SPECIAL ARTICLE

Best Practices Recommendations for Diagnostic Immunohistochemistry in Lung Cancer

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ABSTRACT

Since the 2015 WHO classification was introduced into clinical practice, immunohistochemistry (IHC) has figured prominently in lung cancer diagnosis. In addition to distinction of small cell versus non–small cell carcinoma, patients' treatment of choice is directly linked to histologic subtypes of non–small cell carcinoma, which pertains to IHC results, particularly for poorly differentiated tumors. The use of IHC has improved diagnostic accuracy in the classification of lung carcinoma, but the interpretation of IHC results remains challenging in some instances. Also, pathologists must be aware of many interpretation pitfalls, and the use of IHC should be efficient to spare the tissue for molecular testing. The International Association for the Study of Lung Cancer Pathology Committee received questions on practical application and interpretation of IHC in lung cancer diagnosis. After discussions in several International Association for the Study of Lung Cancer Pathology Committee meetings, the issues and caveats were summarized in terms of 11 key questions covering common and important diagnostic situations in a daily clinical practice with some relevant challenging queries. The questions cover topics such as the best IHC markers for distinguishing NSCLC subtypes, differences in thyroid transcription factor 1 clones, and the utility of IHC in diagnosing uncommon subtypes of lung cancer and distinguishing primary from metastatic tumors. This article provides answers and explanations for the key questions about the use of IHC in diagnosis of lung carcinoma, representing viewpoints of experts in thoracic pathology that should assist the community in the appropriate use of IHC in diagnostic pathology.

Keywords: Lung cancer; Immunohistochemistry; TTF1; p40; Neuroendocrine markers

Introduction

In the past decade, significant progress has been made in the field of immunohistochemistry (IHC). Higher sensitivity and specificity have been provided by staining enhancement techniques, such as signal amplification with and without a linker, development of monoclonal rabbit antibodies, and use of emerging novel markers. Under the current therapeutic strategy algorithm for patients with lung cancer, the diagnosis of lung cancer, including subtyping, is now directly linked to treatment of choice. Accordingly, the 2015 WHO classification of lung cancer first introduced IHC in the classification schema to reflect biological features, and thus IHC is routinely used in clinical practice in diagnosing lung cancer, particularly on small biopsy or cytologic specimens, and poorly differentiated tumors. Currently, adenocarcinoma and squamous cell carcinoma are efficiently separated with thyroid transcription factor 1 (TTF1) and p40 staining, respectively, even in the case of poorly differentiated non–small cell carcinoma (NSCC) with small biopsy and cytologic specimens.[1] However, as with any technique, there are pitfalls and disadvantages in selection of the antibody panel, antibody clones, and interpretation of the staining. In response to these practical issues, the Pathology Committee of the International Association of Lung Cancer Study defined 11 questions that are frequently encountered in daily practice and achieved consensus based on literature review, personal experience of experts, and discussion among the committee members. Some questions

remained challenging in that consensus has not been achieved, but we have tried to describe possible solutions with as many explanations as possible to benefit the practicing community. In this recommendation, we have excluded use of IHC for predictive biomarkers, which has been established elsewhere.[2,3]

Key Questions on Diagnostic IHC of Lung Cancer

Individual members submitted questions based on their experience as experts. The 11 questions that summarized the most pressing issues with IHC were selected for discussion with the entire panel. The consensus was established through three face-to-face committee meetings in 2016 and 2017. The 11 key questions are listed in Table 1.

1. What is the best combination of markers to use in daily practice?

Short Answer. *When IHC is needed for the subtyping of NSCC, TTF1 and p40 are the criterion standard, and these two markers are usually sufficient in clinical*

Table 1. Key Questions and Recommendations for Diagnostic Immunohistochemistry in Lung Cancer

Key Questions	Short Answers
1. What is the best combination of markers to use in daily practice?	When IHC is needed for the subtyping of NSCC, TTF1 and p40 are the criterion standard, and these two markers are usually sufficient in clinical practice if there are no morphologic features of NE differentiation. p40 is preferable to p63 to identify squamous cell carcinoma
2. What extent of TTF1- and p40-positive reactions should we consider to be positive?	Focal positivity for TTF1 is considered a positive reaction indicating pulmonary adenocarcinoma in the proper clinical context, whereas for p40 the cutoff rate should be positivity in more than 50% of tumor nuclei. Focal or weak positivity for p40 is not diagnostic of squamous cell carcinoma
3. Are there any staining differences in lung adenocarcinoma between among TTF1 clones (SPT24, SP141, and 8G7G3/1)?	The staining performance of TTF1 varies among the clones. Among the most commonly used antibodies, 8G7G3/1 is the most specific antibody to identify lung adenocarcinoma
4. Should an NSCC that is diffusely positive for CK7 but negative for TTF1 and p40 be regarded as probably adenocarcinoma?	CK7 is not specific for adenocarcinoma; the marker can be seen in squamous cell carcinoma. The use of CK7 is discouraged for subtyping of NSCC
5. When should NE markers be applied to an NSCC?	NE markers should be applied only in support of NE morphology
6. What is the best antibody panel to differentiate NE tumors from other types of NSCC, and which one is the most reliable?	A panel of chromogranin A, synaptophysin, and CD56 is the best combination to identify NE tumors. The staining significance of each antibody varies among the sample types, histologic subtypes, and extent and/or intensity of positive reactions
7. When should a proliferation marker be used in diagnosis?	The main established role of Ki-67 in lung carcinomas is to help distinguish carcinoids from high-grade NE carcinomas (large cell NE carcinoma and small cell carcinomas), especially in small or crushed biopsy or cytologic samples. The role of Ki-67 in separating typical from atypical carcinoids is not established and needs more investigation
8. Is IHC useful to render a specific diagnosis of uncommon lung cancer subtypes (sarcomatoid carcinoma, salivary gland-type tumors, and NUT carcinoma)?	Currently, IHC and molecular testing are needed to achieve the definitive diagnoses of uncommon lung cancers such as sarcomatoid carcinoma, salivary gland-type tumors, and NUT carcinoma and to distinguish from the mimics.
9. What portion of the cytologic sample is best for immunostaining: the cell block, the air-dried smears, or the ethanol-fixed smears? Can destained smears be used adequately?	All cytologic preparations, including cell blocks and ethanol-fixed and air-dried slides, can principally be used for immunostaining. Formalin-fixed cell blocks are most straightforward, whereas rigorous protocol optimization, validation, and quality control are required in immunostaining in cytologic examination
10. Which IHC panel is recommended to differentiate lung mucinous adenocarcinoma from metastatic mimics?	There is no useful marker to differentiate pulmonary mucinous adenocarcinoma from metastatic mimics. A clinicopathologic tumor board is crucial for this clinical context
11. Are there any IHC or other markers to differentiate between primary lung cancers and metastases; between squamous cell carcinomas of lung primary and metastases from thymic, head and neck, endocervical, and the other cancers; and between adenocarcinomas of primary and metastases from gynecologic, mammary, uroepithelial, nonpulmonary NE, prostate, and liver cancers?	In this clinical context, morphologic comparison with prior tumor is crucial. There are no absolute IHC markers to make the differential diagnosis, and pathologists should be aware of the pitfalls of IHC

CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); CK7, cytokeratin 7; IHC, immunohistochemistry; NE, neuroendocrine; NSCC, non-small cell carcinoma; NUT, nuclear protein in testis; TTF1, thyroid transcription factor 1.

practice if there are no morphologic features of neuroendocrine (NE) differentiation. p40 is preferable to p63 to identify squamous cell carcinoma.

The value of IHC testing depends on the probability of the proposed diagnosis, which is a combination of clinical findings and histologic type. The choice and number of markers are heavily dependent on these assessments. When focused on a tumor of likely lung origin for which the main question is subtyping of adenocarcinoma versus squamous carcinoma, recommendations for a limited panel include TTF1 and p40.

TTF1 is a critical single marker for adenocarcinoma, as is p40 for squamous cell carcinoma,[1] with napsin A also showing some diagnostic utility as a secondary marker for adenocarcinoma. When compared with corresponding surgical resection, TTF1 had slightly better performance than napsin A, whereas a combination of TTF1 and napsin A may yield greater sensitivity for adenocarcinoma.[4] However, based on our experience, most cases do not require both markers; thus, TTF1 is an essential marker for adenocarcinoma in the routine case, whereas a larger panel can be used in challenging cases.

Some reports have shown better performance of napsin A than TTF1,[5] with greater sensitivity[6]; however, TTF1 as a nuclear stain can make interpretation more straightforward. Also, the staining performance of napsin A differs between monoclonal and polyclonal antibodies (as discussed in Key Question 10). In TTF1-positive non–small cell tumors, napsin A may play a role in classification of NE tumors, as it may be positive in a subset of large cell NE carcinomas (LCNECs), which are molecularly similar to adenocarcinoma, helping to separate them from high-grade NE tumors such as small cell carcinoma, which are typically napsin A–negative.[7,8] The use of surfactant protein A is discouraged, as its performance is inferior.[9,10]

As a marker of squamous cell carcinoma, p63 was more frequently used in IHC analysis before introduction of the p40 antibody. A number of studies showed that TTF1 and p63 were the most useful markers in distinguishing adenocarcinoma from squamous cell carcinoma.[11,12] However, the use of p40 IHC, which targets a splice variant of p63, is more specific and has a sensitivity comparable to that of p63 in determination of the squamous histologic type.[13,14] For example, as many as 20% to 30% of lung adenocarcinomas can be immunoreactive with p63. Although this reaction is usually weak to moderate in a minority of cells, rare cases, including cases of ALK receptor tyrosine kinase–positive adenocarcinoma, show more diffuse staining, (Fig. 1).[15] p63 staining can also be seen in some sarcomas, myoepithelial tumors, and lymphomas. p63-positive tumors that are TTF1 negative, even if the staining is diffusely positive, should not be assumed to be squamous cell

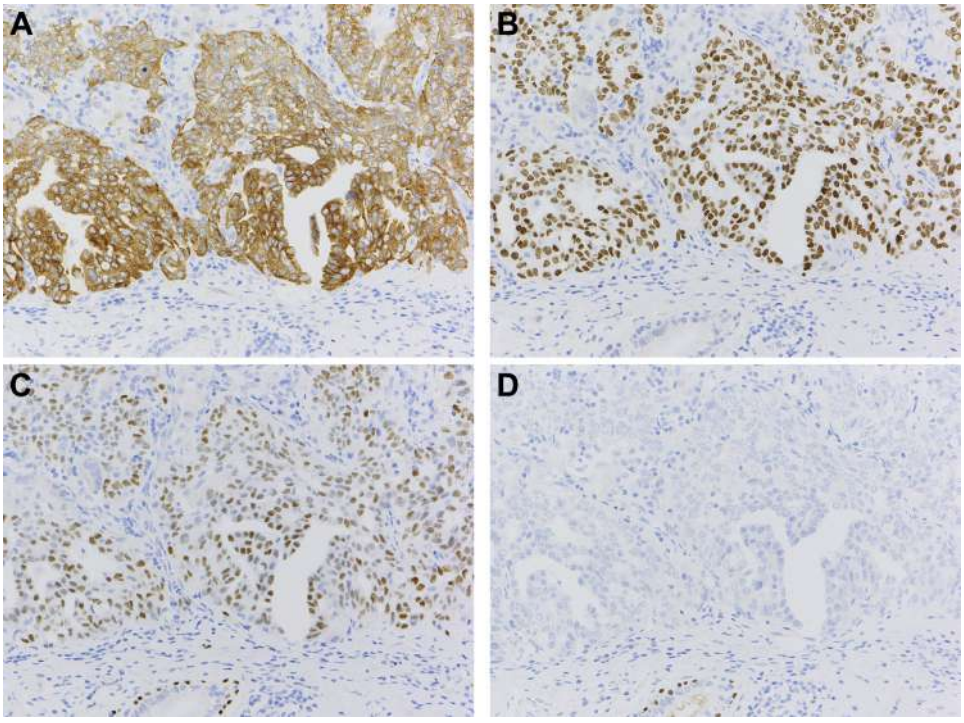

Figure 1. ALK receptor tyrosine kinase (ALK)-positive adenocarcinoma of the lung (*A*). A vast majority of ALK positive lung cancers are also positive for thyroid transcription factor 1, as in this case (*B*). Another characteristic of ALK-positive tumor is discordant expression between p63 (*C*) and p40 (*D*), which can be a pitfall when p63 is used alone as a marker of squamous cell carcinoma.

Table 2. Commonly Used Antibodies in Lung Cancer

A. Markers in the differential diagnosis of carcinoma of likely lung origin
TTF1, p40: best for daily practice
CD56, synaptophysin, chromogranin A: when NE morphologic features are identified
B. Markers in the differential diagnosis of adenocarcinoma (e.g., TTF1-negative or unknown primary)
TTF1, PAX8, GATA3, CDX2, CK7, CK20, (PSMA or NKX3-1 for men)
C. Markers for epithelioid undifferentiated neoplasms
Pan-cytokeratin (AE1/AE3, CAM5.2), S100, desmin, SMA, CD34, CD31, CD45
Calretinin, OCT4 (specific settings: tumor distribution, age)

CD31, an alias for PECAM1 (platelet and endothelial cell adhesion molecule 1); CD34, CD34 molecule; CD45, an alias for PTPRC (protein tyrosine kinase phosphatase, receptor type C); CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); CDX2, caudal; type homeobox 2; CK7, cytokeratin 7; CK20, cytokeratin 20; GATA3, GATA binding protein; NE, neuroendocrine; NKX3-1, NK3 homeobox 1; OCT4, octamer-binding transcription factor 4; PAX8, paired box 8; PSMA, prostatic specific membrane antigen and an alias for FOLH1 (folate hydrolase 1); SMA, smooth muscle actin; TTF1, thyroid transcription factor 1.

carcinomas, as the result of subsequent p40 staining may be negative, which would favor the diagnosis of NSCC not otherwise specified (NOS). It has been observed that p40 IHC is less likely to stain p63-positive lung adenocarcinoma, sarcomas, and lymphomas, with only an occasional adenocarcinoma showing weak and focal p40 staining. The extent of staining required to define positivity is discussed in the next section.

In studies of cases involving diagnostically difficult biopsy samples with resection confirmation, p40 has higher sensitivity and specificity for squamous carcinoma when compared with cytokeratin 5/6 (CK5/6).[16] Therefore, p40 IHC emerges as a critical marker in the classification of carcinomas of lung primary, with p63 as an alternative and CK5/6 in difficult cases (Table 2).

2. What extent of TTF1- and p40-positive reactions should we consider to be positive?

Short Answer. *Focal positivity for TTF1 is considered a positive reaction indicating pulmonary adenocarcinoma in the proper clinical context, whereas for p40 the cutoff rate should be positivity in more than 50% of tumor nuclei. Focal or weak positivity for p40 is not diagnostic of squamous cell carcinoma.*

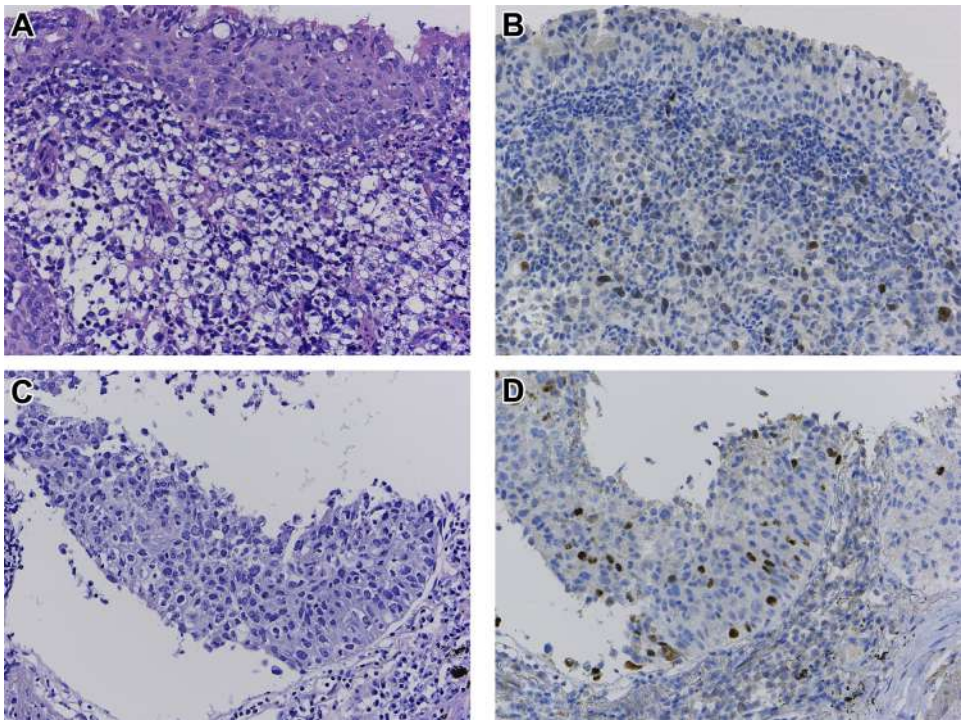

Figure 2. (*A–B*) A case with focal positivity of thyroid transcription factor 1 (TTF1). Histologically, the tumor cells do not show clear morphologic differentiation (*A*). The displayed TTF1 staining (*B*) should be evaluated as positive; thus, the tumor is diagnosed as non-small cell carcinoma, favor adenocarcinoma. (*C–D*) Another case with unclear morphologic differentiation (*C*). As the definition of a positive reaction with p40 is defined as 50% or more positive staining of the tumor cells, the widely scattered but sparse positive reaction with p40 (*D*) should not be considered as a definite diagnosis of squamous cell carcinoma (*D*).

As with other diagnostic immunomarkers, the sensitivity and specificity of TTF1 are dependent on the context in which they are applied, the clone used (see Key Question 3), and the staining techniques and protocols. In most studies, approximately 75% to 80% of adenocarcinomas are positive for TTF1, whereas adenocarcinoma with mucinous features tends to be negative for TTF1.[17,18] Regarding TTF1 immunoreactivity, focal positivity is considered a positive reaction (Fig. 2), which is indicative of adenocarcinoma in the proper clinical context. Indeed, it has been reported that with a cutoff value of more than 5% weak or strong positivity, the reaction reached a sensitivity of 0.8 and a specificity of 0.9.[19] In cases with only focal TTF1 tumor cell staining and a substantial TTF1-negative solid pattern component, p40 staining should be performed to pursue possible adenosquamous differentiation.

Unlike for TTF1, focal and weak positivity for p40 is not diagnostic of squamous cell carcinoma because focal positivity for p40 can be seen in adenocarcinomas and other tumor types. The cutoff value for p40 should be positivity in more than 50% of tumor nuclei. Positivity in less than 10% should not be used for diagnostic classification. A range of 10% to 50% positivity is a matter of discussion and dependent on the clinical context and the intensity of staining (see Fig. 2). Of note, the keratinizing component is often negative for p40, and therefore, negative staining of the component does not exclude the diagnosis of squamous cell carcinoma. However, keratinization is a diagnostic criterion for squamous cell carcinoma, so if it is present, IHC is not required. In indeterminate cases, it is recommended that the 2015 WHO terminology of NSCC NOS be used, but use of NSCC NOS should be minimized.

Another important consideration is the criterion for adenosquamous carcinoma with respect to TTF1 and p40/p63 evaluation. First, this diagnosis cannot be made without a resection specimen, and in small biopsy samples, the possibility can be raised if two distinct cell populations are present. If each component is morphologically differentiated with glandular patterns for adenocarcinoma and keratinizing squamous cell carcinoma, IHC may not be needed to suggest the diagnosis. However, if one or both components consist of solid patterns, immunoreactivity for each marker should be seen in different components or areas of the tumor. Conversely, double positivity (TTF1 and p40/p63) in the same cell does not define adenosquamous carcinoma. It has been reported that such tumors should probably be classified as NSCC, favor adenocarcinoma,[20,21] although selection of the antibody clone may cause such reactions, as discussed later.

Another challenging situation is the recurrence of *EGFR*-mutated adenocarcinomas after targeted therapy, resulting in a pure squamous cell carcinoma that may be p40 positive and TTF1 negative while retaining the original *EGFR* mutation, sometimes with an additional T790M mutation.[22,23] This transition of histologic differentiation may represent a mechanism of resistance to tyrosine kinase inhibitors.

3. Are there any staining differences in lung adenocarcinoma between TTF1 clones (SPT24, SP141, and 8G7G3/1)?

Short Answer. *The staining performance of TTF1 varies among the clones. Among the most frequently used antibodies, 8G7G3/1 is the most specific antibody to identify lung adenocarcinoma.*

A number of different TTF1 clones are commercially available, including rabbit and goat polyclonal antibodies, mouse monoclonal antibodies (including the clones 8G7G3/1, SPT24, BGX-397A, SMP150, and 5S143), and rabbit monoclonal antibodies (including the clones SP141, EP15844, C12-I, and G21-G).[24] However, the mouse monoclonal antibodies 8G7G3/1 and SPT24, and the more recently available rabbit monoclonal antibody SP141, are the most widely used in clinical practice.[4,19,24,25]

There are two clinical benefits of TTF1 staining: the differential diagnosis of lung adenocarcinoma from squamous cell carcinoma, and the distinction of primary lung adenocarcinoma from nonpulmonary carcinoma, both of which require specificity of staining. However, sensitivity and specificity are always part of a trade-off.

Focusing on TTF1 and the distinction between lung adenocarcinoma and squamous cell carcinoma, a review of the current literature[24] revealed that the 8G7G3/1 clone was less sensitive for the detection of lung adenocarcinoma than the SPT24 clone was (Table 3).[24,26,27] However, with regard to TTF1 staining in lung squamous cell carcinoma, the specificity for adenocarcinoma is higher in 8G7G3/1 than in SPT24

Table 3. TTF1 Expression in Lung Adenocarcinoma and Squamous Cell Carcinoma

	8G7G3/1		SPT24	
Carcinoma	n	Positive, n (%)	n	Positive, n (%)
Lung adenocarcinoma[24]	2614	2004 (76.7%)	579	471 (81.3%)
Squamous cell carcinoma[a]				
Kadota et al.[26]	449	0 (0%)	448	27 (6.0%)
Kashima et al.[27]	38	1 (3%) with EnVision[b]	38	5 (13%) with EnVision[a]
	38	4 (11%) with CSA-II[c]	38	20 (53%) with CSA-II[a]

[a]Studies, directly compared with 8G7G3/1 and SPT24 in identical series.
[b]Envision is manufactured by Dako (Copenhagen, Denmark).
[c]CSA-II is manufactured by Roche Ventana (Tucson, Arizona).
TTF1, thyroid transcription factor 1.

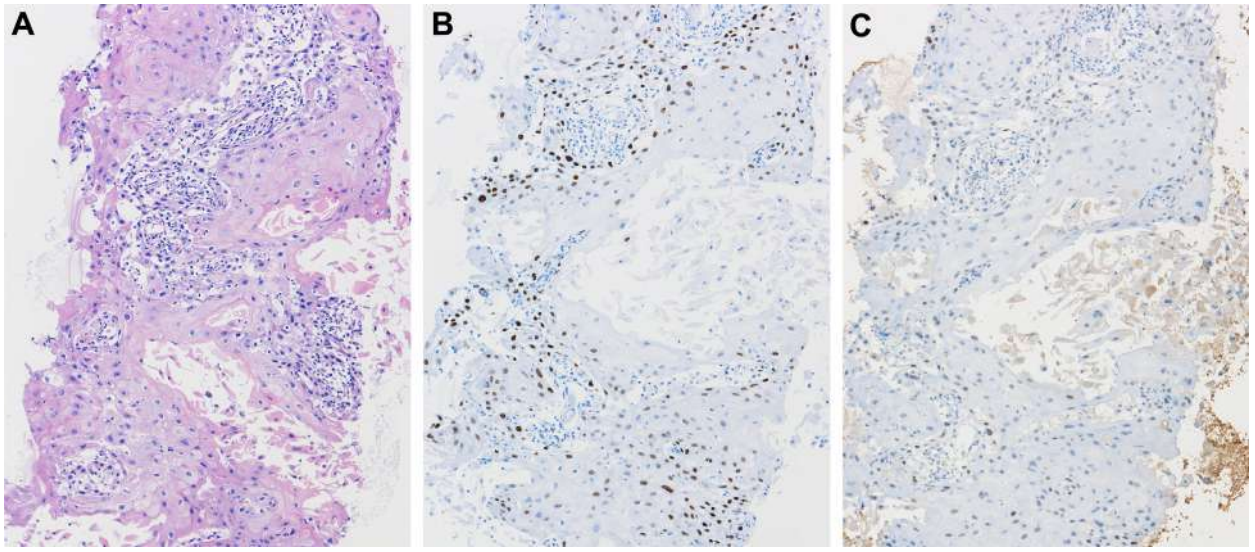

Figure 3. Thyroid transcription factor 1 expression according to antibody clones in primary squamous cell carcinoma of the lung (hematoxylin and eosin staining) (*A*). Positive reactions with clone SPT24 staining (*B*) is contrasted with weak or negative with clone 8G7G3/1 (*C*).

(Fig. 3). It is noted that a certain percentage of squamous cell carcinomas are labeled with TTF1, particularly when a signal amplification system is applied[27]; the frequency of positivity in squamous cell carcinoma is higher with SPT24.

When TTF1 is used for the differential diagnosis between primary lung adenocarcinoma and other cancers, the characteristics of the antibody clone in use should be considered. It has been reported that a small percentage of nonpulmonary tumors can be positive for TTF1 (Table 4) and the SPT24 clone has lower specificity for the detection of lung adenocarcinoma than the 8G7G3/1 clone does (see Table 4).[24] There is not as much literature examining the staining performance of the newer SP141 clone, but some reports have suggested that SP141 has characteristics similar to those of the clone SPT24.[25,28] So even in the case of TTF1-positive adenocarcinomas in the lung, it is important for pathologists to be aware of previous extrathoracic carcinomas and, in particular, tumors with unusual morphologic features, to pursue additional IHC markers to address other primary sites.

Table 4. Results of TTF1 Expression in Tumors from Primary Sites, Including the Female Genital Tract, Breast, Colon, and Stomach in Some Published Studies in the Literature

	8G7G3/1		SPT24	
Primary Carcinoma	n	Positive, n (%)	n	Positive, n (%)
Ovarian carcinoma	615	22 (3.6%)	161	16 (9.9%)
Endometrial adenocarcinoma	215	17 (7.9%)	68	19 (27.9%)
Uterine cervical adenocarcinoma	92	3 (3.3%)	39	6 (15.4% [breast])
Uterine cervical squamous carcinoma	7	0 (0%)		
Breast adenocarcinoma	297	4 (1.5%)	580	13 (2.4%)
Colon adenocarcinoma	594	11 (1.8%)	258	15 (5.8%)
Gastric adenocarcinoma	170	3 (1.8%)	110	1 (0.9%)

Modified based on data by Ordonez.[24]
TTF1, thyroid transcription factor 1.

There are several preanalytic considerations in regard to TTF1 immunostaining that deserve special mention. Gruchy et al. found reduced or absent TTF1 immunostaining in cytologic specimens fixed in alcohol-based fixatives, including CytoLyt (Hologic, Marlborough MA), and in surgical pathologic specimens that are subjected to decalcifying agents such as formic or hydrochloric acid.[29] This reduction in staining with TTF1 was not seen in specimens that were fixed only with routine 10% buffered formalin. It should be stressed that IHC protocols need to be validated on control tissues that are subjected to the same preanalytic conditions as the test tissue, including fixation in alcohol-based fixatives and decalcification treatments even using gentler ethylenediaminetetraacetic acid (EDTA)–based solutions.

4. Should an NSCC that is diffusely positive for CK7 but negative for TTF1 and p40 be regarded as probably adenocarcinoma?

Short Answer. *Cytokeratin 7 (CK7) is not specific for adenocarcinoma; the marker can be seen in squamous cell carcinoma. The use of CK7 is discouraged for subtyping of NSCC.*

CK7 is a 54-KDa protein that is present in simple epithelia and lung alveoli and expressed in 94% to 100% of lung adenocarcinomas. Almost all studies have demonstrated high sensitivity of CK7 for diagnosing

Table 5. Summary of CK7 Expression in Lung Cancer

Studies	Type of Samples	Primary Lung ADC	Primary Lung SqCC	Primary Other NSCC	Sensitivity for ADC	Specificity for ADC	PPV for ADC	NPV for ADCor ADC
Lyda and Weiss[30]	Surgical specimens	31 of 33 (94%)	8 of 37 (22%)	2 of 6 LCNEC (33%), 5 of 9 LCC (56%)				
Johansson[31]	Surgical specimens	11 of 11 (100%)	4 of 12 (33%)	8 of 9 LCC or pleomorphic carcinoma				
Camilo et al.[32]	Surgical specimens	16 of 17 (91%)	1 of 18 (5,5%)	3 of 5 LCC (60%)				
Mukhopadhyay and Katzenstein[33]	Biopsies versus surgical specimens	19 of 19 (100%)	9 of 15 (60%)	3 of 4 LCC (75%)	100%	37%	61%	100%
Warth et al.[18]	TMA from surgical specimens	509 of 530 (96%)	96 of 456 (21%)	47 ASC (89%), 60 LCC (71%), 31 sarcomatoid carcinoma (68%)	96%	79%	84%	94%
Kimbrell et al.[34]	Cytologic vs. surgical specimens	8 of 8 (100%)	7 of 9 (77%)	6 of 6 LCC (100%) 3 of 3 ASC (100%)				
Noh and Shim[35]	TMA (poorly differentiated areas)	32 of 36 (92%)	5 of 38 (13%)	6 of 8 LCC (75%)	92%	76%	75%	92%
Righi et al.[36]	Cell blocks (FNAC examination) versus surgical specimens	66 of 66 (100%)	15 of 24 (62%)	10 of 13 (1 of 1 ASC, 9 of 12 LCC and sarcomatoid carcinoma)	100%	92%	92%	100%
Koh et al.[37]	TMA from surgical specimens	107 of 108 (99%)	15 of 59 (25%)	12 of 17 incl. 4 ASC, 4 pleomorphic carcinoma, 9 LCC	99%	65%	81%	98%
Gurda et al.[38]	Cell blocks (FNAC examination)	45 of 48 (94%)	7 of 14 (50%)		94%	50%	86%	70%
Sekar et al.[39]	Cell blocks (FNAC examination)	15 of 15 (100%)	1 of 15 (6%)	17 of 30 other NSCC (56%)	100%	93%	94%	100%

ADC, adenocarcinoma; ASC, adenosquamous carcinoma; CK7, cytokeratin 7; FNAC, fine needle aspiration cytologic; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; NPV, negative predictive value (true negative/true negative plus false-negative); NSCC, non-small cell carcinoma; PPV, positive predictive value (true positive/true positive plus false-positive); SqCC, squamous cell carcinoma; TMA, tissue microarray.

lung adenocarcinoma, ranging from 92% to 100%, thus contrasting with its low specificity (ranging from 50% to 93%) when compared with other markers such as napsin A and TTF1 (Table 5).[18,30–39] Indeed, according to a recent series, 5% to 77% (mean 25%) of squamous cell carcinoma can display CK7 staining (Fig. 4) that is often weaker than the staining observed in adenocarcinoma.[34] Most adenosquamous carcinomas, large cell carcinomas, pleomorphic carcinomas, and LCNECs can also express CK7.[40–42] Of note, CK7 expression, in contrast to TTF1 expression, is not restricted to adenocarcinoma arising from the lung and is widely expressed by other tumors such as breast carcinoma. For these reasons and to spare the tissue for potential molecular testing, the International Association of Lung Cancer Study pathology panel discourages the use of CK7 as a marker of glandular differentiation or a marker of a lung primary.

According to the 2015 WHO classification, if there are no morphologic findings, mucin stains, or IHC markers supporting adenocarcinoma or squamous cell carcinoma, the tumor should be classified as NSCC NOS in small biopsy specimens. In the absence of TTF1 or p40 expression, CK5/6 and CK7 cannot distinguish between adenocarcinoma and squamous cell carcinoma when used alone.[36] NSCC that demonstrates only CK7 expression and is negative or equivocal for other markers (i.e., TTF1, napsin A, and p40) should be considered as NSCC NOS).[43] However, some pulmonary large cell carcinomas that lacked squamous differentiation (e.g., keratinization and/or intercellular bridge) with negative-TTF1 and equivocal or negative-p40 were reported to be clinicopathologically and genetically indistinguishable from the solid subtype of adenocarcinoma.[44–46] In a series of 315 surgical specimens, TTF1-negative, p63-negative, CK5/6-negative tumors accounted for 10% of adenocarcinomas.[47] For these reasons, molecular testing is still recommended in the 2015 WHO classification in the case of a CK5/6-negative, CK7-positive, TTF1-negative, p40-negative, mucicarmine-negative NSCC.[1] All suspected NSCC NOS should be tested for keratin to confirm carcinoma differentiation and if the result is negative, worked up for metastatic melanoma, lymphoma, sarcoma, and epithelioid hemangioendothelioma.

The incidence of TTF1-negative adenocarcinoma is reported in the literature to range from 0% to 47%, with a mean percentage of 15% (see Table 3).[18,31,33,35–39] TTF1 negativity correlates with invasive mucinous adenocarcinoma and solid adenocarcinoma with mucin (Fig. 5), with only 10% to 15% of mucinous adenocarcinoma being TTF1 positive.

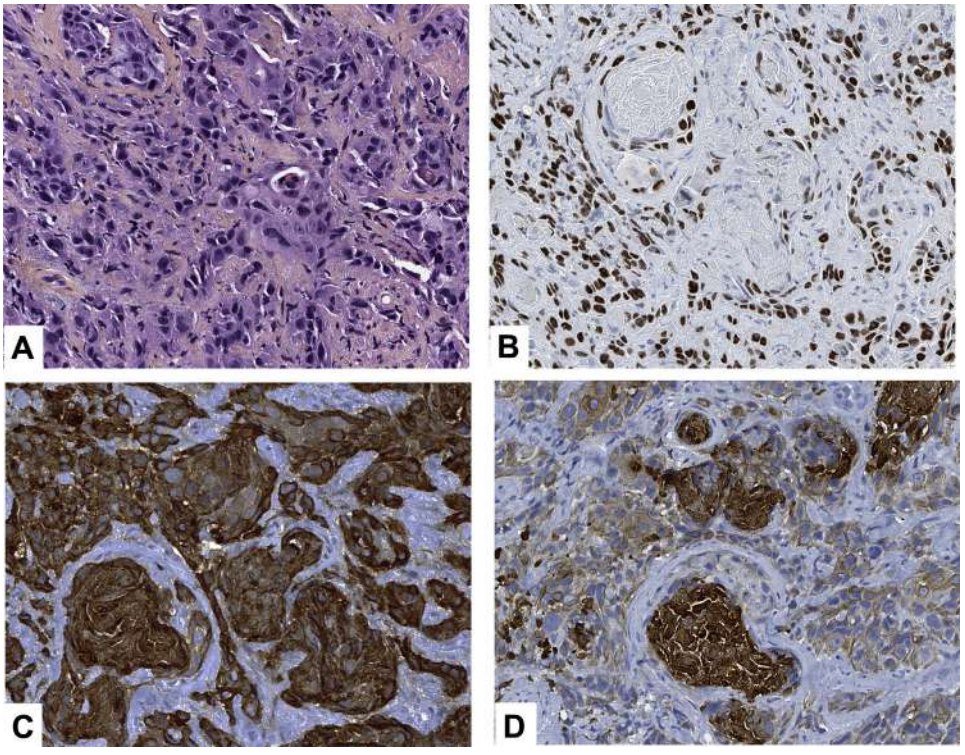

Figure 4. Cytokeratin expression of squamous cell carcinoma in a bronchial biopsy specimen (*A*). Nuclear staining with p40 (BC28 Ab) supports the diagnosis (*B*). The results of staining with both cytokeratin 7 (OV-TL12/30 antibody) (*C*) and cytokeratin 5/6 (D5/16B4 antibody) (*D*) are positive.

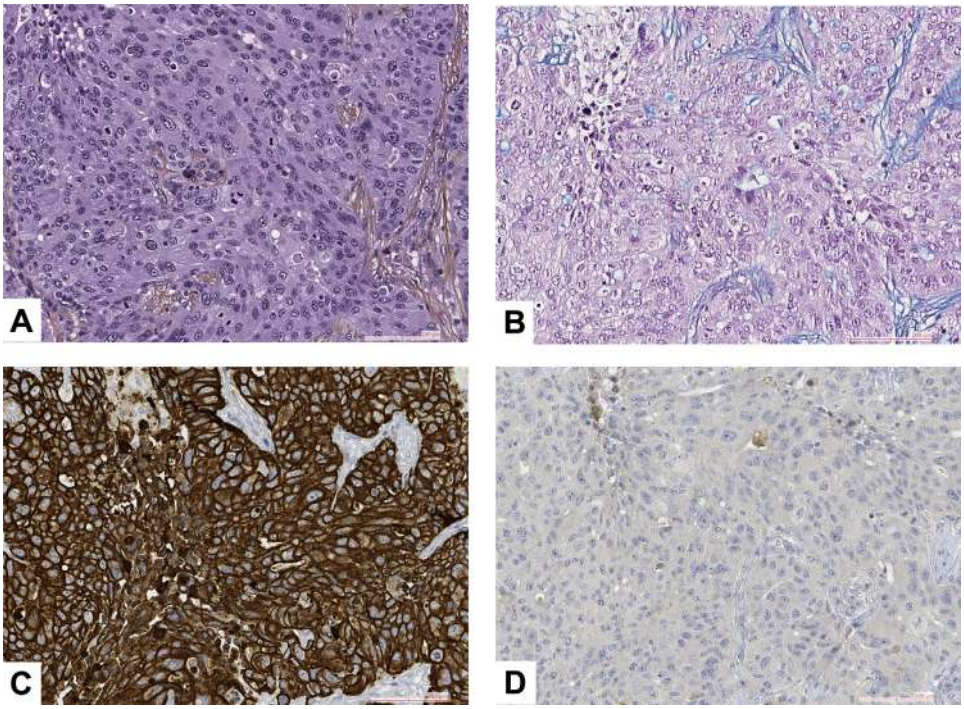

Figure 5. Solid adenocarcinoma of the lung (*A*), which is diagnosed by numerous intracytoplasmic vacuoles of mucus within the cytoplasm of tumor cells with Alcian blue staining (*B*). Diffuse expression of cytokeratin 7 (OV-TL12/30) (*C*) and absence of expression of thyroid transcription factor 1 (8G7G3/1 antibody) (*D*) are noted.

5. When should NE markers be applied to an NSCC?

Short Answer. *NE markers should be applied only in support of NE morphology*

The current WHO classification recommends that staining for NE markers be performed only when NE morphologic features (organoid nesting, rosette-like structures, palisading patterns, etc.) are present.[1,48] Positive NE markers may be detected by light microscopy in approximately 10% to 30% of NSCCs without overt NE morphology. Such tumors may be termed *NSCC with NE differentiation*; however, it is recommended that resected tumors be classified primarily as squamous cell carcinoma, adenocarcinoma, or large cell carcinoma, as applicable, with a comment regarding the positive NE markers (Fig. 6).[1,48] On the basis of a lack of consistent data supporting the clinical relevance of positive NE markers in the absence of NE morphology, NE marker staining is not recommend for tumors lacking NE morphologic features.[49–51] In small biopsy specimens showing NSCC with NE morphology, staining for NE markers should be performed, and if the result is positive, the diagnosis of NSCC, favor LCNEC is recommended.[1,48] If NE morphologic features are present and the results of staining for the markers are negative, the terminology of *NSCC with NE morphology* should be used, with a comment that LCNEC is suspected but stains failed to demonstrate NE differentiation. Given that NE morphologic features may not be appreciated on a small biopsy or cytologic sample, there is a potential for cases of LCNEC to be missed on small specimens. Even so, only when there is a suggestion of NE morphology and markers are positive should the prospect of LCNEC be raised.[1,52] A discussion of issues with NE antibodies, sensitivities, and specificities appears in Key Question 6.

6. What is the best antibody panel to differentiate NE tumors from other types of NSCC, and which one is the most reliable?

Short Answer. *A panel of chromogranin A, synaptophysin, and neural cell adhesion molecule 1 (NCAM 1 [also known by the alias CD56]) is the best combination to identify NE tumors. The staining significance of each antibody varies among the sample types, histologic subtypes, and extent and/or intensity of positive reactions.*

The 2015 WHO classification recognizes three markers for NE differentiation; they are chromogranin A, synaptophysin, and CD56.[48] As there is no clear cutoff for any of these NE markers, the interpretation should be rendered in the context of morphologic features, sample types (cytologic, biopsy, or surgical specimens) and extent of positive reactions.

Chromogranin A and synaptophysin[53] are true markers of NE differentiation, as their epitopes are part of neurosecretory granules or of synaptic vescicles.[54] More chromogranin A staining can be detected in

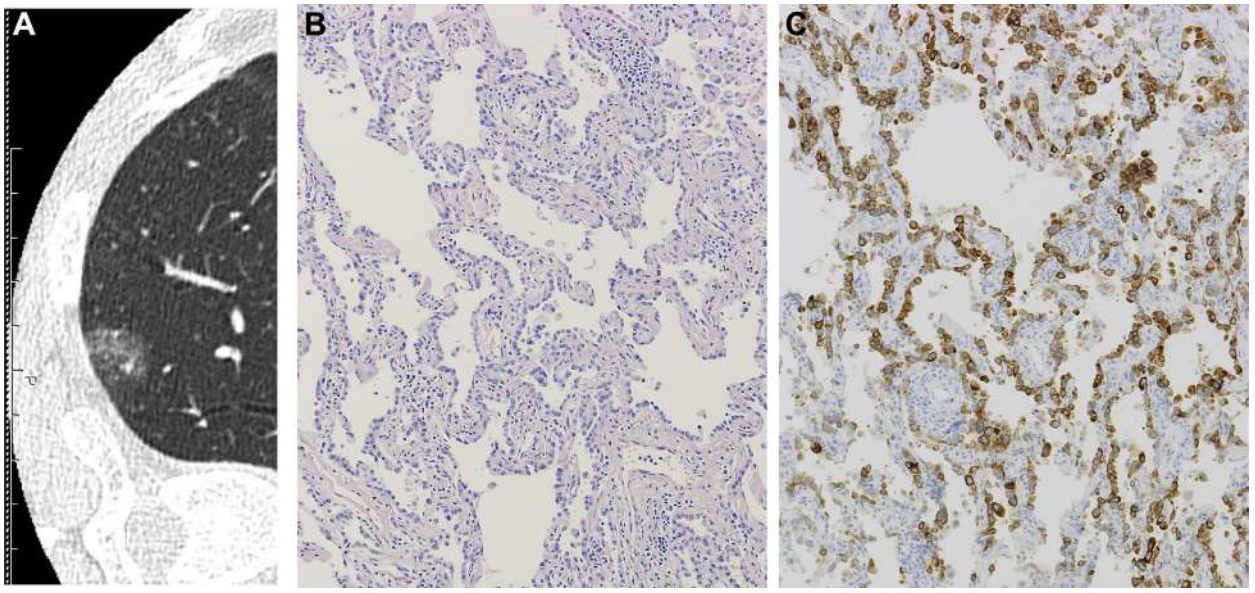

Figure 6. A lesion of typical ground glass attenuation on computed tomography image (*A*) was surgically removed and histologically shows adenocarcinoma (*B*). This tumor has diffuse expression of synaptophysin (*C*). Despite the diffuse expression, the tumor should be diagnosed as adenocarcinoma because it does not have any morphologic neuroendocrine features.

carcinoid tumors than in LCNECs or SCLCs. In carcinoids, chromogranin A usually strongly and diffusely stains the cytoplasm (Fig. 7). In contrast, in SCLCs focal chromogranin A positivity may be present in some but not all tumor cells (Fig. 8). This result should still be called positive for chromogranin A in case of SCLC. This same trend is also seen with synaptophysin (Table 6).[42,55–60] However, some SCLCs and LCNECs will show diffuse and strong expression of multiple NE markers, and this finding does not exclude these diagnoses.

Conversely, CD56 is the most sensitive for the diagnosis of SCLC, although 5% to 10% of SCLCs can be negative for all three NE markers. However, the expression is not specific for NE differentiation, as the protein is expressed on neurons, glia, hematopoietic cells (natural killer cells, $\gamma\delta$-T cells, activated CD8a molecule–positive T cells, and dendritic cells), and skeletal muscle. The lack of specificity implies that CD56 expression as an NE marker should be interpreted in the context of NE morphologic features with hematoxylin and eosin. The IHC pattern for CD56 in most SCLCs is strong membranous staining in all tumor cells (see Fig. 8). In tumors suspected to be SCLC or LCNEC that are TTF1 negative, a p40 stain should be performed to exclude basaloid squamous cell carcinoma.

The combination of NE morphologic features and positivity for any of these NE markers is suggestive of the diagnosis of NE tumor. Currently, there is no consensus as to whether one, two, or three markers should be used.[61] It is noted that between 10% and 20% of NSCCs are positive with one NE marker,[49,55] and it is not recommended that staining for NE IHC markers be routinely performed in poorly differentiated NSCCs that lack NE morphology, as there is no established clinical significance to this finding.[1] Most cases of LCNEC and SCLC are positive for two or more of these three NE markers.[49,55] Also, there is no clear cutoff value for the extent of positive NE marker reactions that we should consider to be positive. Our panel of experts usually accepts any amount of positive staining of any of these NE markers if the NE morphology are apparent. Of note, utilization of IHC in conjunction with morphologic features by hematoxylin and eosin staining increases the concordance between pathologists and their diagnostic confidence.[61]

In addition to these three markers, other NE markers are also known. Human ASH1 like histone lysine methyltransferase is biologically considered as a lineage marker of NE cells,[62,63] and it stains NE tumors specifically.[55,64,65] However, the sensitivity is not sufficiently high (similar to that of Leu7 [CD57]). Polyclonal neuron-specific enolase has high sensitivity but is no longer used because of low specificity. Recent studies of insulinoma-associated protein 1 (INSM1) suggest that it may be a promising addition to the available panel of stains because of its high specificity and sensitivity for labeling an entire spectrum of NE tumors independent of the originating organs and histologic grades.[66–68] Although it is not clear that adding insulinoma-associated protein 1 (INSM1) improves the currently recommended panel of chromogranin A, synaptophysin, and CD56 in detecting NE differentiation, the nuclear staining pattern may enable more straightforward interpretation.[66]

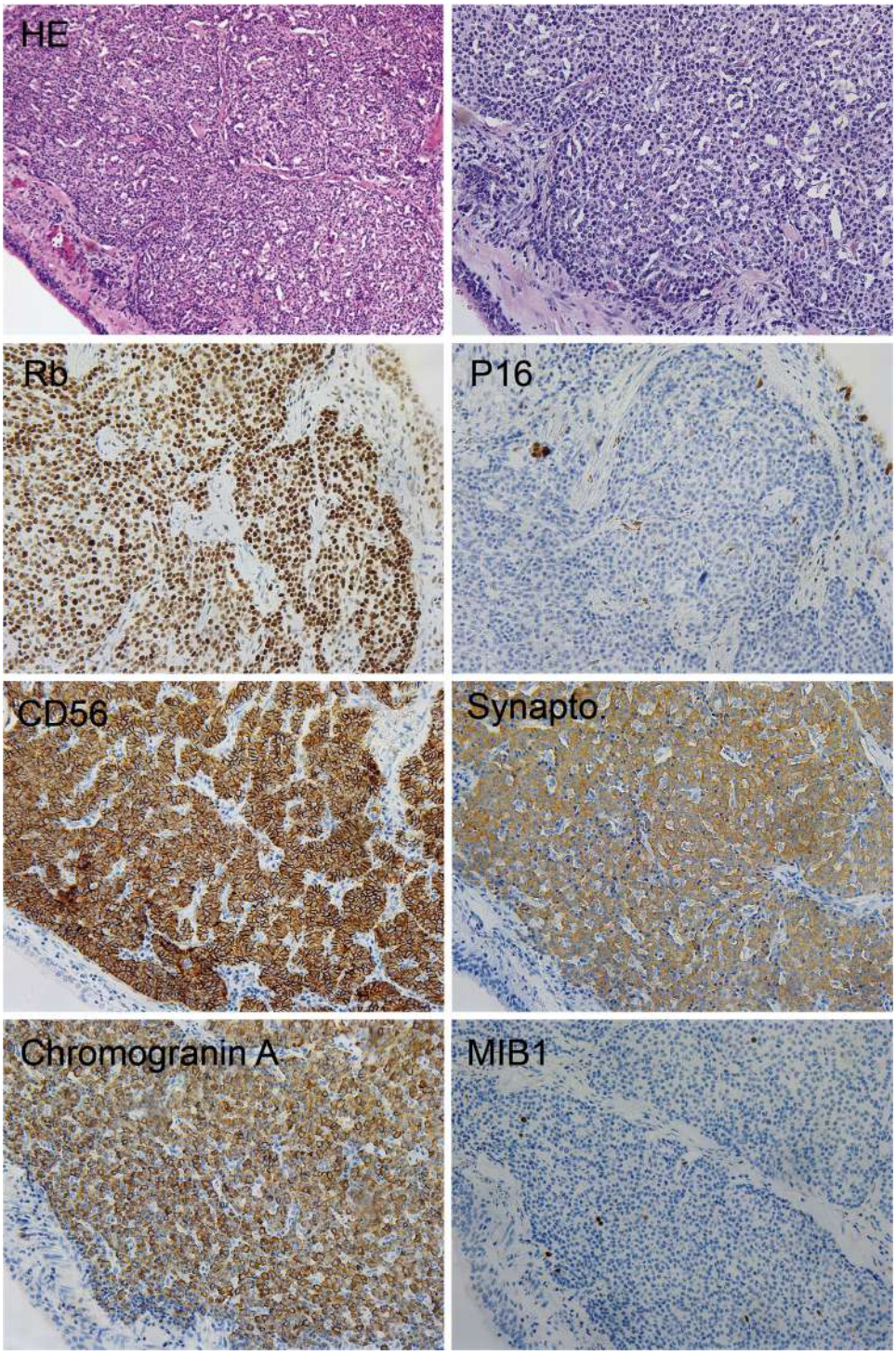

Figure 7. Typical immunoprofile of carcinoid tumor. Note the homogenous distribution of tumor cells, strong staining for three neuroendocrine markers, and MIB1 immunoreactions. HE, hematoxylin and eosin; Synapto, synaptophysin.

7. When should a proliferation marker be used in diagnosis?

Short Answer. *The main established role of Ki-67 in lung carcinomas is to help distinguish carcinoids from high-grade NE carcinomas (LCNEC and small cell carcinomas), especially in small or crushed biopsy and/or cytologic samples. The role of Ki-67 in separating typical from atypical carcinoids is not established and needs more investigation.*

The marker of cell proliferation Ki-67 protein (henceforth simply Ki-67) is the product of marker of proliferation Ki-67 gene (*MKI67*) gene mapping to the 10q26.2 gene, which is involved in all active stages of the

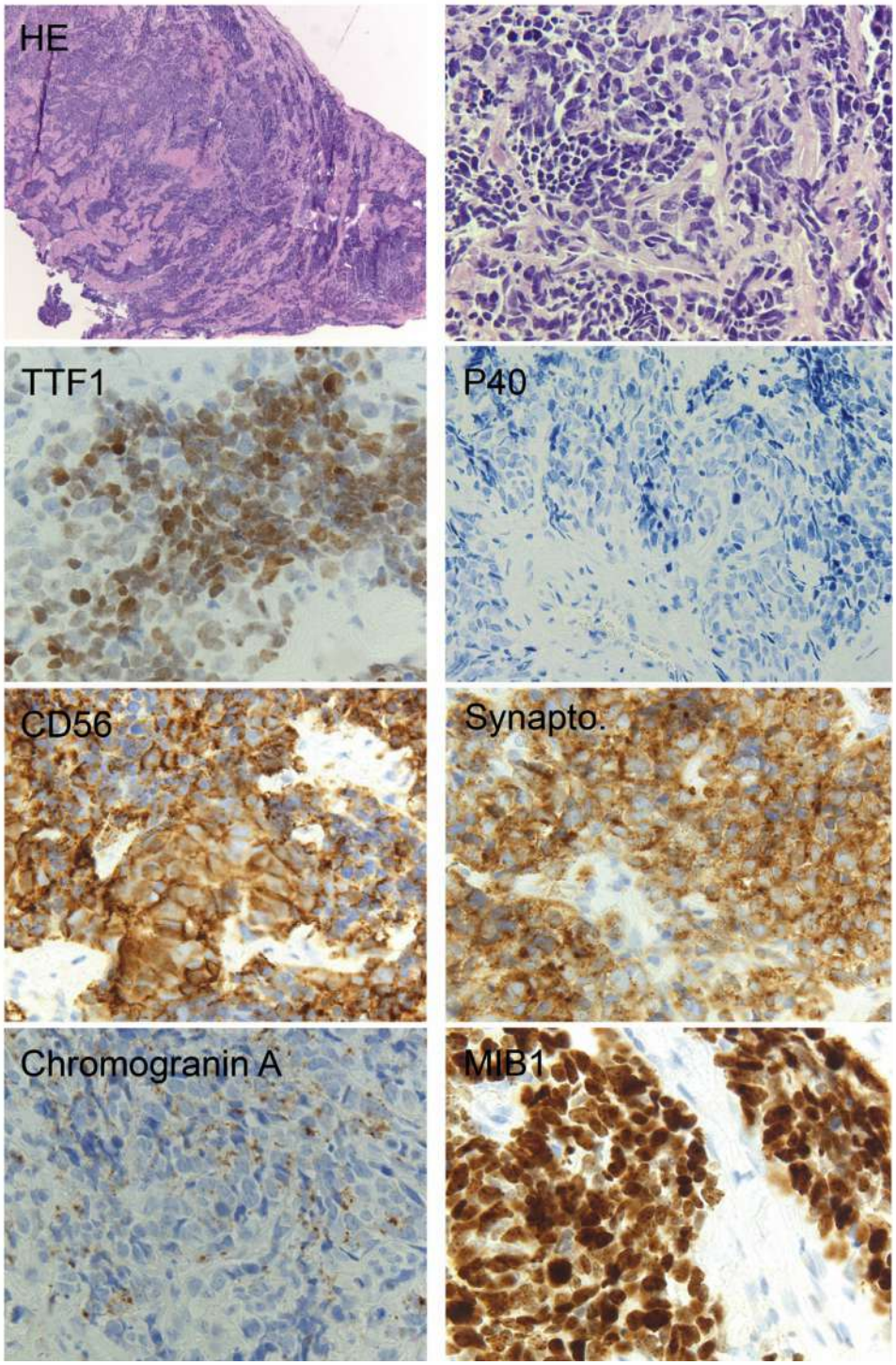

Figure 8. Typical immunoprofile of SCLC. Note the irregular distribution of tumor cells, strong staining for two neuroendocrine markers, and dot-like positivity with chromogranin A. Also, the level of MIB1 labeling is high. HE, hematoxylin and eosin; TTF1, thyroid transcription factor 1; Synapto, synaptophysin.

cell cycle with a maximum in M phase, but not in resting or senescent cells.[69,70] Several antibodies to Ki-67 are available for paraffin-embedded sections, but the MIB-1 clone is ranked as the most widely used reagent after antigenicity recovery systems.[71] Ki-67 expression may be scored semiquantitatively as a percentage of positive cells (labeling index) upon manual counting of 500 to 2000 tumor cells, areas spanning 2 mm^2, or eyeballing estimation on hot spot areas, but a standard scoring method for Ki-67 has not been established for lung cancer.[72]

Table 6. Sensitivity (Median, Range) for the Three Neuroendocrine IHC Markers in the Lung for SCLC, LCNEC, Typical Carcinoids, and Atypical Carcinoids, according to the References with 10 or More Cases of a Histologic Type

IHC Marker	SCLC[55-60]	LCNEC[42,55,58,59]	Typical Carcinoid[55,57,58]	Atypical Carcinoid[55]
Chromogranin A	47% (4%-58%)	41% (9-85%)	97% (93-100%)	79%
Synaptophysin	67% (57%-83%)	69% (62-82%)	97% (96-100%)	79%
CD56	97% (79%-100%)	53% (36-100%)	83% (60-100%)	57%

CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); IHC, immunohistochemistry; LCNEC, large cell neuroendocrine carcinoma;

In lung NE tumor, the main diagnostic role for Ki-67 is in helping to distinguish carcinoids from the high-grade SCLCs and LCNECs in small crushed biopsy specimens (Fig. 9). There are no clear thresholds for separation of typical carcinoid and atypical [AC] carcinoid, with some data suggesting that typical carcinoid has a range of 2.3% to 4.15% and AC ranges from 9% to 17.8%.[71] The cutoff for distinguishing AC from high-grade tumor has also not been firmly established,[71] but a range of 2.5% to 30% has been considered as the cutoff for distinguishing AC from LCNEC.

Although a cutoff of 20% was suggested as an upper limit for AC in the 2015 WHO classification, lower limits of 40% and 50% were suggested for LCNEC and SCLC, so more work is needed to determine how better to use Ki-67 to distinguish AC from high-grade NE carcinomas.

Ki-67 is not currently recommended for rendering a diagnosis or providing managerial information on NSCC. In general, Ki-67 has not consistently been shown to be a poor prognostic factor, although it tends to be increased in poorly differentiated tumors.[73] There may be differences in prognostic impact according to histologic type where the labeling index has been shown to correlate with poor outcomes in adenocarcinoma.[74,75] However, it may not correlate with prognosis in squamous cell carcinoma.[76]

A new marker, anti–phosphohistone H3, has emerged as a mitosis-specific marker that has been investigated in various types of human cancers,[77–79] but large studies on lung cancer are lacking.

8. Is IHC useful to render a specific diagnosis of uncommon lung cancer subtypes (sarcomatoid carcinoma, salivary gland-type tumors, and NUT carcinoma)?

Short Answer. *Currently, IHC and molecular testing are needed to achieve the definitive diagnoses of uncommon lung cancers such as sarcomatoid carcinoma, salivary gland-type tumors, and nuclear protein in testis (NUT) carcinoma and to distinguish from the mimics.*

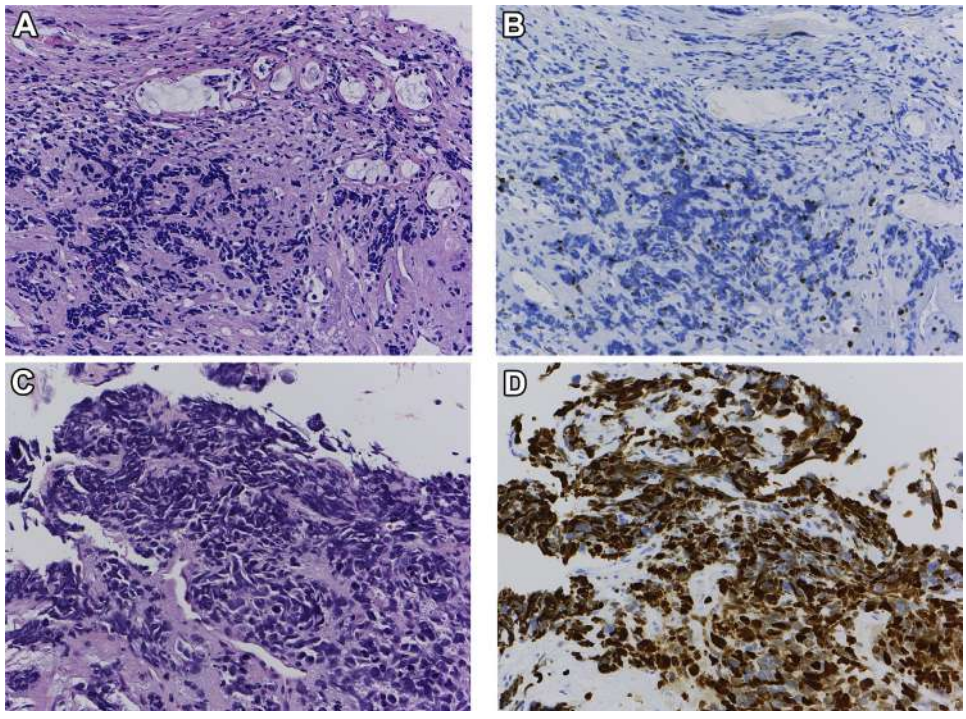

Figure 9. Transbronchial biopsy specimens often have crushed tumor cells as shown (*A* and *C*). In this situation, cytologic specimens, if available, can be more useful for diagnosis, and Ki-67 staining (*B* and *D*) can help with the differential diagnosis between carcinoid tumor (*A* and *B*) and small cell carcinoma (*C* and *D*).

Table 7. Specific Sarcomas That Mimic Sarcomatoid Carcinoma of the Lung in Thoracic Regions

Clinical Context	Sarcoma	Keratin Expression[a]	IHC Markers	References
Primary	Malignant solitary fibrous tumor	+/-	STAT6, CD34, BCL6	89-93
	Inflammatory Myofibroblastic tumor	+/-	ALK, SMA, FISH for *ALK*, *ROS1*, *RET*, *NTRK3* fusions	94-96
	Primary pleural synovial sarcoma	+/-	TLE1, focal keratins, FISH for SS18 fusions	97
Metastatic	Uterine leiomyosarcoma	+/-	SMA, ER, desmin	
	Dedifferentiated liposarcoma	-	CDK4, MDM2	
	Malignant peripheral nerve sheath tumor	-	SOX10, S100, H3K27me3 loss	
	Malignant melanoma	-	SOX10, Melan A, HMB45, S100	

[a]Usually focal and weak, but diffuse positive reactions can occur.
ALK, ALK receptor tyrosine kinase; *ALK*, ALK receptor tyrosine kinase gene; BCL6, B-cell CLL/lymphoma 6; CD34, CD34 molecule; CDK4, cyclin-dependent kinase 4; ER, estrogen receptor; FISH, fluorescence in situ hybridization; HMB45, homatropine methylbromide 45; IHC, immunohistochemistry; MDM2, MDM2 proto-oncogene; *NTRK3*, neurotrophic receptor tyrosine kinase 3 gene; *RET*, ret proto-oncogene; SMA, smooth muscle actin; SOX10, SRY-box 10; TLE1, TLE family member 1, transcriptional corepresser.

Sarcomatoid Carcinoma

Sarcomatoid carcinomas in the lung is an umbrella term encompassing spindle cell carcinomas, giant cell carcinomas, and pleomorphic carcinomas (spindle cell or giant cell carcinomas with one or more conventional carcinoma components) in addition to carcinosarcoma and pulmonary blastoma.[80] The key role of IHC is to distinguish sarcomatoid carcinomas from sarcomatoid mesothelioma, primary or metastatic sarcomas, and metastatic sarcomatoid carcinoma (e.g., renal), as well as to exclude mimics, such as metastatic melanoma. Keratin expression can be quite variable in sarcomatoid carcinomas. The expression is extremely weak and focal in most cases and may be inapparent in small biopsy specimens. Importantly, if a conventional carcinoma component such as acinar adenocarcinoma or keratinizing squamous cell carcinoma is evident in association with a spindle cell or giant cell tumor, documentation of epithelial differentiation by IHC is not necessary. The results of staining for markers of glandular and squamous differentiation, TTF1 and p40, respectively, are frequently negative in sarcomatoid carcinomas but can be positive in a subset, even in cases with minimal keratin expression.[40] It can be useful to utilize a panel of cytokeratins in suspected pleomorphic carcinomas, as some cases show positivity with only one keratin antibody. Cytokeratin 18 is a sensitive marker for epithelial differentiation in sarcomatoid tumors.

Making the distinction between sarcomatoid carcinoma and sarcomatoid mesothelioma can be problematic, as the results of IHC for specific differentiation markers may be negative in both tumor types. Similar to sarcomatoid carcinomas, sarcomatoid mesotheliomas are commonly negative or only weakly or focally positive for mesothelial markers (Wilms tumor 1 [WT1], calretinin, and D2-40). Although loss of BRCA1 associated protein 1 (BAP1) is generally useful for distinguishing between reactive mesothelial proliferations and malignant mesothelioma, the loss occurs in 20% or less of sarcomatoid mesotheliomas[81] and may occur in other tumor types such as sarcomatoid renal cell carcinoma.[82,83] Expression of carcinoma markers, including claudin-4, Ber-EP4, or B72.3, would support the diagnosis of sarcomatoid carcinoma over mesothelioma, but as in the case of keratins, expression of these markers may be extremely weak and focal. Recently, excellent performance of GATA binding protein 3 (GATA3) has been reported in making this distinction, and its 100% sensitivity for sarcomatoid or desmoplastic malignant mesothelioma in particular has suggested that lack of GATA3 expression could be used to exclude the diagnosis of sarcomatoid mesothelioma.[84] Of note, *p16* FISH cannot be used in this differential diagnosis, as both tumors can have homozygous deletions.[85] In many cases, practically, IHC work-up may not be informative, and the final diagnosis requires incorporation of clinicoradiologic information and molecular findings, if available.[86–88]

Distinction of sarcomatoid carcinoma from primary or metastatic sarcoma can be equally problematic, as IHC profiles of these tumors can overlap. Just as sarcomatoid carcinomas may be virtually negative for keratins, some high-grade sarcomas are known to express keratins, usually weakly and focally (Table 7).[89–97] Thus, focal labeling for keratins should not be used as the sole criterion supporting the diagnosis of sarcomatoid carcinoma over sarcoma, and conversely, the lack of detectable keratins, particularly in a small sample, does not favor sarcoma over sarcomatoid carcinoma. It is important to remember that other than a few specific types of mesenchymal neoplasms (see Table 7), primary pulmonary spindle cell or giant cell sarcomas are extremely rare, and even with minimal or absent keratins, such tumors are more likely to represent sarcomatoid carcinoma than primary sarcoma, particularly in a clinical context characteristic of patients with lung

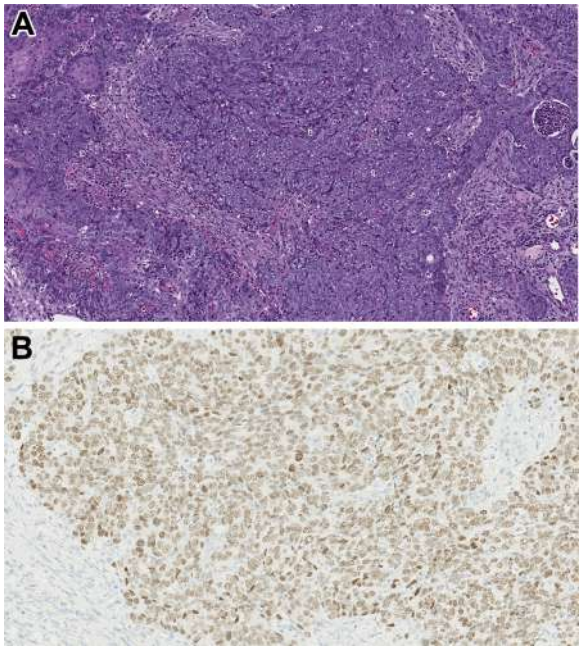

Figure 10. Typical appearance of NUT carcinoma with hematoxylin and eosin staining: undifferentiated, primitive, but monomorphic features with focal abrupt squamous differentiation (*A*). NUT immunohistochemistry shows diffuse nuclear labeling with the characteristic speckled pattern (*B*).

cancer (e.g., older smokers). Molecular testing may support the diagnosis of sarcomatoid carcinoma by identifying alterations typical of NSCC, such as *EGFR*, *KRAS,* or *MET* exon 14 splice site mutations, of which the latter are associated with sarcomatoid histologic type.[98,99]

Salivary Gland–Type Tumors

Salivary gland–type carcinomas can arise in the lung; in this setting, a metastasis from salivary gland primary must be excluded clinically. Their typical location is peribronchial or endobronchial. By far, the most common types of primary pulmonary salivary-type neoplasms include mucoepidermoid carcinoma and adenoid cystic carcinoma.[100] Importantly, all salivary neoplasms lack the expression of lung lineage markers, TTF1 and napsin A; if these markers are detected, this would support primary lung adenocarcinoma over salivary-type neoplasms. Care must be taken to recognize entrapped TTF1-positive cells, which may proliferate extensively in salivary gland tumors that infiltrate the interstitium.

The diagnosis of mucoepidermoid carcinoma can be supported by consistent labeling for p40/p63 in intermediate and squamous cells, positivity for intracytoplasmic mucin with mucin stains, and the demonstration of mastermind like transcriptional coactivator 2 gene (*MAML2*) rearrangements detected in tumors by FISH at a rate of 77% to 100%.[101–103] The result of staining for SRY-box 10 (SOX10) is usually negative in mucoepidermoid carcinoma but can be positive in a subset.[104] The main differential diagnosis in the lung is with adenosquamous carcinoma; expression of TTF1/napsin A in the glandular component would support the former, whereas *MAML2* rearrangement would support the latter. In a case without these features, the distinction remains quite challenging.

For adenoid cystic carcinoma, the main differential diagnosis is with pulmonary basaloid squamous cell carcinoma and other salivary-type tumors. Adenoid cystic carcinoma demonstrates dual luminal epithelial and/or abluminal myoepithelial composition, with luminal cells labeling for low-molecular-weight keratins and c-Kit; in contrast, abluminal myoepithelial cells label with p63/p40; smooth muscle actin; and S100. The tumors are positive for SOX10.[104] In addition, most adenoid cystic carcinoma are positive for v-myb avian myeloblastosis viral oncogene homolog by IHC and harbor v-myb avian myeloblastosis viral oncogene homolog gene (*MYB*) fusions.[105]

Rarer types of salivary neoplasms have been documented in the lung. These include epithelial-myoepithelial carcinoma (p63/smooth muscle actin, telomeric/S100-positive outer myoepithelial cells),[106] acinic cell carcinoma (SOX10- and anoctamin 1–positive),[107,108] hyalinizing clear cell carcinoma (recently renamed in head and neck sites as *clear cell carcinoma*; p63/p40-positive, EWS RNA binding protein 1 gene [*EWSR1*] fusions),[109] myoepithelial carcinoma (SOX10-positive, coexpresses epithelial markers [keratins, or epithelial membrane antigen] plus S100 plus variable myogenic markers, p63, glial fibrillary acidic protein (GFAP); *EWSR1* or FUS fusions),[110,111] and mammary analogue secretory carcinoma (S100, mammaglobin, GCDFP15, SOX10, GATA3, and *ETV6 NTRK3* fusion).[112,113]

NUT Carcinoma

NUT carcinoma is defined by the presence of nuclear protein in testis gene (*NUT*) rearrangement on chromosome 15. A highly specific antibody for NUT protein is commercially available; it demonstrates a distinctive speckled nuclear positivity in NUT carcinoma (Fig. 10).[114,115] The only other neoplasms that label for NUT are germ cell tumors, particularly seminomas; however, their labeling is typically focal and lacks the speckled pattern.[115] NUT carcinomas are usually positive for keratins, although in rare cases they can be negative.[114,116] The are usually positive for p63/p40, supporting squamous differentiation. Notably, NUT

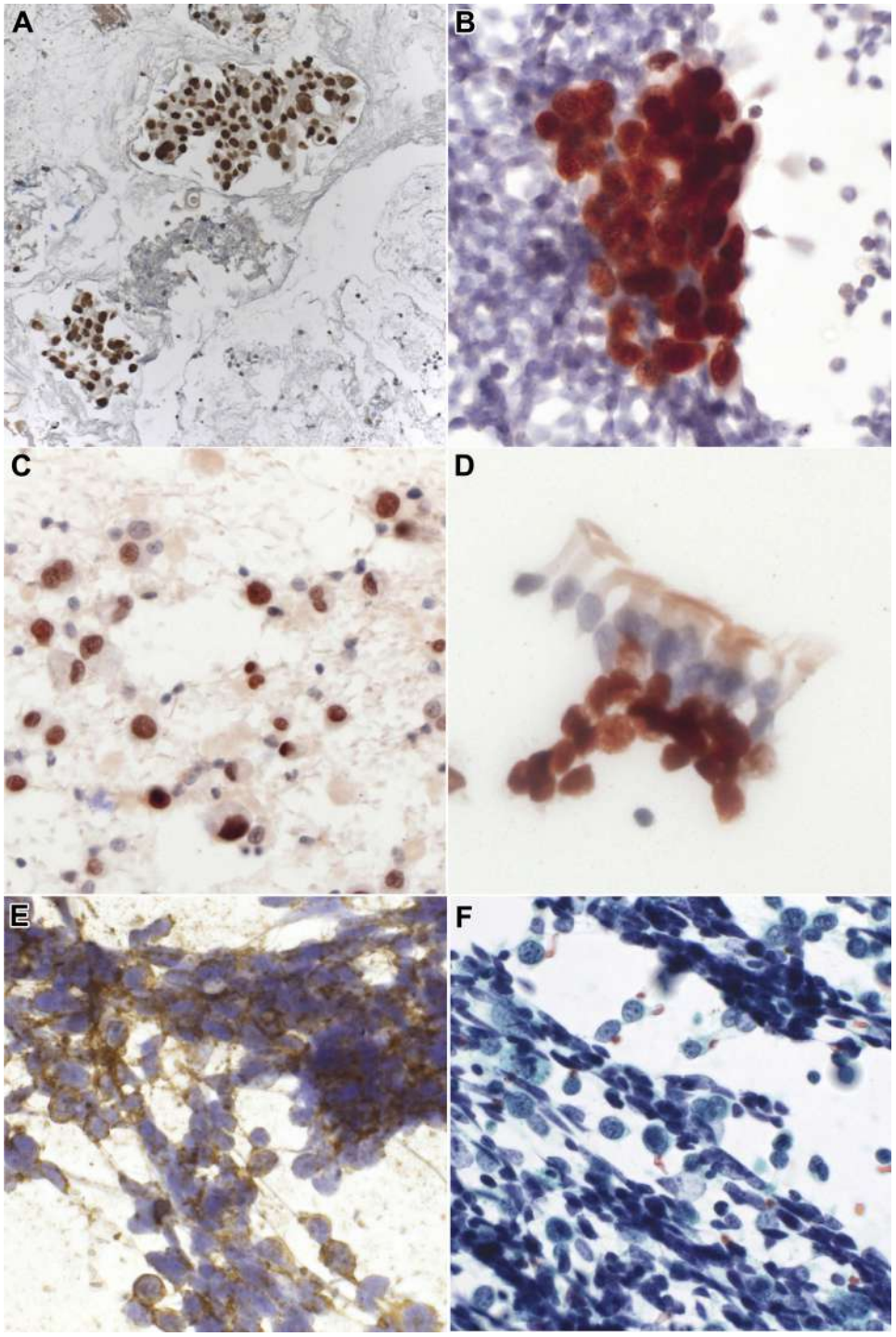

Figure 11. Immunostaining of cytologic specimens. (*A*) Thyroid transcription factor 1 (TTF1)–positive adenocarcinoma in cell block specimen (*brown*) (by 3,3'-diaminobenzidine, Ventana Benchmark XT immunostainer, Ventana Medical Systems, Tucson, AZ). (*B–E*) Immunostaining on Papanicolaou-stained, ethanol-fixed, non–cell block specimens (Leica Bond automated immunostainer, Leica Biosystems, Nussloch, Germany). TTF1–positive adenocarcinoma (*red*) (detection by 3-amino-9 ethylcarbazole) (*B*). p40-positive nonkeratinizing squamous cell carcinoma (*C*). p40-positive benign hyperplastic basal cells underlying ciliated respiratory cells (bronchial brush cytologic examination) (*D*). CD56-positive small cell carcinoma (*E*) with corresponding Papanicolaou-stained specimen (*F*).

carcinomas may be positive for CD34 molecule, which is important in the differential diagnosis with leukemic infiltrates.[117] Other differential diagnosis includes switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling factor–deficient carcinoma or sarcoma, which also displays solid growth pattern of mildly

Table 8. Immunoprofiles of Pulmonary Mucinous Adenocarcinomas and Their Mimics

	TTF1	Napsin-A[a]	CK7	CK20	CDX2
Pulmonary adenocarcinomas					
Invasive mucinous adenocarcinoma[b]	-/+	-/+	++	+/-	+/-
Colloid adenocarcinoma	+/-	+/-	+	+/-	+
Signet ring cell carcinoma[c]	+	+/-	++	-	-
Solid adenocarcinoma with mucin	+	+/-	++	-	-
Mucinous adenocarcinoma of the lung, NOS	+/-	-/+	++	-/+	-/+
Nonpulmonary adenocarcinomas					
GI tract all	-	-	+/-	+	+
Lower GI tract[d]	-	-	-/+	++	++
Upper GI tract[e]	-	-	+	+/-	+/-
Pancreas	-	-	++	+/-	+/-
Breast, mucinous	-	-	++	-	-
Ovary, mucinous	-	-	++	+/-	+/-

Note: Minus sign (-) indicates that less than 10% of the examined tumors exhibited positive expression, minus or plus sign (-/+) indicates that 10% to 40% exhibited positive expression, plus or minus sign (+/-) indicates that 40% or 70% exhibited positive expression, single plus sign (+) indicates that 70% to 90% exhibited positive expression, and double plus sign (++) indicates that more than 90% exhibited positive expression.
Data from references 140–159 and 161.
[a]Immunohistochemistry with monoclonal napsin A antibodies.
[b]Including mixed mucinous sand nonmucinous adenocarcinoma.
[c]Adenocarcinoma with signet ring cell features.
[d]Colorectum and appendix.
[e]Esophagus, stomach, and ampulla.
GI, gastrointestinal; NOS, not otherwise specified.

discohesive epithelioid cells often partly with rhabdoid features. The tumors are reported as SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 gene (*SMARCA4*) or SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 gene (*SMARCB1*)-deficient carcinoma or sarcoma in a small series.[118–122] As these tumors have been recognized recently, a definite entity of this tumor has not been established in the current classification.

9. What portion of the cytologic sample is best for immunostaining: the cell block, the air-dried smears, or the ethanol-fixed smears? Can destained smears be used adequately?

Short Answer. *All cytologic preparations, including cell blocks, ethanol-fixed, and air-dried slides, can principally be used for immunostaining. Formalin-fixed cell blocks are the most straightforward, whereas rigorous protocol optimization, validation, and quality control are required in immunostaining in cytology.*

The ability to perform highly accurate immunostaining of cytologic specimens is crucial given the fact that up to 40% of all lung cancer diagnoses are made by cytologic examination alone. The major difference and challenge in cytology relates to the greater variability of preanalytic conditions and the lack of tissue architecture and/or contexture that might necessitate different scoring strategies. In principle, one can group cytologic preparations into cell block cytologic preparations and non–cell block cytologic preparations. Cell blocks are the most easily accessible cytologic format for immunostaining, because most immunostaining protocols are optimized for formalin-fixed, paraffin-embedded (FFPE) tissue or cell material (Fig. 11*A* and *B*). Principally, it should be possible to apply the same standardized protocols for FFPE tissue on automated immunostainers. This assumption is supported by studies showing highly concordant results for different markers between cell blocks and matched histologic specimens.[123–126] However, the lack of international standards for prefixation methods and preparation protocol is a major issue on cell blocks.[127,128] Currently, more than 10 methods for cell block preparation are in use, the most common ones in the United States being plasma thrombin (33%), Histogel (Thermo Fisher Scientific, Waltham, MA) (27%), the Cellient automated cell block system (Hologic) (27%),[129] and modifications of these.[130] Almost all protocols share the final step of fixing the pellet in 10% buffered formalin and processing it to an FFPE block. The large spectrum of (pre)fixation ranges from fixing the cell material in 10% buffered formalin right to prefixation in ethanol or methanol-based solution before formalin fixation, or even pure fixation in 95% ethanol.

Although the large variety of transport media, prefixatives, and cell block protocols do not appear to cause systematic problems on immunostaining according to a previous survey,[131] recent analyses pinpoint to specific challenges related to preanalytical factors in cell blocks, especially with ethanol or methanol prefixation.[29,132] In addition to absent or near-absent expression of TTF1 with CytoLyt fixative,[29] nearly half (43%) of the 30 antibodies tested on the Cellient cell block system (Hologic) failed initial validation with use of the conditions established for FFPE tissue specimens on the Ventana Benchmark XT immunostainer (Ventana Medical Systems, Tucson, AZ).[133]

Non–cell block cytologic specimens consist of a variety of preparations, which include air-dried and alcohol-fixed smears, cytospins (Thermo Fisher Scientific), ThinPrep (Hologic), or SurePath (Becton Dickinson, Franklin Lakes, NJ) liquid-based preparations. The large variety of preanalytical conditions and preparation methods makes standardization of immunostaining on non–cell block specimens more challenging than in cell blocks. Nevertheless, immunostaining on ethanol-fixed smears or cytospins is principally possible and widely

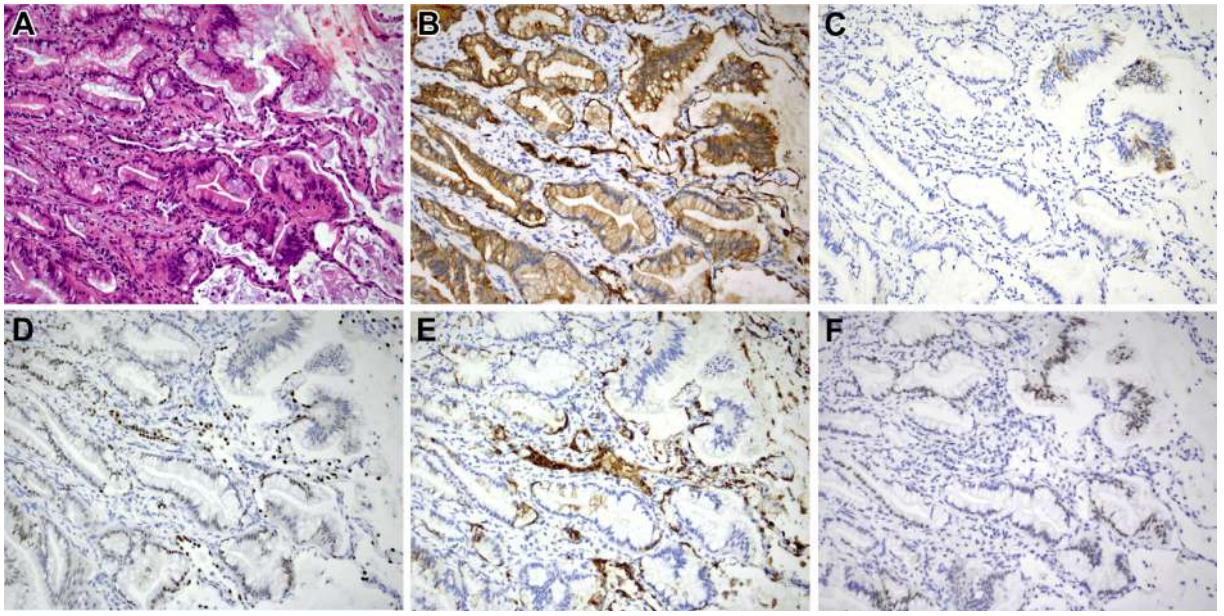

Figure 12. An example of invasive mucinous adenocarcinoma of the lung demonstrating lepidic and acinar patterns (*A*), diffuse expression of cytokeratin 7 (*B*), focal expression of cytokeratin 20 (*C*), scattered foci with weak expression of thyroid transcription factor 1 (TTF1) (*D*) and/or napsin A (*E*), and weak to moderate expression of caudal type homeobox 2 (CDX2) (*F*). Of note, the entrapped type II pneumocytes are reactive to cytokeratin 7 (*B*), TTF1 (*D*), and napsin A (*E*).

practiced; many laboratories that apply immunostaining to non–cell block specimens use the diagnostic Papanicolaou-stained slides (Fig. 11*C–E*).[131,134–136] Prior Papanicolaou staining, which does not negatively interfere with the immunostaining reaction, allows triaging of the available slides for immunostaining and marking of areas of special interest. Alternatively, air-dried and unstained extra slides with postfixation by

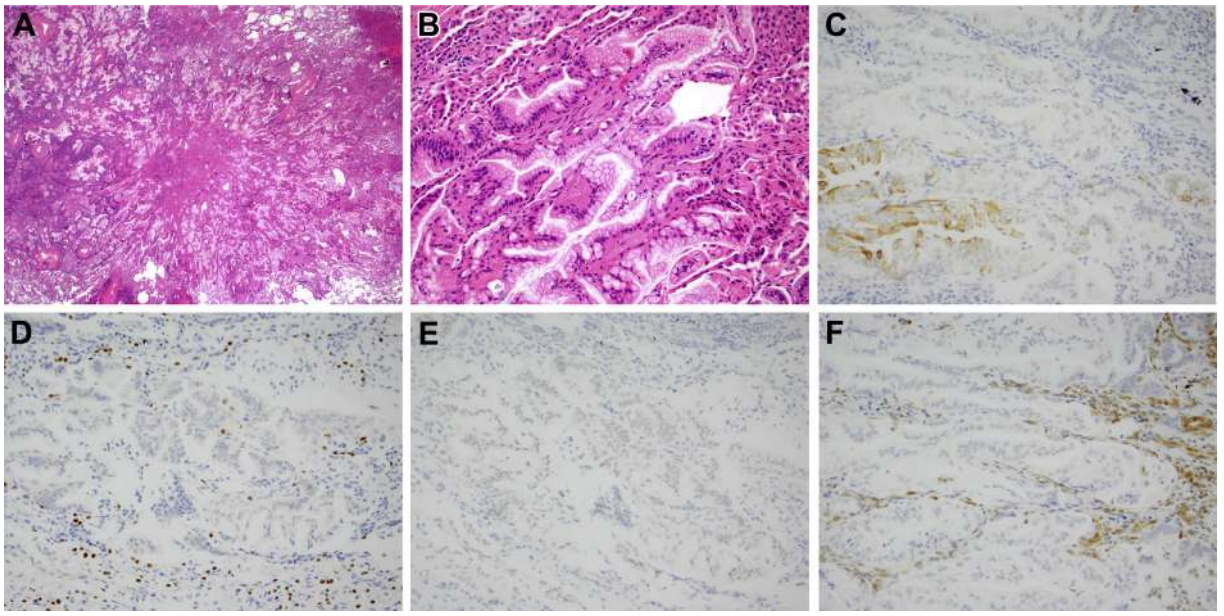

Figure 13. A pancreatic ductal adenocarcinoma metastatic to the lung exhibiting a lepidic pattern at low-power magnification (*A*) and high-power magnification (*B*), focal cytokeratin 7 expression (*C*), thyroid transcription factor 1 (TTF1) negativity (*D*), diffuse weak expression of caudal type homeobox 2 (CDX2) (*E*), and loss of SMAD family member 4 (SMAD4) (*F*). Of note, strong nuclear expression of TTF1 in the entrapped pneumocytes may give the impression of a false-positive result. Also, loss of expression of SMAD4 has been reported as a useful marker for the diagnosis of pancreatic adenocarcinoma, but a significant proportion of invasive mucinous adenocarcinomas of the lung harbor this alteration.[155]

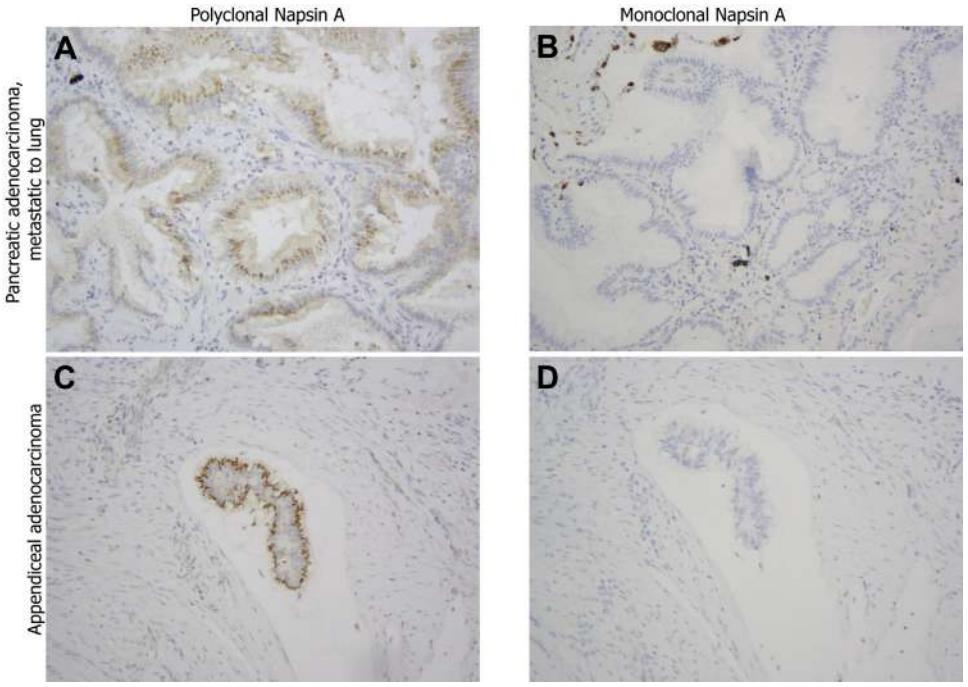

Figure 14. Apical granular reactions of polyclonal napsin A are shown in nonpulmonary carcinoma (*A*), metastasis of pancreatic duct carcinoma, and appendiceal adenocarcinoma (*C*) in contrast to a negative reaction with monoclonal napsin A (clone IP64) (*B* and *D*).

acetone or formalin are also used for immunostaining, but they require extra material not used for primary morphologic diagnosis.[137,138] When slides containing tumor are available, before the initiation of immunostaining, it may well be prudent to photograph or scan stained neoplastic elements for documentation purposes.

In the practical application of immunostaining to either cell block or non–cell block specimens, careful protocol validation and continuous quality control is essential, especially in ethanol-fixed non–cell block preparations, because of the high variability of preanalytic factors and the current lack of standardization. External quality assessment is also important to maintain a high immunostaining quality not only in histologic specimens but also in cytologic specimens. In fact, the UK National External Quality Assessment Service has an external quality assessment program in place to help standardize and improve the quality of immunostaining in cytology.[139]

10. Which IHC panel is recommended to differentiate lung mucinous adenocarcinoma from metastatic mimics?

Short Answer. *There is no useful marker to differentiate pulmonary mucinous adenocarcinoma from metastatic mimics. A clinicopathologic tumor board is crucial for this clinical context.*

When adenocarcinomas of the gastrointestinal (GI) and pancreatobiliary tracts metastasize to the lung, they may exhibit prominent mucinous features. In addition, mucinous carcinomas of the ovary, breast, and other organs may metastasize to the lung. Given that the differentiation of lung adenocarcinomas with mucinous features (pulmonary mucinous adenocarcinomas) from metastatic lesions is often challenging on a morphologic basis alone, multiple groups have studied the role of IHC in this context.[140–160] In particular, distinguishing a metastasis from a pancreatic primary from invasive mucinous adenocarcinoma of the lung is far more challenging, given the similar immunoprofiles (focal caudal type homeobox 2 (CDX2) and cytokeratin 20 [Table 8])[140–159,161]; furthermore, a lepidic growth pattern, which is characteristic of invasive mucinous adenocarcinoma (Fig. 12), is also often identified in pancreatic ductal adenocarcinoma metastatic to the lung (Fig. 13).[155,162] Even with molecular testing, a complete solution for the differential diagnosis remains unsolved[163] unless direct comparison of molecular profiles between the lung and nonlung tumors can be made.[155,162] Notably, a significant proportion of pulmonary mucinous adenocarcinomas, including invasive mucinous adenocarcinomas, are not reactive to TTF1 and/or napsin A. Rather, these immunoreactions typically highlight normal type II pneumocytes entrapped in

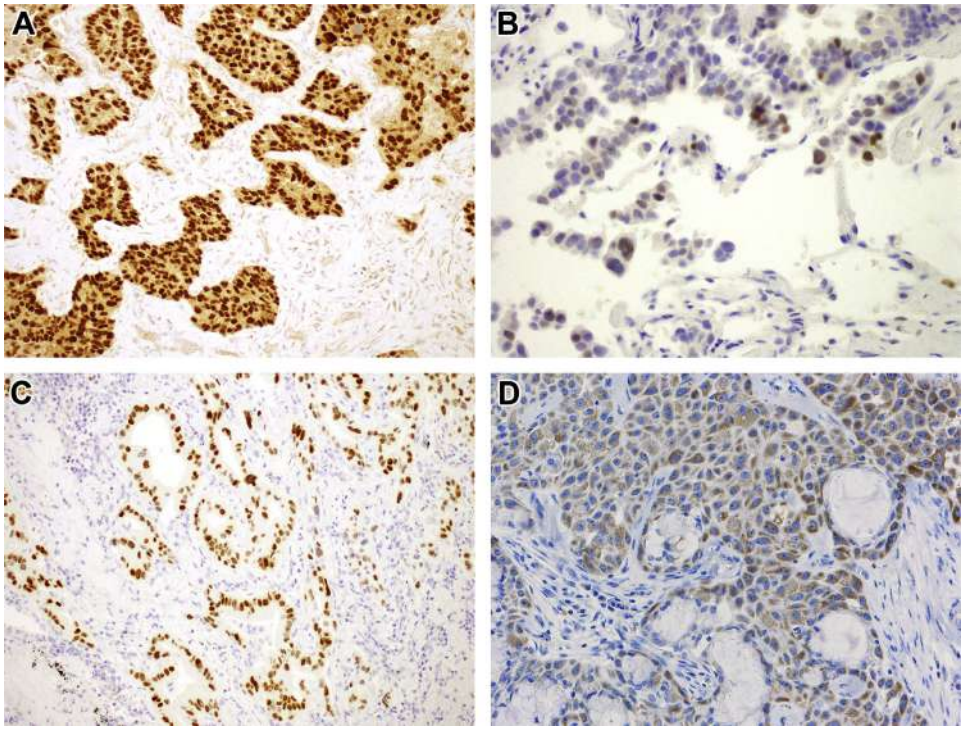

Figure 15. Useful antibodies and pitfalls for some of the more common differential diagnoses of metastatic lung cancer. Paired box 8 (PAX8, monoclonal) positivity in metastatic serous adenocarcinoma from the uterine corpus (*A*). Focal estrogen receptor positivity in primary lung adenocarcinoma (*B*). GATA binding protein 3 (GATA3) positivity in metastatic adenocarcinoma from the breast (*C*). Expression of secretoglobin family 2A member 2 (mammaglobin) in salivary-type adenocarcinoma (mucoepidermoid carcinoma) of the lung (*D*).

the tumor (Figs. 13 and 14), possibly leading to false-positive interpretation in the setting of metastasis. It is also worth mentioning that the high specificity of napsin A for lung origin may not be achieved when a polyclonal antibody is used. One study revealed napsin A expression in 92% of 13 nonpulmonary mucinous adenocarcinomas and 100% of 8 pulmonary mucinous adenocarcinomas by IHC with a polyclonal antibody versus in none of the 13 nonpulmonary and 38% of the eight pulmonary mucinous adenocarcinomas with napsin A expression when a monoclonal antibody was used.[157] Interestingly, other studies using a polyclonal antibody reported napsin A expression in none of 49 nonpulmonary mucinous adenocarcinomas[155,156]; thus, the low specificity reported in the former study may be attributed to its particular IHC platform.[157,164] Further, nonspecific labeling with polyclonal napsin A in mucinous adenocarcinomas appears to have peculiar supranuclear localization, as opposed to the pan-cytoplasmic granular staining present with monoclonal napsin A, possibly owing to cross-reaction with pan-mucin antigen by the polyclonal antibody (see Fig. 14).[157,165]

Most invasive mucinous adenocarcinomas and other pulmonary adenocarcinomas with mucinous features, in particular, those that lack TTF1 expression, react to hepatocyte nuclear factor 4α (HNF4α).[152,161] However, HNF4α is a differentiation transcription factor of the primary gut, including the hepatobiliary and GI tracts, which universally express this transcription factor. Therefore, HNF4α will not help with differentiating between pulmonary mucinous adenocarcinomas and GI and pancreatic primaries.[161]

Among other metastatic mucinous adenocarcinomas, breast and ovarian primaries can be differentiated from a lung primary by their specific markers, including GATA3 and estrogen receptor (ER) for breast colloid carcinoma and paired box 8 (PAX8) for ovarian mucinous carcinoma.[153] Despite encouraging results in the literature, however, the markers cannot be relied on in this situation because not all metastatic tumors are positive for these markers. Only 40% of ovarian mucinous tumors express PAX8, suggesting low sensitivity for this differential diagnosis.

11. Are there any IHC or other markers to differentiate between primary lung cancers and metastases; between squamous cell carcinomas of lung primary and metastases from thymic, head and neck, endocervical, and the other cancers; and

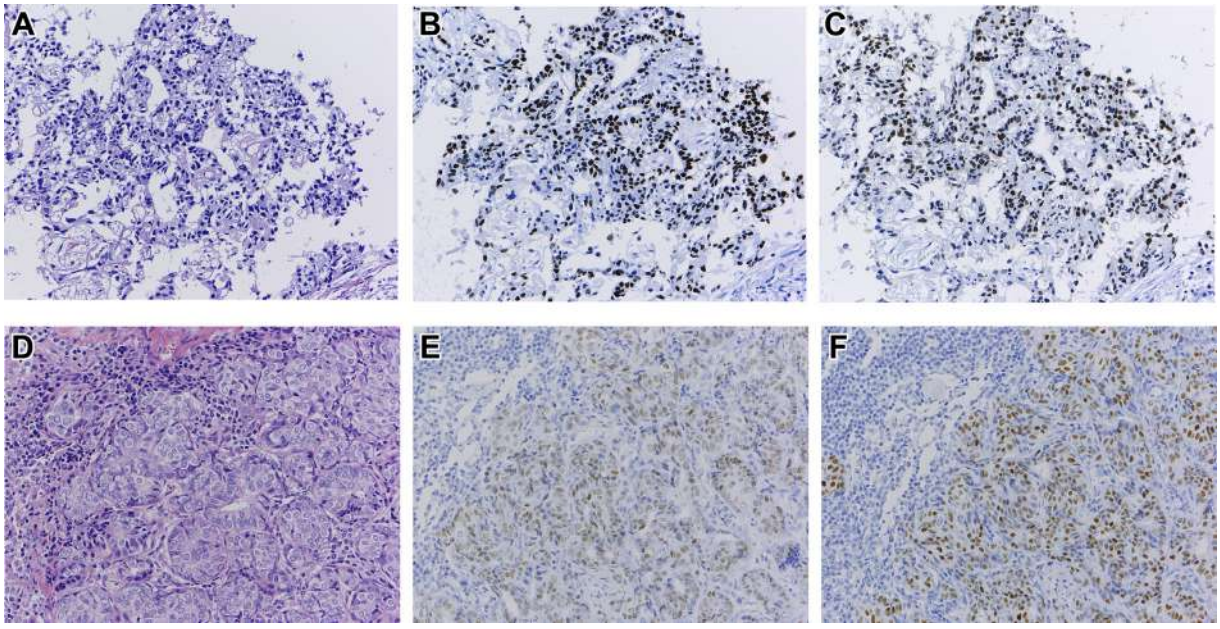

Figure 16. Expression of thyroid transcription factor 1 (TTF1) in nonpulmonary carcinoma. (*A–C*) Metastasis of ovarian endometrioid carcinoma to the lung (*A*) expresses paired box 8 (PAX8) (*B*) and TTF1 (clone SPT24) (*C*). The diagnosis was confirmed with identical *KRAS* mutation (G12A) between lung and ovarian cancer. (*D–F*) Lymph node metastasis of mammary invasive ductal carcinoma (*D*) displays dual expression of estrogen receptor (*E*) and TTF1 (clone SPT24) (*F*).

between adenocarcinomas of primary and metastases from gynecologic, mammary, uroepithelial, nonpulmonary NE, prostate, and liver cancers?

Short Answer. *In this clinical context, morphologic comparison with prior tumor is crucial. There are no absolute IHC markers to make the differential diagnosis, and pathologists should be aware of the pitfalls of IHC.*

Differentiating primary lung carcinoma and metastasis from extrinsic sites is an important practice in diagnostic service. The criterion standard for the decision is based on the morphologic comparison with prior tumors; however, IHC provides strong support for this interpretation, particularly when previous materials are unavailable for review or when morphologic assessment results in equivocal findings.

Squamous Cell Carcinoma

Distinguishing primary lung squamous cell carcinoma from metastasis is challenging particularly when the nodule is solitary. Prior materials should be reviewed whenever possible, because growth pattern and the degree of keratinization may provide clues for the decision. In some instances, it should be kept in mind that metastatic tumors may change their morphologic features, particularly after chemotherapy and/or radiation therapy. Identifying an in situ component may support the primary nature of the tumor, but primary squamous cell carcinoma arising in the peripheral lung may not have such a component. Further, in small samples, an in situ component may not be present or recognized morphologically. Ancillary stains are usually of limited utility, except in the few instances that follow.

Thymic squamous cell carcinoma labels for KIT proto-oncogene receptor tyrosine kinase (also known by the alias CD117) (85%) and CD5 molecule (70%), whereas primary lung squamous cell carcinoma is only infrequently positive for these markers.[166–168] Notably, KIT proto-oncogene receptor tyrosine kinase and CD5 molecule are expressed in approximately 15% of lung adenocarcinoma, and their expression in adenocarcinoma does not suggest a thymic primary. Although the result of staining for PAX8 can be positive for thymic carcinomas when a polyclonal antibody is used, this likely results from cross-reactivity to another *PAX* gene product, which is not reproduced when a monoclonal antibody is used.[169] More than IHC staining, clinical and radiologic correlation is important to confirm whether a tumor is arising in the lung or in the thymus.

Detection of high-risk human papilloma virus (HPV) is helpful when the differential diagnoses include metastatic squamous cell carcinoma from head and neck (especially oropharynx), endocervix, vulva, anus, and penis. Detecting HPV in tumor tissue strongly favors metastasis from these sites[170,171] because HPV infection is considered exceptional in lung squamous cell carcinoma, with the caveat that some geographic difference may exist with regard to the rate of HPV detection reported in lung cancers.[172] Although diffuse p16

immunostaining is an accepted surrogate for high-risk HPV infection in the cervix and oropharynx, about 20% of primary lung NSCCs demonstrate similar p16 positivity despite the lack of HPV infection.[171,173] So, in the event of a diffuse positive result with p16 staining, further molecular testing is recommended to confirm the presence of the HPV genome.

Cancer of Gynecologic Organs

The immunoprofiles of adenocarcinomas arising from the female genital tract (cervix, endometrium, fallopian tube, and ovary) differ depending on the tumor histotype and primary sites. Cervical adenocarcinoma is often associated with high-risk HPV infection and is accordingly characterized by confluent p16 expression; thus, this marker can be utilized for the differential diagnosis as discussed. Serous carcinomas of the ovary and uterus are often positive for WT1, which is usually negative in lung adenocarcinoma. WT1 expression must be nuclear in this context, as cytoplasmic WT1 expression is nonspecific. Staining for PAX8 reveals most adenocarcinomas in the female genital tract, in contrast to being negative in lung adenocarcinoma, and thus is of high utility (Fig. 15*A*).[169,174] The result of staining for TTF1 can be positive in a subset of uterine and ovarian carcinomas (Fig. 16).[175–177] Napsin A is highly expressed in most clear cell carcinomas of the ovary and endometrium, which also frequently express hepatocyte nuclear factor 1β.[177]

Cancer of Other Organs

Breast Cancer. Common breast cancer markers include ER, GATA3, mammaglobin, and GCDFP15, which are expressed in 80%, more than 90%,[178] 40% to 60%,[179–181] and 20% to 40%[179,181] of breast carcinomas, respectively. ER expression does not necessarily support breast primary, as a wide range of ER positivity is reported in lung adenocarcinoma depending on different staining protocols (Fig. 15*B*).[182–184] Nuclear GATA3 staining, usually of a diffuse and strong quality, favors a breast primary (Fig. 15*C*) because its expression is rare to uncommon (0%–8%) in lung adenocarcinoma, with the different results most likely being a consequence of the different antibody clones utilized.[178,183,185] Triple-negative breast cancers may stain with SOX10.[186,187] The result of staining for GCDFP15 is uncommonly positive in lung adenocarcinoma (0%–5.2%).[106,183,188] The result of staining for mammaglobin is usually negative in lung adenocarcinoma.[179–181,183,184] However, the result of staining for GATA3 and mammaglobin can be positive in salivary-type carcinomas in the bronchopulmonary tree (Fig. 15*D*). Breast carcinoma rarely expresses TTF1, which occurs more commonly with the SPT24 clone but rarely with the 8G7G3/1 clone (see Fig. 16).[189–191] Similarly, the result of napsin A staining is usually negative in breast carcinoma, but some labeling has been reported.[6] Utilization of theses IHC markers needs to be made whenever possible in the context of morphologic comparison with the primary breast cancer specimen.

Urothelial Carcinoma. Metastatic urothelial carcinoma may be histologically indistinguishable from poorly differentiated squamous cell carcinoma of the lung. The differentiation can be facilitated by specific urothelial markers, such as uroplakin III and the more sensitive uroplakin II.[192,193] GATA3, the result of staining for which is positive in about 80% of urothelial carcinomas, can be positive in primary lung squamous cell carcinoma, with a rate of positive results in the range from 0% to 20%.[178,184,192,194]

NE Tumors. High-grade NE tumors can express TTF1, regardless of the primary site, and its reactivity should not be interpreted as evidence of pulmonary origin.[195] Other cellular lineage markers, including caudal type homeobox 2 (CDX2), pancreatic and duodenal homeobox 1 (PDX1), ISL LIM homeobox 1 (ISL1), and NK2 homeobox 2 (NKX2.2), are useful for separating carcinoid tumors from metastatic well-differentiated NE tumors of nonpulmonary origin,[196–198] but not for high-grade NE tumors of the lung.

Renal Carcinoma. Renal cell carcinomas express PAX8, whereas PAX8 expression is rare (0%–2%) in lung adenocarcinoma.[169,174] Monoclonal PAX8 antibody is more specific than the polyclonal reagent.[169,199] The result of staining for napsin A can be positive in renal cell carcinomas (about 80% in papillary carcinoma and about 40% in clear cell carcinoma).[6,200]

Prostate Cancer. Prostatic adenocarcinoma metastatic to the lung may be mistaken for lung adenocarcinoma, and sometimes for LCNEC. Prostatic adenocarcinomas express prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and NK3 homeobox 1 (NKX3.1), and lack CK7 expression, unlike lung adenocarcinomas.[201,202]

Liver Cancer. Metastatic hepatocellular carcinoma should be distinguished from lung adenocarcinoma with hepatoid morphologic features.[203] Hepatoid adenocarcinoma of the lung may express alpha fetoprotein, hepatocyte paraffin 1 (HEP-Par1),[203] and arginase-1,[204] and careful clinicopathologic correlation is required for diagnosis.

Summary

We have selected 11 questions on IHC to be the most relevant to current practice. IHC is now an indispensable

tool for diagnostic pathology, but it has many pitfalls, as discussed. As aberrant TTF1 expression in schwannoma was recently reported,[205] we still do not recognize all of them. Therefore, morphologic examination should serve as the foundation of our pathology diagnosis, and all of the aforementioned recommendations are valid only in the proper clinical context. In particular, pathologists should keep in mind that clinical findings, including age, sex, smoking status, also provide an important diagnostic clue.[206,207]

In the next couple of years, development of new technology and emergence of new antibodies may change the current diagnostic situation, and accordingly, our recommendations may not be appropriate at that time. We believe that periodic updates in collaboration with other related organizations are necessary. Similar to the predictive biomarker testing landscape, diagnostic IHC is also directly linked to patients' treatment of choice. All professionals in this field should ensure that all patients receive appropriate diagnoses with the aid of IHC.

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