

Immunohistochemistry in Small Lung Biopsies: Diagnostic Pitfalls and Challenges with Limited Panels

Erik Polsdofer¹, Jian Jing^{1,*}

¹Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, CO 80048, USA

*Correspondence should be addressed to Jian Jing, Jian.jing@caunschutz.edu

Received date: October 03, 2024, **Accepted date:** October 26, 2024

Citation: Polsdofer E, Jing J. Immunohistochemistry in Small Lung Biopsies: Diagnostic Pitfalls and Challenges with Limited Panels. J Exp Pathol. 2024;5(1):61-72.

Copyright: © 2024 Polsdofer E, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

With the increasing need for targeted therapies in non-small cell lung carcinoma (NSCLC), preserving sufficient biopsy material has become essential. As most lung cancer cases are advanced or inoperable at the time of diagnosis, small biopsies, including cytology specimens, often serve as the only source of diagnostic material. Accurate subclassification of adenocarcinoma and squamous cell carcinoma in NSCLC using a limited panel including TTF1 and p63/p40 immunohistochemical (IHC) staining, as well as confirming small cell carcinoma using essential IHC markers, is critical for precise diagnosis, cost-effective treatment, and optimal patient care. This review highlights common challenges in applying IHC to small lung biopsies, focusing on diagnostic pitfalls from real-world cases, including adenocarcinoma, squamous cell carcinoma, and the use of IHC markers to support neuroendocrine carcinoma diagnosis. Together, these entities account for over 94% of all lung cancers, according to 2024 cancer statistics. Relevant clinical information, epidemiology, and molecular mechanisms will also be discussed for comprehensive practice considerations. The goal is to raise awareness among practicing pathologists about the critical role of IHC applications in small lung biopsies, with the aim of improving patient care by minimizing unnecessary IHC use while ensuring accurate diagnoses.

Keywords: Lung biopsy, Adenocarcinoma, Epidemiology, Immunohistochemistry, Cell carcinoma

Introduction

This review examines the common challenges in diagnosing lung cancer using limited IHCs in small biopsies, particularly in the context of adenocarcinoma, squamous cell carcinoma, and neuroendocrine marker applications. Lung cancer subtypes are associated with distinct molecular changes and tailored treatment regimens, including chemotherapy, targeted therapy, and immunotherapy, which are crucial in the era of personalized medicine. Beyond the broad classification of small cell carcinoma and NSCLC, further subclassification into adenocarcinoma and squamous cell carcinoma is critical for initial management and to avoid severe side effects [1].

With the growing demand for targeted therapies in NSCLC, preserving adequate biopsy material for molecular testing is essential. In small biopsies with limited material, the challenge lies in balancing accurate subclassification with the cautious use of IHC to preserve tissue for molecular testing.

Often, lung cancer and its subtypes can be diagnosed based on cytomorphology and histoarchitecture alone. When necessary, the careful application of a limited IHC panel is typically sufficient to confirm the diagnosis in most cases.

In routine subtyping of NSCLC into adenocarcinoma or squamous cell carcinoma, a limited IHC panel, typically including TTF1 and p40, is usually adequate for an accurate diagnosis [4]. This should be reserved for cases where histologic features alone are insufficient, especially in small biopsy specimens or poorly differentiated NSCLC. Neuroendocrine markers should be applied only when neuroendocrine differentiation is suspected based on morphologic features. For neuroendocrine carcinoma including small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC), diagnosis is primarily based on morphology, with IHC support used in select cases [2,3]. Although molecular testing is not required for neuroendocrine carcinomas, the widespread use of neuroendocrine markers in small lung

biopsies often faces the challenge of non-specific staining, leading to inaccurate diagnoses with significant impact. The use of neuroendocrine IHC markers in the setting of appropriate morphologic features is discussed, as well as examples where nonspecific positive staining with these markers can occur.

This conservative diagnostic approach assumes a high likelihood of the cancer being of lung origin, which depends on clinical history, patient demographics, risk factors, the absence of other known malignancies, and consistent radiographic findings. For example, an elderly patient with a smoking history and a solitary lung nodule seen on radiography would warrant a focused pathological workup for primary lung carcinoma. However, if clinical or radiologic information is ambiguous, or if histologic features and IHC patterns are unusual, relying on a limited IHC panel could lead to diagnostic errors with significant prognostic and therapeutic implications.

This review highlights cases that may occasionally arise in routine practice where reliance on a limited IHC panel can lead to misdiagnoses. These errors often occur in small biopsies with unclear clinical histories or subtle pathological features. The goal is to underscore clinical scenarios and nuanced cytohistomorphological features that require heightened suspicion and an expanded diagnostic workup, including thorough history exploration, to avoid inaccurate diagnoses.

Diagnostic Pitfalls of p40/p63

Squamous cell carcinoma (SqCC) of the lung is predominantly seen in current and former heavy smokers, with secondhand smoke exposure and exposure to other carcinogens, age, and family history also contributing as risk factors [5]. The majority arise in the central compartment of the lung and are often endobronchial, although approximately a third can be peripheral [6].

Multiple histologic subtypes are recognized, including keratinizing, non-keratinizing, and basaloid SqCC. Keratinizing SqCC can often be diagnosed by definitive morphologic features alone, including the presence of keratinization, the formation of keratin pearls, and intercellular bridging. In poorly differentiated tumors these findings can be focal and less prominent, particularly on small biopsy specimens. Non-keratinizing SqCC, as the name implies, lacks this distinctive morphology. Basaloid SqCC similarly lacks the presence of keratinization while also consisting of small to intermediate-sized cells. When sufficiently diagnostic morphology is not present, the IHC stains p63 and its more specific counterpart p40 are considered reliable in differentiating SqCC from adenocarcinoma in the workup of a NSCLC [4,7]. This is so long as staining is positive in greater than 50% of tumor cells [8]. The current WHO terminology recommends “NSCLC, favor squamous cell carcinoma,” with consideration for molecular testing beyond adenocarcinoma, particularly in cases where

clinical features such as young age and lack of tobacco exposure suggest a higher likelihood of a targetable mutation.

The molecular signature of SqCC has a predilection for cytosine to adenine nucleotide base substitutions and complex genomic alterations, consistent with its close etiologic ties to cigarette smoking and other carcinogens [9,10]. They can rarely harbor oncogenic driver mutations more commonly identified in adenocarcinoma, including EGFR and ALK [11]. This is more commonly observed in younger patient demographics with no or light smoking history.

The following are a combination of relatively common and rare examples where overlapping morphologic features and a deceptively positive p63/p40 IHC staining pattern might lead the practicing pathologist mistakenly to the diagnosis of lung SqCC. In such scenarios, carefully searching for hidden clinical or radiologic information, incorporation of additional IHC stains, and ancillary molecular testing might be needed to reach the correct interpretation.

Urothelial carcinoma

Invasive urothelial carcinoma (UC) is a relatively common cancer, particularly arising in older men [12]. Although the association is not quite as striking as seen in lung SqCC, urothelial carcinoma is strongly associated with tobacco smoking [13]. Other risk factors include aromatic amines and other occupational carcinogens, certain chemotherapeutics, and radiation exposure. Metastases favor regional lymph nodes, liver, lung, and bone [14]. A study on the patterns of metastasis of muscle-invasive bladder cancers in autopsy cases found that 68% had metastatic disease, 45% demonstrated lung involvement while 16% showed pleural involvement. A broad range of morphology has been reported, but conventional urothelial carcinoma presents as nests, sheets, trabeculae and cords of tumor cells with moderate to abundant cytoplasm that can appear pale to eosinophilic. This creates significant overlap with nonkeratinizing SqCC or moderately differentiated keratinizing SqCC, where keratin pearls and intracellular bridging may not be readily apparent. Divergent differentiation can commonly be seen in urothelial carcinoma, with squamous differentiation being the most common form [15]. In addition, urothelial carcinoma demonstrates positive staining with p63 and p40 in approximately 80.6 and 85.4% of cases, respectively [16]. As shown in **Figure 1**, three cases of metastatic urothelial carcinoma (UC) to the lung, with no clear prior history of UC provided, demonstrated diffuse positivity for p63/p40, with overlapping histomorphological features resembling SqCC. Case 1 (**Figure 1A**) shows a squamous cell carcinoma in situ lesion, while Case 2 (**Figure 1B**) exhibits a vague “intercellular bridging”-like structure. Molecular testing was performed on all cases, revealing mutations commonly associated with UC, including ERBB2 (HER2) p.S310F, TERT promoter mutation, and FGFR3 p.Y373C. GATA3 staining was conducted after molecular findings suggested UC, showing diffuse positivity.

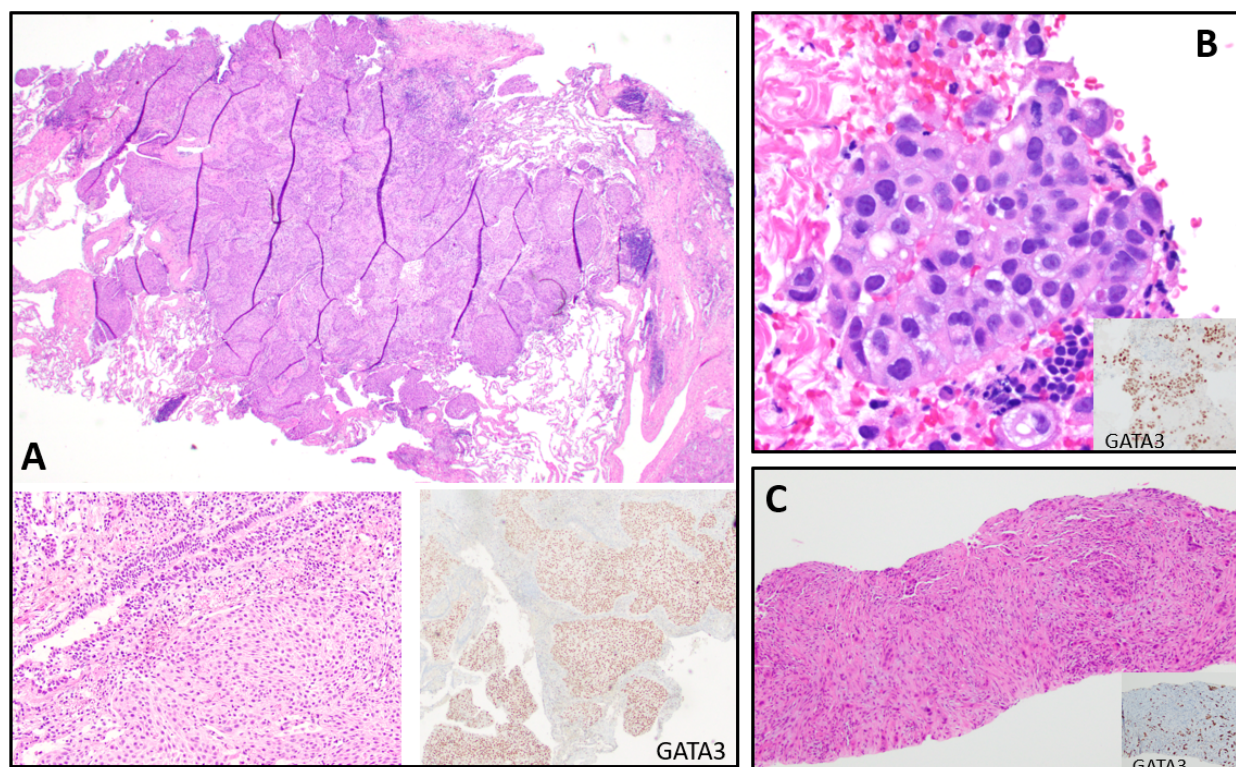


Figure 1. Urothelial carcinoma (UC) mimicking SqCC without a clear prior history. **A.** UC with SCC in situ. **B.** Poorly differentiated carcinoma displaying intracellular bridging-like structures. **C.** A consult case with a pulmonary nodule and lymphadenopathy, showing poorly infiltrating carcinoma with diffuse positive p40 and CK5/6.

The overlap in morphologic features, and immunohistochemical staining pattern can make it difficult to differentiate metastatic urothelial carcinoma from primary lung SqCC, particularly if the pathologist is lacking relevant clinical history about the case. A previous history of urothelial carcinoma would be the most distinctive indicator that additional workup of a new suspected lung SqCC might be warranted. While tobacco smoking has been implicated in both urothelial carcinoma and lung SqCC, a history of light or never smoking would be uncommon for a new primary lung SqCC and would warrant further investigation. A history of regional radiation exposure, particularly for the treatment of cancers from the prostate or uterine cervix would be a predisposing factor raising the risk of having developed urothelial carcinoma that had since metastasized.

In the setting of suspicion for urothelial carcinoma metastatic to the lung, an expanded panel of immunohistochemical stains can help in differentiating from primary lung SqCC. CK7 and GATA-3 have high rates of sensitivity in urothelial carcinoma, staining approximately 100% and 80% of tumors, respectively [16]. However, regarding specificity, approximately 30% of lung SqCC will stain with CK7 and up to 20% will stain with GATA-3. Uroplakin III, and the more sensitive uroplakin II, are positive in urothelial carcinoma while negative in lung SqCC

[17]. Molecularly, bladder cancer shares a high rate of somatic mutations with lung SqCC. Despite tobacco smoke being a recognized risk factor, it does not share lung SCC's prevalence of cytosine to adenine substitution mutations. One of the most common recurrent genetic alterations in urothelial carcinoma are mutations in the TERT promoter region. TERT promoter region mutations are far less frequently identified in NSCLC [18]. Other somatic mutations commonly encountered in urothelial carcinoma include those affecting FGFR3 and ERBB2 [18]. Both are exceedingly rare in NSCLC and in lung SqCC in particular [19].

Basal cell carcinoma (remote BCC)

BCC is the most common malignancy that arises in humans. It more commonly affects older males and has a predilection for sun-exposed skin, with ultraviolet radiation being the closest associated risk factor for its development [20]. Early BCC is commonly small and slow growing but can cause significant morbidity from locally destructive growth, dependent on location and delays in management. Metastasis is exceedingly rare with rates estimated at between 0.0028-0.55% [21-23]. The most common metastatic sites in these cases were lymph nodes at a rate of 60%, followed by hematogenous spread to the lungs at a rate of 42%. Because of the uncommon nature

of BCC to act in a malignant fashion and spread to distant sites, a history of BCC with or without prior therapeutic intervention is frequently missed in pertinent past medical history, even in the context of new lung lesions with suspicion for malignancy.

BCC can share significant morphologic overlap with basaloid and nonkeratinizing SqCC. Keratinization can sometimes be seen, most commonly in nodular BCC. Sclerosing/morphoeic BCC consists of tumor cells in narrow cords compressed by sclerotic stroma that can be difficult to distinguish from poorly differentiated SCC (**Figure 2A**). The IHC stains p40 or p63 are diffusely positive in both entities, staining virtually 100% of BCC, and would not help in differentiating between a primary lung SqCC and a BCC metastatic to the lung [24]. Potentially further confounding factor is BCC demonstrating neuroendocrine

differentiation with positivity of neuroendocrine markers [25].

Separating BCC from SqCC with an extended panel of IHC stains can be nonspecific in small biopsy specimen and should be discouraged. BerEP4 stains are frequently positive in BCC and negative in SqCC [26]. In contrast EMA is frequently negative in BCC while the majority of SqCC stain positive. Most BCC also stains positive with GATA3 [27]. As previously stated in an earlier section, up to 20% of lung SqCC can also stain positive for GATA3. Molecularly, as opposed to lung SqCC's prevalence of cytosine to adenine substitution mutations, BCC has a characteristic "UV signature" with a mutational pattern consisting of mostly cytosine to thymine substitutions [28]. BCC also frequently contains mutations in genes within the hedgehog pathway, with between 70-75% demonstrating

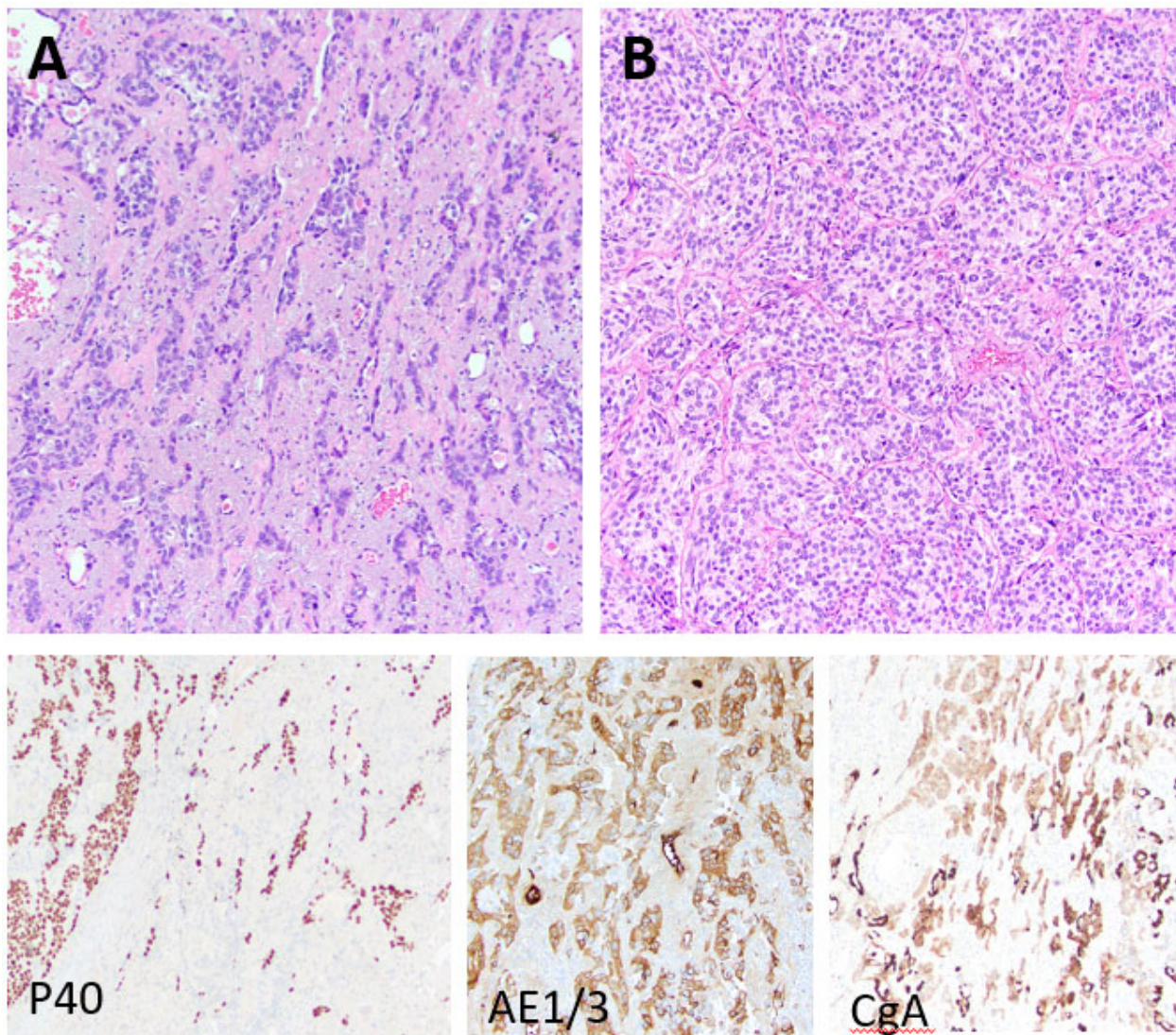


Figure 2. Basal cell carcinoma (BCC) that resembles squamous cell carcinoma, exhibiting positive staining for p40, AE1/AE3, and chromogranin. **A.** Infiltrating poorly differentiated non-small cell carcinoma, 100x. **B.** Region of tumor nests displaying a "zellballen"-like pattern. 200 x.

PTCH1 gene mutations and 10-20% having alterations in Smoothed (SMO), with less frequent mutations being seen in *PTCH2* and suppressor of fused protein (*SUFU*) genes.

In our case, a 69-year-old male former smoker underwent endobronchial ultrasound (EBUS) for a lung mass, with fine needle aspiration (FNA) material demonstrating poorly differentiated NSCLC. An external diagnosis of SCC was initially made. The resection specimen (**Figure 2**) revealed two distinct populations of tumor cells with NSCLC-like morphology: one with an infiltrating pattern (**Figure 2A**) and the other displaying a 'zellballen'-like pattern (**Figure 2B**), which is atypical for SqCC. This prompted further IHC workup, revealing positivity for pancytokeratin AE1/AE3 and p40, alongside positive chromogranin A staining and a high proliferation index (Ki67 >80%). Notably, synaptophysin was negative, which is rare in lung neuroendocrine tumors that usually show strong chromogranin expression.

These findings could easily lead to a misdiagnosis. However, molecular testing revealed a "PTCH1 p. Val908SerfsTer9 (c.2718_2721dup)" mutation and a UV signature. Upon further review of the patient's clinical history, a prior diagnosis of basal cell carcinoma (BCC) in 2012 was identified, which had not been noted in the recent records.

This case highlights the limitations of subclassifying poorly differentiated NSCLC based solely on morphology. In small biopsies showing diffuse positivity for p63/p40 but lacking definitive features of keratinization, such as keratin pearls or intracellular bridging, it is preferable to diagnose 'NSCLC, favor squamous cell carcinoma' rather than making a conclusive diagnosis of SqCC. A limited IHC panel, including TTF-1 and p40/p63 in poorly differentiated NSCLC, should be employed while preserving tissue for molecular testing. Neuroendocrine markers should only be used when there is neuroendocrine morphology present (refer to the section on the application of neuroendocrine markers in small biopsies).

NUT carcinoma

NUT carcinoma of the thorax is a rare and highly aggressive malignancy accounting for a small minority of primary lung carcinomas [29]. One study of primary lung tumors from Kanagawa Children's Medical Center identified only two cases of NUT carcinoma over the span of 41 years, one of which was initially diagnosed as squamous cell carcinoma while the other was initially called unspecified sarcoma or undifferentiated carcinoma [30]. It arises in the central compartment of the thorax, typically presenting at an advanced stage with involvement of the mediastinum and one or both lungs, often with pleural involvement [31]. It has a wide age range of presentations and no known environmental factors. Histologically, it is composed of nests and sheets of small to intermediate-sized cells that may appear monomorphic, with

areas of necrosis. In some cases, keratinization may be present in certain regions. These morphologic features overlap with poorly differentiated lung SqCC and small cell carcinoma. However, the relatively monomorphic nature typical in NUT carcinoma is unusual in a poorly differentiated lung SqCC and small cell carcinoma. It can be a histologic warning that an expanded differential may be warranted. Nearly all cases stain for p63/p40 [29]. Further confounding the diagnosis is the expression of TTF1 and neuroendocrine markers in a subset of NUT carcinomas [31]. Genetically defined by the presence of a nuclear protein in testis (NUTM1) gene rearrangement, characteristically identified is a translocation between NUTM1 and either BRD4 or less commonly to other genes coding for BRD4-interacting proteins [32]. NUT carcinoma can be differentiated from mimickers by detection of this molecular alteration. A diagnostic NUT antibody IHC stain, clone C52B1, is 100% specific and 87% sensitive for NUT carcinoma cases [33]. The staining pattern is nuclear and punctate, with >50% of nuclei showing expression to call a positive result.

A 57-year-old male never-smoker was found to have a left lung mass extending into the bronchus and left lower lobe. On biopsy, the malignant cells were found to be diffusely positive for p63, focally positive for synaptophysin, and negative for TTF1 (**Figure 3**). A diagnosis of "Poorly differentiated NSCLC, favor SqCC" was rendered based on the morphology and IHC staining pattern. However, the unusual cytomorphology, characterized by loosely clustered cells and mild nuclear pleomorphism, combined with the atypical clinical presentation of a non-smoker, raises differential diagnosis other than SqCC. The specimen was sent for molecular testing where a NUTM1 translocation was identified by next generation sequencing. This additional finding rendered an added diagnosis of "NUT carcinoma".

Another rare tumor that can be encountered in practice is *Mucoepidermoid carcinoma (MEC) with predominantly squamoid component*. They derive from the minor salivary glands of the proximal tracheobronchial tree and thus usually arise centrally in an endobronchial fashion, similar to the majority of SqCC. However, development of MEC has a wide age range and they do not have a known association with smoking tobacco. Histologically, MEC comprises of varying proportions of mucocytes, squamoid, and intermediate cells. High-grade tumors can consist predominantly of solid areas comprised of atypical non-keratinizing squamoid cells, making it difficult to differentiate them from lung SqCC. Histologic features that would help distinguish MEC from lung SCC include identification of variable numbers of mucocytes. Histochemical stains for mucicarmine and PASD. The majority of MEC demonstrate recurrent translocations of chromosomes 19p13 and 11q21, resulting in *CRTC1-MAML2* gene fusions [34].

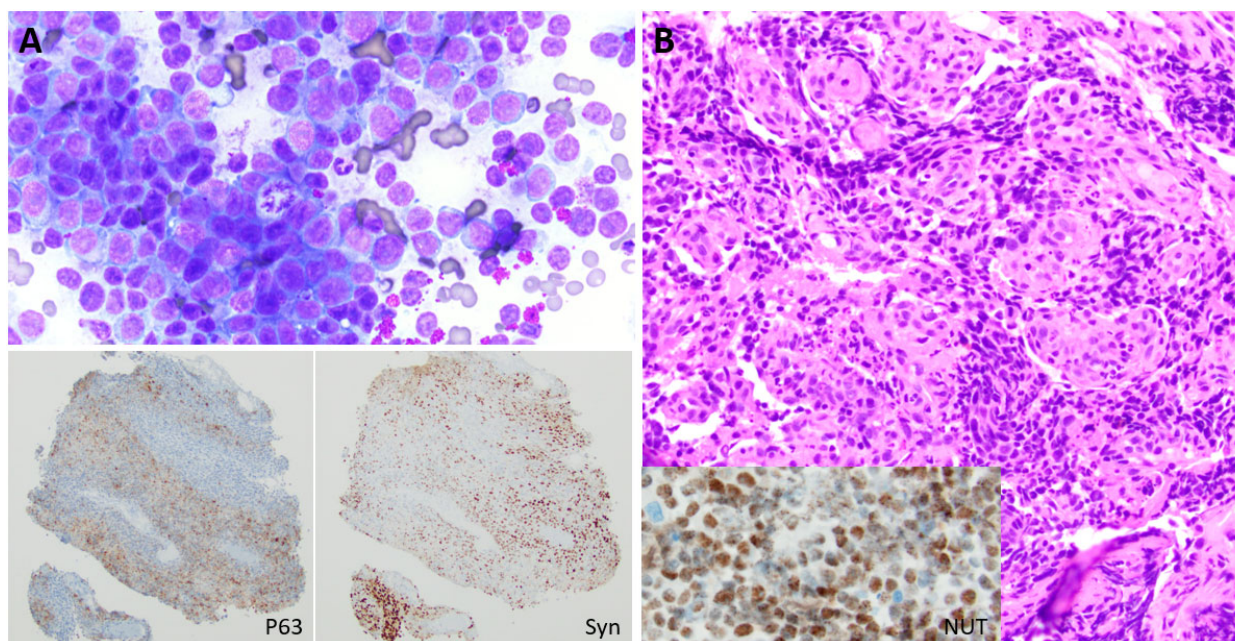


Figure 3. NUT carcinoma in a small biopsy showing diffuse positivity for p63 and focal positivity for synaptophysin. **A.** Diff-Quick smear reveals loosely clustered malignant cells with a high N/C ratio, mild nuclear pleomorphism, and frequent mitotic figures. **B.** Biopsy specimen displays areas of neoplastic cells with moderate to abundant dense cytoplasm. NUT carcinoma is confirmed by positive NUT staining.

Diagnostic Pitfalls of TTF-1

Adenocarcinoma of the lung commonly arises in the periphery but can also be centrally located. Like all lung carcinomas, adenocarcinoma has a strong relationship with tobacco smoking. However, unlike other types of lung carcinoma, adenocarcinoma also has an association with a non-smoking history, particularly in female non-smokers [35]. Unlike the majority of lung SqCC, several genetic alterations have been implicated as oncogenic drivers in lung adenocarcinoma. Mutations in KRAS, NRAS, and MAP2K1 genes are often identified in smokers [36]. Lung adenocarcinoma in non-smokers often harbor mutations in EGFR and ERBB2 genes or fusions in ALK, ROS1, RET, and NTRK genes [37]. BRAF and MET mutations can be seen in both populations.

Lung adenocarcinoma can have a variety of histological subtypes including lepidic, acinar, papillary, micropapillary, and solid. They often consist of a mixture of these and are classified by the predominant pattern. In the setting of differentiating lung adenocarcinoma from a suspected metastasis or of differentiating lung adenocarcinoma from lung SCC when morphologic features are insufficient, at least focal positivity with the IHC TTF1 is often considered adequate to diagnose lung adenocarcinoma in the context of a NSCLC without neuroendocrine morphology [4,38]. TTF1 is typically positive in type 2 pneumocytes but cannot distinguish between benign and malignant cells. A common pitfall of TTF1 staining

includes mistaking reactive pneumocytes or adenomas for malignancies due to an exaggerated dye staining pattern [3]. Conversely, metastatic carcinomas may be misdiagnosed as lung adenocarcinomas when TTF1 is interpreted incorrectly. Several cases illustrate how histological similarities combined with a positive TTF1 IHC stain can result in a misdiagnosis of lung adenocarcinoma. Unusual clinical histories, radiologic findings, and morphologic features should prompt further investigation, including additional IHC stains and molecular studies, to ensure an accurate diagnosis.

Sclerosing pneumocytoma

Sclerosing pneumocytoma is a rare benign epithelial lung neoplasm, typically presenting as a solitary peripheral lesion but occasionally manifesting endobronchially [39]. It can occur across a wide age range, shows a female predominance, and is more common in Asian populations, with most cases arising in never smokers. Sclerosing pneumocytoma is composed of cuboidal surface cells and round stromal cells with subtle cytomorphologic differences. Its growth patterns can be papillary, solid, sclerotic, or hemorrhagic. Both cell types stain positively for TTF1 in as many as 92% of cases in one study [40]. This may lead to overdiagnosis in small biopsies, potentially being misinterpreted as an aggressive form of adenocarcinoma. Additional IHC markers such as pancytokeratin, CAM5.2, CK7, and Napsin can be useful, as only the surface cells show diffuse positivity for these markers,

while stromal cells are either negative or weakly positive. Nearly all cases harbor AKT1 mutations, providing useful ancillary support for diagnosis in challenging cases [41].

As shown in **Figure 4**, FNA and core needle biopsy were performed on a 1.9 cm lobulated right upper lobe nodule in a 53-year-old female non-smoker. The smear reveals small epithelial cells, either singly or in clusters, with mild polymorphisms and fine chromatin (**Figures 4A and 4B**). The biopsy demonstrated sheets of cuboidal cells with scattered acinar formations (**Figure 4C**). TTF1 staining was diffusely positive in the biopsy specimen (**Figure 4D**). Due to the small cell size and mild polymorphisms, pancytokeratin was performed, which highlighted the surface cuboidal cells (**Figure 4C**). The findings were consistent with sclerosing pneumocytoma."

Bronchiolar adenoma/ciliated muconodular papillary tumor

Bronchiolar adenoma/ciliated muconodular papillary tumor (BA/CMPT) is a benign lung tumor that typically arises in the peripheral lung. These tumors are usually identified incidentally in middle-aged to elderly individuals and have no known associated risk factors. Histologically, BA/CMPT consists of a bilayer of bland luminal epithelial cells and basal cells without nuclear atypia. The luminal cells may consist of mucous and ciliated cells (proximal-type BA) or cells resembling type II pneumocytes and club cells (distal-type BA), with growth patterns that are typically papillary or glandular. BA/CMPTs are true neoplasms, often associated with driver mutations such as BRAF, EGFR, KRAS, HRAS, and ALK. However, since these mutations are also reported in lung adenocarcinoma,

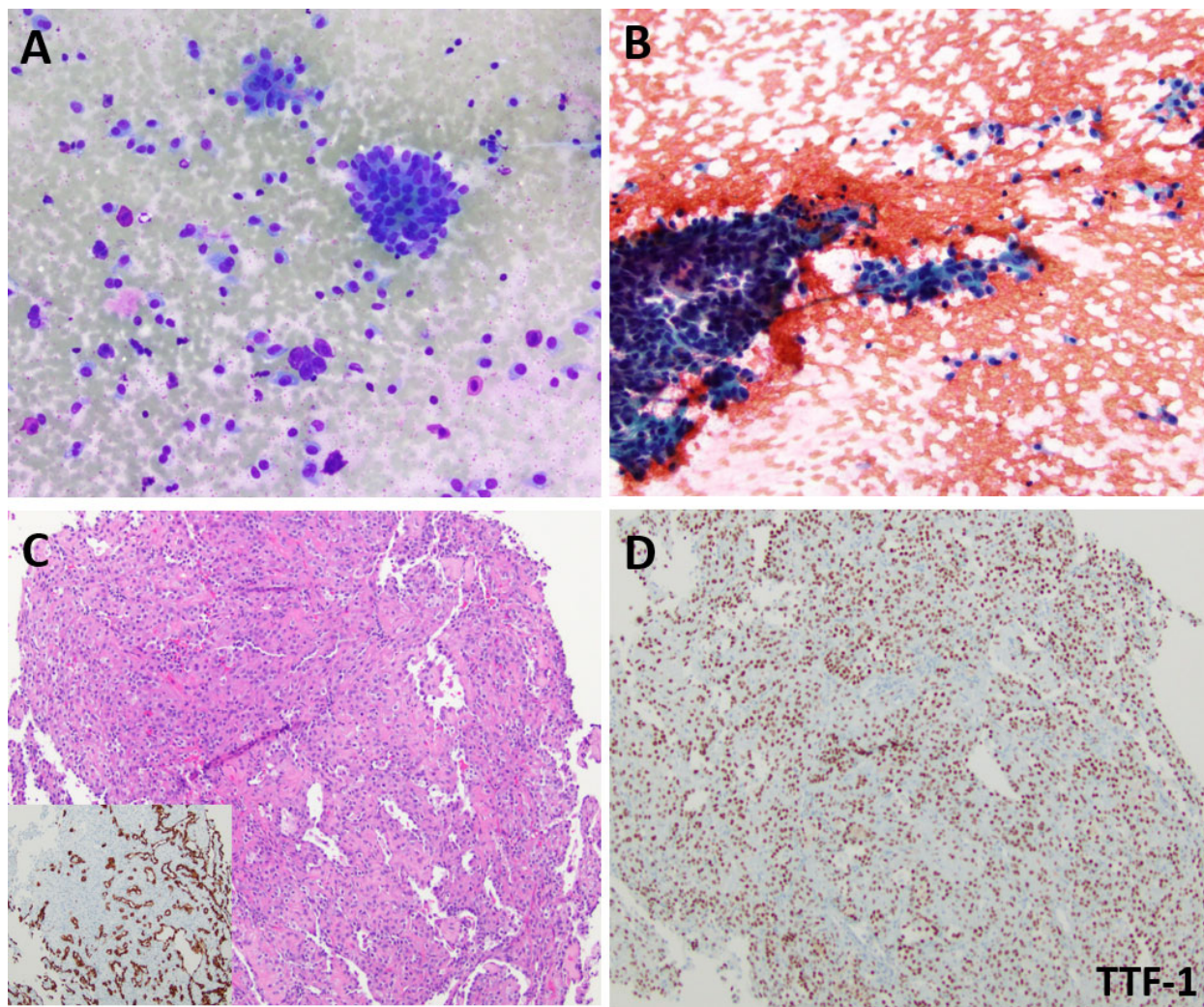


Figure 4. Sclerosing pneumocytoma: **A.** Single or clustered epithelial cells showing mild nuclear pleomorphism and slightly increased size (Diff-Quick, 200x). **B.** Fine chromatin observed in Pap smear (200x). **C.** Biopsy (H&E, 100x) reveals sheets of neoplastic cells with relatively uniform morphology and scattered acinar formation. Insert: AE1/AE3. **D.** Diffuse positivity for TTF-1.

their presence is not sufficient for differentiating between BA/CMPT and adenocarcinoma.

TTF1 staining can be positive in the luminal cells and basal cells. One study demonstrated diffuse positivity in both cell types on all tested distal-type BA, while proximal-type BA showed weak/focal positivity in luminal cells in two of seven cases and similar weak/focal positivity in basal cells in five of seven cases [42]. In lung adenocarcinoma, even focal TTF1 positivity can be considered diagnostic, making TTF1 staining in BA/CMPT potentially misleading. As shown in **Figure 5**, a biopsy of an endobronchial lesion from a 53-year-old male, with atypical cytologic appearance with abundant background mucin (**Figure 5A**) and glandular formation in sclerotic stroma (**Figure 5B**), could easily be misinterpreted as mucinous adenocarcinoma. However, the minimal nuclear atypia and the presence of the characteristic bilayer prompted the use of p40/63 staining, which revealed linear staining with p63/p40 (**Figure 5B**), helping clarify the diagnosis.

Papillary thyroid carcinoma (PTC)

PTC is the most common endocrine malignancy and typically presents as a thyroid nodule, with or without cervical lymphadenopathy. Although distal metastasis is rare, one of the most common sites when it occurs is the lung, where PTC can be confused morphologically and immunohistochemically with lung adenocarcinoma. PTC may also be considered in the differential diagnosis of a new adenocarcinoma metastatic to a lymph node (regional or nonregional) without an identifiable primary, due to similar features. As illustrated in **Figure 6**, a 76-year-old female, under surveillance for slowly growing lung nodules since 2015, was found to have a suspicious left peri-aortic retroperitoneal

lymph node on imaging, which was sampled via fine needle biopsy. The lymph node contained clusters of atypical cells with nuclear pleomorphism, suggesting malignancy (**Figure 6A**). A cell block showed papillary growth (**Figure 6B**). TTF1 staining was positive in the atypical cells, initially suggesting lung adenocarcinoma due to the patient's known lung nodules. However, the presence of cleared chromatin and nuclear grooves, along with abundant papillary structures, led to the addition of Pax8 staining, which was diffusely and strongly positive in the tumor cells, supporting a diagnosis of metastatic PTC. A subsequent review of the patient's history revealed a prior remote history of thyroid cancer. Common molecular alterations in PTC include somatic mutations in BRAF and RAS genes, as well as RET fusions, which have also been reported in lung adenocarcinoma [43].

Another rare tumor that can be encountered in TTF-1 pitfalls is thoracic SMARCA4-deficient undifferentiated tumor (SMARCA4-UT), which is an aggressive malignancy involving the mediastinum and lung. It often arises in young to middle-aged adults with a male predominance and a strong association with heavy tobacco smoking. Histologically, the tumor is comprised of sheets of large and relatively monotonous round to epithelioid cells, occasionally with rare rhabdoid features. Approximately 5% contain a component of conventional NSCLC. SMARCA4-UT can rarely show focal staining for TTF1 in as many as one of ten cases in one study, as well as p63/p40 [44]. While the staining for p63/p40 might not be expressed enough to meet the threshold for what is considered positive in lung SqCC, focal positivity with TTF1 could lead to an incorrect diagnosis of lung adenocarcinoma. There can also be strong synaptophysin positivity, emphasizing the importance of relying on neuroendocrine markers only in the presence of appropriate morphology. Most SMARCA4-UT

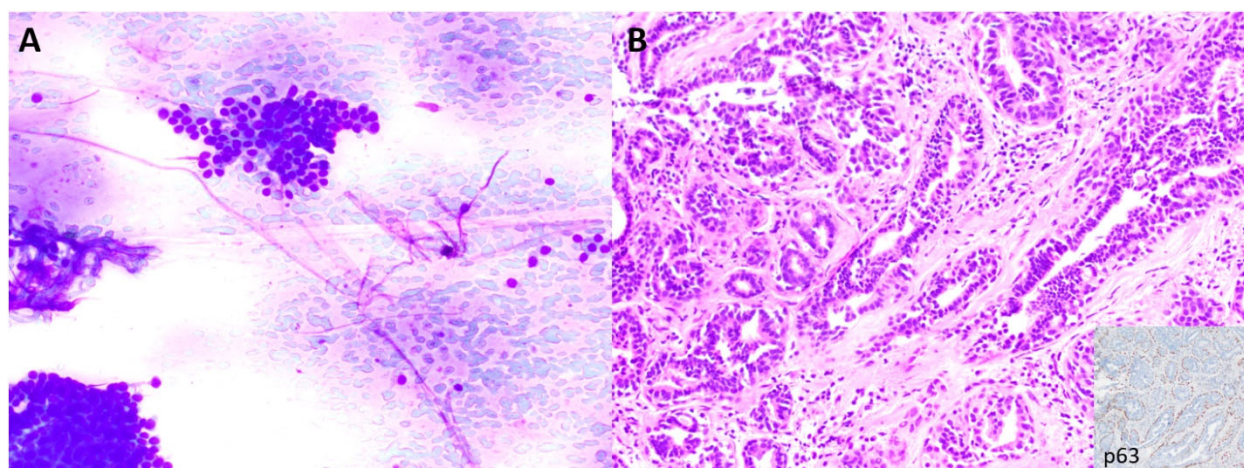


Figure 5. A. Clusters of epithelial cells with crowded arrangements and mild nuclear pleomorphism, set against a background of abundant mucinous material (Diff-Quick, 200x). **B.** The biopsy shows neoplastic cells forming glands of varying sizes and shapes, with focal TTF-1 positivity. P63 highlights the linear basal cells.

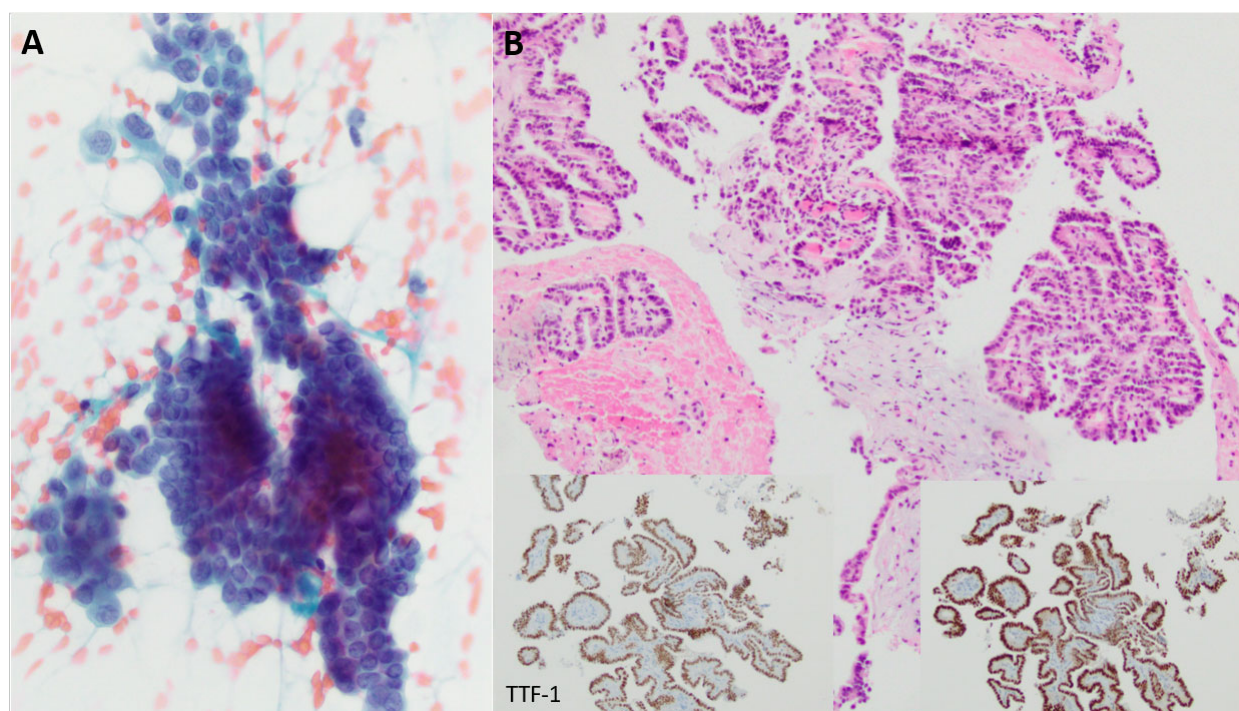


Figure 6. A. Clusters of atypical cells displaying disorganization, marked nuclear pleomorphism, and irregular nuclear membranes, suggestive of cytological malignancy (Pap smear, 200x). **B.** The cell block shows neoplastic cells positive for TTF-1 in a papillary arrangement, set against a background of myxoid stroma. Additional staining shows positivity for PAX8.

share a high somatic mutation burden and genomic smoking signature with conventional NSCLC. However, as their name implies, they molecularly differ by the presence of biallelic inactivation of the SMARCA4 gene. SMARCA4 (BRG1) IHC stain demonstrates complete loss in 75% of cases or severe global reduction of expression in the other 25% of cases. This can be confirmed with molecular sequencing.

Neuroendocrine Markers

Neuroendocrine tumors of the lung are classified into low-grade (typical carcinoid), intermediate-grade (atypical carcinoid), and high-grade categories, which include LCNEC and SCLC. The traditional immunohistochemical (IHC) markers used to detect neuroendocrine differentiation are synaptophysin, chromogranin A, and CD56. Carcinoid tumors typically exhibit strong and diffuse positivity for synaptophysin and chromogranin A. However, in high-grade neuroendocrine carcinomas, 10% of resection specimens may be negative for these traditional markers [43]. SCLC often shows only weak and focal positivity for synaptophysin and chromogranin A, and approximately 25% of cases may be negative for both [46]. Insulinoma-associated protein 1 (INSM1), a newer IHC marker, is highly specific for neuroendocrine differentiation

[47]. According to the current NCCN (National Comprehensive Cancer Network) Guidelines for SCLC, fewer than 5% of SCLCs are negative for all neuroendocrine markers, including INSM1.

The application of neuroendocrine markers should be limited to instances where neuroendocrine morphological features are present [3,4,48,49]. Key cytomorphological features for SCLC include cells with a high N/C ratio, fine chromatin, and a background of abundant necrosis, apoptotic bodies, and mitotic figures. For LCNEC, the defining features include organoid clusters of tumor cells with nuclear palisading, prominent nucleoli, at least a moderate amount of cytoplasm, and a background of abundant necrosis and mitotic activity.

This approach minimizes unnecessary use of tissue, preserves material for ancillary testing, and reduces the risk of diagnostic errors. Neuroendocrine markers can stain positively in some NSCLC cases, complicating interpretation. Additionally, certain entities can nonspecifically stain positive for neuroendocrine markers, increasing the likelihood of misdiagnosis. As we discussed above, BCC can express chromogranin A, SMARCA4-UT may show strong synaptophysin staining, and NUT carcinomas can also be positive for neuroendocrine markers.

Discussion

As the need for accurate diagnosis and molecular testing in small biopsies of lung nodules or mass lesions increases, uncommon entities are being encountered more frequently in clinical practice, with significant implications for patient management. This review highlights challenging cases where subtle cytohistomorphological features and limited IHC markers led to diagnostic pitfalls.

Cytohistomorphology primarily distinguishes between benign and malignant entities, relying on key features such as gland formation, mucin production, and keratinization. Minor cytohistomorphological features in adenocarcinoma include crowded 3D clusters with eccentric nuclei and foamy cytoplasm, while in SqCC, they include 2D sheets of tumor cells with dense eosinophilic cytoplasm. IHC is primarily used to determine tumor origin and for subclassification. It is recommended to avoid IHC when definite morphological features are present or to use either TTF1 or p40/p63 to support adenocarcinoma or SqCC when definite features are lacking but minor cytohistomorphological features are observed, especially in cases where clinical indications suggest a primary tumor without a history of cancer from another site or suspicion of metastasis. For adenocarcinoma, caution is advised when applying TTF1 to benign lesions with reactive changes. In cases of TTF1-negative adenocarcinoma without a clear history or radiological indication of a lung primary, tissue preservation for molecular testing is crucial. For (SqCC), the terminology 'NSCLC, favor SqCC' should be used in poorly differentiated NSCLC cases with diffuse p40/p63 positivity, as multiple deceptive entities discussed in this review can complicate the diagnosis. Tissue preservation remains essential, as molecular alterations may not only impact clinical management but also help clarify mimicking entities. Neuroendocrine markers should be applied to support neuroendocrine morphology, with caution advised in cases involving nonsmokers, uniform small blue cells, and pancytokeratin negativity.

The goal of this review is to recognize potential traps and explore clues such as molecular alterations, clinical history, and expanded IHC workups to prevent misinterpretation and its impact on patient care. Interobserver variability is a common challenge in pathology, especially in small biopsies, due to differences in training, experience, and practice. There is a lack of literature on 'diagnosis inter-observer variability in small lung biopsies,' which is a limitation of this review. The cases presented are drawn from the authors' personal collections, accumulated over years of training and practice, combined with insights from the WHO 5th edition, updated NCCN guidelines for SCLC and NSCLC, and recent review papers on related topics. With the increasing use of digital pathology, Artificial Intelligence (AI) has gained attention for its potential to minimize human error and shows promise in certain objective pathological assessments of IHC quantities. However, providing accurate morphological data for AI remains

a significant challenge. We believe that as more pathologists become aware of the pitfalls in small lung biopsies, more 'correct' data will be accumulated for future reference, helping to reduce errors with the aid of advanced tools.

Conclusion

Recognizing common IHC pitfalls in small lung biopsies is vital for accurate diagnosis and for minimizing unnecessary IHC to preserve tissue for additional molecular tests. Pathologists should remain highly vigilant for atypical clinical or morphological features, use IHC panels judiciously, and integrate molecular findings when appropriate. Continuous quality assurance and staying updated with evolving diagnostic criteria are essential for delivering optimal patient care.

References

1. Zakowski M. Analytic inquiry: Molecular testing in lung cancer. *Cancer Cytopathol.* 2017;125(56):470-76.
2. WHO Classification of Tumours. Editorial Board. Thoracic Tumours (5th ed), International Agency for Research on Cancer, Lyon, France (2021). Page 140-1.
3. Xiong J, Polsdofer E, Jing J. Common practice issues in pulmonary cytology/small biopsy: Diagnostic pitfalls and appropriate use of immunohistochemical stains. *Human Pathology Reports.* 2024 Jun 1;36:300735.
4. Yatabe Y, Dacic S, Borczuk A, Warth A, Russell P, Lantuejoul S, et al. Best Practices Recommendations for Diagnostic Immunohistochemistry in Lung Cancer. *J Thorac Oncol.* 2019 Mar;14(3):377-407.
5. Molina J, Yang P, Cassivi S, Schild S, Adjei A. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. *Mayo Clin Proc.* 2008; 83(5):584-94.
6. Krinsky W, Mugańlinskaya N, Sarkar S, Vulchi M, Patel P, Rao S, et al. The changing anatomic position of squamous cell carcinoma of the lung - a new conundrum. *J Community Hosp Intern Med Perspect.* 2016; 6(6):33299.
7. Bishop J, Teruya-Feldstein J, Westra W, Pelosi G, Travis W, Rekhtman N. p40 (Δ Np63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. *Mod Pathol.* 2012; 25(3):405-15.
8. WHO Classification of Tumours. Editorial Board. Thoracic Tumours (5th ed), International Agency for Research on Cancer, Lyon, France (2021). Page 91.
9. Alexandrov L, Nik-Zainal S, Wedge D, Aparicio S, Behjati S, Biankin A, et al. Signatures of mutational processes in human cancer. *Nature.* 2013; 500(7463):415-21.
10. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature.* 2012; 489(7417):519-25.

11. Lindeman NI, Cagle PT, Aisner DL, Arcila ME, Beasley MB, Bernicker EH, et al. Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors: Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med.* 2018 Mar;142(3):321-46.
12. Saginala K, Barsouk A, Aluru J, Rawla P, Padala S, Barsouk A. Epidemiology of Bladder Cancer. *Med Sci (Basel).* 2020; 8(1):15.
13. Pelucchi C, Bosetti C, Negri E, Malvezzi M, La Vecchia C. Mechanisms of disease: The epidemiology of bladder cancer. *Nat Clin Pract Urol.* 2006; 3(6):327-40.
14. Wallmeroth A, Wagner U, Moch H, Gasser T, Sauter G, Mihatsch M. Patterns of metastasis in muscle-invasive bladder cancer (pT2-4): An autopsy study on 367 patients. *Urol Int.* 1999; 62(2):69-75.
15. Wasco M, Daignault S, Zhang Y, Kunju L, Kinnaman M, Braun T, et al. Urothelial carcinoma with divergent histologic differentiation (mixed histologic features) predicts the presence of locally advanced bladder cancer when detected at transurethral resection. *Urology.* 2007; 70(1):69-74.
16. Hoang L, Tacha D, Bremer R, Haas T, Cheng L. Uroplakin II (UPII), GATA3, and p40 are Highly Sensitive Markers for the Differential Diagnosis of Invasive Urothelial Carcinoma. *Appl Immunohistochem Mol Morphol.* 2015; 23(10):711-6.
17. Gruver A, Amin M, Luthringer D, Westfall D, Arora K, Farver C, et al. Selective immunohistochemical markers to distinguish between metastatic high-grade urothelial carcinoma and primary poorly differentiated invasive squamous cell carcinoma of the lung. *Arch Pathol Lab Med.* 2012; 136(11):1339-46.
18. Kamoun A, de Reyniès A, Allory Y, Sjö Dahl G, Robertson A, Seiler R, et al. Bladder Cancer Molecular Taxonomy Group. A Consensus Molecular Classification of Muscle-invasive Bladder Cancer. *Eur Urol.* 2020; 77(4):420-33.
19. Chuang J, Stehr H, Liang Y, Das M, Huang J, Diehn M, et al. ERBB2-Mutated Metastatic Non-Small Cell Lung Cancer: Response and Resistance to Targeted Therapies. *J Thorac Oncol.* 2017; 12(5):833-42.
20. Lee J, Soung Y, Kim S, Nam S, Park W, Wang Y, et al. ERBB2 kinase domain mutation in the lung squamous cell carcinoma. *Cancer Lett.* 2006; 237(1):89-94.
21. Verkouteren J, Ramdas K, Wakkee M, Nijsten T. Epidemiology of basal cell carcinoma: scholarly review. *Br J Dermatol.* 2017; 177(2):359-72.
22. Seo S, Shim W, Shin D, Kim Y, Sung H. Pulmonary metastasis of Basal cell carcinoma. *Ann Dermatol.* 2011; 23(2):213-6.
23. Nongrum H, Bhuyan D, Royte V, Dkhar H. Metastatic basal cell carcinoma to the lungs: Case report and review of literature. *Indian Dermatol Online J.* 2014; 5(Suppl 1):S26-9.
24. Andruszkow J, Oll M, Förster S, Knüchel R, Jäkel J. p40 in Conjunction With CK20 and E-Cadherin Distinguishes Primary Adnexal Neoplasms of the Skin. *Appl Immunohistochem Mol Morphol.* 2016; 24(6):414-21.
25. Houcine Y, Chelly I, Zehani A, Belhaj Kacem L, Azzouz H, Rekik W, et al. Neuroendocrine differentiation in basal cell carcinoma. *J Immunoassay Immunochem.* 2017; 38(5):487-93.
26. Beer T, Shepherd P, Theaker J. Ber EP4 and epithelial membrane antigen aid distinction of basal cell, squamous cell and basosquamous carcinomas of the skin. *Histopathology.* 2000; 37(3):218-23.
27. Miettinen M, McCue P, Sarlomo-Rikala M, Rys J, Czapiewski P, Wazny K, et al. GATA3: a multispecific but potentially useful marker in surgical pathology: a systematic analysis of 2500 epithelial and nonepithelial tumors. *Am J Surg Pathol.* 2014; 38(1):13-22.
28. Dika E, Scarfi F, Ferracin M, Broseghini E, Marcelli E, Bortolani B, et al. Basal Cell Carcinoma: A Comprehensive Review. *Int J Mol Sci.* 2020; 21(15):5572.
29. Sholl L, Nishino M, Pokharel S, Mino-Kenudson M, French C, Janne P, et al. Primary Pulmonary NUT Midline Carcinoma: Clinical, Radiographic, and Pathologic Characterizations. *J Thorac Oncol.* 2015; 10(6):951-9.
30. Tanaka M, Kato K, Gomi K, Yoshida M, Niwa T, Aida N, et al. NUT midline carcinoma: report of 2 cases suggestive of pulmonary origin. *Am J Surg Pathol.* 2012; 36(3):381-8.
31. Chau N, Ma C, Danga K, Al-Sayegh H, Nardi V, Barrette R, et al. An Anatomical Site and Genetic-Based Prognostic Model for Patients With Nuclear Protein in Testis (NUT) Midline Carcinoma: Analysis of 124 Patients. *JNCI Cancer Spectr.* 2019; 4(2):pkz094.
32. French C, Ramirez C, Kolmakova J, Hickman T, Cameron M, Thyne M, et al. BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. *Oncogene.* 2008; 27(15):2237-42.
33. Haack H, Johnson L, Fry C, Crosby K, Polakiewicz R, Stelow E, et al. Diagnosis of NUT midline carcinoma using a NUT-specific monoclonal antibody. *Am J Surg Pathol.* 2009; 33(7):984-91.
34. Tonon G, Modi S, Wu L, Kubo A, Coxon A, Komiya T, et al. t(11;19) (q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nat Genet.* 2003; 33(2):208-13.
35. Rivera G, Wakelee H. Lung Cancer in Never Smokers. *Adv Exp Med Biol.* 2016; 893:43-57.
36. Arcila M, Drilon A, Sylvester B, Lovly C, Borsu L, Reva B, et al. MAP2K1 (MEK1) Mutations Define a Distinct Subset of Lung Adenocarcinoma Associated with Smoking. *Clin Cancer Res.* 2015; 21(8):1935-43.
37. Farago A, Taylor M, Doebele R, Zhu V, Kummer S, Spira A, et al. Clinicopathologic Features of Non-Small-Cell Lung Cancer Harboring an NTRK Gene Fusion. *JCO Precis Oncol.* 2018;

-
- 2018:PO.18.00037.
38. Russell P, Rogers T, Solomon B, Alam N, Barnett S, Rath V, et al. Correlation between molecular analysis, diagnosis according to the 2015 WHO classification of unresected lung tumours and TTF1 expression in small biopsies and cytology specimens from 344 non-small cell lung carcinoma patients. *Pathology.* 2017; 49(6):604-10.
 39. Devouassoux-Shisheboran M, de la Fouchardière A, Thivolet-Béjui F, Sourisseau-Millan M, Guerin J, Travis W. Endobronchial variant of sclerosing hemangioma of the lung: histological and cytological features on endobronchial material. *Mod Pathol.* 2004; 17(2):252-7.
 40. Devouassoux-Shisheboran M, Hayashi T, Linnoila R, Koss M, Travis W. A clinicopathologic study of 100 cases of pulmonary sclerosing hemangioma with immunohistochemical studies: TTF-1 is expressed in both round and surface cells, suggesting an origin from primitive respiratory epithelium. *Am J Surg Pathol.* 2000; 24(7):906-16.
 41. Yeh Y, Ho H, Wu Y, Pan C, Wang Y, Chou T. AKT1 internal tandem duplications and point mutations are the genetic hallmarks of sclerosing pneumocytoma. *Mod Pathol.* 2020; 33(3):391-403.
 42. Chang J, Montecalvo J, Borsu L, Lu S, Larsen B, Wallace W, et al. Bronchiolar Adenoma: Expansion of the Concept of Ciliated Muconodular Papillary Tumors With Proposal for Revised Terminology Based on Morphologic, Immunophenotypic, and Genomic Analysis of 25 Cases. *Am J Surg Pathol.* 2018; 42(8):1010-26.
 43. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell.* 2014; 159(3):676-90.
 44. Yoshida A, Kobayashi E, Kubo T, Kodaira M, Motoi T, Motoi N, et al. Clinicopathological and molecular characterization of SMARCA4-deficient thoracic sarcomas with comparison to potentially related entities. *Mod Pathol.* 2017; 30(6):797-809.
 45. Travis W. Update on small cell carcinoma and its differentiation from squamous cell carcinoma and other non-small cell carcinomas. *Mod Pathol.* 2012; 25 Suppl 1:S18-30.
 46. Kriegsmann K, Zgorzelski C, Muley T, Christopoulos P, Thomas M, Winter H, et al. Role of Synaptophysin, Chromogranin and CD56 in adenocarcinoma and squamous cell carcinoma of the lung lacking morphological features of neuroendocrine differentiation: a retrospective large-scale study on 1170 tissue samples. *BMC Cancer.* 2021; 21(1):486.
 47. Mukhopadhyay S, Dermawan J, Lanigan C, Farver C. Insulinoma-associated protein 1 (INS1) is a sensitive and highly specific marker of neuroendocrine differentiation in primary lung neoplasms: an immunohistochemical study of 345 cases, including 292 whole-tissue sections. *Mod Pathol.* 2019; 32(1):100-9.
 48. Hiroshima K, Iyoda A, Shida T, Shibuya K, Iizasa T, Kishi H, et al. Distinction of pulmonary large cell neuroendocrine carcinoma from small cell lung carcinoma: a morphological, immunohistochemical, and molecular analysis. *Mod Pathol.* 2006; 19(10):1358-68.
 49. Jing J, Konopka KE. Diagnosis of Lung Carcinoma on Small Biopsy. *Surg Pathol Clin.* 2020 Mar;13(1):1-15.
-