

# Advances in the Application of Immunohistochemical Markers in the Subtyping of Non-Small Cell Lung Cancer

Bohui ZHAN, Cong GUO\*

Department of Pathology, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen 518107, Guangdong, China

\*Corresponding author, [guocong@sysush.com](mailto:guocong@sysush.com)

## Abstract

Lung cancer is the most common malignancy worldwide in terms of both incidence and mortality, with non-small cell lung cancer (NSCLC) accounting for approximately 80%–85% of all cases. As most patients are diagnosed at an advanced stage with limited biopsy material, hematoxylin and eosin (H&E) staining alone is often insufficient for precise histotyping. Immunohistochemistry (IHC), by detecting specific molecular markers, can significantly improve the accuracy and reproducibility of NSCLC subtyping. This review systematically summarizes the diagnostic utility and expression characteristics of commonly used IHC markers—including TTF-1, Napsin A, Ki-67, CK7, CK5/6, p63, and p40—in the differential diagnosis between lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC), and discusses the clinical strategies of multi-marker combinatorial testing. Current evidence indicates that the four-antibody panel combining TTF-1/Napsin A (for adenocarcinoma) and p40/CK5/6 (for squamous cell carcinoma) effectively distinguishes ADC from SCC, and is of great significance in guiding individualized therapy.

**Keywords** Immunohistochemistry; Non-small cell lung cancer; Lung adenocarcinoma; Squamous cell carcinoma; Differential diagnosis

## 1 Introduction

Lung cancer ranks first among all cancers in both incidence and mortality worldwide<sup>[1–2]</sup>. Non-small cell lung cancer (NSCLC) constitutes approximately 80%–85% of all lung cancer cases, with major histologic subtypes including adenocarcinoma (ADC), squamous cell carcinoma (SCC), and adenosquamous carcinoma (ASC)<sup>[3]</sup>. Accurate discrimination between ADC and SCC is of critical clinical importance, as it directly influences treatment selection and patient prognosis. The diagnostic criteria for lung cancer have undergone several significant updates. In the early World Health Organization (WHO) classification systems, the diagnosis relied primarily on H&E staining. In 1999, immunohistochemistry (IHC) was first incorporated into the WHO diagnostic criteria. However, in the 2004 revision, its application remained limited to the differential diagnosis of uncommon entities such as large cell neuroendocrine carcinoma, sarcomatoid carcinoma, and malignant mesothelioma. In 2015, the WHO, in collaboration with the International Association for the Study of Lung Cancer (IASLC), issued an updated classification that emphasized the necessity of precise histologic subtyping of NSCLC, the value of molecular markers in guiding targeted therapy, the standardization of diagnostic terminology for small biopsy specimens, and the pivotal role of IHC in accurate subtyping<sup>[3]</sup>. Since approximately two-thirds of lung cancer patients

are diagnosed at an advanced stage, specimens are often limited to small biopsies or cytological materials, rendering H&E staining alone diagnostically insufficient. The application of IHC can significantly improve diagnostic accuracy and reduce the frequency of the ambiguous diagnosis “non-small cell lung cancer-not otherwise specified” (NSCLC-NOS). Currently, IHC markers including thyroid transcription factor-1 (TTF-1), Napsin A, Ki-67, and p53 have become routine tools in the pathological diagnosis and differential diagnosis of lung cancer.

## 2 Commonly Used Immunohistochemical Markers in Non-Small Cell Lung Cancer

### 2.1 TTF-1

Thyroid transcription factor-1 (TTF-1, also known as NKX2-1) is a homeodomain-containing nuclear protein transcription factor encoded by a gene located on chromosome 14q13.3. This factor exhibits dual functions in lung adenocarcinoma: it can act as a lineage-survival oncogene subject to specific amplification<sup>[4]</sup>, while also mediating tumor-suppressive effects through downstream target genes. At the molecular level, TTF-1 regulates the differentiation and maturation of pulmonary epithelial cells through its DNA-binding domain, playing a central role in alveolar morphogenesis and functional maintenance. Specifically, it regulates the expression of lung-specific genes such as surfactant proteins (SP-A, SP-B, SP-C) and Clara cell secretory protein (CCSP), while also participating in the regulatory network of lung branching morphogenesis and thyroid follicular formation. Owing to these pleiotropic functions, TTF-1 has become an important diagnostic marker and a potential therapeutic target for lung adenocarcinoma. In terms of expression dynamics, TTF-1 is expressed at low levels during the embryonic stage, gradually increases with lung maturation, and ultimately localizes predominantly to the nuclei of alveolar cells. Of note, this protein has relatively low chemical stability and is susceptible to degradation under suboptimal pre-analytical conditions, such as delayed fixation or prolonged fixation<sup>[5]</sup>, imposing specific requirements on detection protocols.

### 2.2 Napsin A

Napsin A is an aspartic protease encoded by a gene on chromosome 19q13.3. Its precursor is a single-chain protein of 420 amino acids with a molecular weight of 45 kDa, and its primary structure consists of a signal peptide, a pro-peptide region, a catalytic domain, and a C-terminal region. Giordano et al.<sup>[6]</sup> demonstrated that this enzyme begins to express in fetal pulmonary glandular structures as early as the 12th week of gestation, with expression levels declining by the 30th week to approximate the adult pulmonary pattern; under pathological conditions (such as inflammation and pulmonary hypoplasia), its expression can be reactively upregulated, possibly as a compensatory response. Regarding tissue distribution, Napsin A exhibits notable specificity: in the kidney, it is predominantly localized to proximal tubular epithelial cells, participating in lysosomal protein degradation; in the lung, it is selectively expressed in type II alveolar epithelial cells, playing a critical role in the biosynthesis of pulmonary surfactant. As a diagnostic marker, Napsin A is significantly overexpressed in primary lung adenocarcinoma and atypical adenomatous hyperplasia, while it is almost absent or expressed at very low levels in small cell lung carcinoma and squamous cell carcinoma, demonstrating high specificity for differential diagnosis.

### 2.3 Ki-67

Ki-67 is a nuclear proliferation-associated antigen expressed in all active phases of the cell cycle (G1, S, G2, and M), but completely absent in quiescent (G0) cells, making it a reliable marker of cellular proliferative activity. The Ki-67 labeling index (percentage of positive cells) determined by IHC can objectively reflect the proliferation status of tumor tissues. Studies have shown that the Ki-67 labeling index is positively correlated with the degree of tumor malignancy; higher expression levels generally indicate stronger

proliferative activity and greater invasiveness<sup>[7]</sup>. In NSCLC, high Ki-67 expression is generally associated with poor clinical outcomes and an elevated risk of tumor recurrence, and is closely correlated with patient prognosis<sup>[8]</sup>. These findings support the utility of Ki-67 as a prognostic biomarker in routine clinical practice, particularly for identifying patients at high risk of recurrence who may benefit from more intensive adjuvant therapy.

## 2.4 CK7

Cytokeratins (CK) are major components of the epithelial cytoskeleton, playing a key role in maintaining epithelial integrity and continuity. Their expression is highly tissue-specific and closely associated with epithelial cell proliferation and differentiation. To date, over 20 CK subtypes have been identified in epithelial tissues<sup>[9]</sup>. CK7 is primarily expressed in the glandular epithelium and transitional epithelium of the lung and ovary, while it is typically negative in the epithelium of the colon, prostate, and gastrointestinal tract. As a diagnostic marker, CK7 demonstrates a positivity rate of nearly 100% in lung adenocarcinoma, indicating extremely high sensitivity; however, its specificity is suboptimal, as approximately 30%–60% of pulmonary SCCs may also express CK7<sup>[10]</sup>. Furthermore, adenocarcinomas arising from the breast, stomach, pancreas, ovary, and endometrium are also frequently CK7-positive. Therefore, in the diagnosis and differential diagnosis of lung adenocarcinoma, CK7 should be used in combination with more specific antibodies such as TTF-1 and Napsin A. The combination of CK7 with TTF-1 and Napsin A can effectively confirm pulmonary adenocarcinoma in most cases, while the addition of CK5/6 and p40 helps exclude squamous differentiation. Currently, studies on the association between CK7 expression and the prognosis or gene mutations of lung cancer remain insufficient, and its clinical value is still largely limited to the field of auxiliary diagnosis.

## 2.5 CK5/6

Cytokeratin 5/6 (CK5/6) belongs to the type II keratin heterodimer family and is encoded by the KRT5 and KRT6A/B/C genes. Its immunohistochemical localization is mainly in the basal cell layer of stratified squamous epithelium and the cytoplasm and cell membrane of metaplastic squamous epithelium. This marker has a sensitivity of approximately 73% for pulmonary SCC and is almost entirely negative in lung adenocarcinoma, with a specificity approaching 100%<sup>[11]</sup>, making it an effective indicator for distinguishing pulmonary SCC from ADC. The sensitivity of CK5/6 is higher in well-to-moderately differentiated SCC than in poorly differentiated variants, where expression may be reduced or lost. However, it should be noted that approximately 50%–70% of pleural epithelioid mesotheliomas may also show CK5/6 positivity; therefore, in practical differential diagnosis, it should be used in combination with antibodies such as Calretinin, WT-1, and BerEP4 for distinction. Compared with p40, CK5/6 is less specific for SCC, but it remains valuable as a complementary marker in the squamous panel, particularly in cases with equivocal p40 staining.

## 2.6 p63 and p40

p63 is a member of the p53 gene family and is expressed to varying degrees in cells with squamous differentiation potential, including myoepithelial cells, basal cells, and parabasal cells<sup>[12]</sup>. This marker shows a positivity rate exceeding 90% in pulmonary SCC, but is also positive in 10%–33% of lung adenocarcinomas, indicating reasonable sensitivity but insufficient specificity; moreover, p63 is expressed in various other tumors including oral squamous cell carcinoma, cutaneous squamous cell carcinoma, esophageal squamous cell carcinoma, and urothelial carcinoma, further limiting its discriminatory value. p40 specifically recognizes the p63 isoform lacking the N-terminal transactivation domain, which is more closely associated with squamous differentiation. Both markers show roughly equivalent sensitivity in pulmonary SCC; however, p40 exhibits a significantly lower positivity rate in lung adenocarcinoma, demonstrating

superior specificity. Consequently, p40 has largely replaced p63 as the preferred squamous marker in most diagnostic laboratories. Therefore, p40 has greater clinical utility than p63 in the immunohistochemical diagnosis of pulmonary SCC.

### 3 Multi-Marker Combinatorial Testing Strategies and Clinical Practice

Owing to the limitations in sensitivity, specificity, and histologic variability of single IHC markers, no single marker can ensure absolute diagnostic accuracy in NSCLC subtyping. International guidelines recommend the adoption of multi-marker combinatorial testing strategies<sup>[13]</sup>. A commonly used clinical panel comprises adenocarcinoma markers (TTF-1 + Napsin A) and squamous markers (p40 + CK5/6)—a four-antibody panel. TTF-1 and Napsin A are highly specific for lung adenocarcinoma, while p40 and CK5/6 are sensitive markers for squamous differentiation. The typical expression patterns are as follows: lung adenocarcinoma presents as TTF-1+/Napsin A+/p40-/CK5/6- (with rare CK5/6 weak+), while pulmonary SCC presents as TTF-1-/Napsin A-/p40+/CK5/6+. This four-antibody approach has been validated in large cohorts and is now considered the standard of care for NSCLC subtyping on small biopsies. Two special scenarios warrant particular attention: (1) In cases of TTF-1/p40 double positivity, adenocarcinoma is favored because p40 may show focal positivity in adenocarcinomas, whereas TTF-1 is essentially negative in SCC; (2) In TTF-1/p40 double-negative cases, adenocarcinoma cannot be ruled out directly because TTF-1 expression may be lost in 10%–20% of poorly differentiated adenocarcinomas; nor can SCC be excluded—here, the auxiliary value of Napsin A and CK5/6 becomes particularly critical. In such challenging cases, the incorporation of additional markers—including mucin stains (e.g., PAS-D) or other lineage-specific antibodies—may aid in reaching a definitive diagnosis. For NSCLC-NOS cases, supplementary testing of SOX2 (as an adjunct squamous marker) and CK7/CK20 expression profiles, along with driver gene mutation testing (EGFR, ALK, ROS1, etc.), may further improve diagnostic precision. The use of a well-validated IHC panel not only reduces interobserver variability but also ensures that patients receive appropriate therapy in a timely manner. Accurate pathological subtyping not only guides first-line chemotherapy in advanced NSCLC (e.g., pemetrexed is more effective in adenocarcinoma than in SCC), but also constitutes a prerequisite for initiating targeted therapy and immunotherapy<sup>[14]</sup>. Thus, IHC-based subtyping serves as the foundation upon which molecular testing and treatment decisions are built.

### 4 Conclusions

Immunohistochemistry has become a cornerstone for precise pathological subtyping of NSCLC. The combined use of adenocarcinoma markers (TTF-1 and Napsin A) and squamous markers (p40 and CK5/6) significantly improves diagnostic accuracy in distinguishing lung adenocarcinoma from SCC, reduces the proportion of NSCLC-NOS diagnoses, and provides reliable pathological evidence for individualized treatment. The standardized application of this four-antibody panel in routine diagnostic practice ensures consistent and reproducible results across different laboratories.

Looking forward, artificial intelligence-assisted digital pathology offers promise for standardizing IHC interpretation and reducing interobserver variability<sup>[15]</sup>. Emerging high-throughput technologies, such as single-cell RNA sequencing and spatial transcriptomics, may facilitate the discovery of subtype-specific markers to complement the existing panel. Ultimately, the integration of IHC-based histotyping with molecular testing and liquid biopsy is expected to establish a multidimensional precision diagnostic system, enabling holistic management of lung cancer patients.

In summary, IHC-based multi-marker combinatorial testing remains the cornerstone of NSCLC subtyping. Continued optimization of marker panels and incorporation of emerging technologies will further enhance diagnostic precision and improve clinical outcomes.

**To Cite This Article** Bohui ZHAN and Cong GUO (2026). Advances in the Application of Immunohistochemical Markers in the Subtyping of Non-Small Cell Lung Cancer. *Medical Research*, 8(3), 1–5. <https://doi.org/10.6913/mrhk.080301>

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