained more benefit from gemcitabine (Scagliotti et al., 2008; Ciuleanu et al., 2009; Syrigos et al., 2010; Paz-Ares et al., 2012). The occurrence rate of epidermal growth factor receptor (EGFR) mutation was higher in ADC than SQCC (Park et al., 2009; Leighl, 2012) which caused ADC patients had better outcomes using EGFR tyrosine kinase inhibitors

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(EGFR-TKIs) (Mok et al., 2009; Maemondo et al., 2010; Mitsudomi et al., 2010; Shukuya et al., 2011; Zhou et al., 2011; Han et al., 2012; Rosell et al., 2012). In addition, prior studies revealed increased risk of serious hemorrhage with bevacizumab in SQCC patients (Sandler et al., 2006; Cohen et al., 2007; Hapani et al., 2010). Therefore, it had highlighted the importance of specific pathological NSCLC subtyping before treatment.

Although the necessity of histologic subtyping had been increased, hematoxylin-eosin (HE) staining showed lower accuracy and interobserver reproducibility in diagnosis of poorly differentiated NSCLC, especially in small biopsy samples (Cataluna et al., 1996; Feinstein et al., 1970; Grilley-Olson et al., 2009; Travis et al., 2010; Sigel et al., 2011). Hence, many auxiliary methods, such as immunohistochemistry (IHC), gene expression profiling and micro RNA, had been studied to assist with this dilemma and IHC was the most convenient and practical solution (Johansson, 2004; Camilo et al., 2006; Kargi et al., 2007; Rossi et al., 2009). Several studies had shown that ADC and SQCC had virtually non-overlapping coexpression profiles of thyroid transcription factor 1 (TTF-1) and P63—the master transcriptional regulators in glandular and squamous cell lineages, respectively (Loo et al., 2010; Nicholson et al., 2010). Another research indicated that the best single marker for ADC and SQCC, based on sensitivity and specificity, would be Napsin A and P63, respectively (Noh et al., 2012). The latest studies showed an isoform of P63, P40, suggested to be equivalent to P63 in sensitivity for SQCC, but it is markedly superior to P63 in specificity (Bishop et al., 2012; Nobre et al., 2013). The combination of these IHC markers might be optimal.

Besides above IHC markers, there were many serum tumor markers (TMs) which indicated pulmonary ADC, such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), CA153 and CA199 (Diez et al., 1991; Diez et al., 1994; Picardo et al., 1994; Mitsuhashi et al., 1995; Picardo et al., 1996; Alatas et al., 2001). Nevertheless, some SQCC cases diagnosed by HE staining showed elevated expression of these adenocarcinoma-specific tumor markers (AD-TMs). It was possible that a potential misinterpretation considering ADC with high-level AD-TMs as SQCC based on HE staining might be existed.

In this study, we performed IHC testing using TTF-1, p63, Napsin A and p40 to verify the pathological type of pulmonary SQCC with poor differentiation or high-level AD-TMs diagnosed previously by HE staining. We also collected genetic data of these patients with known EGFR mutation status to compare the difference of EGFR gene mutations between patients with confirmed pathological diagnosis and those with revised pathological subtype.

Materials and Methods

Patients

2958 patients diagnosed with pulmonary SQCC who were treated at the Cancer Center of Sun Yat-Sen University (Guangzhou, China) during January 2007 to October 2012 were screened in the study. Only 132 patients with poor differentiation or high-level AD-TMs

diagnosed previously by HE staining were enrolled onto the research ultimately if they had enough tissue sample. All of the records such as clinical and pathological features were collected with no patients having received any treatment. The clinicopathological features of the patients included age, gender, smoking history, UICC stage, degree of differentiation, level of serum AD-TMs, type of tissue sample and EGFR mutation status. All patients were restaged by seventh edition of UICC Staging System for NSCLC. Categories of each characteristic were divided as following: for age, patients more than 55 years old were considered as the older group. Smoking history was noted as yes or no. High AD-TMs level was defined, if serum CEA, CA125, CA153 or CA199 ≥ 5 ng/ml, 35 U/ml, 25 U/ml, 35 U/ml, respectively. Tissue samples were collected from surgery or biopsy. EGFR exon 19 deletions or exon 21 alterations were considered as EGFR mutants by fluorescent quantitative polymerase chain reaction (PCR). The study was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (Guangzhou, China). All the patients had provided written informed consent before samples were collected.

IHC analyses

IHC staining was performed using mouse monoclonal anti-human antibodies for reconfirming the pathological diagnosis, including P63 (DAKO, 1:100), TTF-1 (DAKO, 1:100), P40 (DAKO, 1:100) and Napsin A (DAKO, 1:50). Sections with 5- μ m-thick were cut from the formalin-fixed, paraffin-embedded (FFPE) tumor block and then routinely deparaffinized and rehydrated. For antigen retrieval, slides were heated in a microwave oven for 30 minutes in citrate buffer solution (pH=7.4) and cooled slowly at room temperature for 20 minutes. After blocking the activity of endogenous peroxidase with 3% hydrogen peroxide for 8 minutes, the sections were treated with primary antibodies and incubated for 12 hours. Subsequently, the slides were rinsed in PBS three times and incubated in biotinylated secondary antibodies. After incubation, slides were washed again with PBS and then visualized using diaminobenzidine. Finally, Mayer's hematoxylin was used to counterstain the sections, which were then dehydrated and mounted.

P40 and P63 were the IHC markers which indicated SQCC, while Napsin A and TTF-1 supported ADC. As long as one of SQCC markers was positive and ADC markers were double-negative, the profile was considered as SQCC. Similarly, only if one of ADC markers was positive and SQCC markers were double-negative, the profile was further confirmed as ADC. If the markers of SQCC and ADC were co-expressed, the case was regarded as ADSQC. Besides, the quadruple-negative profile was diagnosed as NSCLC-not otherwise specified (NSCLC-NOS). The reassessment process was shown in Figure 1.

Two pathologists who don't know the information of the patients were asked to independently assess the expression. Histologic (H) scores were assigned by multiplying the percentage of stained cells (0, absent; 1, <25%; 2, 25%-50%; 3, 51%-75% and 4, >75%) by an intensity score (0, absent; 1, weak; 2, moderate; and 3, strong). After analyzing the distribution of H scores,

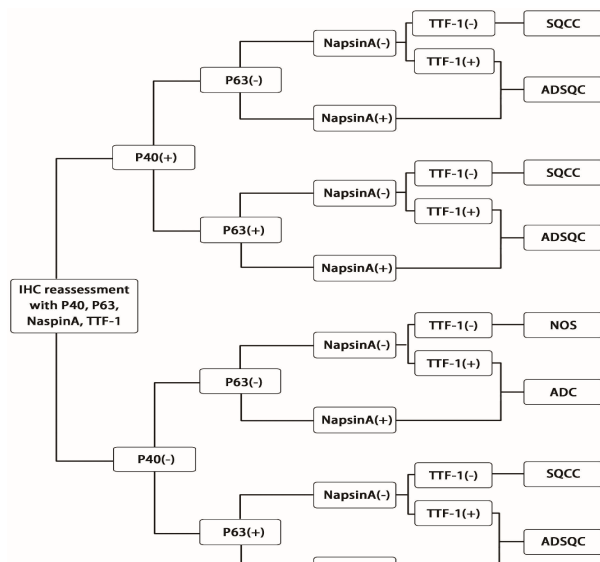


Figure 1. IHC Reassessment Chart for Pathological Diagnosis with P40, P63, Napsin A and TTF-1. Abbreviation: IHC=immunohistochemistry; TTF-1=thyroid transcription factor-1; SQCC=squamous cell carcinoma; ADC=adenocarcinoma; ADSQC=adenosquamous carcinoma; NOS=not otherwise specified

Table 1. Baseline Data of Enrolled 132 Pulmonary SQCC Patients

Characteristics	Confirmed pathological diagnosis	Revised pathological diagnosis
Age [years]		
≤55	36	6
>55	76	14
Gender		
Male	100	18
Female	12	2
Smoking status		
Yes	94	15
No	18	5
UICC stage		
I-III A	78	12
IIIB-IV	34	8
Degree of differentiation		
High & moderate	17	2
Low	95	18
Level of AD-TMs		
Normal	63	8
High	49	12
Type of tissue sample		
Biopsy	24	11
Surgery	88	9
EGFR mutation status		
Mutant	4	3
Wild-type	87	15
Unknown	21	2

*Abbreviations: AD-TMs=adenocarcinoma-specific tumor markers; SQCC=squamous cell carcinoma

we set the results of IHC for positivity if the H-score ≥ 4 (Noh et al., 2012).

Diagnostic standard of pathological type

The combination of HE staining and IHC reassessment was the diagnostic standard of pathological subtype of

NSCLC. HE staining was the main methods to access the profile diagnosed as NSCLC-NOS. The final diagnosis will be discussed by two pathologists to reach consensus in case of disagreement.

Statistical analysis

SPSS 17.0 software was used for the statistical analysis. Continuous variables were divided into different categories as mentioned above. All the cut-off values were obtained by X-tile software (Version 3.6.1, Yale University, New Haven, CT), taking clinical expertise into consideration. The confirming rate of pathological diagnosis was the number of cases confirmed to be SQCC by the diagnostic standard divided by the number of cases diagnosed only by HE staining. Univariate and multivariate logistic regression analysis were used to test the association between the different factors and diagnostic accuracy. Results were reported with odds ratio (OR), corresponding 95% confidence intervals (CI). A two sided p-value < 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 132 patients with poor differentiation or high-level AD-TMs diagnosed as SQCC by HE staining previously were included in this study. The first cohort consisted of 61 patients with high-level AD-TMs who were divided into four subgroups according to the degree of differentiation and type of sample. The second cohort included 71 patients with normal-level AD-TMs and poor differentiation classified as two subgroups based on the type of sample (Figure 2). The median age of all the enrolled patients was 60 years (range: 30-90 years). Most of the patients were male (n=118, 89.4%) and smokers (n=109, 82.6%). More than half of the patients were in UICC III or IV stage (n=78, 59.1%). The majority of the tissue sample was from surgery (n=97, 73.5%). Most of the patients had known EGFR mutation status (n=109, 82.6%). The basic clinical characteristics of the patients were presented in Table 1.

Pathological reassessment based on the combination of HE staining and IHC reassessment

132 patients in the two cohorts had adequate specimens for IHC reassessment. The pathological subtype diagnosed as SQCC was 112 cases (Figure 3A), while the revised diagnosis were 20 cases with ADC (n=18) (Figure 3B), ADSQC (n=1) (Figure 3C) and LCC (n=1) (not displayed) respectively. The pathological confirming rate of all cases was 84.8%.

In the first cohort, the confirming rate in poor differentiated subgroup (84.0%, 64.7%) was lower than high/moderate differentiated subgroup (92.3%, 83.3%) whatever the specimen was collected from surgery or biopsy. In addition, biopsy samples had lower confirming rate (83.3%, 64.7%) than surgical samples (92.3%, 84.0%) both in high/moderate and poor differentiated subgroups, while the result was similar in the second cohort (confirming rate of biopsy samples and surgical samples=66.7% and 93.2%). Besides, patients with high-

Table 2. Pathological Reassessment of 61 Pulmonary SQCC Patients with Gigh-level AD-TMs

Pathological reassessment	Samples from surgery (n=38)		Samples from biopsy (n=23)	
	High/moderate differentiation (n=13)	Poor differentiation (n=25)	High/moderate differentiation (n=6)	Poor differentiation (n=17)
SQCC	12	21	5	11
ADC	1	4	0	6
ADSQC	0	0	1	0
Confirming rate	92.30%	84.00%	83.30%	64.70%

*Abbreviations: AD-TMs=adenocarcinoma-specific tumor markers; SQCC=squamous cell carcinoma; ADC=adenocarcinoma; ADSQC=adenosquamous carcinoma

Table 3. Pathological Reassessment of 71 Pulmonary SQCC Patients with Normal-level AD-TMs and Poor Differentiation

Pathological reassessment	Samples from surgery (n=59)	Samples from biopsy (n=12)
SQCC	55	8
ADC	4	3
LCC	0	1
Confirming rate	93.20%	66.70%

*Abbreviations: AD-TMs=adenocarcinoma-specific tumor markers; SQCC=squamous cell carcinoma; ADC=adenocarcinoma; LCC=large-cell carcinoma

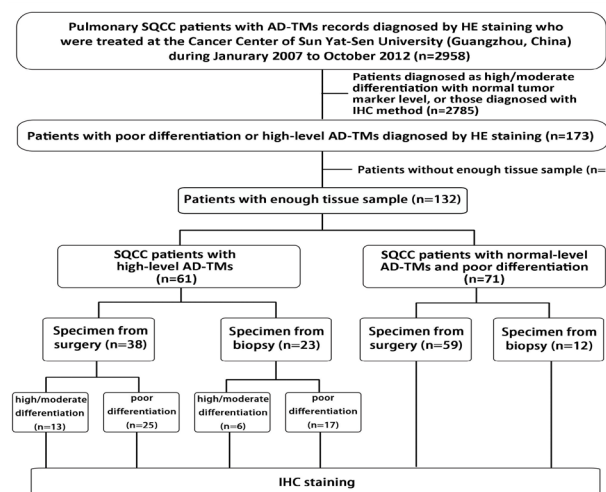


Figure 2. Flowing Chart of the Enrollment. Abbreviation: AD-TMs=adenocarcinoma-specific tumor markers; HE=hematoxylin-eosin; SQCC=squamous cell carcinoma; IHC=immunohistochemistry

level AD-TMs had lower confirming rate (84.0%, 64.7%) than those with normal-level AD-TMs (93.2%, 66.7%) in poor differentiated cases whatever the specimen was from surgery or biopsy (Table 2, 3).

Univariate logistic regression analysis showed that biopsy sample was a significant factor which lowered diagnostic accuracy of pathological subtype (OR, 4.481; $p=0.002$), while the poor differentiated sample (OR, 1.611; $p=0.737$) or cases with high-level AD-TMs (OR, 1.929; $p=0.179$) were not. Additionally, multivariate logistic regression analysis testing the association between the different factors and pathological diagnostic accuracy showed the similar results (biopsy sample: OR, 4.037 95% CI 1.446-11.267, $p=0.008$; poor differentiated sample: OR, 2.478 95% CI 0.464-13.226, $p=0.288$; cases with

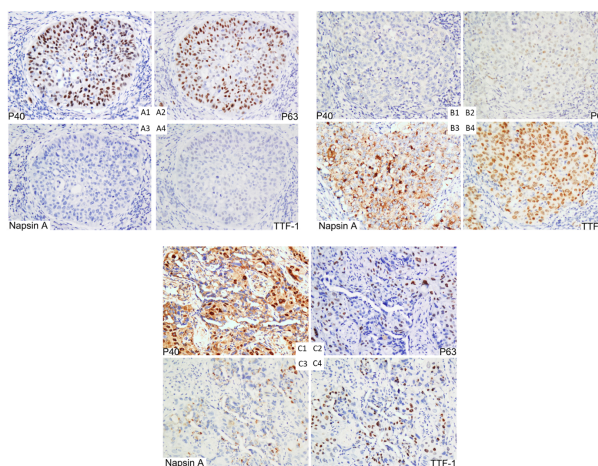


Figure 3. IHC Reassessment Of Pathological Diagnosis In Patients to be SQCC (A), ADC (B), ADSQC (C) (20x). (A1) P40 positive with deep-brown stained nuclei; (A2) P63 positive with brown stained nuclei; (A3) negative Napsin A without stained; (A4) negative TTF-1 sample without stained. (B1) P40 negative without stained; (B2) P63 negative without stained; (B3) positive Napsin A with deep-brown stained cytoplasm; (B4) positive TTF-1 sample with brown stained nuclei. (C1) positive P40 with brown stained nuclei; (C2) P63 positive with brown stained nuclei; (C3) positive Napsin A with slight-brown stained cytoplasm; (C4) positive TTF-1 sample with brown stained nuclei. Abbreviation: IHC=immunohistochemistry; TTF-1=thyroid transcription factor-1; SQCC=squamous cell carcinoma; ADC=adenocarcinoma; ADSQC=adenosquamous carcinoma

high-level AD-TMs: OR, 1.766 95% CI 0.597-5.233, $p=0.304$) (Table 4).

Pathological reassessment and EGFR mutation

There were seven cases tested as EGFR mutants according to data of 109 patients with known EGFR mutation status through all the 132 included cases. Four EGFR mutants were in patients with revised pathological subtype and three mutants were in patients with confirmed pathological subtype. EGFR mutation rate was higher in patients with pathological diagnostic changes than those with confirmed pathological subtype after pathological reassessment (16.7%, 3/18 vs 4.4% 4/91, $p=0.157$).

The pathological confirming rate in EGFR mutants was lower than EGFR wild-type patients (57.1%, 4/7 vs 85.3% 87/102). However, multivariate logistic regression analysis based on data of 109 patients with known EGFR mutation status showed that only biopsy sample was a significant factor which lowered diagnostic accuracy

Table 4. The Association between the different Factors and Diagnostic Accuracy in Univariate and Multivariate Logistic Regression Analysis

Parameter	Confirmed pathological diagnosis	Revised pathological diagnosis	Univariate analysis		Multivariate analysis		
			OR	p value	OR	95% CI	p value
Degree of differentiation							
Low	95	18	1.611	0.737	2.478	0.464-13.226	0.288
High & moderate	17	2	1		1	(-)	
Level of AD-TMs							
High	49	12	1.929	0.179	1.766	0.597-5.223	0.304
Normal	63	8	1		1	(-)	
Type of tissue sample							
Biopsy	24	11	4.481	0.002	4.037	1.446-11.267	0.008
Surgery	88	9	1		1	(-)	

*Abbreviations: AD-TMs = adenocarcinoma-specific tumor markers; OR = odd ratio; CI = confidence interval

of pathological subtype (OR, 4.540 95% CI 1.466-14.062, $p=0.009$) if taking EGFR mutation status into consideration.

Discussion

Evidence demonstrated that accurate pathological subtype classification was pivotal in selecting optimal chemotherapy (Scagliotti et al., 2008; Ciuleanu et al., 2009; Syrigos et al., 2010; Paz-Ares et al., 2012) and/or target therapy (Sandler et al., 2006; Cohen et al., 2007; Mok et al., 2009; Park et al., 2009; Hapani et al., 2010; Maemondo et al., 2010; Mitsudomi et al., 2010; Shukuya et al., 2011; Zhou et al., 2011; Han et al., 2012; Leighl, 2012; Rosell et al., 2012). Previous study reported that the agreement of pathological diagnosis of NSCLC based on HE-staining was correlated with differentiated degree of carcinoma, pathologist experience, pulmonary pathology expertise, pathologist diagnostic confidence and slide quality (Grilley-Olson et al., 2013). Another literature showed that 10% of SQCCs, 14% of ADCs and 50% of LCCs were misclassified on bronchial biopsies (Cataluna et al., 1996). We also found some lung SQCC cases diagnosed by HE staining showed elevated expression of AD-TMs indicating ADC in clinical practice. Based on above all, we considered that some pulmonary SQCCs with poor differentiation, high-level AD-TMs or biopsy specimens diagnosed previously by HE staining might be doubtful in terms of pathological subtype.

Advances in IHC had brought great benefits to the field of pathological diagnosis of NSCLC. The latest research revealed that IHC testing was a significant factor associated with attaining a specific pathological diagnosis (Sulpher et al., 2013). However, too many articles tried to identify the most sensitive and specific IHC markers for pathological diagnosis (Loo et al., 2010; Nicholson et al., 2010; Noh et al., 2012; Bishop et al., 2012; Nobre et al., 2013) while few studies were focused on the practical effects reducing misinterpretation of pathological subtype by IHC tests in combination of the most important and reliable markers. Therefore, we use TTF-1, p63, Napsin A and p40 to verify the pathological type of pulmonary SQCC with poor differentiation or high-level AD-TMs diagnosed previously by HE staining.

Our results showed the occurrence of pathological

subtype uncertainty in poor differentiated subgroup, biopsy samples and high-level AD-TMs cases was more than those with high/moderate differentiation, surgical specimens and normal-level AD-TMs. Moreover, biopsy sample was the significant factor decreasing diagnostic accuracy of pathological subtype. These results admit of following interpretations. Firstly, poor differentiated cell morphological features both in SQCC and ADC were immature and similar without obvious structures, such as intercellular bridge or acinus, to estimate, which might cause misinterpretation. Secondly, the specimen of biopsy was partial that could not represent general view of tumor. For example, if we only obtained limited tissue of ADSQC to test, either SQCC or ADC might be diagnosed. Other studies also reported that mixed cell types were seen in a small number of lung carcinoma cases (Rogli et al., 1985). Heterogeneity was identified by extensive sampling of the entire tumor and was seldom recognized in biopsy specimens (Rogli et al., 1985). Additionally, high-level AD-TMs were indicators of pulmonary ADC (Diez et al., 1991; Diez et al., 1994; Picardo et al., 1994; Mitsuhashi et al., 1995; Picardo et al., 1996; Alatas et al., 2001), which made the diagnosis of SQCC more questionable.

Another result in our study revealed that EGFR mutation rate was numerically higher in patients with pathological diagnostic changes than those with confirmed pathological subtype as SQCC after pathological reassessment. Previous studies reported that EGFR mutation rate was 12%-13.3% in Asian pulmonary SQCC (Tanaka et al., 2010; Hata et al., 2013), which was similar to the result of a meta-analysis that 10% of Chinese pulmonary SQCC had EGFR mutation (Wu et al., 2007). However, updated data of Catalogue of Somatic Mutations in Cancer (COSMIC) showed that EGFR mutation rate was only 5.1% in pulmonary SQCC (Forbes et al., 2011). Another research even suggested that EGFR mutation do not occur in pure pulmonary SQCC. Occasional detection of these mutations in samples diagnosed as SQCC is due to challenges with the diagnosis of ADSQC and ADC, which can be largely resolved by pathological reassessment by IHC biomarkers (Rekhtman et al., 2012). Based on previous results above and our result in terms of EGFR mutation rate of pulmonary SQCC (4.4%), we considered that the occurrence of EGFR mutations in pulmonary SQCC might be overestimated because of confounding

pathological subtype in ADC.

There are several major limitations of our study. First, this is a retrospective study. All the data were collected retrospectively. Second, small sample size might result in no statistically significant association between degree of differentiation/levels of AD-TMs and pathological diagnostic accuracy. Besides, EGFR mutation status was unknown in a small portion of included patients which might affect the result in the difference of EGFR gene mutations between patients with confirmed pathological diagnosis and those with revised pathological subtype. Nonetheless, regardless of above limitations, this study showed that HE staining was restricted in diagnosis of pathological subtype of NSCLC. Patients with high-level AD-TMs or diagnosed as poor differentiated pulmonary SQCC by HE staining might be recommended to have IHC testing to confirm pathological diagnosis, especially those with biopsy samples. Nevertheless, because all the IHC markers could not avoid false positive or negative diagnosis, the combination of HE staining and IHC should be diagnostic standard of pathological subtype of NSCLC.

In conclusion, it is vital to confirm pathological subtype of NSCLC which is associated with the treatment and survival of patients. HE staining might cause pathological doubtful interpretation in pulmonary SQCC patients with poor differentiation or high-level AD-TMs, especially those with biopsy samples, while IHC tests can enhance diagnostic accuracy of pathological subtype. In clinical practice, HE staining and IHC should be combined as diagnostic standard of pathological subtype of NSCLC.

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