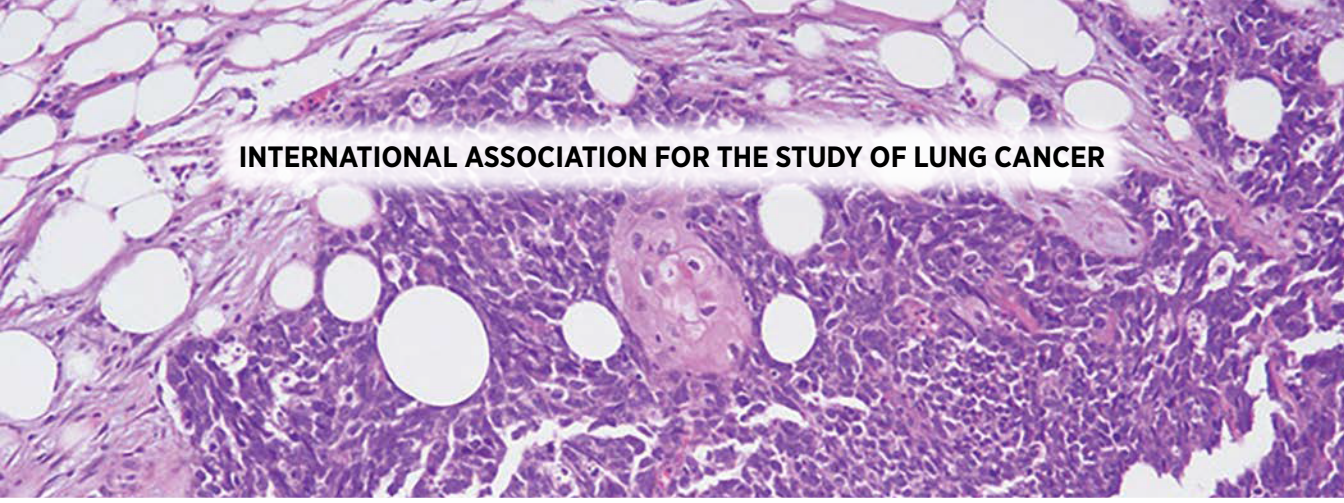

INTERNATIONAL ASSOCIATION FOR THE STUDY OF LUNG CANCER

IASLC ATLAS OF DIAGNOSTIC IMMUNOHISTOCHEMISTRY

EDITED BY

Yasushi Yatabe, MD, PhD
Alain C. Borczuk, MD
Wendy A. Cooper, MBBS, Bsc(Med), FRCPA, PhD
Sanja Dacic, MD, PhD
Keith M. Kerr, MD, FRCPATH, FRCPE
Andre L. Moreira, MD, PhD
Ming Sound Tsao, MD, FRCPC

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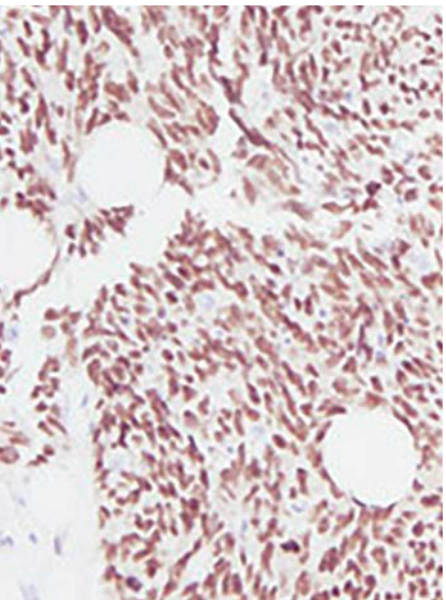

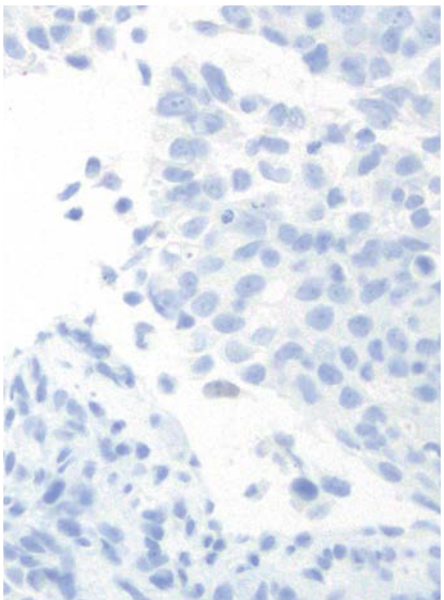

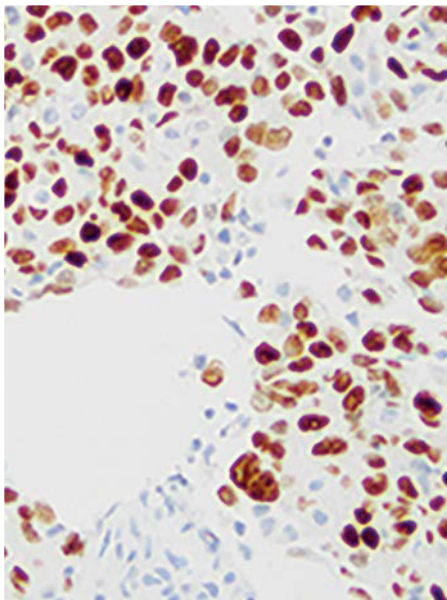

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International Association for the Study of Lung Cancer, Denver, CO, USA

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Andre L. Moreira, MD, PhD
Ming Sound Tsao, MD, FRCPC

IASLC Office:
IASLC, 999 17th Street, Suite 200, Denver, Colorado 80202, USA
www.iaslc.org

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YASUSHI YATABE, MD, PhD
ALAIN C. BORCZUK, MD
WENDY A. COOPER, MBBS, Bsc(Med), FRCPA, PhD
SANJA DACIC, MD, PhD
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ANDRE L. MOREIRA, MD, PhD
MING SOUND TSAO, MD, FRCPC

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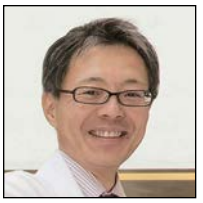
Contributors

Editors



Yasushi Yatabe, MD, PhD
Chief
Department of Diagnostic Pathology
National Cancer Center
Tokyo, Japan

Alain C. Borczuk, MD
Vice Chairman for Anatomic Pathology
Director of Anatomic Pathology
Chief of Thoracic Pathology
Department of Pathology
Weill Cornell Medicine
New York, New York, United States

Wendy A. Cooper, MBBS, Bsc(Med), FRCPA, PhD
Associate Professor
Tissue Pathology and Diagnostic Oncology
NSW Health Pathology
Royal Prince Alfred Hospital
Sydney, New South Wales, Australia

Sanja Dacic, MD, PhD
Professor
Department of Pathology
University of Pittsburgh Medical Center
Pittsburgh, Pennsylvania, United States

Keith M. Kerr, BSc, MB ChB, FRCPath, Hon FRCPEd, Hon FRCSI
Consultant Pathologist
Department of Pathology
Aberdeen Royal Infirmary
Professor of Pulmonary Pathology
Aberdeen University Medical School
Aberdeen, Scotland, United Kingdom

Andre L. Moreira, MD, PhD
Professor of Pathology
Director, Center of Biospecimen Research and Development (CBRD)
Director of Surgical Pathology and Cardiothoracic Pathology
New York University Langone Health
New York, New York, United States

Ming Sound Tsao, MD, FRCPC
Pathologist, Senior Scientist, and Professor
M. Qasim Choksi Chair in Lung Cancer
Translational Research
Princess Margaret Cancer Centre, University Health Network
Department of Laboratory Medicine and Pathobiology
University of Toronto
Toronto, Ontario, Canada

Authors

Mary Beth Beasley, MD
Professor of Pathology
Ichan School of Medicine at Mount Sinai
New York, New York, United States

Sabina Berezowska, MD
Associate Professor
Institute of Pathology
Lausanne University Hospital and Lausanne University
Lausanne, Switzerland

Lukas Bubendorf, MD
Professor and Head of Cytopathology
Institute of Medical Genetics and Pathology
University Hospital Basel
Basel, Switzerland

Teh-Ying Chou, MD, PhD, MBA
Professor and Chair
Department of Pathology and Laboratory Medicine
Taipei Veterans General Hospital
Taipei, Taiwan

Jin-Haeng Chung, MD, PhD
Professor
Department of Pathology and Translational Medicine
Seoul National University Bundang Hospital
Seoul, South Korea

Balazs Halmos, MD
Section Chief, Thoracic/Head and Neck Oncology
Director, Clinical Cancer Genomics
Professor Clinical Medicine/Albert Einstein College of Medicine
Bronx, New York, United States

Yuchen Han
Professor
Department of Pathology
Shanghai Chest Hospital
Shanghai, China

Fred Hirsch, MD, PhD
Executive Director
Center for Thoracic Oncology
Mount Sinai Cancer Center, Mount Sinai Health System
Professor of Medicine
Ichan School of Medicine
Joe Lowe and Louis Price Professor of Medicine
Associate Director
Tisch Cancer Institute
New York, New York, United States

David M. Hwang, BSc, MD, PhD, FRCPC
Chief, Department of Laboratory Medicine and Molecular Diagnostics
Sunnybrook Health Sciences Centre
Professor, Department of Laboratory Medicine and Pathology
University of Toronto
Toronto, Ontario, Canada

Deepali Jain, MD, FIAC
Additional Professor
Department of Pathology
All India Institute of Medical Sciences
New Delhi, India

Philippe Joubert, MD, PhD
Institut Universitaire de Cardiologie et de Pneumologie de Quebec
Universite Laval
Quebec City, Quebec, Canada

Sylvie Lantuejoul, MD, PhD
Department of Biopathology
Research Pathology Platform CRCL
Centre de Lutte Contre le Cancer UNICANCER Leon Berard
Universite Grenoble Alpes
Grenoble, France

Fernando Lopez-Rios, MD, PhD, FIAC
Professor of Pathology & Molecular Pathology
Pathology-Targeted Therapies Laboratory
HM Hospitales
Universidad CEU San Pablo
Madrid, Spain

Diasuke Matsubara, MD, PhD
Department of Pathology
Division of Integrative Pathology
Jichi Medical University
Shimotsuke, Tochigi, Japan

Yuko Minami, MD, PhD
Director, Pathology Department
National Organization Hospital Ibarakihigashi National Hospital
The Center of Chest Diseases and Severe Motor and Intellectual Disease
Ibaraki, Japan

Mari Mino-Kenudson, MD
Professor of Pathology
Harvard Medical School
Massachusetts General Hospital
Boston, Massachusetts, United States

Andrew G. Nicholson, DM, FRCPath
Consultant Histopathologist
Royal Brompton and Harefield Hospitals NHS Foundation Trust
Honorary Professor of Respiratory Pathology, National Heart and Lung Division
Imperial College
London, United Kingdom

Masayuki Noguchi, MD
Professor of Diagnostic Pathology
Faculty of Medicine
University of Tsukuba
Tsukuba, Japan

Mauro Papotti, MD
Professor of Pathology
Department of Oncology
University of Turin
Torino, Italy

Harvey I. Pass, MD
Stephen E. Banner Professor of Thoracic Oncology
Director, Division of General Thoracic Surgery
Vice-Chair, Research, Cardiothoracic Surgery
NYU Langone Health
New York, New York, United States

Claudia Poleri, MD
Office of Pathology Consultants
Buenos Aires, Argentina

Anja C. Roden, MD
Professor of Laboratory Medicine and Pathology
Consultant
Department of Laboratory Medicine and Pathology
Mayo Clinic
Rochester, Minnesota, United States

Prudence A. Russell, MBBS (Hons), FRCPA
Associate Professor
St. Vincent's Hospital and the University of Melbourne
Melbourne, Victoria, Australia

Kurt A. Schalper, MD, PhD
Department of Pathology
Yale School of Medicine
New Haven, Connecticut, United States

Lynette M. Sholl, MD
Department of Pathology
Brigham and Women's Hospital and Harvard Medical School
Boston, Massachusetts, United States

Erik Thunnissen, MD, PhD
Amsterdam University Medical Center VUmc
Amsterdam, The Netherlands

William Travis, MD
Attending Thoracic Pathologist
Department of Pathology
Memorial Sloan Kettering Cancer Center
New York, New York, United States

Ignacio I. Wistuba, MD
Professor and Chair
Department of Translational Molecular Pathology
The University of Texas MD Anderson Cancer Center
Houston, Texas, United States

Akihiko Yoshida, MD, PhD
Department of Diagnostic Pathology
National Cancer Center Hospital
Tokyo, Japan

Abbreviations

The following abbreviations are used in the text:

3-D: three-dimensional
ABC: avidin-biotin complex
AC: atypical carcinoid
ADC: adenocarcinoma
AEC: 3-amino-9-ethylcarbazole
AFP: α-fetoprotein
ALK: anaplastic lymphoma kinase
AMP: Association for Molecular Pathology
AP: acid [or alkaline] phosphatase
AUC: area under the curve
BALT: bronchial-associated lymphoid tissue
BAP1: BRCA1-associated protein 1
BET: bromodomain and extraterminal
β-HCG: beta human chorionic gonadotropin
BRAF: B-raf proto-oncogene
BRD: bromodomain and extraterminal (BET) domain-containing protein
CA IX: carbonic anhydrase IX
CAMTA1: calmodulin-binding transcription activator 1
CAP: College of American Pathologists
CDK4: cyclin-dependent kinase 4
CE*: Conformité Européenne*
CEA: carcinoembryonic antigen
CK5/6: cytokeratin 5/6
CLSI: Clinical and Laboratory Standards Institute
CMPT: ciliated muconodular papillary tumor
CMV: cytomegalovirus
CRC: colorectal cancer
CSA: catalyzed signal amplification
CSA-II: catalyzed signal amplification
CT: computed tomography
ctDNA: circulating-tumor DNA
DAB: 3,3'-diaminobenzidine
DAPI: 4',6-diamidino-2-phenylindole

EBUS-FNA: endoscopic ultrasound-guided transbronchial fine-needle aspiration
ECT2: epithelial cell transforming 2
EDTA: ethylenediaminetetraacetic acid
EGFR: epidermal growth factor receptor
EHE: epithelioid hemangioendothelioma
ELISA: enzyme-linked immunosorbent assay
EMA: epithelial membrane antigen
EQA: external quality assessment
ER: estrogen receptor
ETV4: ETS variant transcription factor 4
EZH2: enhancer of zeste homolog 2
FDA: U.S. Food and Drug Administration
FFPE: formalin-fixed paraffin-embedded
FISH: fluorescence in situ hybridization
FNA: fine-needle aspiration
FOB: flexible fiberoptic bronchoscopy
GCDFP: gross cystic disease fluid protein
GFAP: glial fibrillary acidic protein
GI: gastrointestinal
H&E: hematoxylin and eosin
hASH1: human achaete-scute homolog-1
HB_s: hepatitis B surface antigen
HCC: hepatocellular carcinoma
HepPar1: hepatocyte paraffin 1
HER2: human epidermal growth factor receptor 2
HIAR: heat-induced antigen retrieval
HiDAC: high-dose cytarabine
HMW: high molecular weight
HNF4α: hepatocyte nuclear factor 4 alpha
HPV: human papilloma virus
HQ: 3-hydroxy-2-quinoxaline
HRP: horseradish peroxidase
IC: immune cell
ICAPC: immunohistochemistry critical assay performance control
IFN-γ: interferon gamma
IHC: immunohistochemistry
IL-8: interleukin-8
IMA: invasive mucinous adenocarcinoma
IMT: inflammatory myofibroblastic tumor
INI1: integrase interactor 1
INSM1: insulinoma-associated protein 1
irPRC: immune-related pathologic response criteria
ISH: in situ hybridization
IVD: in vitro diagnostic(s)
KRAS: Kirsten rat sarcoma viral oncogene homolog
LAG-3: lymphocyte-activation gene 3
LCA: leukocyte common antigen
LCNEC: large cell neuroendocrine carcinoma

LDT: laboratory-developed test
LMW: low molecular weight
LSAB: labeled streptavidin-biotin
MDM2: mouse double minute 2 homolog/E3 ubiquitin-protein ligase
MEK: mitogen-activated protein kinase (MAPK) kinase
MET: MET proto-oncogene
MIBI: multiple ion beam–based ionization
MiTF: microphthalmia transcription factor
MPM: malignant pleural mesothelioma
MPNST: malignant peripheral nerve sheath tumor
MPR: major pathologic response
mRNA: messenger RNA
MSA: muscle specific actin
MSI: microsatellite instability
MTAP: methylthioadenosine phosphorylase
MYB: myeloblastosis proto-oncogene, transcription factor
NCAM1: neural cell adhesion molecule 1
NE: neuroendocrine
NEC: neuroendocrine carcinoma
NEN: neuroendocrine neoplasm
NGS: next-generation sequencing
NKX3.1: NK3 homeobox 1 gene
NOS: not otherwise specified
NRG1: neuregulin
NSCC: non-small cell carcinoma
NSCLC: non-small cell lung carcinoma
NSE: neuron-specific enolase
NTRK: neurotrophic tyrosine receptor kinase gene
NUT: nuclear protein in testis
OTP: orthopedia homeobox protein
P13KCA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α gene
PAS: periodic acid–Schiff
PBMC: peripheral blood mononuclear cell
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction
PD-1: programmed cell death protein-1
PD-L1: programmed death ligand-1
PEComa: perivascular epithelioid cell tumor
PET: positron emission tomography
PMA: phorbol myristate acetate
PR: progesterone receptor
PSA: prostatic-specific antigen
PSAP: prostatic-specific acid phosphatase
PSMA: prostate specific membrane antigen
RB: retinoblastoma
RET: ret proto-oncogene
ROC: receiver operating characteristic
ROS1: c-ros oncogene 1

RT-PCR: reverse-transcriptase polymerase chain reaction
SALL4: spalt-like transcription factor 4 (or sal-like protein 4)
SCC: small cell carcinoma
SCLC: small cell lung carcinoma
SFT: solitary fibrous tumor
SLBx: surgical lung biopsy
SMA: smooth muscle actin
SP-A, SP-B: surfactant protein A, surfactant protein B
SQCC: squamous cell carcinoma
STAS: spread through alveolar spaces
STAT6: signal transducer and activator of transcription 6
SUVmax: maximum standardized uptake value
TBBx: transbronchial biopsy
TBNA: transbronchial needle aspiration
TC: typical carcinoid
TdT: terminal deoxynucleotidyl transferase
TFE3: transcription factor E3
TIM-3: mucin domain-containing protein 3
TKI: tyrosine kinase inhibitor
TLE1: transducin-like enhancer protein 1
TMB: tumor mutational burden
TNBC: triple-negative breast carcinoma
TPS: tumor proportion score
TTF1: thyroid transcription factor-1
UIP: usual interstitial pneumonia
UK NEQAS: U.K. National External Quality Assessment Service
VEGF: vascular endothelial growth factor
WHO: World Health Organization
WT: wild type
WT1: Wilms tumor protein
ZEB1: zinc-finger E-box binding protein 1

Summary of Key Questions and Short Answers

Title	Key questions	Short answers
	Chapter 5	
Immunohistochemistry for Small Specimens	5.1 Should immunohistochemistry (IHC) be performed in all small biopsies?	The pathologist's morphologic impression should drive the initial diagnostic approach: If the biopsy shows clear-cut morphologic differentiation of a squamous cell carcinoma (ie, keratinization) or adenocarcinoma (ie, glandular formation), the pathologist is encouraged to render the diagnosis accordingly and is not mandated to apply confirmatory IHC.
	5.2 When should IHC be performed to classify non-small cell carcinoma (NSCC)?	IHC should be performed in poorly differentiated carcinoma (often solid growth pattern).
	5.3 What are the best first markers to classify NSCC?	The combination of p40 and thyroid transcription factor-1 (TTF1) has the best sensitivity and specificity to separate NSCC into adenocarcinoma and squamous cell carcinoma.
	5.4 What other tumor markers can be helpful in the classification of NSCC?	Napsin A and cytokeratin 5/6 (CK5/6) may improve the classification in some NSCC, not otherwise specified (NOS). A pankeratin should be added in case of a poorly differentiated tumor that is negative for TTF1, p40, CK5/6, and napsin A.
	5.5 What are the best markers for neuroendocrine (NE) neoplasms?	A panel of NE markers (chromogranin, synaptophysin, CD56, and/or insulinoma-associated protein 1 (INSM1) should be added to confirm NE phenotype. In addition, a proliferation marker is helpful in small biopsies with significant crush artifact.
	5.6 What are the challenging diagnostic scenarios in small biopsies?	The artifacts associated with small biopsies, including poor cellular preservation, crush artifact, and distortion, are as likely to confound interpretation of benign structures as they are to mask the morphologic features of tumor cells. Careful correlation of histology and immunohistochemical stains is necessary.
	5.7 How should biopsy specimens be handled to optimize predictive biomarker testing results?	A detailed protocol for tissue utilization should be established in each laboratory for determination of predictive markers
	Chapter 6	
Immunomarkers in the Classification of Resected Major Lung Cancers	6.1 What is the best combination of markers to use in daily practice to distinguish adenocarcinoma and squamous cell carcinoma?	In daily practice, TTF1 and p40 will suffice for subtyping most NSCCs without defining morphologic characteristics or NE morphology. This panel may be expanded when metastases or rarer variants are suspected.
	6.2 What is the role of immunomarkers in the diagnosis of adenosquamous carcinoma?	IHC for TTF1 and p40 can be helpful in the diagnosis of adenosquamous carcinoma when they highlight 2 distinct tumor cell populations.
	6.3 What is the utility of IHC in sarcomatoid carcinoma?	Immunoreactivity for cytokeratin can be helpful in supporting a malignant spindle or giant cell carcinoma pattern in pleomorphic carcinoma. IHC in rare subtypes can confirm heterologous elements, or in a blastoma, a fetal adenocarcinoma component.
	6.4 What is the role of IHC in the diagnosis of large cell carcinoma?	In mucicarmine negative undifferentiated carcinomas without TTF1, napsin A, or p40 staining, a diagnosis of large cell carcinoma can be rendered, after consideration of *SMARCA4*-deficient carcinoma.

Title	Key questions	Short answers
	Chapter 7	
Thyroid Transcription Factor-1	7.1 Are there any staining differences in lung adenocarcinoma between TTF1 clones?	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.
	7.2 What extent of TTF1 staining is considered a positive test result?	Focal positivity for TTF1 is considered a positive reaction indicating pulmonary adenocarcinoma in the proper clinical context, and the optimal cutoff values vary among the clones.
	7.3 Are there any pre-analytic considerations for TTF1 immunostaining?	There are some specific pre-analytic considerations regarding TTF1 immunostaining, mostly in relation to reduced or absent staining in specimens fixed in alcohol-based fixatives and subjected to certain decalcifying agents.
	Chapter 8	
Immunohistochemistry for p40 and p63 in Lung Cancer	8.1 In what cases should p40 be used rather than p63?	p40 should be used for identification of morphologically undifferentiated squamous cell carcinomas as it demonstrates superior accuracy to p63 in this setting.
	8.2 In what cases should p63 be used instead of p40?	If nuclear protein in testis (NUT) carcinoma is considered in the differential diagnosis of a tumor, then p63 may be more useful than p40.
	8.3 What extent of p40/p63 positive reactions should be considered positive?	There is usually diffuse strong positivity for p40 (and p63) in squamous cell carcinomas, and expression in at least 50% of nuclei should be considered a positive result.
	Chapter 9	
Cytokeratin Markers	9.1 What are pancytokeratin stains and what is their role in the diagnosis of lung cancer?	Pancytokeratin stains can establish a diagnosis of carcinoma when the tumor is morphologically undifferentiated.
	9.2 Are CK5 or CK5/6 sensitive and specific markers for squamous cell carcinoma of the lung?	CK5/6 does not have adequate sensitivity and specificity, thus should not be used alone to diagnose pulmonary squamous cell carcinoma.
	9.3 Should CK7 be used to differentiate lung adenocarcinoma from squamous cell carcinoma?	CK7 should not be used to distinguish between pulmonary adenocarcinoma and squamous cell carcinoma.
	9.4 Should an NSCC that is diffusely positive for CK7 but negative for TTF1 and p40 be regarded as "probably adenocarcinoma"?	CK7 alone is insufficient to establish a diagnosis of pulmonary adenocarcinoma.
	9.5 Is CK7 a helpful stain in differentiating pulmonary adenocarcinoma from mesothelioma?	CK7 cannot differentiate pulmonary adenocarcinoma from malignant mesothelioma.
	9.6 Which cytokeratin antibody is preferred to stain small cell lung carcinoma (SCLC)?	Pancytokeratin antibodies are preferred when being used to recognize SCLC.
	9.7 What is the utility of CK20 in the diagnosis of lung cancer?	CK20 positivity does not exclude mucinous adenocarcinoma of lung origin.
	9.8 Which cytokeratin antibody should be used for mesothelioma?	Pancytokeratin and CK5/6 are useful in diagnosing mesothelioma, in conjunction with other mesothelioma markers.
	9.9 What is the role of cytokeratins in the diagnosis of thymoma?	Pancytokeratins are very useful in the differential diagnosis of thymomas from other mediastinal lesions.
	Chapter 10	
Neuroendocrine Markers	10.1 What IHC markers are useful to support NE morphology in the classification of NE neoplasms?	Chromogranin, synaptophysin, CD56, and INSM1 are useful NE markers in support of NE morphology.
	10.2 What non-NE markers can assist in the classification of tumors in the differential diagnosis of neuroendocrine neoplasm (NEN)?	Pankeratin and low-molecular-weight keratins rather than high-molecular-weight keratins should be positive in NENs.
	10.3 When should NE markers be applied to an NSCC?	NE markers should only be used when morphologic features of NE differentiation are present. In small samples where NE morphology may be difficult to assess, there may be greater specificity when 2 or more markers are positive.

Title	Key questions	Short answers
	Chapter 14	
Immunomarkers for Other Rare Tumors	14.1 Does IHC aid in the diagnosis of alveolar adenoma?	Although primarily a hematoxylin and eosin (H&E) diagnosis, IHC can be valuable to highlight the neoplastic pneumocytes and mesenchymal stroma.
	14.2 Does p40 IHC have a role in the diagnosis of ciliated muconodular papillary tumor (CMPT), distinguishing it from adenocarcinoma?	Basal cell markers, such as p40, p63, and CK5/6, help identify the double epithelial cell layer in CMPT.
	14.3 What is the immunoprofile of NUT carcinomas?	Apart from NUT expression, keratins and basal cell markers are often expressed, but TTF1 and epithelial membrane antigen (EMA) may also be found.
	14.4 What is the pattern of NUT expression in NUT carcinomas?	A nuclear speckled pattern in more than 50% of tumor cells is characteristic and diagnostic for NUT carcinoma. NUT immunostain is sensitive (87%) and specific (100% after exclusion of seminoma).
	14.5 Are immunomarkers useful in distinguishing sclerosing pneumocytoma from adenocarcinoma?	Immunostains may help in the diagnosis of sclerosing pneumocytoma, by assisting in the identification of the 2 cellular compartments with different immunoprofiles; keratins and TTF1 are key elements in this distinction.
	14.6 Are immunomarkers helpful in diagnosing pulmonary mucoepidermoid carcinomas?	Of only limited use, IHC for p63, p40, or CK5/6 may highlight the epidermoid cell component.
	14.7 Do immunostains aid in the diagnosis of pulmonary adenoid cystic carcinomas and their distinction from both non-small cell and small cell carcinoma?	As with mucoepidermoid carcinoma, the diagnosis of adenoid cystic carcinoma is normally based on an H&E stain. IHC may, however, help in identifying the dual cell population, especially in morphologically challenging samples, when CD117 can highlight ductal/luminal cells while p63 and S100 stain the myoepithelial/peripheral cells.
	14.8 Can immunostains aid in the diagnosis of epithelial-myoepithelial carcinoma in the lung?	As with other salivary-type tumors, IHC only assists in identifying the different cell populations defining the lesions in the correct morphologic context of the H&E stained section.
	Chapter 15	
Immunomarkers for Thoracic Sarcoma	15.1 Which immunomarkers are useful in the diagnosis of so-called *SMARCA4*-deficient thoracic malignant tumor?	Thoracic *SMARCA4*-deficient undifferentiated malignant tumor shows absence or marked reduction of immunostaining for SMARCA4 (BRG1).
	15.2 When should SMARCA4 staining be considered in the assessment of thoracic tumors?	Staining for SMARCA4 should be performed in morphologically undifferentiated, relatively monotonous, discohesive, or rhabdoid pattern tumors, and not more generally in morphologically undifferentiated carcinomas.
	15.3 Which immunomarkers are useful to prove vascular endothelial differentiation?	ERG and CD31 are useful endothelial markers. Other markers, such as CD34 and FLI1, are potentially useful but less definitive.
	15.4 Which immunomarkers are useful in subtyping malignant vascular endothelial tumors?	IHC for calmodulin-binding transcription activator 1 (CAMTA1) and transcription factor E3 (TFE3) is a surrogate for translocation and can be useful in vascular tumor subclassification.
	15.5 Which immunomarkers are useful for distinguishing synovial sarcoma from its mimics?	IHC for cytokeratin and transducin-like enhancer protein 1 (TLE1), with relevant negatives, can be helpful in the diagnosis of synovial sarcoma. However, difficult cases can be confirmed with fluorescence in situ hybridization (FISH) or molecular testing.
	15.6 Which immunomarkers are useful for assessing solitary fibrous tumors (SFTs)?	Signal transducer and activator of transcription 6 (STAT6), using a monoclonal antibody, is a sensitive and relatively specific marker for SFT.
	15.7 Are immunomarkers helpful in diagnosing inflammatory myofibroblastic tumors (IMTs)?	In the correct morphologic setting, IHC for ALK or ROS1 can be helpful in the diagnosis of IMT.
	15.8 Which immunomarkers are useful for assessing pleomorphic spindle cell sarcomas?	Various markers can help classify spindle cell sarcoma, including MDM2, muscle markers, and H3K27me3, but all can have reactivity in more than 1 tumor type.

Title	Key questions	Short answers
	15.9 Which immunomarkers are useful in assessing round cell sarcoma?	Markers of round cell sarcoma may be helpful in the diagnosis, but some overlap remains with more common entities such as small cell carcinoma. Epidemiologic considerations (age) and tumor location can be helpful, as well as FISH and molecular testing, as needed.
	15.10 Which immunomarkers help to distinguish perivascular epithelioid cell tumor (PEComa) from its mimics?	PEComa are generally negative for cytokeratin and positive for HMB45, melan A, and tyrosinase A.
Chapter 16		
Immunomarkers for Differentiation from Metastatic Tumors	16.1 Is IHC useful for distinguishing metastatic squamous cell carcinoma and primary lung squamous cell carcinoma?	There is no IHC marker that can reliably assist in distinction of metastatic from primary squamous cell carcinoma in the lung. Clinicopathologic correlation is required.
	16.2 What IHC markers are useful in distinguishing metastatic tumors of GI tract origin from primary lung tumors?	A combination of IHC for cytokeratins (CK7/CK20), lung (TTF1/napsin A), and GI (CDX2) tract markers is useful to confirm a metastasis from a GI tract origin. In TTF1/napsin A negative tumors, positivity for CDX2 points toward a metastasis from the GI tract. In TTF1/napsin A/CDX2 negative tumors, the CK7/CK20 profile may be helpful, but clinical and radiologic correlation is usually required to confirm the origin.
	16.3 What IHC markers are useful to distinguish metastatic carcinomas of breast origin from primary lung carcinoma?	In any patient with a history of a breast carcinoma, comparison with histologic features of the primary breast tumor is recommended where possible. A combination of lung and breast markers that include TTF1 and/or napsin A and estrogen receptor/progesterone receptor (ER/PR) or GATA3 can provide a definite answer in most cases. When clinically relevant, GATA3 should be combined with mammaglobin to rule out an extramammary malignancy such as bladder carcinoma. In triple-negative breast carcinoma (TNBC), a combination of lung markers with both GATA3 and SOX10 is recommended to distinguish metastatic breast carcinoma from primary lung carcinoma.
	16.4 What IHC markers are useful to distinguish metastatic carcinoma of female genital tract origin from primary lung carcinoma?	When the differential diagnosis of a lung tumor includes a metastatic female genital tract carcinoma, TTF1 should be used with caution as with primary lung adenocarcinomas, these tumors may also express TTF1. PAX8 staining is useful to help identify metastatic tumors of female genital tract origin.
	16.5 What IHC markers are useful to distinguish metastatic carcinomas of urothelial origin from primary lung carcinoma?	A combination of CK7, CK20, and GATA3 are most useful in the distinction of metastatic urothelial carcinoma from pulmonary squamous cell carcinoma.
	16.6 What IHC markers are useful to distinguish metastatic carcinomas of renal origin from primary lung carcinoma?	PAX8 and TTF1 are useful to distinguish metastatic renal cell carcinoma (PAX8+/TTF1–) from primary lung adenocarcinoma (PAX8–/TTF1±). Napsin A is not useful in this setting as it is expressed in a variable proportion of renal cell carcinomas.
	16.7 What IHC markers are useful to distinguish metastatic carcinomas of prostate origin from primary lung carcinoma?	A combination of negative CK7, CK20, and TTF1 together with positive staining for a prostate marker such as NK3 homeobox 1 (NKX3.1) can be used to identify metastatic prostatic carcinoma.
	16.8 What IHC markers are useful to distinguish metastatic carcinoma of hepatic origin from primary lung carcinoma?	A combination of hepatocellular markers, such as arginase-1 and hepatocyte paraffin 1 (HepPar1), together with negative CK7 and pulmonary markers can be used to identify metastatic hepatocellular carcinoma.
	16.9 What IHC markers are useful to distinguish metastatic carcinoma of thyroid origin from primary lung carcinoma?	A combination of PAX8 and TTF1 positivity together with absence of napsin A is useful to identify metastatic thyroid carcinomas.
Chapter 17		
Mesothelioma and Immunohistochemistry	17.1 What are the best markers to distinguish epithelioid malignant pleural mesothelioma (MPM) from carcinoma?	Calretinin and Wilms tumor protein (WT1) are the best positive mesothelial markers for diagnosis of epithelioid MPM. Claudin 4, MOC31, monoclonal CEA, B72.3, and Ber-EP4 in combination with site-specific markers are best in differentiating carcinoma from epithelioid MPM.

Title	Key questions	Short answers
	17.2 What are the best markers to distinguish sarcomatoid MPM from sarcomatoid carcinoma?	Sarcomatoid MPM can be diagnosed with positive cytokeratin and mesothelial markers, most frequently D2-40 and calretinin, while carcinoma markers are negative. If cytokeratins are negative, the differential diagnosis should include sarcomas, and expanded work-up for specific gene fusions/rearrangements should be considered.
	17.3 What is the role of cytokeratins in the diagnosis of MPM?	Cytokeratin stains are helpful in highlighting full-thickness pleural cellularity, lack of zonation, and presence of invasion of mesothelial cells into chest wall adipose tissue.
	17.4 What immunohistochemical markers can be used to distinguish between benign and malignant mesothelial proliferations?	BRCA1-associated protein 1 (BAP1) IHC and methylthioadenosine phosphorylase (MTAP) (as a surrogate for *CDKN2A* homozygous deletion) can be helpful in distinguishing benign from malignant mesothelial proliferation in surgical and fluid specimens.
Chapter 18		
Thymic Tumors and Immunohistochemistry	18.1 What are the best markers for the diagnosis of thymoma?	A combination of keratin and terminal deoxynucleotidyl transferase (TdT) is the best panel for the diagnosis of thymoma. CD1a or CD99 can also be used to mark thymic immature lymphocytes.
	18.2 What other markers can be used to highlight thymic epithelial cells?	Polyclonal PAX8 and p40/p63 can be added to the panel of keratin and TdT for the characterization of thymomas.
	18.3 How can IHC help in the classification of thymomas?	A keratin stain can reveal the pattern of epithelial cells, which helps in the classification between B1 and B2 thymoma.
	18.4 How can thymoma be differentiated from thymic carcinoma?	The differential diagnosis of World Health Organization (WHO) type B3 thymoma and thymic carcinoma can be challenging in small biopsy specimens. The presence of TdT-positive lymphocytes is in favor of the diagnosis of thymoma. Positive CD5 and/or CD117 expression helps the diagnosis of thymic carcinoma as well as the differential diagnosis from lung squamous cell carcinoma.
	18.5 Which stains are useful in diagnosing germ cell tumors?	SALL4 is a pan-germ cell tumor marker that should be included in a panel to work up these tumors. Once positive SALL4 suggests germ cell tumor, OCT3/4, KIT (CD117), CD30, and glypican 3 can be used as classifiers for seminoma and non-seminomatous tumors. Other markers should be included after histologic examination and added accordingly.
Chapter 19		
Use of Immunohistochemistry in Predictive Biomarker Testing	19.1 Does IHC have a role in detecting epidermal growth factor receptor (EGFR) alterations?	EGFR WT IHC has no current routinely recommended clinical use. IHC to detect a limited range of mutant EGFR proteins is occasionally used in particular circumstances.
	19.2 What is the role of IHC in detecting tumors bearing *ALK* gene rearrangements?	ALK IHC has a pivotal role in *ALK* predictive biomarker testing in patients with advanced stage non-small cell lung carcinoma (NSCLC).
	19.3 What is the role of IHC in detecting tumors bearing *ROS1* gene rearrangements?	ROS1 IHC is an established technique for the enrichment of a patient population with advanced stage NSCLC to have confirmation of *ROS1* gene rearrangement by an alternative molecular method.
	19.4 Does IHC have a role in the identification of tumors with neurotrophic tyrosine receptor kinase (*NTRK1-3*) gene rearrangements?	IHC testing to assist the detection of *NTRK1-3* gene rearrangements is an emerging technique but without an established role.
	19.5 Does IHC testing have any role in predictive biomarker testing for any other targetable genomic alterations?	There are no established roles for IHC testing for the identification of patients bearing other targetable alterations.
	19.6 What is the role of PD-L1 IHC in selecting patients with NSCC for immunotherapy?	Although alternative biomarkers for use with immunotherapy in NSCC are actively being sought, it seems highly likely that PD-L1 IHC will stay as part of the required assessment of NSCC clinical samples in relation to anti–programmed cell death protein-1 (PD1) and PD-L1 immunotherapy. Questions, however, remain about different assays, sample types, expression in tumor versus immune cells, and how the IHC data should be used for clinical decision-making.
	19.7 Can IHC be used to assess the tumor microenvironment to select patients for immunotherapy?	How tumor microenvironmental factors might, in the future, be assessed and used remains to be determined. This must be based on sound evidence and clinical trials.

Introduction

1

By Yasushi Yatabe, Keith M. Kerr, Alain C. Borczuk, Wendy A. Cooper
Sanja Dacic, Andre L. Moreira, and Ming Sound Tsao

Immunohistochemistry (IHC) is a cornerstone of pathologic diagnosis, by far the most widely used ancillary technique to assist with the identification and classification of disease. For the last 30 years, pathologists have harnessed this powerful technology to transform the way we make diagnoses across the spectrum of pathologic medicine but especially in tumor pathology. Histochemical techniques and electron microscopy still have their place, but IHC is the go-to technique to answer a problem.

Immunohistochemical markers are now key in providing more accurate diagnosis in lung cancer and other thoracic malignancies. Many of the issues are common to many areas of cancer diagnosis, such as the use of keratins and other epithelial markers to identify and diagnose carcinoma, the use of lymphoid markers to identify and classify lymphoproliferative disorders, and the use of organ-specific markers in the diagnosis of tumors that are metastatic to the lung, where the immunophenotype can be crucial in securing the correct diagnosis when the morphology is insufficient. Neuroendocrine markers are important in the diagnosis of the spectrum of neuroendocrine tumors that occur in the lung, for example, in differentiating cases of small cell lung carcinoma (SCLC) as opposed to a tumor in the non-small cell family (see the following discussion), as their treatments are vastly different. Neuroendocrine markers are also required for the diagnosis of large cell neuroendocrine carcinoma, in order to confirm neuroendocrine differentiation in a tumor with appropriate morphology. IHC is a fundamental part of the diagnosis of malignant mesothelioma, has allowed accurate diagnosis in even very small samples, and provides greater diagnostic security in an area where there are particular medicolegal implications for this diagnosis. A better understanding of the morphologic classification of thymoma is underpinned by the expression of some cytokeratins and also antigens associated with thymic T cells, and IHC also allows a more secure diagnosis in small diagnostic samples in this setting.

The evolution of personalized medicine in lung cancer and the emergence of a number of different anti-cancer therapies that are prescribed on the basis of particular pathologic

features of the patient's tumor has, however, given IHC a very special and extra role, beyond the traditional view of IHC as a diagnostic adjunct.

As mentioned earlier, IHC can be useful in separating SCLC, which in the advanced setting is treated with platinum/etoposide drug combinations, as opposed to alternative regimens given in advanced non-small cell lung carcinoma (NSCLC). The approval of platinum/pemetrexed combination therapy for patients with advanced non-squamous NSCLC gave IHC a pivotal role in determining therapy choice for patients with advanced stage NSCLC. In the small biopsy and cytology type samples that are all that is available in most patients with advanced stage disease, between 25% and 40% of cases of NSCLC cannot be accurately subtyped beyond a label of NSCLC-not otherwise specified (NOS) on morphologic grounds alone. Prior to the introduction of different chemotherapy regimens depending on NSCLC subtype, this was no impediment to therapy, and a diagnosis of NSCLC-NOS was clinically acceptable. When therapy choice became predicated on specific diagnosis, IHC became the key tool in refining the diagnosis of NSCLC-NOS, reducing the prevalence of these cases to under 10% by the judicious use of thyroid transcription factor-1 (TTF1) to predict adenocarcinoma histology and p63 or p40 to predict squamous histology. This principle is now embedded in the World Health Organization (WHO) classification of lung tumors.

In the WHO classification published in 2015, IHC was also given a more fundamental role. The presence of these same markers, p40 and TTF1, was given equal status with classical morphologic features in the *definition* of both squamous cell and adenocarcinoma respectively, in the surgically resected tumor setting. Thus, IHC has been responsible for re-diagnosis of probably two-thirds of cases, which would formerly have been called large cell carcinoma, as either squamous cell or adenocarcinoma purely on the basis of a positive p40 or TTF1 IHC stain.

Finally, although not the focus of this atlas, IHC has a crucial role in the identification of therapy predictive biomarkers that allow patients to be selected for treatment with an ever-expanding range of targeted therapies aimed at addictive oncogenic drivers and other molecular factors important for the growth of a particular tumor. Given the propensity for these targets to occur in adenocarcinoma, the initial subtype diagnosis of NSCLC is therefore also crucial in ensuring the accurate triage of cases for molecular testing. The evolution of immunotherapy in lung cancer has given a further important role for IHC in determining the appropriate therapy for patients.

It is very clear that in order to render the best and most accurate diagnosis for our patients with thoracic malignancy, pathologists must understand how IHC works, how to use it, when to use it, and how to appropriately interpret the results of the assays performed. In this atlas, members of the Pathology Committee of the International Association for the Study of Lung Cancer have provided a succinct but comprehensive review of many aspects of IHC that are relevant to thoracic tumor diagnosis, building on a review article published in the *Journal of Thoracic Oncology* (Yatabe et al 2019). We very much hope that readers will find this atlas a useful tool to aid their work.

Reference

Yatabe Y, Dacic S, Borczuk AC, et al. Best practice recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Clinical Relevance of Accurate Diagnosis of Thoracic Neoplasms Using Immunohistochemistry

2

By Harvey I. Pass and Balazs Halmos

Value for Immunohistochemistry in the Work-Up of Pulmonary Nodules and Lung Cancer Staging

Lung Cancer Diagnoses for the Indeterminate Pulmonary Nodule

Not every patient needs histologic verification of malignancy for a surgeon to deem the nodule actionable. A preoperative biopsy is seldom used for part-solid nodules in high-risk individuals with solid components that are developing or growing, especially if there are other characteristics pointing to malignancy, that is, high standardized uptake value (SUVmax) with positron emission tomography/computed tomography (PET/CT). There are cases, however, where absolute confirmation of histology is useful, specifically to rule out a benign process, or in a patient with a previous extrathoracic malignancy where documentation of a metastasis before resection is suggested. In these, fine-needle aspiration or core biopsies and supplementation with specific lung cancer immunohistochemistry (IHC) panels may be invaluable to decide appropriate management. IHC supplementation may also become crucial in determining whether one is dealing with an unusual situation on the biopsy, including whether a tumor is a benign nodule masquerading as lung cancer, or in differentiating small cell lung cancer from other entities in the neuroendocrine spectrum. Differentiating cavitary lesions, which could be of fungal or mycobacterial origin from cavitating carcinoma, are another example where histology and special stains, including IHC, can drastically change prescribed therapy.

Lung Cancer Diagnosis

IHC is crucial for the diagnosis and definitive classification of lung cancers in some instances. The purview of the surgeon is to operate for the correct diagnosis and to be able to assure the patient that a complete resection was performed. Errors in management can obviously be avoided if the surgeon has an unequivocal preoperative diagnosis of malignancy, and if the type of malignancy as well as any associated staging details related to the pathology of the

abnormality are correctly diagnosed. Surgeons are confronted with percutaneous core and fine-needle aspirations for a variety of conditions in the chest, including lung cancer, benign nodules masquerading as lung cancer, and mediastinal as well as pleural tumors. To guide a surgeon's decision, the specificity of a biopsy not only depends on the size of the specimen that the pathologist is working with and the pattern recognition on a hematoxylin-eosin stain, but also on specific IHC panels differentiating benign conditions from those that are malignant and the type of malignant tumors.

Lymph Node Status and IHC

Immunohistochemical examination of suspicious adenopathy by size or PET-avidity in a patient with presumed or diagnosed lung cancer may have significant applicability. Determination of micrometastases detected using either cytokeratin cocktails or other IHC markers of lung cancer may alert the surgeon to decide whether to refer the patient for induction regimens or to operate first. Moreover, in the patient with a history of chronic lymphocytic leukemia/small lymphocytic lymphoma or other indolent lymphomas, confirmation that the suspicious adenopathy is related to the lymphoma and not to lung cancer may require flow cytometry as well as lymphoma IHC panels.

Mistaken and Masquerading Identity

The published thoracic oncology literature is replete with surgical cases that were "near misses" or determined to be "surprises" after resection. There have been multiple reports differentiating infection, such as *Klebsiella* (McCartney et al 2014), actinomycosis (Papakonstantinou et al 2019), and cytomegalovirus (CMV) (Allen et al 2005), from tumor by IHC. Desquamative interstitial pneumonias have been confused with pulmonary adenocarcinomas that have discohesive zones with large numbers of cells in the airspaces (Raparia et al 2014; Mutton et al 1998). IHC with cytokeratin staining has been invaluable in differentiating the diagnosis in such cases.

Mesothelioma

The differential diagnosis of the patient with suspected pleural disease demands the use of multi-antibody panels in order to decide whether surgery is appropriate. The use of BRCA1-associated protein 1 (BAP1) staining loss, as well as the presence of staining for calretinin and Wilms tumor protein (WT1) among other antibodies, coupled with the lack of staining for adenocarcinoma and squamous markers, help a thoracic surgeon specializing in the management of mesothelioma (see Chapter 17). Patients with pleural effusion with a history of malignancy may also be confused for mesothelioma, and IHC for the primary tumor matching that of the pleural disease can rule out cytoreductive surgery. Although controversy surrounds the use of surgery for mesothelioma, making the correct diagnosis with IHC is paramount. Distinguishing mesotheliomas from other intrathoracic malignancies yields a path to initiating the proper treatment unique to this disease and also might allow patients to understand the etiology of their disease—with potential legal and financial implications. Recognizing the more aggressive sarcomatoid histology also allows the clinical team to avoid treatment interventions, such as extensive surgeries, which have limited benefit in this subset and might only expose patients to undue treatment morbidity.

Neuroendocrine Carcinomas

A very important pathologic distinction driving clinical decision-making is that of subtyping neuroendocrine carcinomas (see Chapter 10). Proper IHC tests to assess neuroendocrine (NE) differentiation and Ki-67 staining may assist in proper classification and thereby optimal tailoring of therapy. Typical (low-grade NE tumors) and atypical (intermediate grade) carcinoids are generally treated along guidelines established for low-grade gastrointestinal (GI) NE tumors with somatostatin analogs, mTOR inhibitors (everolimus) (Yao et al 2011), vascular endothelial growth factor (VEGF)–targeting drugs (sunitinib) (Raymond et al 2011), and more recently, peptide receptor radionuclide therapy (Strosberg et al 2017). The mainstay of therapy of more aggressive, high-grade NE malignancies, such as large cell NE carcinomas and small cell carcinomas, has traditionally been combination chemotherapy with the recent integration and demonstrated activity of checkpoint inhibitor therapy. Such combinations led to the approval of atezolizumab in combination with carboplatin/etoposide chemotherapy and pembrolizumab for the management of extensive-stage small cell lung cancer (Horn et al 2018; Chung et al 2020).

Other Thoracic Malignancies

Proper subtyping of thymic malignancies, including invasive thymomas, thymic carcinomas, and carcinoids, permit optimal treatment selection, prognostication, and further experimental study participation. The recent World Health Organization (WHO) classification acknowledges the important role of IHC studies in proper subtype assignment (Marx et al 2014; see Chapter 18). A unique aspect in the management of thymic malignancies is that of paraneoplastic syndromes, and recent studies suggest a high frequency of potentially severe immune adverse events with checkpoint inhibitor therapy in this class of tumors (Lippner et al 2019). Other emerging subsets with potential treatment relevance given potential targetable molecular alterations are nuclear protein in testis (NUT) carcinomas (Stathis et al 2016; see Chapter 14) and *SMARCA4*-deficient intrathoracic malignancies (Le Loarer et al 2015; see Chapter 15). Although listed under non-small cell lung cancers, recognizing pulmonary sarcomatoid carcinomas is important, given their aggressive nature and also their high frequency of harboring *MET* exon 14 alterations (Liu et al 2016) and treatment responsiveness to both *MET*-targeting as well as immunotherapy (Schrock et al 2017).

Metastatic Disease

A key distinction in the differential diagnoses of lung lesions or hilar/mediastinal/supraclavicular adenopathy is that of metastatic disease from other organs (see Chapter 16). Careful communication between clinician and pathologist is pivotal to ensure that key historical, clinical, and radiographic elements of the case are relayed to the pathologist to guide the extent of the work-up. Although appropriate diagnosis of a metastatic malignancy is critically important, wasteful use of tissue for a series of unneeded IHC studies in a case of a known pulmonary malignancy where biopsy is done to allow biomarker studies to guide therapy can be of significant negative consequence. Recent studies suggest that besides IHC studies, expanded molecular testing might assist in the recognition of primary site/tumor synchronicity (Chang et al 2019).

Special Considerations on the Use of IHC for Treatment Selection

Prognostic Markers for Adjuvant Treatment Selection

The current WHO histologic classification of adenocarcinoma defines subtypes, which are associated with an increased chance of recurrence and death (Warth et al 2012; Tsao et al 2015). Nevertheless, not all patients with micropapillary or solid disease have early recurrence, and other methods for more accurate prognostication must be investigated. Certain microscopic morphologic issues, such as lymphovascular invasion, visceral pleural invasion, and spread through alveolar spaces (STAS), and high-grade nuclear features have also been associated with a more aggressive phenotype; however, the inability to successfully predict recurrence with these features is one of the reasons they are not considered absolute indications for adjuvant therapy. This interrelationship between matching features, which consistently are associated with recurrence and/or death, and a justified need for adding potentially beneficial therapies is also influenced by the possibility of overtreating patients and causing toxic complications. The hope that single or multiple antibody IHC prognostication can increase the accuracy of recurrence prediction started more than 25 years ago with oncoprotein staining for erbB-2 (human epidermal growth factor receptor 2 [HER2]/neu), p53, and Ki-67 in 271 early stage lung cancer patients (Harpole et al 1995). Since then, there have been hundreds of published reports on IHC-based prognostic markers without clear impact in practice (Zhu et al 2006; Woodard et al 2016; Seymour et al 2019). This can largely be accounted for by the lack of standardization in the IHC methods used, including the source and quality of the antibodies used, staining protocol, scoring algorithm and "cutoff," and statistical approach to analyze the data. Inconsistent results can also be caused by the small sample size in some studies, for which cases included are less representative. Institutional and publication biases can also play an important role (Zhu and Tsao 2014). Most of these studies lacked validation sets and failed to perform multivariate analyses to prove that the panel of single IHC test is an independent predictor of events.

Predictive IHC Biomarkers

A review by Hung and Sholl (2018) detailed which of the targetable fusions and mutations have accompanying specific IHC antibodies. Anaplastic lymphoma kinase (*ALK*) rearrangements can be detected with clones D5F3 (Roche Tissue Diagnostics) and 5A4 (Leica) with sensitivities and specificities greater than 95% and can be used as stand-alone assays for selecting patients for ALK-based therapies (Lindeman et al 2018; Tsao et al 2016). c-ros oncogene 1 (*ROS1*) rearrangement, like *ALK*, can be diagnosed most commonly by fluorescence in situ hybridization (FISH); and Hung and Sholl (2018) emphasize that despite a sensitivity of 95% with clone D4D6 (Cell Signaling Technology, Inc.), the specificity varies among studies. Hence, ROS1 IHC should have confirmation of a positive result by other platforms (Lindeman et al 2018); however, a negative ROS1 IHC result can be considered reliable to rule out a *ROS1* translocation. Epidermal growth factor receptor (EGFR) mutant–specific antibodies for L858R and for exon 19 E746-A750 deletion, have variable clinical performance, and an overall sensitivity of 59% and specificity of 98% is generally agreed on (Ragazzi et al 2016). False-positive IHC results have been recorded (Kitamura et al 2010), and tumors with negative EGFR mutant–specific IHC results should be retested for EGFR mutations using

molecular methods. Hence, EGFR mutant–specific antibodies should not be used as a standalone test for therapy (Lindeman et al 2018). Further details are discussed in Chapter 19.

Squamous Cell Lung Cancer

Squamous cell carcinomas continue to make up a substantial proportion of non-small cell lung cancers and are highly associated with smoking history, generally demonstrating intermediate/high tumor mutation burden and significant benefit from checkpoint inhibitor therapy. Pathologic distinction remains highly important for several considerations. First, initial studies of the anti-VEGF monoclonal antibody, bevacizumab, in patients with advanced non-small cell lung cancers highlighted substantial toxicity, namely a high risk of hemoptysis, potentially fatal and particularly common in patients with squamous cell tumors (Johnson et al 2004; Table 2-1). Whether this high risk is related to biologic or anatomic features (usually central large masses with cavitation are at highest risk, which are more common with squamous histology) is unclear; however, as the approval of bevacizumab remains limited to non-squamous tumors, and indeed this is a real toxicity concern, histologic confirmation is important if the use of bevacizumab is contemplated. Second, the molecular genetics of squamous cell lung cancers is quite distinct from adenocarcinomas, and there is generally very low likelihood for the identification of the common actionable findings notable for adenocarcinomas. Therefore, in general, upfront molecular testing is not recommended for squamous cell carcinomas. Key caveats here are that the rare nonsmoker subset of squamous cell lung cancers requires adenocarcinoma type testing as yield and actionability is high (Sholl 2017). Furthermore, if there is a reasonable potential for mixed histology (squamous histology identified from a very small sample), then molecular testing might be prudent although clear guidelines for this subset are difficult to generate because of the great heterogeneity of sample types. Squamous cell carcinomas quite uniformly are

Table 2-1. Potential Treatments According to Genetic Alterations

Diagnosis (test)	Treatment potentially indicated	Treatment potentially excluded
Squamous NSCLC	Necitumumab (anti-EGFR mAb)	Bevacizumab (anti-VEGF mAb)
Non-squamous NSCLC	Pemetrexed, bevacizumab	
EGFR mutation	EGFR TKIs	Immunotherapy
ALK translocation	ALK TKIs	Immunotherapy
ROS1 translocation	ROS1 TKIs	
BRAF V600E	BRAF/MEK combination therapy	
*NTRK*1/2/3 translocation	TRK inhibitor therapy	
MET exon 14 skipping alteration	MET TKIs	
RET translocation	RET TKIs	
ERBB2/HER2 mutation	Experimental ERBB2 inhibitors	
PD-L1 positive	Single-agent immunotherapy	
NUT carcinoma	Experimental BET/HiDAC inhibitors	
SMARCA4-deficient thoracic sarcomas	Experimental EZH2 inhibitors	

Abbreviations: *ALK* = anaplastic lymphoma kinase; *BET* = bromodomain and extraterminal; *BRAF* = B-raf proto-oncogene; *EGFR* = epidermal growth factor receptor; EZH2 = enhancer of zeste homolog 2; *HER2* = human epidermal growth factor receptor 2; HiDAC = high-dose cytarabine; MEK = mitogen-activated protein kinase (MAPK) kinase; *MET* = MET proto-oncogene; NSCLC = non-small cell lung carcinoma; *NTRK* = neurotrophic tyrosine receptor kinase; NUT = nuclear protein in testis; PD-L1 = programmed death ligand-1; *RET* = ret proto-oncogene; *ROS1*= c-ros oncogene 1; TKI = tyrosine kinase inhibitor; VEGF = vascular endothelial growth factor.

strongly positive for EGFR receptor expression and indeed the SQUIRE (Standards for Quality Improvement Reporting Excellence) study demonstrated a statistically significant benefit of the anti-EGFR monoclonal antibody, necitumumab, in combination with platinum-based doublet chemotherapy in advanced squamous non-small cell lung carcinoma (NSCLC) leading to U.S. Food and Drug Administration (FDA) approval; although, because of limited clinical benefit and high cost, this treatment is generally not utilized (Thatcher et al 2015). Recent genomics and proteomic studies highlight key molecularly defined tumor subsets among squamous cell tumors, for example, characterized by alterations in oxidative pathways of potential treatment significance (Stewart et al 2019).

Non-Squamous Non-Small Cell Lung Cancer

From a treatment perspective, the proper classification of a "non-squamous" non-small cell lung cancer—principally consisting of majority adenocarcinomas with a shrinking percentage of large cell carcinomas over time—has gained importance with the introduction of the anti-multitargeted anti-folate, pemetrexed. Pemetrexed was demonstrated to have excellent activity and tolerance through a series of studies in both the second-line and the first-line settings in patients with advanced NSCLC with subset analyses of several studies demonstrating histologic differences in activity with inferior activity in patients with squamous cell carcinomas (Scagliotti et al 2008). Conversely, in patients with advanced non-squamous tumors, platinum/pemetrexed chemotherapy doublets have become the most commonly used frontline chemotherapy regimens worldwide and are also extensively used in the adjuvant setting (Scagliotti et al 2011). Ultimately based on the preceding studies, the approval of this widely used and favored chemotherapeutic agent is restricted to non-squamous tumors; therefore for the clinician and patient alike, it remains important that this distinction is made by the pathologist as much as feasible, although admittedly, this construct is artifactual and continues to be debatable. The introduction of immunotherapies has not made this distinction any less important because the most commonly used chemo/immunotherapy combination for non-squamous non-small cell lung cancers based on the highly positive KEYNOTE-189 study remains pemetrexed-based (Gandhi et al 2018). Maintenance pemetrexed therapy is also generally used in this context only for patients with advanced non-squamous non-small cell lung cancer as opposed to no maintenance chemotherapy recommended in squamous cell tumors (Ciuleanu et al 2009). In addition, given the much higher frequency of actionable molecular alterations in adeno and large cell carcinomas, the continued need for proper histologic classification and distinction is highlighted (Chan et al 2019).

Molecular Testing and Tissue Stewardship

Since the discovery of activating *EGFR* gene mutations to identify a molecularly defined tumor subset with exquisite sensitivity to EGFR tyrosine kinase inhibitors (Lynch et al 2004), there has been an explosion of knowledge demonstrating that for the optimal upfront management of advanced non-small cell lung cancer, broad molecular genotyping needs to be completed. This includes at a minimum *EGFR/ALK/ROS*/B-raf proto-oncogene (*BRAF*) testing, where high-level evidence exists for the upfront use of highly effective molecularly targeted therapies (Lindeman et al 2018; Halmos 2018). With the recent exciting data as to excellent activity

and consequent FDA approvals for neurotrophic tyrosine receptor kinase (*NTRK*) inhibitors for *NTRK*1/2/3 fusion–positive cancers, testing for such actionable albeit rare alterations has become highly important as well (Drilon et al 2018a). Emerging data on the actionability of MET exon 14 skipping alterations (Drilon et al 2020) and RET translocations (Subbiah et al 2018) rounds out the presently targetable group). Finally, HER2 alterations (Pillai et al 2017), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) G12C mutations (Lanman et al 2020), and neuregulin (NRG1) fusions (Drilon et al 2018b) are now the focus of ongoing studies with promising data to suggest that testing for these at a minimum could allow patient participation in ongoing clinical trials. All in all, completing genotyping properly with confident positive and negative results obtained in a timely manner to guide patient management requires the development of institutional reflex testing protocols. This suggests the potential superiority of multiplex, ideally next-generation sequencing (NGS) testing over single gene testing approaches. These issues also call for dedicated coordination and extreme care as to tissue stewardship to maximize the yield of clinically relevant tests over unnecessary tissue wastage, which lead to incomplete genotyping and lost opportunities or potential added risks and/or costs for patients, resulting in further invasive procedures. However, even in the era of NGS testing, IHC may have some roles in real practice (Tsao and Yatabe 2019).

PD-L1 Testing

During the last few years, checkpoint inhibitors have transformed the landscape of lung cancer management. Currently, the standard of care for essentially all patients with advanced non-small cell lung cancer and extensive-stage small cell lung cancer includes anti-programmed cell death protein-1 (PD1) or anti-programmed death ligand-1 (PD-L1) therapy in combination with chemotherapy generally or as single-agent pembrolizumab for patients with high PD-L1 tumor proportion score (TPS) score–positive non-small cell lung cancer (Alexander et al 2020). In addition, immunotherapy, namely the anti-PD-L1 antibody, durvalumab, is approved and widely used in the context of definitive therapy for unresectable stage III non-small cell lung cancer following delivery of concurrent chemoradiation (Antonia et al 2018). A vast number of clinical studies are currently being conducted to expand on the utility of immunotherapy in these settings and furthermore to assess the role of immunotherapy in earlier stage scenarios where the impact on cure might be anticipated to be even more significant.

Although the benefits brought about through checkpoint inhibition are clear, how to enrich patient populations for enhanced benefits or lesser toxicity remains ill-defined. From the large variety of biomarkers being assessed, PD-L1 IHC remains the sole validated biomarker. The current approved use is for determining candidacy for single-agent pembrolizumab in the upfront advanced NSCLC setting. A PD-L1 IHC TPS score of 50% or greater defines a patient population in which pembrolizumab is superior to doublet chemotherapy. A score between 1% and 49% determines an intermediate patient population where efficacy of pembrolizumab appears similar to doublet chemotherapy but with lesser toxicities (Lantuejoul et al 2020). As the standard of care is no longer doublet chemotherapy but chemo/immunotherapy for most patients based on results of the KEYNOTE-189/407 and IMpower-150/130 studies, these results need to be put in perspective and interpreted with caution (Gandhi et al 2018; Paz-Ares et al 2018; Horn et al 2018). There currently is no

defined role for PD-L1 testing in the management of small cell lung cancer, and durvalumab is approved by the FDA irrespective of PD-L1 IHC test results in stage III NSCLC (albeit subset analyses suggest limited benefit if any in patients with PD-L1 TPS scores of 0) (Gray et al 2020). The IMpower series of studies assessing the use of the anti-PD-L1 antibody, atezolizumab, focused on a different PD-L1 IHC scoring system including immune cell positivity (TC/IC) (Vennapusa et al 2019). Emerging results suggest similarly enriched benefits with single-agent immunotherapy in patients with high TC/IC scores.

Although there are many issues with PD-L1 IHC testing as to antibody selection, scoring system, and temporal/intratumoral heterogeneity (Rimm et al 2017; Lantuejoul et al 2020), it remains a clinically relevant assay recommended for all patients with advanced NSCLC, squamous and non-squamous alike, based on the preceding considerations. There remains tremendous hope for further biomarkers to emerge to optimize patient selection based on (1) tumor genetics, such as tumor mutational burden (TMB) (tissue or circulating-tumor DNA [ctDNA] based) (Vokes et al 2019), and microsatellite instability (MSI) (less impact in lung cancer because of low frequency) as well as single gene markers (eg, *STK11*/*KEAP1* as negative predictors of immunotherapy efficacy [Skoulidis et al 2018]); or (2) immune signatures by means of RNA expression of selective immune-related genes (Socinski et al 2018). However, none have yet reached the level of validation to be recommended for everyday use.

Emerging Considerations for IHC Studies in the Evolving Treatment Paradigms of Lung Cancer

Histologic Assessment Following Neoadjuvant Therapy

With the increased use of neoadjuvant therapy in the management of selective groups of higher stage patients or in the evaluation of efficacy for window-of-opportunity trials, a more standardized approach for pathologic interpretation of response to therapy at the time of post-induction resection is crucial. Excellent reviews by Hellman and colleagues (2014) and Blumenthal and coworkers (2018) have emphasized the accuracy of the category of 0 to 10% residual viable cells as a major pathologic response (MPR), originally described by Pataer and colleagues (2012) as a surrogate for survival in neoadjuvant trials. Quantitation of residual viable disease may require specific consideration of the type of treatment that the individual had, that is, chemotherapy versus immunotherapy. The characterizations of immunotherapy-related responses have led to the proposal of specific immune-related pathologic response criteria (irPRC) (Cottrell et al 2018). Obviously, there must be standardization of MPR assessment, possibly including response in regional lymph nodes, and the International Association for the Study of Lung Cancer (IASLC) recommendation on MPR assessment has just been published (Travis et al 2020). The standardization, however, may be fine-tuned using multiplexed immunofluorescent platforms (Parra et al 2018) as well as digital spatial profiling (Beechem 2020) of specific regions of interest in the interpretation of the residual tumor bed.

Acquired Resistance and Histologic Transformations

Targeted therapeutics have dramatically improved the care of patients with advanced non-small cell lung cancer harboring actionable alterations; however, acquired resistance is a

uniform issue in this context. Whereas acquired resistance is best understood in the setting of EGFR-mutated lung cancer, the same paradigms appear to apply to all other disease subsets where effective molecularly targeted therapeutics are available (Attarian et al 2017; Lim and Ma 2019). Acquired resistance in general is driven by secondary genetic or epigenetic changes in the tumor that quite predictably impact the following three things:

1. Pathway alterations, typically secondary mutations of the target gene, frequently affect the drug-binding characteristics of the target protein. The best-known example of this is the common emergence of the gatekeeper *EGFR* T790M mutation on first- or second-generation EGFR tyrosine kinase inhibitors (Kobayashi et al 2005). Such secondary mutations might be sensitive to next-generation agents and thereby might guide treatment tailoring.
2. Bypass alterations, for example, MET amplification, represent a potentially actionable alteration with the use of combination targeted therapy blocking both pathways (Nguyen et al 2009).
3. Histologic transformation, which most commonly is seen as small cell transformation in tumors that usually harbor TP53, retinoblastoma (RB), or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) co-alterations. Such small cell-like tumors can respond to small-cell directed treatment regimens, which call for proper detection.

More recently, other histologic transformations to squamous cell and sarcomatoid carcinoma are also being observed with increasing frequency (Schoenfeld et al 2020). Overall, molecular resampling has increasing value, leading to better understanding, optimized experimental trial designs, and most importantly, improved day-to-day patient care. Although molecular testing is currently often initially pursued via ctDNA testing, tissue testing is still commonly called for when ctDNA testing is not informative, or suspicion exists for histologic transformation. In these settings, careful communication between oncologists and pathologists is of paramount importance for appropriate prioritization of the available tissue for conventional IHC tests to properly diagnose malignancy and histologic transformation, preserving tissue for usually NGS-based testing platforms to carefully define the growing spectrum of potential acquired resistance alterations.

Conclusions

There is an ever-growing need for tissue sparing for practicing prudent tissue stewardship during diagnostic work-up. Furthermore, some of the tissue cores are viewed as ideal to meet the research studies calling for best practices to be applied throughout the diagnostic continuum as to obtaining tissue as long as safely feasible. Cautious utilization of IHC studies is a key element in maximizing the diagnostic yield, minimizing the need for repeat procedures, and thereby optimizing yield and turnaround times to treatment initiation.

References

Alexander M, Ko B, Lambert R, et al. The evolving use of pembrolizumab in combination treatment approaches for non-small cell lung cancer. *Expert Rev Respir Med*. 2020;14(2):137-147.

Allen TC, Bag R, Zander DS, Cagle PT. Cytomegalovirus infection masquerading as carcinoma in a lung transplant patient. *Arch Pathol Lab Med*. 2005;129(1):e1-3.

Antonia SJ, Villegas A, Daniel D, et al. Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. *N Engl J Med*. 2018;379(24):2342-2350.

Attarian S, Rahman N, Halmos B. Emerging uses of biomarkers in lung cancer management: molecular mechanisms of resistance. *Ann Transl Med*. 2017;5(18):377.

Beechem JM. High-plex spatially resolved RNA and protein detection using digital spatial profiling: a technology designed for immuno-oncology biomarker discovery and translational research. *Methods Mol Biol*. 2020;2055:563-583.

Blumenthal GM, Bunn PA Jr., Chaft JE, et al. Current status and future perspectives on neoadjuvant therapy in lung cancer. *J Thorac Oncol*. 2018;13(12):1818-1831.

Chan AW, Chau SL, Tong JH, et al. The landscape of actionable molecular alterations in immunomarker-defined large-cell carcinoma of the lung. *J Thorac Oncol*. 2019;14(7):1213-1222.

Chang JC, Alex D, Bott M, et al. Comprehensive next-generation sequencing unambiguously distinguishes separate primary lung carcinomas from intrapulmonary metastases: comparison with standard histopathologic approach. *Clin Cancer Res*. 2019;25(23):7113-7125.

Chung HC, Piha-Paul SA, Lopez-Martin J, et al. Pembrolizumab after two or more lines of previous therapy in patients with recurrent or metastatic SCLC: results from the KEYNOTE-028 and KEYNOTE-158 studies. *J Thorac Oncol*. 2020;15(4):618-627.

Ciuleanu T, Brodowicz T, Zielinski C, et al. Maintenance pemetrexed plus best supportive care versus placebo plus best supportive care for non-small-cell lung cancer: a randomised, double-blind, phase 3 study. *Lancet*. 2009;374(9699):1432-1440.

Cottrell TR, Thompson ED, Forde PM, et al. Pathologic features of response to neoadjuvant anti-PD-1 in resected non-small-cell lung carcinoma: a proposal for quantitative immune-related pathologic response criteria (irPRC). *Ann Oncol*. 2018;29(8):1853-1860.

Drilon A, Clark JW, Weiss J, et al. Antitumor activity of crizotinib in lung cancers harboring a MET exon 14 alteration. *Nat Med*. 2020;26(1):47-51.

Drilon A, Laetsch TW, Kummar S, et al. Efficacy of larotrectinib in TRK fusion-positive cancers in adults and children. *N Engl J Med*. 2018;378(8):731-739. (a)

Drilon A, Somwar R, Mangatt BP, et al. Response to ERBB3-directed targeted therapy in NRG1-rearranged cancers. *Cancer Discov*. 2018;8(6):686-695. (b)

Gandhi L, Rodríguez-Abreu D, Gadgeel S, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N Engl J Med*. 2018;378(22):2078-2092.

Gray JE, Villegas A, Daniel D, et al. Three-year overall survival with durvalumab after chemoradiotherapy in stage III NSCLC-update from PACIFIC. *J Thorac Oncol*. 2020;15(2):288-293.

Halmos B. Molecular testing in lung cancer: where to draw the line?. *Arch Pathol Lab Med*. 2018;142(7):787-789.

Harpole DH Jr., Herndon JE 2nd, Wolfe WG, et al. A prognostic model of recurrence and death in stage I non-small cell lung cancer utilizing presentation, histopathology, and oncoprotein expression. *Cancer Res*. 1995;55(1):51-56.

Hellmann MD, Chaft JE, William WN Jr., et al. Pathological response after neoadjuvant chemotherapy in resectable non-small-cell lung cancers: proposal for the use of major pathological response as a surrogate endpoint. *Lancet Oncol*. 2014;15(1):e42-50.

Horn L, Mansfield AS, Szczęsna A, et al. First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. *N Engl J Med*. 2018;379(23):2220-2229.

Hung YP, Sholl LM. Diagnostic and predictive immunohistochemistry for non-small cell lung carcinomas. *Adv Anat Pathol*. 2018;25(6):374-386.

Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol*. 2004;22(11):2184-2191.

Kitamura A, Hosoda W, Sasaki E, et al. Immunohistochemical detection of EGFR mutation using mutation-specific antibodies in lung cancer. *Clin Cancer Res*. 2010;16(13):3349-3355.

Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2005;352(8):786-792.

Lanman BA, Allen JR, Allen JG, et al. Discovery of a covalent inhibitor of KRASG12C (AMG 510) for the treatment of solid tumors. *J Med Chem*. 2020;63(1):52-65.

Lantuejoul S, Sound-Tsao M, Cooper WA, et al. PD-L1 Testing for lung cancer in 2019: perspective from the IASLC Pathology Committee. *J Thorac Oncol*. 2020;15(4):499-519.

Le Loarer F, Watson S, Pierron G, et al. SMARCA4 inactivation defines a group of undifferentiated thoracic malignancies transcriptionally related to BAF-deficient sarcomas. *Nat Genet*. 2015;47(10):1200-1205.

Lim ZF, Ma PC. Emerging insights of tumor heterogeneity and drug resistance mechanisms in lung cancer targeted therapy. *J Hematol Oncol*. 2019;12(1):134.

Lindeman NI, Cagle PT, Aisner DL, 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. *J Thorac Oncol*. 2018;13(3):323-358.

Lippner EA, Lewis DB, Robinson WH, et al. Paraneoplastic and therapy-related immune complications in thymic malignancies. *Curr Treat Options Oncol*. 2019;20(7):62.

Liu X, Jia Y, Stoopler MB, et al. Next-generation sequencing of pulmonary sarcomatoid carcinoma reveals high frequency of actionable MET gene mutations. *J Clin Oncol*. 2016;34(8):794-802.

Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350(21):2129-2139.

Marx A, Ströbel P, Badve SS, et al. ITMIG consensus statement on the use of the WHO histological classification of thymoma and thymic carcinoma: refined definitions, histological criteria, and reporting. *J Thorac Oncol*. 2014;9(5):596-611.

McCartney C, Moghadam A, Sriram KB. Lung adenocarcinoma masquerading as refractory *Klebsiella pneumoniae*. *BMJ Case Rep*. 2014;2014.

Mutton AE, Hasleton PS, Curry A, et al. Differentiation of desquamative interstitial pneumonia (DIP) from pulmonary adenocarcinoma by immunocytochemistry. *Histopathology*. 1998;33(2):129-135.

Nguyen KS, Kobayashi S, Costa DB. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. *Clin Lung Cancer*. 2009;10(4):281-289.

Papakonstantinou NA, Vlachou G, Vourlakou C, et al. Pulmonary actinomycosis masquerading as lung cancer: keep it in mind. *ANZ J Surg*. 2019;89(7-8):966-968.

Parra ER, Villalobos P, Behrens C, et al. Effect of neoadjuvant chemotherapy on the immune microenvironment in non-small cell lung carcinomas as determined by multiplex immunofluorescence and image analysis approaches. *J Immunother Cancer*. 2018;6(1):48.

Pataer A, Kalhor N, Correa AM, et al. Histopathologic response criteria predict survival of patients with resected lung cancer after neoadjuvant chemotherapy. *J Thorac Oncol*. 2012;7(5):825-832.

Paz-Ares L, Luft A, Vicente D, et al. Pembrolizumab plus chemotherapy for squamous non-small-cell lung cancer. *N Engl J Med*. 2018;379(21):2040-2051.

Pillai RN, Behera M, Berry LD, et al. HER2 mutations in lung adenocarcinomas: a report from the Lung Cancer Mutation Consortium. *Cancer*. 2017;123(21):4099-4105.

Ragazzi M, Tamagnini I, Bisagni A, et al. Diamond: immunohistochemistry versus sequencing in EGFR analysis of lung adenocarcinomas. *J Clin Pathol*. 2016;69(5):440-447.

Raparia K, Ketterer J, Dalurzo ML, et al. Lung tumors masquerading as desquamative interstitial pneumonia (DIP): report of 7 cases and review of the literature. *Am J Surg Pathol*. 2014;38(7):921-924.

Raymond E, Dahan L, Raoul JL, et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors [published correction appears in *N Engl J Med*. 2011;364(11):1082]. *N Engl J Med*. 2011;364(6):501-513.

Rimm DL, Han G, Taube JM, et al. A prospective, multi-institutional, pathologist-based assessment of 4 immunohistochemistry assays for PD-L1 expression in non-small cell lung cancer. *JAMA Oncol*. 2017;3(8):1051-1058.

Scagliotti G, Brodowicz T, Shepherd FA, et al. Treatment-by-histology interaction analyses in three phase III trials show superiority of pemetrexed in nonsquamous non-small cell lung cancer. *J Thorac Oncol*. 2011;6(1):64-70.

Scagliotti GV, Parikh P, von Pawel J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol*. 2008;26(21):3543-3551.

Schoenfeld AJ, Chan JM, Kubota D, et al. Tumor analyses reveal squamous transformation and off-target alterations as early resistance mechanisms to first-line osimertinib in EGFR-mutant lung cancer. *Clin Cancer Res*. Published online January 7, 2020. doi:10.1158/1078-0432.CCR-19-3563

Schrock AB, Li SD, Frampton GM, et al. Pulmonary sarcomatoid carcinomas commonly harbor either potentially targetable genomic alterations or high tumor mutational burden as observed by comprehensive genomic profiling. *J Thorac Oncol*. 2017;12(6):932-942.

Seymour L, Le Teuff G, Brambilla E, et al. LACE-Bio: Validation of predictive and/or prognostic immunohistochemistry/histochemistry-based biomarkers in resected non-small-cell lung cancer. *Clin Lung Cancer*. 2019;20(2):66-73.e6.

Sholl L. Molecular diagnostics of lung cancer in the clinic. *Transl Lung Cancer Res*. 2017;6(5):560-569.

Skoulidis F, Goldberg ME, Greenawalt DM, et al. STK11/LKB1 mutations and PD-1 inhibitor resistance in KRAS-mutant lung adenocarcinoma. *Cancer Discov*. 2018;8(7):822-835.

Socinski MA, Jotte RM, Cappuzzo F, et al. Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. *N Engl J Med*. 2018;378(24):2288-2301.

Stathis A, Zucca E, Bekradda M, et al. Clinical response of carcinomas harboring the BRD4-NUT oncoprotein to the targeted bromodomain inhibitor OTX015/MK-8628. *Cancer Discov*. 2016;6(5):492-500.

Stewart PA, Welsh EA, Slebos RJC, et al. Proteogenomic landscape of squamous cell lung cancer. *Nat Commun*. 2019;10(1):3578.

Strosberg J, El-Haddad G, Wolin E, et al. Phase 3 trial of 177lu-dotatate for midgut neuroendocrine tumors. *N Engl J Med*. 2017;376(2):125-135.

Subbiah V, Velcheti V, Tuch BB, et al. Selective RET kinase inhibition for patients with RET-altered cancers. *Ann Oncol*. 2018;29(8):1869-1876.

Thatcher N, Hirsch FR, Luft AV, et al. Necitumumab plus gemcitabine and cisplatin versus gemcitabine and cisplatin alone as first-line therapy in patients with stage IV squamous non-small-cell lung cancer (SQUIRE): an open-label, randomised, controlled phase 3 trial. *Lancet Oncol*. 2015;16(7):763-774.

Travis WD, Dacic S, Wistuba I, et al. IASLC multidisciplinary recommendations for pathologic assessment of lung cancer resection specimens after neoadjuvant therapy. *J Thorac Oncol*. 2020;15(5):709-740.

Tsao MS, Hirsch FR, Yatabe Y, eds. *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer*. 2nd ed. IASLC; 2016.

Tsao MS, Marguet S, Le Teuff G, et al. Subtype classification of lung adenocarcinoma predicts benefit from adjuvant chemotherapy in patients undergoing complete resection. *J Clin Oncol*. 2015;33(30):3439-3446.

Tsao MS, Yatabe Y. Old soldiers never die: is there still a role for immunohistochemistry in the era of next-generation sequencing panel testing?. *J Thorac Oncol*. 2019;14(12):2035-2038.

Vennapusa B, Baker B, Kowanetz M, et al. Development of a PD-L1 complementary diagnostic immunohistochemistry assay (SP142) for atezolizumab. *Appl Immunohistochem Mol Morphol*. 2019;27(2):92-100.

Vokes NI, Liu D, Ricciuti B, et al. Harmonization of tumor mutational burden quantification and association with response to immune checkpoint blockade in non-small-cell lung cancer. *JCO Precis Oncol*. 2019;3:10.1200/PO.19.00171.

Warth A, Muley T, Meister M, et al. The novel histologic International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification system of lung adenocarcinoma is a stage-independent predictor of survival. *J Clin Oncol*. 2012;30(13):1438-1446.

Woodard GA, Jones KD, Jablons DM. Lung cancer staging and prognosis. *Cancer Treat Res*. 2016;170:47-75.

Yao JC, Shah MH, Ito T, et al. Everolimus for advanced pancreatic neuroendocrine tumors. *N Engl J Med*. 2011;364(6):514-523.

Zhu CQ, Shih W, Ling CH, Tsao MS. Immunohistochemical markers of prognosis in non-small cell lung cancer: a review and proposal for a multiphase approach to marker evaluation. *J Clin Pathol*. 2006;59(8):790-800.

Zhu CQ, Tsao MS. Prognostic markers in lung cancer: is it ready for prime time? *Transl Lung Cancer Res*. 2014;3(3):149-158.

Principles of Immunohistochemistry

3

By Erik Thunnissen and Alain C. Borczuk

Introduction

Immunohistochemistry (IHC) is a technique that allows visualization of proteins in histologic sections; a similar approach on cells in cytologic specimens is called *immunocytochemistry*. With IHC, the variable domain of the primary antibody recognizes and binds to an antigen, which is usually a protein epitope. These epitopes can reflect secondary and tertiary protein structures, but monoclonal antibodies are now more frequently developed against peptides of 4-25 amino acids (Saper 2009), making detection in formalin-fixed tissue after antigen retrieval more feasible. A second antibody that binds to the primary antibody and subsequent chemical reactions are used to visualize the localization of the epitope, a process known as *signal enhancement*. The location of the IHC staining is detected in the tissue context with use of a microscope. IHC staining may be located on or in one or more subcellular areas, such as on the cell membrane, in the cytoplasm, or in the nucleus. IHC is a rapid and relatively inexpensive method that is preferred by most pathologists primarily because it allows for the evaluation of tissue architecture and tumor cells (Tsao et al 2017).

Protocols and Procedures for Immunohistochemistry

The major steps belonging to the analytical part of IHC are (1) epitope retrieval, (2) incubation with the primary antibody, and (3) signal enhancement and the visualization system (Taylor and Rudbeck 2013). The histologic sections are usually mounted on the glass slide with a special coating, keeping the section sticking to the glass during the IHC procedure. The epitope retrieval is performed to recover (unmask) the antigens that may have been masked during the fixation procedure. Most of these techniques involve a combination of heat with either high or low pH to reverse protein crosslinks caused by fixation; more aggressive methods use proteases to cleave proteins into smaller peptide epitopes. As exogenous enzymes are used during the signal enhancement and visualization step, the effect of possible, functionally similar, endogenous enzymes is blocked by a blocking step.

The variable domains of the primary antibody bind to the epitope of the protein of interest. The primary antibody is diluted in a buffer facilitating the binding during incubation. Standardizing the temperature and time of this incubation step is important for stable results.

In the *direct* IHC method (see Chapter 4) the primary antibody also carries a label for visualization. However, the concentration of epitopes in the histologic section needs to be very high to microscopically detect a signal using this technique.

In the *indirect* IHC method (see Chapter 4), a second incubation is needed, where the variable domains of a secondary antibody bind against the constant domains of the primary antibody. The secondary antibody carries an enzyme that is used for visualization. The indirect IHC approach provides stronger signal enhancement, implying that proteins with a lower epitope concentration in a section may be detected than with the direct method (Prinsen et al 2003). A relatively recent development is the use of a dextran polymer containing several secondary antibodies, as well as enzymes, for visualization. The effect of this signal enhancement approach (like multiple lightbulbs rather than a single lightbulb) is 10 to 20 times more intense than the indirect IHC with an enzyme on the secondary antibody, allowing an even lower epitope concentration to become visible.

After incubation with enhancement moiety and washing, a chromogen solution is added. The soluble chromogen in the solution is usually colorless. The enzyme added in the enhancement step (eg, horseradish peroxidase or alkaline phosphatase) transfers the chromogen in an insoluble substrate that precipitates at the spot of the primary–secondary antibody complex, that is, at the localization of the protein. The size of this precipitate depends of the amount and localization of epitope in a cell and, when visible, is minimally equal to the resolution of the light microscope (500 nm or 0.5 μm) but may also cumulatively fill the complete cytoplasm (20–30 μm).

After the IHC visualization step and washing, a nuclear counterstain is performed, which facilitates recognition of nuclei and the underlying tissue architecture. The washing steps in between the major steps aim to wash away unbound products and solvents, keeping the background of the histologic section clean.

Subsequently, the sections on the microscopic glass slides are dehydrated and covered with a mounting medium (with a refractory index of 1.5, the same as glass) and a thin glass cover slip. This results in parallel glass planes above and below the histologic section, avoiding the angle effect of a "stick in water."

Evaluation and Interpretation

The relationship between epitope concentration and signal intensity for different enhancement systems was shown in 2003 by Prinsen and colleagues and is graphically illustrated in Figure 3-1. Differences in intensity of the S-shape curve may be categorized semiquantitatively as negative, positive (+), double positive (++), or triple positive (+++). Once the plateau of maximal intensity (+++) is reached, further increase in epitope concentration will not lead to higher intensity. The range between negative and +++ is quite narrow and spans an epitope concentration of two- to fourfold, depending on the amplification system. Although a parallel may be drawn with enzyme-linked immunosorbent assay (ELISA) as a quantitative protein detection assay, IHC is only quantitative in the linear dynamic range (the steep part of the S-shape curve, ie, the range between + and ++). Thus, IHC is semiquantitative

at best and only in this range of the curve. Therefore, the signal can become saturated and non-quantitative.

Using an additional tyramide signal amplification in a commercial assay on anaplastic lymphoma kinase (ALK) IHC resulted in a qualitative IHC (consequently with an almost vertical line in the steep S-shape curve): The IHC test outcome in this setup is purely qualitative—either negative or positive (Wynes et al 2014). A stronger amplification system may occasionally lead to increased background staining, and possibly to false-positive results (Ibrahim et al 2016).

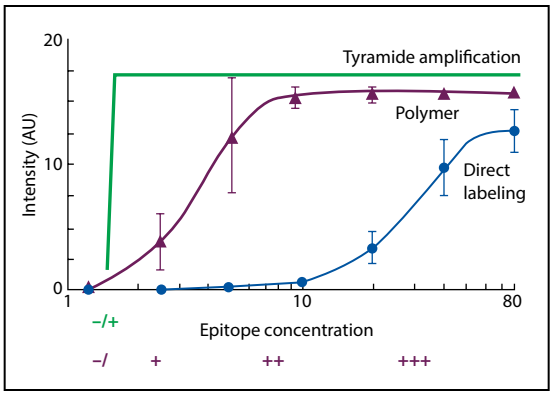


Figure 3-1. Relation between epitope concentration and signal enhancement in immunohistochemistry (IHC). AU = arbitrary unit. (Modified with permission from Prinsen et al 2003)

The determination of staining intensity has a subjective element, which may in practice be reduced with the use of successive microscope objective lenses with inherent related spatial resolution as a physical aid in establishing the intensity level. This approach, first applied to human epidermal growth factor receptor 2 (HER2) testing, may lead to more uniform intensity scoring (Ruschoff et al 2010). Strong staining (+++) is clearly visible with use of an ×2.5 or ×5 microscope objective lens; moderate staining (++) requires a ×10 or ×20 objective lens to be clearly seen; and weak staining (+) can be seen only with an ×40 objective lens (Ruschoff et al 2013).

Rabbit and Mouse Monoclonal Antibodies

Although mouse monoclonal antibodies are widely used, rabbit monoclonal antibodies have performance characteristics that increasingly favor their use. Specifically, the rabbit immune system responds to human peptides that are not reactive in mouse systems, and in general, they produce antibodies with higher affinity. This is especially true of smaller peptides, which are being used as antigens for antibody production (Rief et al 1998; Weber et al 2017).

Laboratory-Developed Tests

For diagnostic and predictive testing, many commercial assays are available. Pricing of predictive testing is dramatically higher than for diagnostic testing, and diagnostic commercial assays are generally more expensive than laboratory-developed tests (LDTs). In times with limited budgets for most laboratories, the economic challenge with predictive testing encourages use of an LDT in place of a commercial assay.

The advantage of a commercial assay is that most aspects of the IHC assay/kit have been rigorously tested, and the conditions for the assay have been chosen to lead to stable test outcomes in time. LDTs need to be developed to perform at the same standard.

Standarization of IHC

Ideally, similar to the ELISA, a reference or calibration standard should be available in IHC testing, but this is lacking for IHC in daily pathology. Standardization is required because

inconsistency in sample preparation (pre-analytical variables) and inadequate validations of reagents (analytical variables) may influence (usually diminish) IHC test results.

The list of pre-analytical variables comprises at least: fixative type; time in fixative; reagents and conditions of dehydration, clearing, and paraffin impregnation; and conditions of slide drying and storage (Engel and Moore 2011). During fixation, the penetration rate of neutral buffered formaldehyde may be relatively quick in small samples (1 mm/h) (Howat and Wilson 2014) but is much slower (0.2 mm/h) in the collapsed lung with multiple thin layers of air between the alveolar walls (van Seijen et al 2019). Delay in fixation may reduce protein stability and thereby hamper IHC (van Seijen et al 2019). Detrimental effects of delayed fixation may also be seen in hematoxylin and eosin–stained sections, such as epithelial detachment of the basement membrane, as well as pyknotic or close to pyknotic nuclei (Radonic et al 2019). These samples should not be used for analytical validation of IHC. Tissue handling is discussed in more detail in Chapter 5.

To begin developing an LDT, a control sample with high epitope concentration is useful as this should be positive under possibly initial suboptimal conditions. When this control is positive, and negative control (eg, complete procedure except for the step with the primary antibody) negative, samples with lower epitope concentration are useful for fine-tuning LDTs.

The optimal sample for monitoring daily performance, as well as for validation of an IHC assay, is a sample with sufficient epitope concentration close to the threshold to call such a sample positive (usually +). Automation of the IHC procedure on specific instruments (eg, control incubation temperatures and times) increases stability of conditions and the robustness of the IHC result to a great extent, helping to obtain consistent results.

Validation of IHC

According to the College of American Pathologists (CAP) guidelines for initial analytic validation of nonpredictive (*diagnostic*) IHC assays, laboratories should test a minimum of 10 positive and 10 negative tissues (Fitzgibbons et al 2014). When the laboratory medical director determines that fewer than 20 validation cases are sufficient for a specific marker (eg, rare antigen, implying rare positive cases), the rationale for that decision needs to be documented. For diagnostic purposes, when samples of sufficient quality are available for the IHC assay, the concordance with the test assay should be high (preferably 100%, implying sensitivity of 100% and specificity of 100%).

When (consecutive) clinical samples are taken into account, not always controllable pre-analytical factors may come into play, explaining the recommendation that "for initial validation of every assay used clinically, laboratories should achieve at least 90% overall concordance between the new test and the comparator test or expected results" (Fitzgibbons et al 2014).

For initial analytic validation of all laboratory-developed *predictive* marker assays, laboratories should test a minimum of 20 positive and 20 negative cases according to CAP (Fitzgibbons et al 2014) and 50 positive and 50 negative samples according to the Clinical and Laboratory Standards Institute (CLSI) (Garrett et al 2008). These numbers have an arbitrary component with lower confidence intervals in the latter. Other guidelines do not touch on this issue or are not as explicit. Both (CAP and CLSI) guidelines mention the 90% concordance with the comparator test. Nevertheless, both guidelines have a flaw: The concordance

rate is dependent on the composition of the cases. The difference between *analytical/technical* validation of a diagnostic test and *clinical* validation of a predictive test is that the latter is associated with a predicted response to a specific treatment. In this regard, an LDT IHC test validation has to establish a purpose for the test, as correct validation requires that the control samples and the working range for antigen detection is appropriate for the assay. This concept of "fit for purpose" has great importance as the same antibody may need different validation based on this principle. For example to be in the correct working range for diagnostic utility, detection of GATA3 in breast carcinoma versus lymphocytes might require a different protocol based on differences in antigen level (Cheung et al 2017a). In addition to the initial validation, an LDT also is subject to revalidation potentially with new antibody lots, new antibody clones, or changes in protocol or testing equipment.

In the case of programmed death ligand-1 (PD-L1) IHC in non-small cell lung carcinoma (NSCLC), any intensity of membranous staining in at least 50% of tumor cells is considered to be positive as this predictive test is directly validated by clinical data from a phase 3 randomized clinical trial (Reck et al 2016). Conceptually, clinical validation using the samples from the phase 3 studies (with known patient outcome) and comparison with the performance of the new PD-L1 assay is a way forward. However, the availability of tumor tissue from these studies (with mainly small biopsies) is limited and prohibits extensive testing. An alternative approach is to compare the test to be validated with the same commercial PD-L1 assay used in the phase 3 study, because the study-validated threshold is associated with the clinical outcome. The assumption with this approach is that the commercial assay is robust in time and place.

Recently, more specific guidance on how to perform *clinical* validation of PD-L1 IHC was provided for the first time (Thunnissen 2019a). To this end, critical samples (Thunnissen et al 2018; Thunnissen 2019b), which have an epitope concentration close to the threshold of the validated assay are suitable. A practical approach is to stain the concept LDT and compendium diagnostic test in a pairwise fashion on approximately 20 to 40 consecutive clinical samples (Thunnissen 2019b). If both assays are not too deviant, approximately 80% to 90% of the samples will be concordant throughout the whole slide. The 2 or 3 samples that show at least focal discordancy (including focal differences in intensity) can be used for further titration of the LDT; for example, by increasing or reducing the primary antibody concentration. Thus, the new PD-L1 test should become positive at the same intensity as the clinically validated test (comparator test). This procedure may be called *indirect clinical validation of predictive testing*.

If the deviation between both PD-L1 assays is larger, usually the concept LDT stains less intensely or not at all compared to the commercial assay. More discordant samples are then available for further improvement of the concept LDT, and more rigorous adaptions may then also be considered, such as modifying the epitope retrieval and/or signal enhancement steps of the IHC method. To facilitate this process, careful tissue management is helpful (Bubendorf et al 2017), such as cutting several spare sections upfront for additional PD-L1 testing. Subsequently, any repeated testing on suitable samples can be performed within a few days or weeks after initial cutting.

In fact, in this selection process of critical samples, the heterogeneity of PD-L1 is exploited: Some of the tumor cells may be negative and other tumor cells, not far from each

other, positive in the same section. Likewise, some tumor cells may show PD-L1 expression with a higher intensity, whereas others may be less strongly stained.

Samples with high epitope concentration reach the maximum level of staining (+++) and are likely to be positive in comparison with most other PD-L1 assays (Fitzgibbons et al 2014). The notion that such a sample is a less useful sample for detection of variation in daily practice may also hold for the high epitope concentration in placenta as an external positive control (Dodson et al 2019) even though placenta is the recommended positive control in at least 1 U.S. Food and Drug Administration (FDA)-approved assay (VENTANA PD-L1 [SP263] assay, package insert). For validating an IHC test, using only samples with a high epitope concentration may give a false sense of safety. Samples to be used as controls must fit the purpose of the test (Cheung et al 2017a).

Samples with a very low epitope concentration will be negative with any PD-L1 assay. As such, cases with high and very low or absent epitope concentration are not informative for comparison of PD-L1 test performance. The a priori chance for 90% concordance between 2 tests will be high, if 50% PD-L1 negative samples and 40% PD-L1 strongly positive samples are examined. Similarly, the kappa value for this comparison of 2 IHC tests will be very high (>0.8) compared to kappa values in diagnostic surgical pathology studies (0.6-0.7). Thus, this type of case selection will lead to concordance of most assays, while this remains to be seen with a wider range of samples.

Previously, for analytical assay validation "immunohistochemistry critical assay performance controls (ICAPCs)" were described (Torlakovic et al 2015), as part of a series of 4 papers covering several aspects of the diagnostic IHC setting (Cheung et al 2017a, 2017b; Torlakovic et al 2015, 2017). The difference between ICAPCs and the "critical" samples is that the latter have thresholds associated with clinical response, while this (unintentionally) may also hold for some ICAPCs, it is not likely to be true for all ICAPCs. Moreover, critical samples can be detected in a small series (~ n = 20-40) of NSCLC cases in most laboratories, whereas ICAPCs, such as xenografts, are not always easy to obtain for each laboratory. The terminology of indirect clinical validation of predictive testing is more appropriate for predictive IHC validation than the older term *diagnostic validation* not only because of the clinical treatment association, but also for discussion with hospital management: Predictive testing within each diagnostic category is an "add-on" to the diagnostic tests (requiring extra budget on the existing diagnostic budget) to advise on essentially different treatment options.

Quality Assurance

In addition to internal quality assurance measures as discussed earlier, external quality assurance is essential for ensuring adequate performance of IHC. The aim of external quality assessment (EQA) is to establish with the support of an independent organization (EQA provider) the performance of a diagnostic or predictive test. Basically, the EQA provider distributes test samples across different laboratories and evaluates the test outcomes, for example, true positive, false positive, true negative, and false negative. To be successful, a laboratory needs to score a certain number of correct test outcomes (van Krieken et al 2013).

In certain countries, laboratories have an obligation to participate and perform at an acceptable level. Participation in EQA has the advantage of an independent validation of the assay. Occasionally, a slow deterioration of IHC testing may occur unnoticed, especially if an

optimal test sample (ie, stable low positive [+] sample) is lacking. In such cases, this underperformance may be detected by participation in EQA.

Initially, the providers for predictive EQA acted on a local scale (Thunnissen et al 2011; Normanno et al 2011, 2013; Scheel et al 2016). For more than a decade, EQA has also been performed at the multinational level by the European Society of Pathology (Tembuyser et al 2014; Keppens et al 2018), the U.K. National External Quality Assessment Service (UK NEQAS) (Ibrahim et al 2016; Dodson et al 2019), NordiQC (Vyberg and Nielsen 2016), CAP, and others (Patton et al 2014). In the initial EQA rounds, LDT tests may have lower scores, but after a learning period, LDTs can perform equally to FDA-approved assays in subsequent EQA rounds (Adam et al 2018).

Conclusions

IHC is a powerful diagnostic tool, and protocols have become robust in the detection of antigens that are relevant to pathology tumor classification and the organ of origin. The basic technique, however, is more qualitative than quantitative, but semiquantitative assessment can be achieved through careful protocol construction and validation. Methods of validation are becoming better defined and can support LDTs in the diagnostic as well as the predictive arena.

References

Adam J, Le Stang N, Rouquette I, et al. Multicenter harmonization study for PD-L1 IHC testing in non-small-cell lung cancer. *Ann Oncol*. 2018;29(4):953-958.

Bubendorf L, Lantuejoul S, de Langen AJ, et al. Nonsmall cell lung carcinoma: diagnostic difficulties in small biopsies and cytological specimens. In: Dorfmuller P, Cavazza A, eds. *Pathology for the Clinician*, vol 2. *Eur Respir Rev*. 2017;26(144):170007.

Cheung CC, D'Arrigo C, Dietel M, et al. Evolution of quality assurance for clinical immunohistochemistry in the era of precision medicine: part 1: fit-for-purpose approach to classification of clinical immunohistochemistry biomarkers. *Appl Immunohistochem Mol Morphol*. 2017;25(1):4-11. (a)

Cheung CC, D'Arrigo C, Dietel M, et al. Evolution of quality assurance for clinical immunohistochemistry in the era of precision medicine: part 4: tissue tools for quality assurance in immunohistochemistry. *Appl Immunohistochem Mol Morphol*. 2017;25(4):227-230. (b)

Dodson A, Parry S, Lissenberg-Witte B, et al. External quality assessment demonstrates that PD-L1 22C3 and SP263 assays are systematically different. *J Pathol Clin Res*. Published online December 17, 2019. doi:10.1002/cjp2.153

Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med*. 2011;135(5):537-543.

Fitzgibbons PL, Bradley LA, Fatheree LA, et al. Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med*. 2014;138(11):1432-1443.

Garrett PE, Lasky FD, Meier KL. *User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline*. 2nd ed. Clinical and Laboratory Standards Institute (CLSI); 2008.

Howat WJ, Wilson BA. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods*. 2014;70(1):12-19.

Ibrahim M, Parry S, Wilkinson D, et al. ALK immunohistochemistry in NSCLC: discordant staining can impact patient treatment regimen. *J Thorac Oncol*. 2016;11(12):2241-2247.

Keppens C, Tack V, Hart N, et al. A stitch in time saves nine: external quality assessment rounds demonstrate improved quality of biomarker analysis in lung cancer. *Oncotarget*. 2018;9(29):20524-20538.

Normanno N, Pinto C, Castiglione F, et al. KRAS mutations testing in colorectal carcinoma patients in Italy: from guidelines to external quality assessment. *PLoS One*. 2011;6(12):e2916.

Normanno N, Pinto C, Taddei G, et al. Results of the first Italian external quality assurance scheme for somatic EGFR mutation testing in non-small cell lung cancer. *J Thorac Oncol*. 2013;8(6):773-778.

Patton S, Normanno N, Blackhall F, et al. Assessing standardization of molecular testing for non-small-cell lung cancer: results of a worldwide external quality assessment (EQA) scheme for EGFR mutation testing. *Br J Cancer*. 2014;111(2):413-420.

Prinsen CF, Klaassen CH, Thunnissen FB. Microarray as a model for quantitative visualization chemistry. *Appl Immunohistochem Mol Morphol*. 2003;11(2):168-173.

Radonic T, Dickhoff C, Mino-Kenudson M, et al. Gross handling of pulmonary resection specimen: maintaining the 3-dimensional orientation. *J Thorac Dis*. 2019;11(suppl 1):S37-S44.

Reck M, Rodriguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med*. 2016;375(19):1823-1833.

Rief N, Waschow C, Nastainczyk W, et al. Production and characterization of a rabbit monoclonal antibody against human CDC25C phosphatase. *Hybridoma*. 1998;17(4):389-394.

Ruschoff J, Dietel M, Baretton G, et al. HER2 diagnostics in gastric cancer-guideline validation and development and standardized immunohistochemical testing. *Virchows Arch*. 2010;457(3):299-307.

Ruschoff J, Kerr KM, Grote HJ, et al. Reproducibility of immunohistochemical scoring for epidermal growth factor receptor expression in non-small cell lung cancer: round robin test. *Arch Pathol Lab Med*. 2013;137(9):1255-1261.

Saper CB. A guide to the perplexed on the specificity of antibodies. *J Histochem Cytochem*. 2009;57(1):1-5.

Scheel AH, Dietel M, Heukamp LC, et al. Harmonized PD-L1 immunohistochemistry for pulmonary squamous-cell and adenocarcinomas. *Mod Pathol*. 2016;29(10):1165-1172.

Taylor CR, Rudbeck L. *Immunohistochemical Staining Methods*. Dako; 2013.

Tembuyser L, Tack V, Zwaenepoel K, et al. The relevance of external quality assessment for molecular testing for ALK positive non-small cell lung cancer: results from two pilot rounds show room for optimization. *PLoS One*. 2014;9(11):e112159.

Thunnissen E. How to validate predictive immunohistochemistry testing in pathology? A practical approach exploiting the heterogeneity of programmed death ligand-1 present in non-small cell lung cancer. *Arch Pathol Lab Med*. 2019;143(1):11-21. (a)

Thunnissen E. In Reply. *Arch Pathol Lab Med*. 2019;143(8):909-910. (b)

Thunnissen E, Allen TC, Adam J, et al. Immunohistochemistry of pulmonary biomarkers: a perspective from members of the pulmonary pathology society. *Arch Pathol Lab Med*. 2018;142(3):408-419.

Thunnissen E, Bovee JV, Bruinsma H, et al. EGFR and KRAS quality assurance schemes in pathology: generating normative data for molecular predictive marker analysis in targeted therapy. *J Clin Pathol*. 2011;64(10):884-892.

Torlakovic EE, Cheung CC, D'Arrigo C, et al. Evolution of quality assurance for clinical immunohistochemistry in the era of precision medicine. Part 3: technical validation of immunohistochemistry (IHC) assays in clinical IHC laboratories. *Appl Immunohistochem Mol Morphol*. 2017;25(3):151-159.

Torlakovic EE, Nielsen S, Francis G, et al. Standardization of positive controls in diagnostic immunohistochemistry: recommendations from the International Ad Hoc Expert Committee. *Appl Immunohistochem Mol Morphol*. 2015;23(1):1-18.

Tsao MS, Kerr KM, Dacic S, et al, eds. *IASLC Atlas of PD-L1 Immunohistochemistry Testing in Lung Cancer*. IASLC; 2017.

van Krieken JH, Normanno N, Blackhall F, et al. Guideline on the requirements of external quality assessment programs in molecular pathology. *Virchows Arch*. 2013;462(1):27-37.

van Seijen M, Brcic L, Gonzales AN, et al. Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen. *Virchows Arch*. 2019;475(2):191-199.

Vyberg M, Nielsen S. Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC). *Virchows Arch*. 2016;468(1):19-29.

Weber J, Peng H, Rader C. From rabbit antibody repertoires to rabbit monoclonal antibodies. *Exp Mol Med*. 2017;49(3):e305.

Wynes MW, Sholl LM, Dietel M, et al. An international interpretation study using the ALK IHC antibody D5F3 and a sensitive detection kit demonstrates high concordance between ALK IHC and ALK FISH and between evaluators. *J Thorac Oncol*. 2014;9(5):631-638.

Techniques and Technologies in Immunohistochemistry

4

By David M. Hwang, Kurt A. Schalper, and Sanja Dacic

Introduction

Recent decades have seen significant advances in immunohistochemistry techniques and in technologies to automate and enhance the information gained by immunohistochemical staining. This chapter reviews detection and amplification systems, automated stainers, applications of digital imaging and morphometry, and emerging technologies in immunohistochemistry.

Basic Principles of Immunohistochemistry

Antibody–antigen binding may be detected using either fluorescent labels (ie, immunofluorescence) or chromogenic substrates. As the vast majority of clinical immunohistochemical staining in thoracic oncology uses chromogenic rather than fluorescent labels, this chapter focuses on chromogenic techniques.

Earlier iterations of immunohistochemistry typically used antibodies conjugated to enzymes such as horseradish peroxidase (HRP) or acid (or alkaline) phosphatase (AP) (Nakane and Pierce 1966, 1967), enabling enzymatic deposition of a chromogen at sites of antibody binding. While multiple chromogenic substrates are now available, the most commonly used are 3,3'-diaminobenzidine (DAB), which produces brown-colored deposits with HRP; and 3-amino-9-ethylcarbazole (AEC), which produces red deposits.

Target detection may be performed by either direct or indirect methods. In direct detection, the primary antibody against the antigen is labeled for detection (Figure 4-1A). However, as direct detection is of limited sensitivity and typically applicable only to highly expressed proteins, indirect detection techniques are more commonly used for clinical applications. In indirect methods, the primary antibody is unlabeled, while a secondary antibody targeting the species in which the primary antibody was generated is labeled for detection (Figure 4-1B). Multiple secondary antibody molecules may bind to a single primary antibody molecule, allowing for a degree of signal amplification and increased sensitivity of detection. Use of indirect methods also allows for detection of many different antigens using a small number of species-directed secondary antibodies (eg, anti-mouse, anti-rabbit), without the

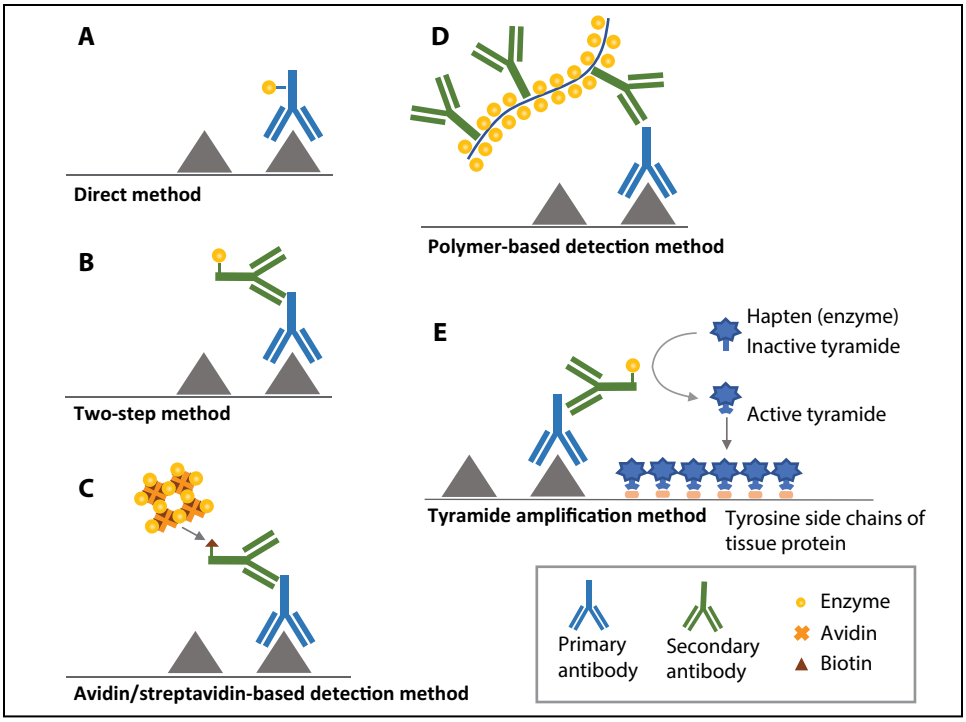


Figure 4-1. Various methods of immunohistochemical detection. **(A)** Direct method, using an enzyme-conjugated primary antibody. **(B)** Two-step method, using a hapten-labeled secondary antibody, which is specific to the primary antibody. **(C)** Avidin/streptavidin-based detection method, using a biotinylated secondary antibody to link the primary antibody to a large complex of avidin, streptavidin, and enzyme. **(D)** Polymer-based detection method, via a polymer-conjugated secondary antibody. **(E)** Tyramide amplification method, using a deposition of biotinylated tyramide to tissue tyrosine side chains, which is activated by free radical formation with antibody-labeled peroxidase.

need to label each primary antibody, and without the potential for negative effects on antigen binding resulting from the labeling of primary antibodies.

A number of strategies for further enhancing sensitivity through additional signal amplification have also been developed and include the following.

Avidin/Streptavidin-Biotin-Detection Enzyme Complexes

The avidin-biotin complex (ABC) and the labeled streptavidin-biotin (LSAB) methods are included in these complexes (Figure 4-1C). In both methods, binding of a biotinylated secondary antibody to the primary antibody is followed by addition of biotinylated detection enzyme (eg, HRP, AP) complexed with avidin (ABC method) or of streptavidin-conjugated detection enzyme (LSAB method), greatly increasing the number of detection enzyme molecules and chromogen deposition at sites of antigen binding. A limitation of these methods is the binding of avidin or streptavidin to endogenous biotin or lectin-like molecules within the tissue, resulting potentially in higher nonspecific background staining.

Polymer and Other Non–Biotin-Based Detection Complexes

To circumvent some of the issues related to biotin-based approaches, a number of non–biotin-based detection methods were developed (Figure 4-1D). Polymer-based approaches,

in which multiple secondary antibody and detection enzyme molecules are attached to a dextran polymer backbone, form the basis of widely used detection kits, including the EnVision (Agilent Dako) kit and *ultra*View Universal DAB detection kit (Roche Tissue Diagnostics). As the large size of the polymeric complexes may inhibit tissue penetration, some protocols use a second antibody (linker) between the primary antibody and the polymer to improve sensitivity—an approach used in the Dako PD-L1 IHC 22C3 pharmDx assay.

A different non–biotin-based approach was taken by the OptiView detection system (Roche Tissue Diagnostics), in which secondary antibodies are labeled with multiple molecules of a non-endogenous hapten (3-hydroxy-2-quinoxaline [HQ]), followed by detection using HRP-conjugated anti-HQ antibodies (Nitta et al 2013).

Catalyzed Signal Amplification Methods

In catalyzed signal amplification (CSA) methods, the HRP enzyme conventionally used to generate the chromogen is first used to catalyze another chemical reaction that deposits more binding sites for recruitment of additional enzyme molecules (Figure 4-1E). For example, in the presence of hydrogen peroxide, HRP oxidizes biotinylated tyramide, generating a free radical species that reacts with and results in deposition of biotin in the surrounding tissue. This deposited biotin is then used to recruit additional detection enzyme molecules through addition of avidin or streptavidin-biotin-enzyme complexes, resulting in up to 1000-fold signal amplification compared to the conventional ABC method described earlier (Ramos-Vara 2017). A variation of this strategy, in which HQ-tyramide is substituted for biotinylated tyramide, is used by the OptiView amplification system (Roche Tissue Diagnostics), which together with the OptiView detection system is used in the VENTANA ALK (D5F3) CDx Assay (Nitta et al 2013).

Automated Immunohistochemistry Stainers

Performed manually, immunohistochemistry is a time- and labor-intensive process, with up to 100 manual interactions required with each slide stained, each with potential for variability and error. Automation of most or all of these steps reduces costs by significantly decreasing the technologists' time required for staining, while improving quality by ensuring greater intra- and inter-individual consistency.

Since the report of the first robotic workstation for immunocytochemical staining in the late 1980s (Brigati et al 1988), there has been widespread adoption of automated immunohistochemistry stainers for use in clinical laboratories. Multiple platforms are currently available, each with its own advantages and disadvantages, with no single best autostainer for all applications (Table 4-1). Rather, laboratories must choose the autostainer(s) that best address their specific needs and requirements (Prichard 2014; Myers 2008). In this regard, considerations may include the following.

Size

Autostainers come in a range of sizes and may be either benchtop or floor units, depending on the available space.

Table 4-1. Comparison of Commonly Used Automated Immunohistochemistry Platforms

	Benchtop		Floor			
	Agilent Dako	Leica	Agilent Dako	Leica	Roche	Roche
	Autostainer	Bond Max	Omnis	Bond III	BenchMark XT	BenchMark ULTRA
Automated staining steps						
Slide baking	No	Yes	No	Yes	Yes	Yes
Dewaxing	No	Yes	Yes	Yes	Yes	Yes
Onboard heating	No	Yes	Yes	Yes	Yes	Yes
Onboard in situ hybridization	No	DNA/RNA	DNA/RNA	DNA/RNA	DNA/RNA	DNA/RNA
Dimensions						
Size (width × depth × height), cm	89 × 66 × 68	76 × 77.5 × 70.3	150 × 80 × 177	79 × 80.6 × 137.8	89 × 66 × 153	112 × 84 × 159
Slide management						
Slide capacity	48	30	60	30	30	30
Batch size, slides	4 × 12	3 × 10	12 × 5	3 × 10	1 × 30	30 × 1
Processing capacity/24 h for immunohistochemistry (IHC)	144	90	165	90/120	90	90
Reagents						
Positions	42	36	60	36	35	35
Temperature control	No	No	Yes	No	No	No
Open system	Yes	Primary antibody	Primary antibody	Primary antibody	Primary antibody + enzyme	Primary antibody + enzyme

Adapted from De Wiest and van Hecke 2017.

Open Versus Closed Systems

Some autostainers provide greater flexibility with reagent choice or protocols (*open* systems), while others limit the types or sources of reagents and protocols that can be used (*closed* systems). Whereas open systems may be more useful in settings where there is a need to develop and optimize new staining protocols for large numbers of markers (eg, research and design settings), closed systems are more geared toward maximizing reproducibility and minimizing hands-on time through the application of standardized reagents and protocols.

Throughput and Batch Sizes

Throughput is generally a primary consideration when evaluating automated platforms and is primarily often thought of as a function of slide capacity and run time. However, in addition to the sheer volume of slides processed, other considerations may include the number of different tests and protocols that need to be run, as different platforms offer varying degrees of flexibility for batch sizes, in which the staining protocol for all slides in the batch should be similar, so as to optimize the ability to load new slides for staining and run times.

Other Functionalities

Different autostainers may offer varying other functionalities, for example, onboard slide baking, deparaffinization, heating (for antigen retrieval), and in situ hybridization (ISH). Although these additional functionalities may increase consistency and reduce hands-on

time, they may lengthen run times. Additional details regarding automated stainers can be found in the literature (Prichard 2014; Myers 2008).

Digital Imaging and Morphometric Analysis

Prominent developments for fast and accurate digitalization of whole-slide histology preparations have supported increasing roles of morphology and immunohistochemistry in pathology practice. Digital slides from hematoxylin and eosin stains or immunohistochemistry (also called *virtual slides*) are currently being used for multiple purposes including primary diagnosis, biomarker evaluation, clinical conferences, remote case evaluation, second opinion consultations, education and research, and case storage (Pantanowitz et al 2013; Evans et al 2018; Liu and Pantanowitz 2019). Multiple digital slide imaging platforms have become commercially available, and some have received regulatory approval and/or clearance for clinical use (Liu and Pantanowitz 2019). This has allowed increasing adoption of digital pathology worldwide and some institutions to operate exclusively on virtual slides. The availability of digital samples has also prompted the use of computational tools for more detailed morphometric analyses and quantitative assessment of variables. These developments have expanded the scope of immunohistochemistry, but have also posed new questions and challenges.

Digital Immunohistochemistry for Primary Diagnosis and Biomarker Assessment

The use of digital slides in the clinic is associated with the regulatory landscape governing the respective site or institution, and differences in the definitions and requirements have been seen between authorities across world regions (Pantanowitz et al 2013; Evans et al 2018; Garcia-Rojo et al 2019; Zhao et al 2015). As an example, digital pathology received the first regulatory approval by the U.S. Food and Drug Administration (FDA) in 2017 for primary diagnosis in surgical pathology based on non-inferiority studies relative to microscopic diagnosis of hematoxylin-eosin preparations (Evans et al 2018). The use of digital immunohistochemistry slides represents another potential clinical use, but it is not considered to be for primary diagnosis. In general, immunochemistry is used to refine or complement the diagnosis via identification of differentiation markers or for detection of prognostic or predictive biomarkers (see the following "Qualitative Versus Quantitative Analysis" subsection). Digital analysis of immunohistochemistry slides can therefore be conducted using either an FDA-cleared or non-FDA-cleared platform such as a laboratory-developed test, as long as proper laboratory and assay validation and quality control requirements are accomplished. In the United States, digital immunohistochemistry testing requires the fulfillment of College of American Pathologists (CAP) standards including evaluation of accuracy, precision, and reproducibility (Evans et al 2018). Digital pathology systems have received in vitro diagnostic (eg, *Conformité Européenne* [CE]) designation in the European Union allowing their broad clinical use including immunohistochemistry and specific software for automated assessment of biomarkers (Garcia-Rojo et al 2019).

Qualitative Versus Quantitative Analysis

Interpretation of immunohistochemistry slides is usually based on qualitative or semiquantitative visual estimation of the chromogenic reaction under light microscopy analyzed in

the context of the morphologic features. Most differentiation markers, such as keratins, thyroid transcription factor-1 (TTF1), and p63/p40, require a relatively simple binary assessment (eg, positive or negative). Predictive or companion biomarkers, such as programmed death ligand-1 (PD-L1), anaplastic lymphoma kinase (*ALK*), c-ros oncogene 1 (*ROS1*), human epidermal growth factor receptor 2 (*ERBB2*, also known as *HER2*), or hormone receptors, require more complex evaluation including expressions of quantity and/or staining intensity. The subjective nature of the pathologist evaluation provides numerous advantages relative to simplicity, efficiency, and cost-effectiveness of the analysis. However, the consistency across laboratories and interpathologist agreement show limitations.

Strategies to overcome such limitations include the creation of expert-based standardized analysis and scoring guidelines (Lindeman et al 2018; Wolff et al 2018; Lantuejoul et al 2019; Jain et al 2019; Tsao et al 2016). Quantitative image analysis, when performed correctly, can generate tissue marker readouts that are precise and highly reproducible (Aeffner et al 2019). Most advanced image analysis tools use feature extraction and machine-learning segmentation algorithms to obtain localized information based on specific sample areas, cells, or noncellular objects. Although achieving accurate tissue segmentation and event quantification across cases poses numerous challenges, multiple automated quantitative analysis algorithms for digital immunohistochemistry slide scoring have long been established and regulatory (eg, FDA) cleared to assess biomarkers in breast cancer (eg, estrogen receptor [ER], progesterone receptor [PR], *HER2*, and Ki-67). To the best of our knowledge, no clinical-grade automated immunohistochemistry scoring device or algorithm has been established for biomarker testing in thoracic/airway malignancies. However, multiple commercial and open source software applications are currently available to conduct quantitative tissue biomarker analysis (eg, Aperio ImageScope, InForm, Halo, QuPath, Cyto-Mine, Orbit, ImageJ/SlideJ, and Visiopharm). Examples of quantitative PD-L1 scoring in non-small cell lung cancer using tissue cell/tissue segmentation and automated cell enumeration with 2 different commercial platforms are shown in Figure 4-2. In support of the feasibility of conducting automated PD-L1 immunohistochemistry assessment, recent studies using commercial instrumentation or custom software reported relatively high concordance with pathologist-based scoring in lung cancer specimens (Taylor et al 2019; Althammer et al 2019; Widmaier et al 2020).

Machine Learning, Artificial Intelligence, and Slide Interpretation

Machine-learning strategies allow the analysis of complex data in an iterative, fast, objective and statistically controlled manner; permit the identification of patterns with minimal human intervention; facilitate the integration of variables with different units/dimensions; and increase the value of data sets using *in silico* augmentation strategies (Esteva et al 2019; Bera et al 2019). Machine learning can be applied to digital pathology to achieve high-level tasks beyond tissue segmentation and marker/cell counting, including the identification of subvisual morphometric patterns, integrated diagnostics, and acquisition of prognosis/predictive results. Specifically, deep-learning analysis using convolutional neural networks has shown prominent potential for pattern recognition of image representations achieving human-level performance in object classification. In support of this, a deep-learning algorithm was recently shown to be able to accurately predict the PD-L1 status of non-small

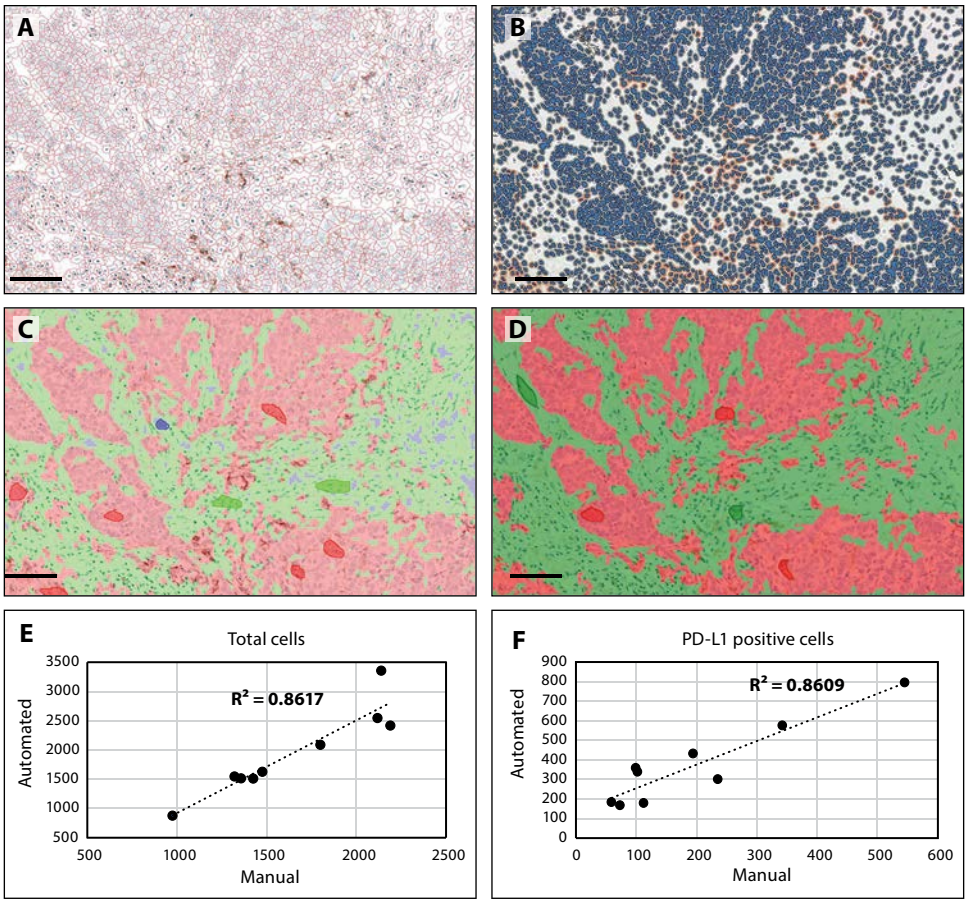


Figure 4-2. Automated tissue analysis of non-small cell lung cancer stained for programmed cell death ligand-1 **(PD-L1). (A-C)** Non-small cell lung cancer specimens stained with PD-L1 (clone E1L3N) and submitted to **(A)** automated cell segmentation or **(C)** tissue segmentation using InForm software (Akoya Biosciences). **(B-D)** Non-small cell lung cancer specimens stained with PD-L1 and submitted to **(B)** automated cell segmentation **(D)** or tissue segmentation using Halo software (Indica Labs). In **C and D,** *red* depicts the tumor compartment and *green* indicates the stromal compartment. Bar = 300 µm. **(E-F)** Charts showing the correlation of the cell counts using manual counting or with automated image analysis software **(E)** for all cells in the caption or **(F)** for those cells positive for PD-L1. R^2 = linear regression coefficient.

cell lung cancer cases through analysis of digital hematoxylin-eosin stained preparations (Sha et al 2019). In addition, generative adversarial networks have been used for automated digital PD-L1 immunohistochemical scoring in lung cancer core needle biopsies (Kapil et al 2018). Efforts to establish artificial intelligence-based automated diagnostics of digital slides, including immunohistochemistry, are ongoing, and multiple companies exploiting this concept have been founded (eg, PathAI, Paige, Proscia, and SpIntellX, among others).

Emerging Technologies and Platforms in Immunohistochemistry

Recent developments have focused on increasing the throughput of immunohistochemistry assays by performing simultaneous staining/detection of multiple targets. Multiplexed immunofluorescence has emerged as a dominant platform because it can overcome the dynamic range limitations of chromogenic immunohistochemistry and avoid the difficulties to accurately separate the signal from different colorimetric substrates under light

Table 4-2. Properties of Tissue Analysis Platforms

Platform	Immunohistochemistry	Immunofluorescence	Imaging mass cytometry
Signal production	Light absorbance	Light emission	Mass tag ion current
Suitable FFPE samples	Yes	Yes	Yes
Linear/quantitative output	No	Yes	Yes
Optimal number of markers	1-2	1-6	30-35
Dynamic range	1 log	2.5 logs	4-5 logs
Maximal resolution	200 nm	200 nm	1 μm
Signal spillover	+++	++	+
Signal amplification	~300-3000 tags/ab (HRP-TSA)	~300-3000 tags/ab (HRP-TSA)	~160 atoms/ab
Analysis throughput	<0.5 min/mm^2 tissue	0.5-1 min/mm^2 tissue	1-2 h/mm^2 tissue

Abbreviations: ab = antibody; FFPE = formalin-fixed paraffin-embedded; HRP = horseradish peroxidase; TSA = tyramide signal amplification.

microscopy (Table 4-2). In addition, immunofluorescence allows the selective acquisition of marker-specific signal from different fluorescent channels using bandpass filters and spectral unmixing strategies that favor linear/quantitative target measurement (Carvajal-Hausdorf et al 2015). Current multiplexed quantitative immunofluorescence protocols allow the simultaneous detection and signal quantification of up to 6 to 7 markers in different wavelengths using commercial reagents and/or instruments. Automated multiplexed immunofluorescence platforms have been successfully used to perform objective and localized measurement of tumor and immune-related markers in formalin-fixed paraffin-embedded lung cancer specimens (Velcheti et al 2014; Carvajal-Hausdorf et al 2015; Schalper et al 2015). In addition, multiplexed immunofluorescence protocols can accommodate the simultaneous identification of different analytes including protein and messenger RNA (mRNA) transcripts. In support of this, localized detection of tumor protein markers and PD-L1 or interleukin-8 (IL-8) mRNA transcripts have been successfully measured in lung cancer biopsy tissue (Velcheti et al 2014; Sanmamed et al 2017). Representative images from multiplexed fluorescence panels simultaneously mapping 5 immune-related protein or mRNA targets are shown in Figure 4-3. Recently, mass spectrometry technology was adapted for use in a microscope format to quantitatively study tissue samples using 30 to 40 targets (theoretically, >150) with 1 μm resolution (Giesen et al 2014). This technology, termed *imaging mass cytometry* (Table 4-2) and also referred to as *elemental immunohistochemistry*, uses primary antibodies conjugated with lanthanide series elements and is commercially available (Hyperion, Fluidigm Corporation). A similar technology using metal-conjugated antibodies and multiple ion beam–based ionization (MIBI, IONpath) has also been reported and is being commercialized (Angelo et al 2014). It is expected that the integration of high-throughput, quantitative, and spatially resolved analysis of targets with advanced computational strategies will prominently enhance the pathologist armamentarium for enhanced diagnostics and biomarker assessment in the near future.

Conclusions

Recent advances in techniques of immunohistochemistry expand a range of detection in epitope concentration (sensitivity) and a variety of molecules. Furthermore, automated stainers

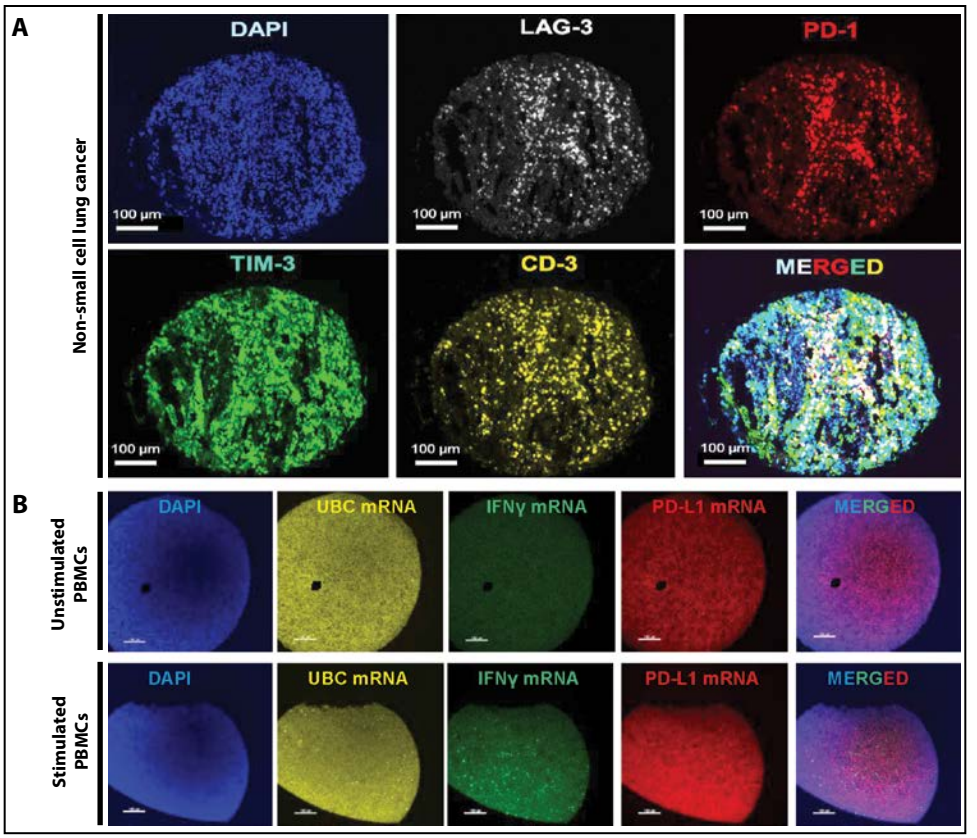


Figure 4-3. Simultaneous detection of multiple protein or messenger RNA (mRNA) targets using immunofluorescence in lung cancer. **(A)** Representative fluorescence images showing the expression of 4',6-diamidino-2-phenylindole (DAPI) (all cells, *blue channel*), CD3 (T cells, *yellow channel*), PD-1 (*red channel*), lymphocyte-activation gene 3 (*LAG-3*) (*white channel*), and mucin-domain containing-3 (TIM-3) protein (*green channel*) in formalin-fixed paraffin-embedded human non-small cell lung cancer. **(B)** Representative fluorescence images showing the expression of DAPI (all cells, *blue channel*), ubiquitin C (UBC) messenger RNA (mRNA) (*yellow channel*), interferon gamma (IFN-γ) mRNA (*green channel*), and PD-L1 mRNA (*red channel*) in formalin-fixed paraffin-embedded preparations from unstimulated human peripheral blood mononuclear cells (PBMCs) or PBMCs stimulated for 4 h with phorbol myristate acetate (PMA) plus ionomycin. Bar = 300 µm.

facilitate high-throughput, reliable, and reproducible staining, which are needed in clinical practice. Currently, various digital imaging technologies are emerging, and many of them show promising results, suggesting that immunohistochemistry is being further developed.

References

Aeffner F, Zarella MD, Buchbinder N, et al. Introduction to digital image analysis in whole-slide imaging: a white paper from the Digital Pathology Association. *J Pathol Inform*. 2019;10(1):9.

Althammer S, Tan TH, Spitzmuller A, et al. Automated image analysis of NSCLC biopsies to predict response to anti-PD-L1 therapy. *J Immunother Cancer*. 2019;7(1):121.

Angelo M, Bendall SC, Finck R, et al. Multiplexed ion beam imaging of human breast tumors. *Nat Med*. 2014;20(4):436-442.

Bera K, Schalper KA, Rimm DL, et al. Artificial intelligence in digital pathology—new tools for diagnosis and precision oncology. *Nat Rev Clin Oncol*. 2019;16(11):703-715.

Brigati DJ, Budgeon LR, Unger ER, et al. Immunocytochemistry is automated: development of a robotic workstation based upon the capillary action principle. *J Histotechnol*. 1988;11(3):165-183.

Carvajal-Hausdorf DE, Schalper KA, Neurmeister VM, et al. Quantitative measurement of cancer tissue biomarkers in the lab and in the clinic. *Lab Invest*. 2015;95(4):385-396.

De Wiest B, van Hecke D. IHC stainer platforms: overview, pros and cons. NordiQC database. 2017. Accessed January 31, 2020. https://www.nordiqc.org/downloads/documents/81.pdf

Esteva A, Robicquet A, Ramsundar B, et al. A guide to deep learning in healthcare. *Nat Med*. 2019;25(1):24-29.

Evans AJ, Bauer TW, Bui MM, et al. US Food and Drug Administration approval of whole slide imaging for primary diagnosis: a key milestone is reached and new questions are raised. *Arch Pathol Lab Med*. 2018;142(11):1383-1387.

Garcia-Rojo M, De Mena D, Muriel Cueto P, et al. New European Union regulations related to whole slide image scanners and image analysis software. *J Pathol Inform*. 2019;10:2.

Giesen C, Wang HA, Schapiro D, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods*. 2014;11(4):417-422.

Jain D, Nabirajan A, Borczuk A, et al. Immunocytochemistry for predictive biomarker testing in lung cancer cytology. *Cancer Cytopathol*. 2019;127(5):325-339.

Kapil A, Meier A, Zuraw A, et al. Deep semi supervised generative learning for automated tumor proportion scoring on NSCLC tissue needle biopsies. *Sci Rep*. 2018;8(1):17343.

Lantuejoul S, Sound-Tsao M, Cooper WA, et al. PD-L1 testing for lung cancer in 2019: perspective from the IASLC pathology committee. *J Thorac Oncol*. 2019;S1556-0864(19)33847-X.

Lindeman NI, Cagel PT, Aisner DL, 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;142(3):321-346.

Liu Y, Pantanowitz L. Digital pathology: review of current opportunities and challenges for oral pathologists. *J Oral Pathol Med*. 2019;48(4):263-269.

Myers J. A review of automated slide stainers for immunohistochemistry and in situ hybridization. *Med Lab Obs*. 2008;40(1):41-44.

Nakane PK, Pierce GB Jr. Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens. *J Cell Biol*. 1967;33(2):307-318.

Nakane PK, Pierce GB Jr. Enzyme-labeled antibodies: preparation and application for the localization of antigens. *J Histochem Cytochem*. 1966;14(12):929-931.

Nitta H, Tsuta K, Yoshida A, et al. New methods for ALK status diagnosis in non-small-cell lung cancer: an improved ALK immunohistochemical assay and a new, brightfield, dual ALK IHC-in situ hybridization assay. *J Thorac Oncol*. 2013;8(8):1019-1031.

Pantanowitz L, Sinard JH, Hendricks WH, et al. Validating whole slide imaging for diagnostic purposes in pathology: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med*. 2013;137(12):1710-1712.

Prichard JW. Overview of automated immunohistochemistry. *Arch Pathol Lab Med*. 2014;138(12):1578-1582.

Ramos-Vara JA. Principles and methods of immunohistochemistry. *Methods Mol Biol*. 2017;1641:115-128.

Sanmamed MF, Perez-Gracia JL, Schalper KA, et al. Changes in serum interleukin-8 (IL-8) levels reflect and predict response to anti-PD-1 treatment in melanoma and non-small-cell lung cancer patients. *Ann Oncol*. 2017;28(8):1988-1995.

Schalper KA, Brown J, Carvajal-Hausdorf D, et al. Objective measurement and clinical significance of TILs in non-small cell lung cancer. *J Natl Cancer Inst*. 2015;107(3):dju435.

Sha L, Osinksi BL, Ho IY, et al. Multi-field-of-view deep learning model predicts nonsmall cell lung cancer programmed death-ligand 1 status from whole-slide hematoxylin and eosin images. *J Pathol Inform*. 2019;10:24.

Taylor CR, Jadhav AP, Gholap A, et al. A multi-institutional study to evaluate automated whole slide scoring of immunohistochemistry for assessment of programmed death ligand 1 (PD-L1) expression in non-small cell lung cancer. *Appl Immunohistochem Mol Morphol*. 2019;27(4):263-269.

Tsao MS, Hirsch FR, Yatabe Y, eds. *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer*. 2nd ed. IASLC; 2016.

Velcheti V, Schalper KA, Carvajal DE, et al. Programmed death ligand-1 expression in non-small cell lung cancer. *Lab Invest*. 2014;94(1):107-116.

Widmaier M, Wiestler T, Walker J, et al. Comparison of continuous measures across diagnostic PD-L1 assays in non-small cell lung cancer using automated image analysis. *Mod Pathol*. 2020;33(3):380-390.

Wolff AC, Hammond MEH, Allison KH, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. *Arch Pathol Lab Med*. 2018;142(11):1364-1382.

Zhao C, Wu T, Ding X, et al. International telepathology consultation: three years of experience between the University of Pittsburgh Medical Center and KingMed Diagnostics in China. *J Pathol Inform*. 2015;6:63.

Immunohistochemistry for Small Specimens

5

By Lynette M. Sholl and Claudia Poleri

Introduction

It is estimated that 70% of primary lung cancers are diagnosed and staged using small biopsies and/or cytology specimens. Technical approaches to tumor tissue acquisition in the lung include transthoracic needle biopsy and/or fine-needle aspiration (FNA), endobronchial or transbronchial biopsy and/or FNA, and pleural fluid aspiration in the context of malignant effusions (Ofiara et al 2012). Principles specific to cytology specimens are discussed in more detail in Chapter 12. Small biopsies as just described are derived from minimally invasive procedures that are associated with reduced discomfort and risk to the patient relative to surgical biopsies. However, small biopsies pose several potential challenges to optimal diagnosis and downstream biomarker testing. First, the small size of these specimens can contribute to challenges around insufficient number of evaluable tumor cells and nucleic acid quantity for downstream molecular testing. Second, lung primary-site biopsies can be substantially contaminated by benign cells, such as normal lung parenchyma, bronchial epithelium, or pleural tissue, with implications both for diagnosis and molecular test sensitivity. Third, small biopsies often suffer from artifact-like crushing and tissue distortion as a function of the procedure used to obtain the tissue. Finally, these specimens represent only a small fraction of the overall tumor and may not capture the extent of tumor heterogeneity. This chapter focuses on approaches to small biopsy handling, with an emphasis on the role of immunohistochemistry (IHC) for diagnosis and therapeutic prediction and suggestions for optimizing this tool in practice.

Should IHC Be Performed in All Small Biopsies?

Because of the essential nature of molecular profiling to select advanced-stage non-small cell carcinoma (NSCC) patients for targeted therapy, pathologists must be cognizant of the need to preserve tumor tissue for biomarker testing while still generating the most accurate and specific diagnosis possible. Diagnostic strategies originally proposed in 2011 have formed the basis of current nomenclature (Travis et al 2011, 2013, 2015) and emphasize the judicious use of IHC in patients with suspected NSCC (Figure 5-1).

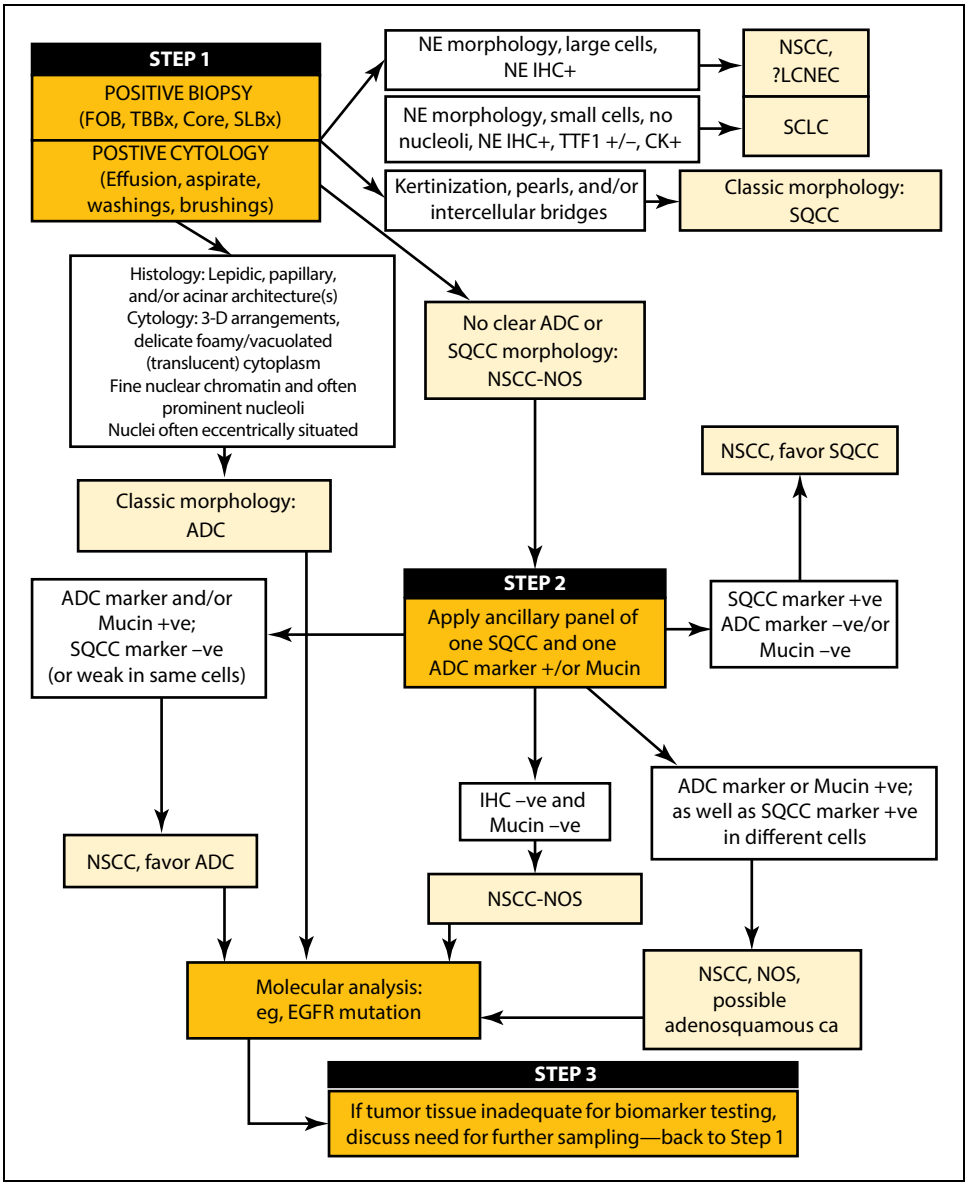


Figure 5-1. Algorithm for the work-up of small biopsies and/or cytology samples with lung cancer. Immunohistochemistry (IHC) does not have to be performed on the samples with classical morphology of adenocarcinoma or squamous cell carcinoma. If there is neuroendocrine morphology, the tumor may be classified according to standard criteria. IHC should be performed in cases with no clear adenocarcinoma, squamous, or neuroendocrine (NE) morphology. ADC = adenocarcinoma; CK = cytokeratin; EGFR = epidermal growth factor receptor; FOB = flexible fiberoptic bronchoscopy; LCNEC = large cell neuroendocrine carcinoma; NOS = not otherwise specified; NSCC = non-small cell carcinoma; SCLC = small cell lung carcinoma; SLBx = surgical lung biopsy; SQCC = squamous cell carcinoma; TBBx = transbronchial biopsy; TTF1 = thyroid transcription factor-1. (Adapted from Travis et al 2011)

Summary Answer

Briefly, the pathologist's morphologic impression should drive the initial diagnostic approach: If the biopsy shows clear-cut morphologic differentiation of a squamous cell carcinoma (ie, keratinization) or adenocarcinoma (ie, glandular formation), the pathologist is encouraged to render the diagnosis accordingly and is not mandated to apply confirmatory

IHC. This assumes, of course, that the clinical context supports a diagnosis of primary lung cancer and that potential mimics (metastatic disease, mesothelioma) have been considered when appropriate.

When Should IHC Be Performed to Classify NSCC?

It can be difficult to render a confident morphologic diagnosis on a biopsy with poorly differentiated carcinoma (no morphologic evidence of adenocarcinoma or squamous cell carcinoma). In this context, assuming at a minimum that small cell carcinoma has been excluded morphologically, the pathologist is left with a diagnosis of NSCC, not otherwise specified (NOS). At this point, IHC should be leveraged to clarify whether the tissue is more likely to represent adenocarcinoma or squamous cell carcinoma (or other). The use of IHC at this juncture is associated with a substantial reduction in the frequency of an NSCC-NOS diagnosis (Loo et al 2010; Nicholson et al 2010; Mukhopadhyay and Katzenstein 2011; Zhao et al 2014) and provides the treating clinician with greater confidence in choosing therapy and/or requesting predictive biomarker testing.

Summary Answer

IHC should be performed in poorly differentiated carcinoma (often solid growth pattern). This pattern can be seen in adenocarcinomas and non-keratinizing squamous cell carcinoma.

What Are the Best First Markers to Classify NSCC?

The rationale for selecting certain antibodies and clones are covered in detail elsewhere in this atlas. In brief, the optimal first-line IHC panel for NSCC diagnosis includes only thyroid transcription factor-1 (TTF1) and p40 (Travis et al 2013; Yatabe et al 2019). A guide to interpretation of these stains based on the extent of positivity is provided in Table 5-1. Examples of morphologically undifferentiated tumors further characterized by IHC are shown in Figures 5-2 and 5-3.

Summary Answer

The combination of p40 and TTF1 has the best sensitivity and specificity to separate NSCC into adenocarcinoma and squamous cell carcinoma.

What Other Tumor Markers Can Be Helpful in the Classification of NSCC?

Napsin A is relatively comparable to TTF1 in identification of adenocarcinoma (Tran et al 2016), and as a cytoplasmic marker, it can be multiplexed with the nuclear stain p40 so that

Table 5-1. Initial Diagnostic Immunohistochemical Panel for Non-Small Cell Carcinoma (NSCC) Diagnosis on Small Biopsies or Cytology Specimens

TTF1	p40	Diagnosis	Comments
+[a] to +++	– or +	NSCC, favor adenocarcinoma	When same cell population staining
–	+ in >50%	NSCC, favor squamous cell carcinoma	
–	+ in 10% to <50%	NSCC, NOS	
+[a] to ++	Any extent	NSCC, NOS, possible adenosquamous carcinoma	When separate cell populations staining

Abbreviations: NOS = not otherwise specified; TTF1 = thyroid transcription factor-1.

[a] Focal tumor cell staining.

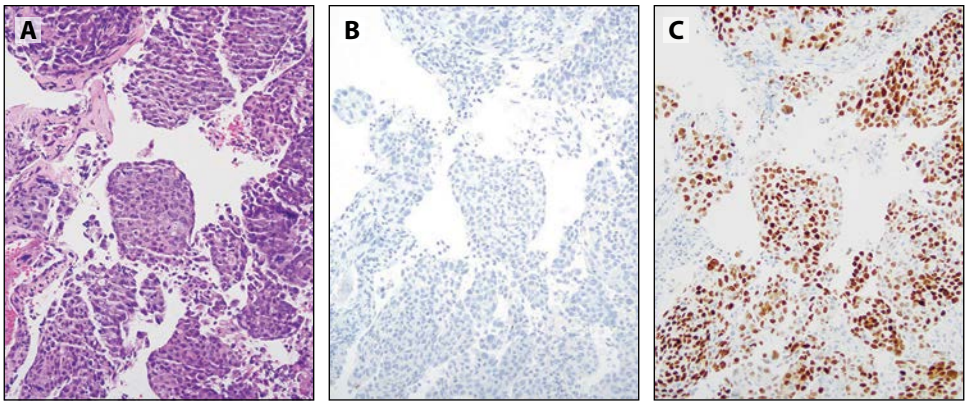


Figure 5-2. Non-small cell carcinoma (NSCC), favor adenocarcinoma. **(A)** Note poorly differentiated carcinoma with a solid growth pattern and without morphologic evidence of glandular, squamous, or neuroendocrine differentiation on routine H&E sections. The carcinoma cells are **(B)** negative for p40 and **(C)** diffusely positive for thyroid transcription factor-1 (TTF1).

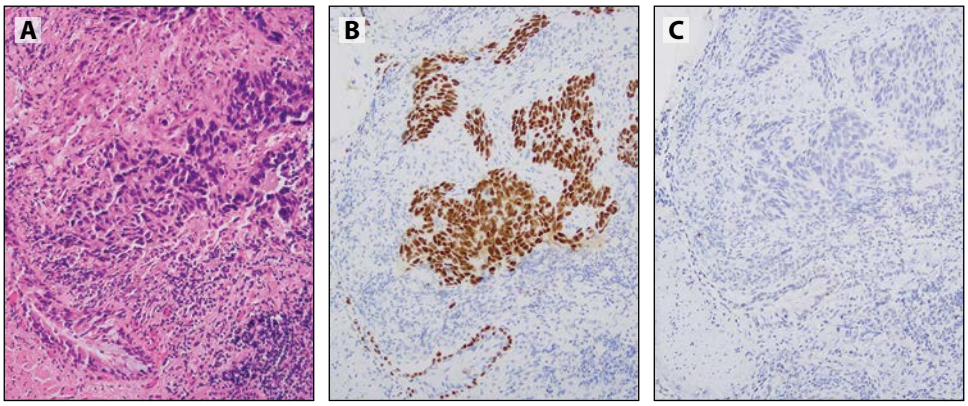


Figure 5-3. Non-small cell carcinoma (NSCC), favor squamous cell carcinoma. **(A)** Note a poorly differentiated carcinoma with a solid growth pattern and extensive necrosis on routine H&E sections. The carcinoma cells are **(B)** diffusely positive for p40 and **(C)** negative for thyroid transcription factor-1 (TTF1).

only a single slide is required for IHC analysis (Nishino et al 2016). However, because of challenges interpreting napsin A staining, it is not generally recommended for first-line use (Yatabe et al 2019). If TTF1 and p40 stains are uninformative or equivocal, a limited panel of second-line IHC markers including napsin A, cytokeratin 5/6 (CK5/6) may occasionally inform the diagnosis (Travis et al 2015). Cytokeratin 7 is a poor discriminator of adenocarcinoma and squamous carcinoma of the lung. It is not recommended for this purpose (Yatabe et al 2019). Molecular studies should be considered for NSCC-NOS, as the diagnosis of adenocarcinoma cannot be ruled out. A pankeratin stain should be added to a NSCC-NOS, null type, that shows no staining for TTF1 or p40.

Summary Answer

Napsin A and CK5/6 may improve the classification in some NSCC-NOS. A pankeratin should be added in case of a poorly differentiated carcinoma that is negative for TTF1, p40, CK5/6, and napsin A.

What Are the Best Markers for Neuroendocrine Neoplasms?

Neuroendocrine tumors, in particular carcinoid family tumors and small cell carcinoma, commonly arise from the central airways and are amenable to endobronchial or transbronchial biopsy approaches. The tumor cells are delicate and prone to artifactual crushing during the sampling process. In addition, the morphology of small cell carcinoma, carcinoid, benign structures (such as lymphoid tissue), and other small round blue cell tumors (basaloid squamous carcinoma, lymphoma, sarcomas) can show substantial overlap. As a result, IHC is often essential to a confident diagnosis when presented with this differential. Details of relevant markers are covered in detail later in this atlas. Some basic considerations are presented here.

If the cells resemble lymphocytes, and bronchial-associated lymphoid tissue (BALT) is suspected, staining with leukocyte common antigen alone may suffice to confirm this diagnosis (Figure 5-4). BALT tissue is common in the adult lung and may be prominent at airway branch points (Churg et al 2005).

A keratin stain can be used to confirm a diagnosis of an epithelial neoplasm; however, small cell carcinomas may rarely be negative or only very focally positive. Neuroendocrine marker stains including synaptophysin, chromogranin, CD56, and/or insulinoma-associated protein 1 (INSM1) may be used to confirm neuroendocrine differentiation, but are negative in up to 10% of cases (Travis et al 2015) (Figure 5-5).

Carcinoid tumors lack the overt features of malignancy (atypia, frequent mitoses, sheet-like necrosis) characteristic of small cell carcinoma. On limited and poorly preserved samples, Ki-67 IHC may be warranted to confirm a low proliferation rate in carcinoid tumors, or very high rate in small cell carcinomas (for neuroendocrine markers, see Chapter 10).

In the differential with other lung primary carcinomas, small cell carcinoma can morphologically resemble basaloid squamous cell carcinoma; positive p40 expression will support the latter diagnosis.

Summary Answer

A panel of neuroendocrine markers (chromogranin, synaptophysin, CD56, and/or INSM1) should be added to confirm neuroendocrine phenotype. In addition, a proliferation marker

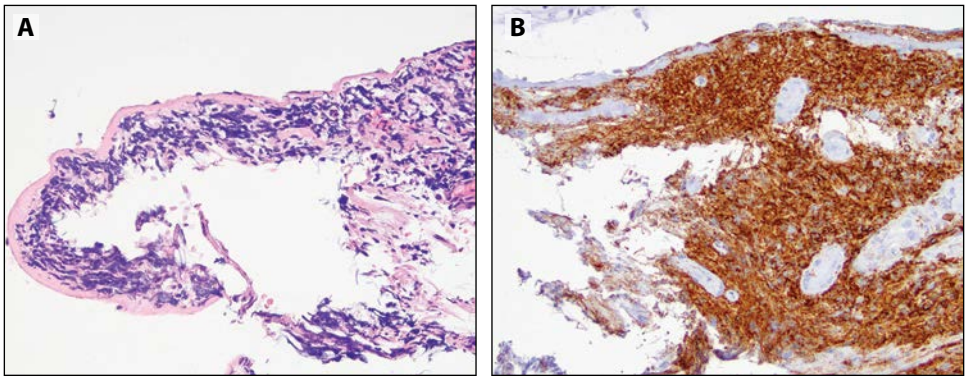


Figure 5-4. (A) Small crushed and denuded airway biopsy with poorly preserved small blue cells on routine H&E. **(B)** Diffuse staining for leukocyte common antigen (LCA)-CD45 confirms the morphologic impression of bronchial-associated lymphoid tissue (BALT).

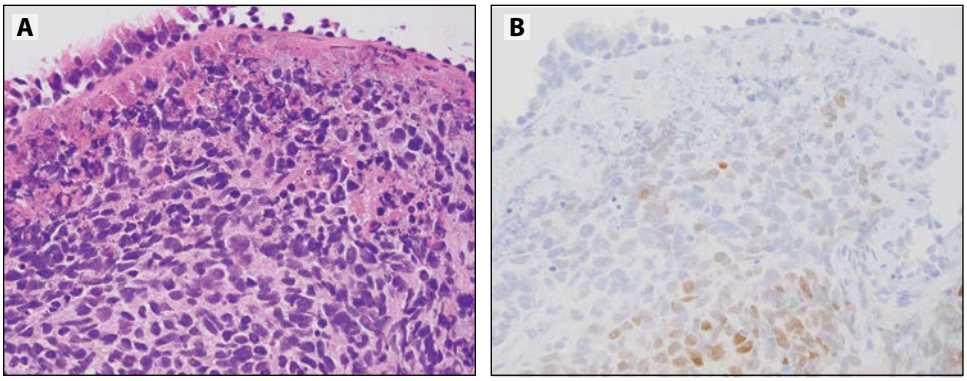


Figure 5-5. (A) Endobronchial biopsy containing small cell carcinoma on H&E. **(B)** Multifocal positive nuclear staining for insulinoma-associated protein 1 (INSM1).

is helpful in small biopsies with significant crush artifact. Inclusion of pankeratin markers can differentiate between a neuroendocrine tumor and lymphoma. Inclusion of p40 can avoid a pitfall of mistaking basaloid squamous cell carcinoma with a high-grade neuroendocrine carcinoma.

What Are the Challenging Diagnostic Scenarios in Small Biopsies?

The artifacts associated with small biopsies—including poor cellular preservation, crush artifact, and distortion—are as likely to confound interpretation of benign structures as they are to mask the morphologic features of tumor cells. In some scenarios, IHC may help highlight benign or malignant cell populations. In others, the pathologist must rely entirely on morphologic clues and context. Distinguishing between poorly preserved submucosal glands or reactive endothelium and infiltrating adenocarcinoma on an endobronchial biopsy can be challenging. TTF1 staining highlights infiltrating adenocarcinoma, but should be negative in benign airway wall components when using a high-specificity clone (8G7G3/1) (Figure 5-6).

Conversely, the pathologist should not overinterpret entrapped or adjacent TTF1-positive reactive pneumocytes as indicative of an adenocarcinoma diagnosis (Figure 5-7). Careful cross referencing of the hematoxylin and eosin (H&E) stain in a serial section ensures that the appropriate population is evaluated for immunoreactivity.

Summary Answer

Careful correlation of histology and immunohistochemical stains is necessary to avoid misinterpretation of the latter by biopsy artifacts.

How Should Biopsy Specimens Be Handled to Optimize Predictive Biomarker Testing Results?

The accuracy of molecular testing depends in large part on the quality of the specimens received for testing. Sample quality, for example, protein and nucleic acid preservation and amount, depends both on the fixed attributes of the tumor (size, infiltrative nature, extent of inflammatory infiltrates, tumor necrosis, etc) as well as more controllable variables such as sample handling in the biopsy suite and pathology laboratory. Many of the critical pre-analytic variables are discussed elsewhere in this atlas; biopsies are relatively unique because of their

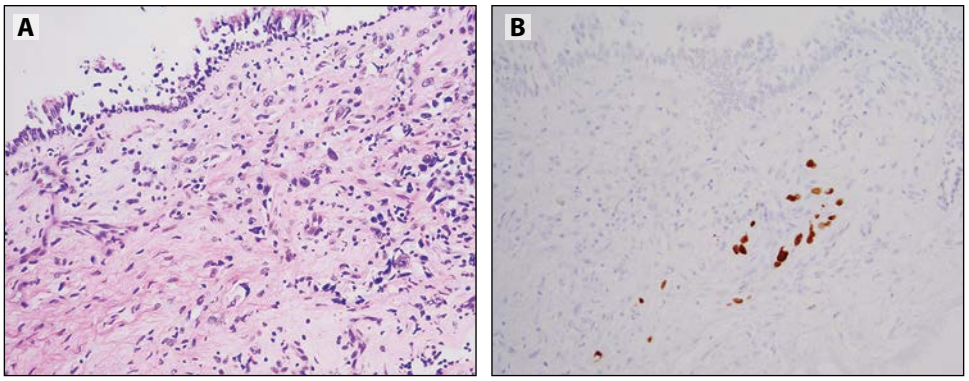


Figure 5-6. (A) Airway wall with rare highly atypical cells admixed with chronic inflammation; **(B)** thyroid transcription factor-1 (TTF1) stain highlights infiltrating tumor cells.

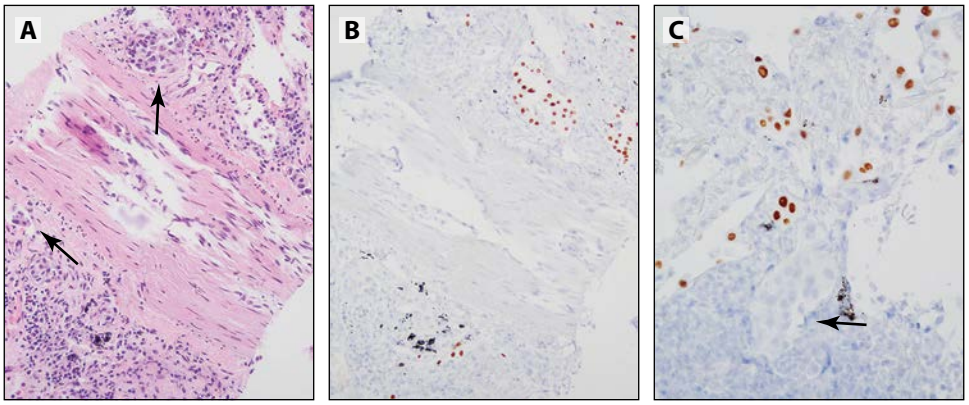


Figure 5-7. (A) Non-small cell lung carcinoma, not otherwise specified, present extensively within lymphatics (*arrows*); **(B)** adjacent reactive pneumocytes are highlighted by thyroid transcription factor-1 (TTF1) staining. **(C)** Note negative cells within lymphatics (*arrow*) (best seen in the ×400 magnification in panel A). Stain for pankeratin-highlighted tumor cells (not shown).

delicate nature and need for rapid fixation to prevent sample drying and degradation. Bone biopsies often require a decalcification step in acid or chelating solutions prior to routine slide processing in histology laboratories. Acid solutions (hydrochloric acid, formic acid) degrade proteins and nucleic acids and can severely impair reactivity by IHC, polymerase chain reaction (PCR), and sequencing-based molecular tests (Lindeman et al 2013; Maclary et al 2017). Chelating solutions, such as those containing ethylenediaminetetraacetic acid (EDTA), are relatively effective for decalcification of small biopsies, with improved preservation of protein antigenicity and nucleic acid integrity and more modest negative effects on IHC and molecular studies (Schrijver et al 2016).

It is paramount in the work-up of a suspected non-small cell lung carcinoma that the pathologist considers the potential need for subsequent predictive biomarker testing, including programmed death ligand-1 (PD-L1) IHC, anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1) fluorescence in situ hybridization (FISH) or IHC, and molecular profiling for sequence variation in epidermal growth factor receptor (EGFR) and B-raf proto-oncogene (*BRAF*), and a range of other potential therapeutic targets (Lindeman et al 2018). Judicious use of diagnostic IHC is, therefore, essential. The laboratory workflow should be adapted

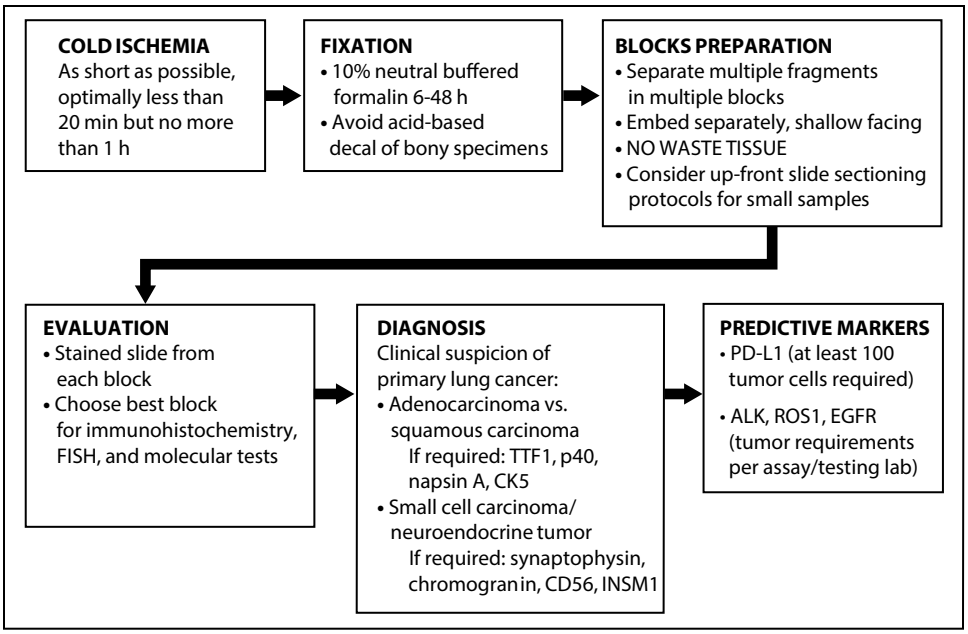


Figure 5-8. Overview of suggested laboratory processes for small biopsy preparation. ALK = anaplastic lymphoma kinase; CK5 =cytokeratin 5; EGFR = epidermal growth factor receptor; FISH = fluorescence in situ hybridization; INSM1 = insuloma-associated protein 1; PD-L1 = programmed death ligand-1; ROS1 = c-ros oncogene 1 protein; TTF1 = thyroid transcription factor-1.

to manage small specimens that may demand a large number and diversity of clinical tests (Figure 5-8).

Some laboratories have advocated for establishment of a "molecular priority" biopsy protocol (Aisner et al 2016) that flags a sample for dedicated handling in the histology laboratory to include separate embedding and superficial facing of the individual biopsy fragments, with up-front slide-cutting protocols to ensure that adequate unstained slides are available for diagnostic and predictive IHC and molecular and/or cytogenetic tests. Touch imprints or smears made from the fresh core biopsy may also be used for molecular analysis (Roh 2019). Because this approach is more time and labor intensive, it is essential that the pathology laboratory partner with the clinicians obtaining these biopsies to ensure that the indication and potential need for genomic testing is made clear at the time of specimen receipt.

Minimum predictive biomarker testing requirements vary depending on the target and assay in question. The companion diagnostic label for PD-L1 pharmDx IHC testing requires at least 100 tumor cells; the PD-L1 tumor proportion score may be underestimated in specimens with fewer than 100 tumor cells (Gagne et al 2019). *ALK* FISH testing (Abbott/Vysis) requires 50 tumor nuclei. ALK IHC, however, does not require a minimum number of tumor nuclei; the same is true for ROS1 IHC, which may be employed as a screening tool for *ROS1* fusion detection. Increasingly, molecular methods, such as DNA and RNA-based next-generation sequencing assays, can detect fusion events even in small samples with significant benign cell contamination. RNA-based fusion detection (including for *ALK*, *ROS1*, ret proto-oncogene [*RET*], neurotrophic tyrosine receptor kinase [*NTRK*1-3]) may be a particularly powerful tool in limited samples as it is optimized to detect expressed transcripts,

which may be present at a very high level in tumor cells and thus detectable even in suboptimal specimens (Davies et al 2018; Benayed et al 2019).

Summary Answer

Small biopsies play a crucial role in patient management. Therefore, a detailed protocol for tissue utilization should be established in each laboratory for determination of predictive markers. Awareness of limitations, pitfalls, and requirements for each biomarker is paramount in establishing an effective work flow.

Conclusions

Immunohistochemical stains are a powerful tool to classify poorly differentiated carcinoma, especially in biopsy specimens where procedural artifacts and sampling may become an issue.

Pathologists should apply recommended panels and algorithms for classification and determination of predictive markers to improve utilization of small biopsy material, leading to an accurate diagnosis as well as identification of predictive biomarkers.

References

Aisner DL, Rumery MD, Merrick DT, et al. Do more with less: tips and techniques for maximizing small biopsy and cytology specimens for molecular and ancillary testing: the University of Colorado experience. *Arch Pathol Lab Med*. 2016;140(11):1206-1220.

Benayed R, Offin M, Mullaney K, et al. High yield of RNA sequencing for targetable kinase fusions in lung adenocarcinomas with no mitogenic driver alteration detected by DNA sequencing and low tumor mutation burden. *Clin Cancer Res*. 2019;25(15):4712-4722.

Churg AM, Myers JL, Tazelaar HD, et al. *Thurlbeck's Pathology of the Lung*. 3rd ed. Thieme; 2005.

Davies KD, Le AT, Sheren J, et al. Comparison of molecular testing modalities for detection of ROS1 rearrangements in a cohort of positive patient samples. *J Thorac Oncol*. 2018;13(10):1474-1482.

Gagne A, Wang E, Bastien N, et al. Impact of specimen characteristics on PD-L1 testing in non-small cell lung cancer: validation of the IASLC PD-L1 testing recommendations. *J Thorac Oncol*. 2019;14(12):2062-2070.

Lindeman NI, Cagel PT, Aisner DL, 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;142(3):321-346.

Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *J Thorac Oncol*. 2013;8(7):823-859.

Loo PS, Thomas SC, Nicolson MC, et al. Subtyping of undifferentiated non-small cell carcinomas in bronchial biopsy specimens. *J Thorac Oncol*. 2010;5(4):442-447.

Maclary SC, Mohanty SK, Bose S, et al. Effect of hydrochloric acid decalcification on expression pattern of prognostic markers in invasive breast carcinomas. *Appl Immunohistochem Mol Morphol*. 2017;25(2):144-149.

Mukhopadhyay S, Katzenstein AL. Subclassification of non-small cell lung carcinomas lacking morphological differentiation on biopsy specimens: utility of an immunohistochemical panel containing TTF-1, napsin A, p63, and CK5/6. *Am J Surg Pathol*. 2011;35(1):15-25.

Nicholson AG, Gonzalez D, Shah P, et al. Refining the diagnosis and EGFR status of non-small cell lung carcinoma in biopsy and cytologic material, using a panel of mucin staining, TTF-1, cytokeratin 5/6, and P63, and EGFR mutation analysis. *J Thorac Oncol*. 2010;5(4):436-441.

Nishino M, Hoang MP, Della Pelle P, et al. Napsin A/p40 antibody cocktail for subtyping non-small cell lung carcinoma on cytology and small biopsy specimens. *Cancer Cytopathol*. 2016;124(7):472-484.

Ofiara LM, Navasakulpong A, Ezer N, et al. The importance of a satisfactory biopsy for the diagnosis of lung cancer in the era of personalized treatment. *Curr Oncol*. 2012;19(suppl 1):S16-23.

Roh MH. The utilization of cytologic and small biopsy samples for ancillary molecular testing. *Mod Pathol*. 2019;32(suppl 1):77-85.

Schrijver WA, van der Groep P, Hoefnagel LD, et al. Influence of decalcification procedures on immunohistochemistry and molecular pathology in breast cancer. *Mod Pathol*. 2016;29(12):1460-1470.

Tran L, Mattsson JS, Nodin B, et al. Various antibody clones of napsin A, thyroid transcription factor 1, and p40 and comparisons with cytokeratin 5 and p63 in histopathological diagnostics of non-small cell lung carcinoma. *Appl Immunohistochem Mol Morphol*. 2016;24(9):648-659.

Travis WD, Brambilla E, Burke AP, et al. *WHO Classification of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015.

Travis WD, Brambilla E, Noguchi M, et al. Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. *Arch Pathol Lab Med*. 2013;137(5):668-684.

Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol*. 2011;6(2):244-285.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Zhao W, Wang H, Peng Y, et al. ΔNp63, CK5/6, TTF-1 and napsin A, a reliable panel to subtype non-small cell lung cancer in biopsy specimens. *Int J Clin Exp Pathol*. 2014;7(7):4247-4253.

Immunomarkers in the Classification of Resected Major Lung Cancers

6

By Sabina Berezowska, Andrew G. Nicholson, William D. Travis, Alain C. Borczuk, and Ming Sound Tsao

Introduction

The presence of defining morphologic patterns and features is sufficient for the diagnosis of the major types of non-small cell carcinoma (NSCC) in around 60% of biopsies (Nicholson et al 2010; Loo et al 2010) and 80% of resections. For adenocarcinoma, these patterns are lepidic, acinar, papillary, micropapillary, cribriform architecture(s), and/or mucin production and features such as signet ring morphology (Travis et al 2011). For squamous cell carcinoma, these are keratinization and squamous pearl formation with intercellular bridges. In cases where these features are lacking and mucin stains are negative, immunohistochemical stains should be applied to enable further typing of NSCCs. This concept of typing morphologically undifferentiated tumors according to their immunohistochemical expression profiles was recommended in the 2015 World Health Organization (WHO) classification (Travis et al 2015). In small biopsies and cytology specimens, the number of stains utilized for diagnosis or predictive marker testing should be kept to a minimum to preserve as much tissue as possible for molecular analysis (as discussed in Chapter 5) and in resections limited to the minimum needed for accurate classification.

What Is the Best Combination of Markers to Use in Daily Practice to Distinguish Adenocarcinoma and Squamous Cell Carcinoma?

In morphologically undifferentiated resected non-small cell carcinoma without neuroendocrine morphology (Figure 6-1A and B), the most useful and frequently sufficient panel differentiating adenocarcinoma and squamous cell carcinoma consists of thyroid transcription factor-1 (TTF1; clone 8G7G3/1) and p40 (Yatabe et al 2019). Excluding the question of metastatic disease, TTF1 expression characterizes the tumor as an adenocarcinoma of the lung over the other major non-squamous cell carcinoma categories (Figure 6-1C and D). TTF1 is a nuclear marker expressed in 75% to more than 80% of non-mucinous primary pulmonary adenocarcinomas, depending on the clone, with higher specificity but lower sensitivity of clone 8G7G3/1 than clone SPT24 (Ordonez 2012; Kadota et al 2015; Kashima et al 2014).

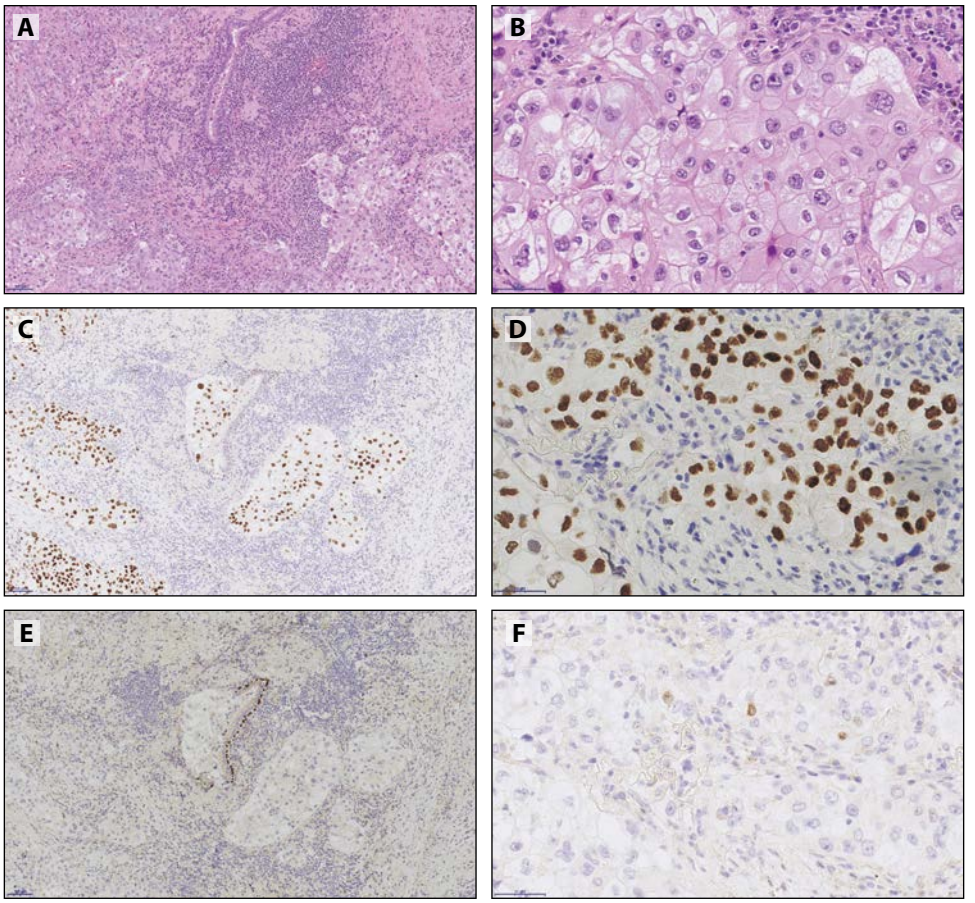


Figure 6-1. Use of thyroid transcription factor-1 (TTF1) and p40 to classify poorly differentiated carcinoma in resected lung cancers. This adenocarcinoma of solid pattern stains with TTF1 and not p40. The weak p40 staining in a TTF1-positive tumor does not indicate adenosquamous histology: **(A, C, E)** ×10; **(B, D, F)** ×40; **(A and B)** H&E, **(C and D)** TTF1-8G7G3/1, and (**E and F)** p40.

Focal TTF1 (clone 8G7G3/1) positivity is sufficient for calling the tumor TTF1 positive, and reactivity can be weak. (TTF1 is discussed in detail in Chapter 7.) p40 (ΔNp63) is a nuclear marker of squamous differentiation. Focal or weak positivity for p40 may be observed in TTF1 positive tumors; these cases should still be classified as adenocarcinomas.

Napsin A may be a valuable marker for diagnosing adenocarcinoma in TTF1-negative cases of primary lung tumors, as squamous cell carcinomas are consistently reported to be napsin A–negative (Kadota et al 2015; Whithaus et al 2012). Napsin A (monoclonal) shows a granular, cytoplasmic pattern (Figure 6-2). It is expressed in type II pneumocytes, alveolar macrophages, renal tubules, exocrine glands, and pancreatic ducts. The monoclonal antibody is preferred over the polyclonal antibody because of its higher specificity (Mukhopadhyay and Katzenstein 2012). However, its usage is not recommended if TTF1 is positive, as most lung adenocarcinomas co-express TTF1 and napsin A.

In the case of a TTF1 and p40 co-expression in the same tumor cells of a morphologically undifferentiated non-small cell carcinoma, even a weak TTF1 positivity is sufficient to type tumors as adenocarcinomas.

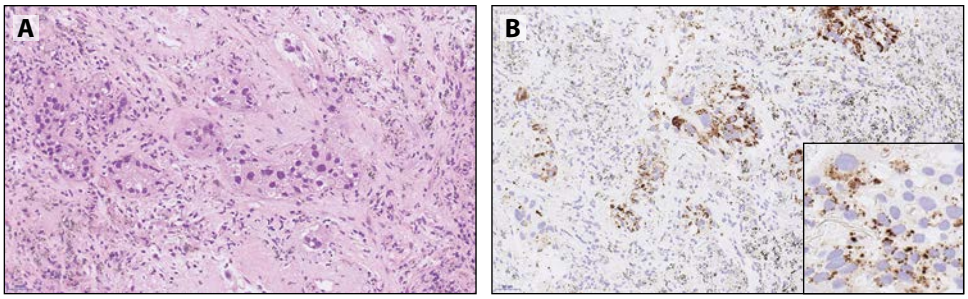


Figure 6-2. Napsin A may be a valuable marker for diagnosing adenocarcinoma in thyroid transcription factor-1 (TTF1)-negative cases of primary lung tumors. Napsin A (monoclonal) show a granular, cytoplasmic pattern (*inset* ×40). ×20 **(A)** H&E and **(B)** napsin A.

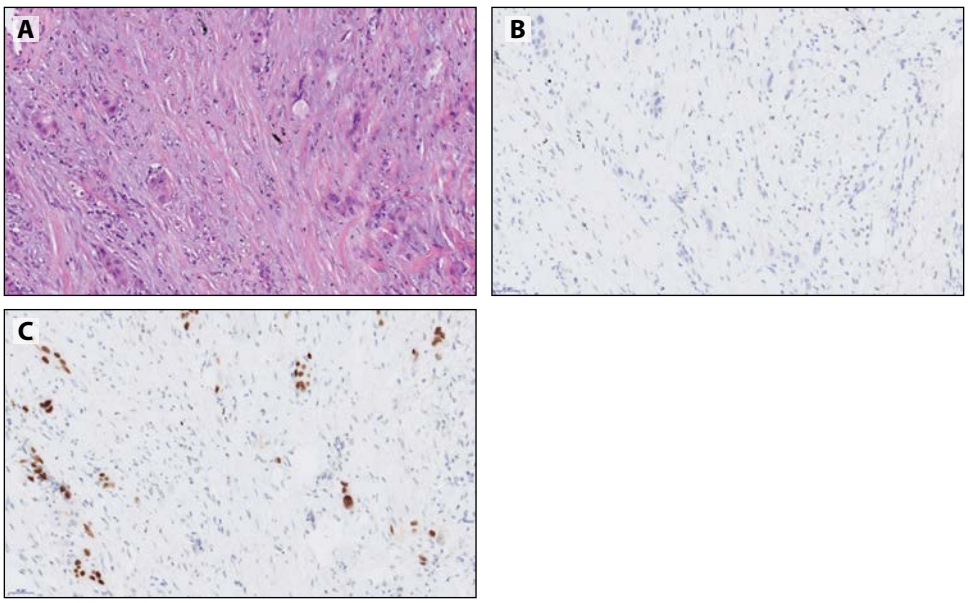


Figure 6-3. Highly infiltrative, morphologically undifferentiated tumors without **(A)** keratinization or **(B)** thyroid transcription factor-1 (TTF1) expression can be typed as squamous cell carcinomas if they show strong p40 positivity as in the case shown here. ×20 **(A)** H&E, **(B)** TTF1, and **(C)** p40.

Squamous cell carcinomas strongly express p40 in over 50% of tumor cells and must be TTF1 negative (Figure 6-3). p63 shows similar sensitivity to p40 but is less specific (Bishop et al 2012) and should not be used if p40 is available. Cytokeratin 5/6 (CK5/6) may also be used as an additional marker of squamous differentiation, but it cannot distinguish between adenocarcinoma and squamous cell carcinoma when used alone (Righi et al 2011; Rekhtman et al 2011; Warth et al 2012).

Acantholytic squamous cell carcinoma can be confused with adenocarcinoma and in some instances, epithelioid angiosarcoma. In these cases, a combination of TTF1 and p40 can be very useful to confirm squamous cell carcinoma (Figure 6-4), with an expanded panel if both are negative.

Because of its low specificity, cytokeratin 7 (CK7) positivity should not be used to differentiate adenocarcinomatous from squamous differentiation (Figure 6-5). Although CK7 is

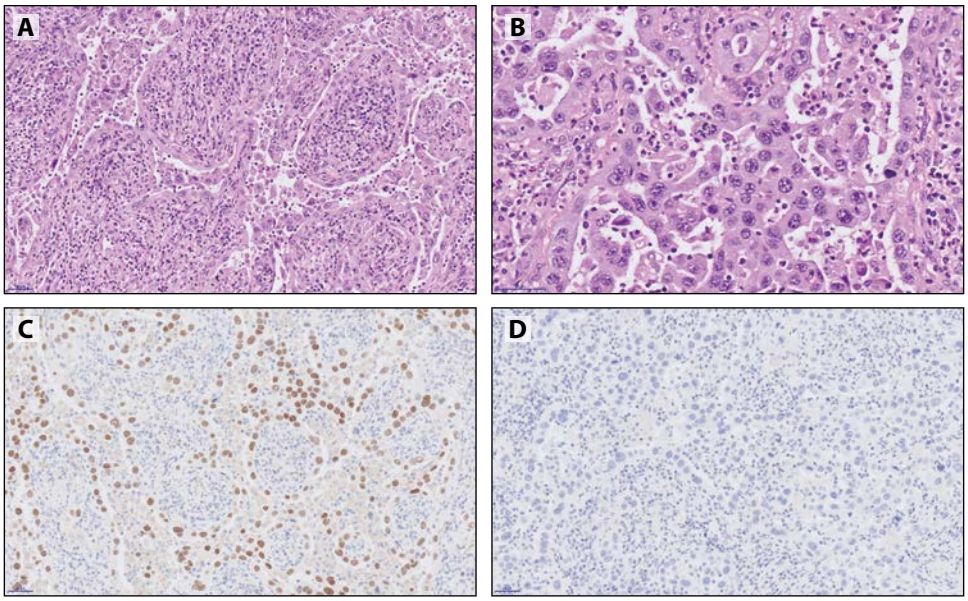


Figure 6-4. Because of the therapeutic consequences, the threshold for applying immunohistochemistry to validate subtyping of non-small cell carcinoma (NSCC) in uncertain cases should be low. **(A and B)** The acantholytic growth pattern of this tumor pointed toward an adenocarcinoma. However, the immunohistochemical expression pattern with strong **(C)** p40 positivity and **(D)** negativity for thyroid transcription factor-1 (TTF1) confirmed a squamous cell carcinoma. **(A, C,** and **D)** ×20; **(B)** ×40; **(A and B)** H&E, **(C)** p40, and **(D)** TTF1.

positive in 91% to 100% of lung adenocarcinomas, squamous cell carcinomas may show strong and diffuse CK7 positivity, reported in 5% to 77% of cases (Warth et al 2012; Mukhopadhyay and Katzenstein 2012; Johansson 2004; Noh and Shim 2012; Righi et al 2011; Koh et al 2014; Gurda et al 2015). See Chapter 9 for an in-depth discussion on cytokeratin usage.

Not all primary lung carcinomas with squamous differentiation as evidenced by strong p40 expression or keratinization represent squamous cell carcinomas. Some morphologically undifferentiated tumors with strong p40 expression may represent nuclear protein in testis (NUT)-carcinomas (Haack et al 2009). The typical foci of keratinization may be only focally present in a resection specimen (Figure 6-6) (see Chapter 14). Intrapulmonary thymomas can also show diffuse strong p40 expression.

In a morphologically undifferentiated tumor, focal p40 in less than 10% of the tumor or weak immunoreactivity should not be interpreted as squamous differentiation. In such cases, it should be classified as large cell carcinoma with uncertain immunohistochemical features, not as non-keratinizing squamous cell carcinoma.

Summary Answer

In daily practice, TTF1 and p40 suffice for subtyping most non-small cell carcinomas without defining morphologic characteristics or neuroendocrine morphology. This panel may be expanded when metastases or rarer variants are suspected.

What Is the Role of Immunomarkers in the Diagnosis of Adenosquamous Carcinoma?

The diagnosis of adenosquamous carcinoma requires a resection specimen with at least 10% of each component. As with histomorphologically diagnosed adenocarcinomas and

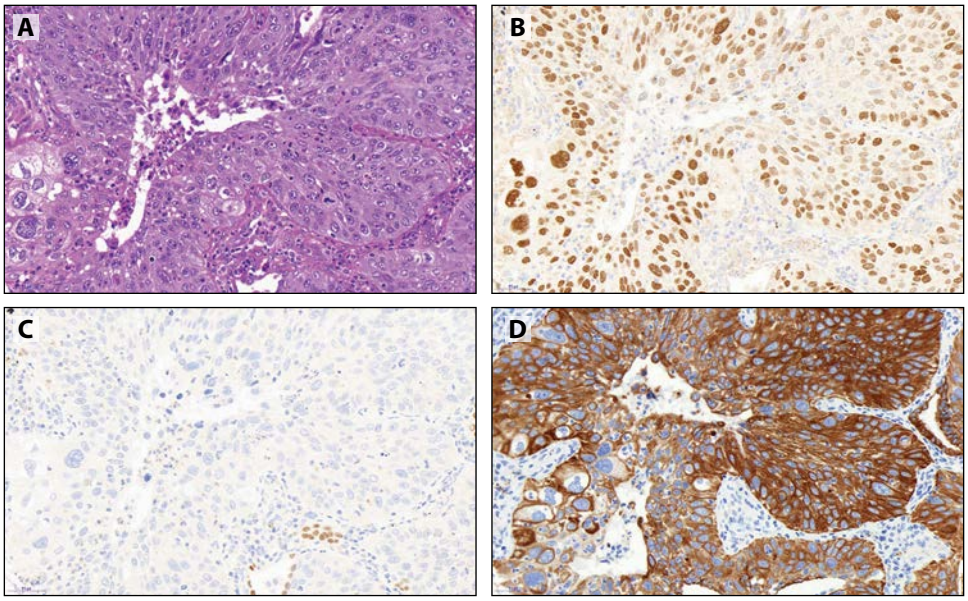


Figure 6-5. A non-keratinizing squamous cell carcinoma with expression of **(B)** p40, **(C)** absence of thyroid transcription factor-1 (TTF1), and **(D)** strong and homogeneous cytokeratin 7 (CK7) expression. A metastasis from urothelial carcinoma was ruled out using additional immunomarkers and history. **(A-D)** ×30; **(A)** H&E, **(B)** p40, **(C)** TTF1, and **(D)** CK7.

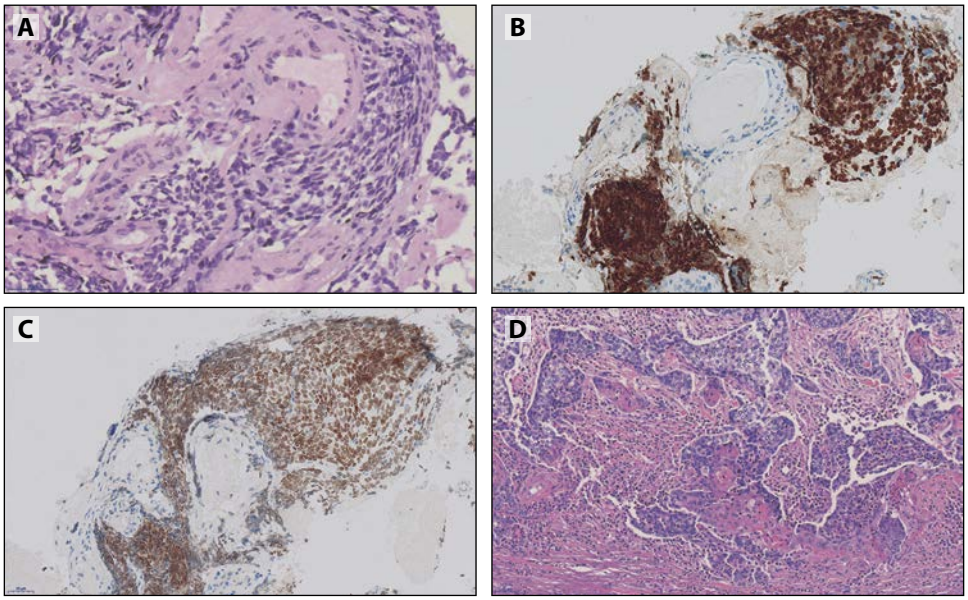


Figure 6-6. Nuclear protein in testis (NUT) carcinoma is defined by the occurrence of *NUT* gene rearrangement, detectable using the available monoclonal NUT-antibody with speckled nuclear positivity. Keratinization may be only focal. **(A)** ×40; **(B-D)** ×20; **(A and D)** H&E, **(B)** p40, and **(C)** NUT.

squamous cell carcinomas, there is no need for immunohistochemical confirmation of the 2 different components in cases of adenosquamous carcinoma when consisting of morphologically unequivocal components of squamous cell carcinoma and adenocarcinoma (Travis et al 2015). When one or both of the components are poorly differentiated, immunohistochemistry is necessary to support this diagnosis in demonstrating 2 clearly delineated components:

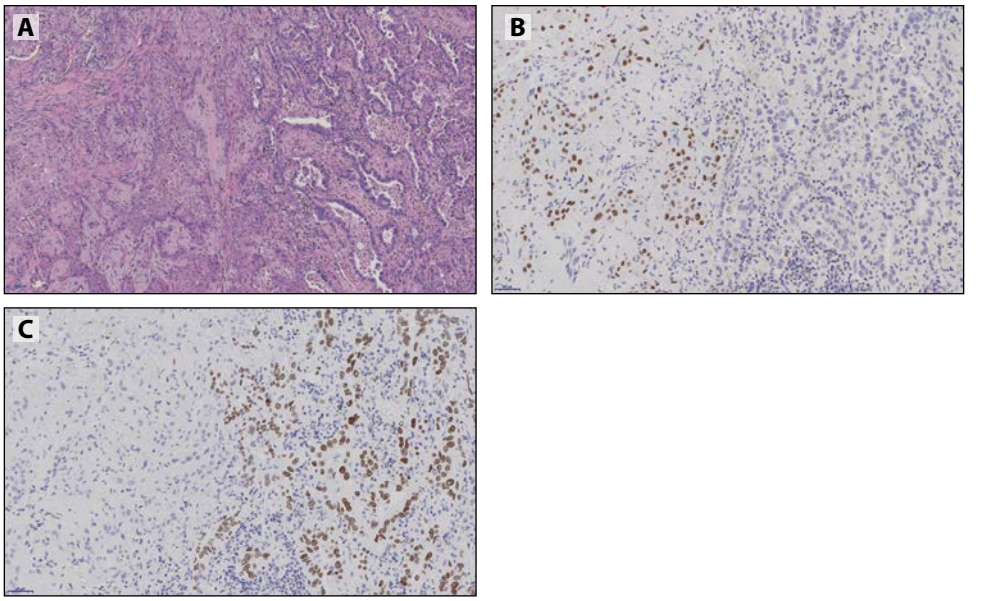


Figure 6-7. The 2 tumor components are clearly delimitated, with the squamous cell carcinoma component with p40 expression/thyroid transcription factor-1 (TTF1) negativity on the *left*, and the adenocarcinoma component with p40 negativity/TTF1 expression on the *right*. ×20; **(A)** H&E, **(B)** p40, and **(C)** TTF1.

1 TTF1 and 1 p40 positive (Figure 6-7). When co-expression of TTF1 and p40 occurs in the same tumor cells, such cases should be classified as adenocarcinomas.

Summary Answer

Immunohistochemistry for TTF1 and p40 can be helpful in the diagnosis of adenosquamous carcinoma when they highlight 2 distinct tumor cell populations.

What Is the Utility of Immunohistochemistry in Sarcomatoid Carcinoma?

The diagnosis of pleomorphic carcinoma requires the identification of adenocarcinoma, squamous cell carcinoma, or large cell carcinoma in conjunction with either spindle or giant cell carcinoma (Figure 6-8A and C). In this setting, the use of TTF1 and p40 may help characterize the carcinomatous component. The spindle cell or giant cell carcinoma component is often positive for only cytokeratin, and this may be of utility in the classification of pure spindle or giant cell carcinoma (Figure 6-8B and D). Zinc-finger E-box binding protein 1 (ZEB1) nuclear immunoreactivity has been reported in the sarcomatoid component of these tumors (Matsubara et al 2014; Viswanathan et al 2019), but its precise role in diagnostics remains uncertain (Figure 6-8E). A broad spectrum of cytokeratins rather than a single cytokeratin may be needed in some cases to confirm the epithelial origin of the tumor.

The diagnosis of blastoma requires a combination of fetal-type adenocarcinoma with a sarcomatoid, usually heterologous component of malignant cartilage or skeletal muscle. Because this is a β-catenin mutated tumor, it shows β-catenin nuclear immunoreactivity, as is seen in fetal-type adenocarcinoma (see Figure 13-5).

The diagnosis of carcinosarcoma includes a combination of carcinoma and a heterologous sarcomatoid component. The use of immunohistochemistry in this setting may be

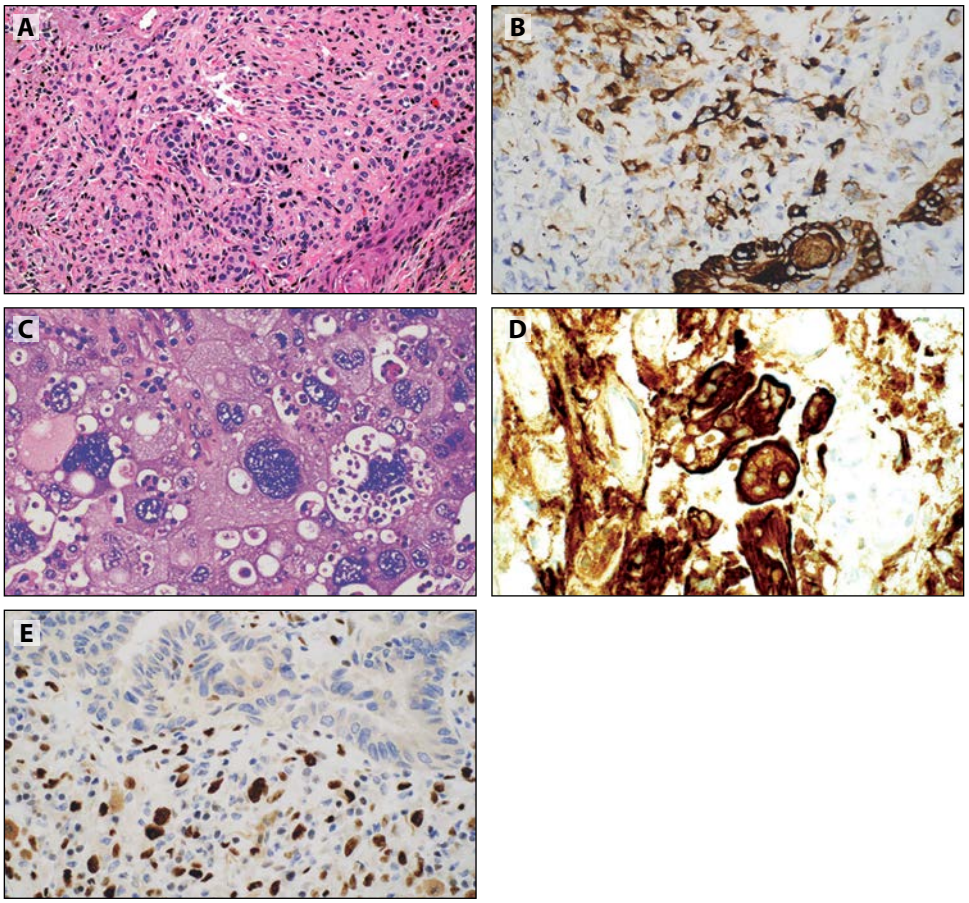


Figure 6-8. Immunohistochemistry in sarcomatoid carcinoma. **(A)** The pleomorphic carcinoma shows cytokeratin reactivity in the squamous component as well as **(B)** the spindle cell component. **(C)** A giant cell carcinoma is confirmed as a carcinoma using **(D)** cytokeratin immunoreactivity. Although its diagnostic use remains uncertain, ZEB1 immunohistochemistry shows nuclear staining in the sarcomatoid component of a pleomorphic carcinoma. **(A, B, E)** ×20; **(C and D)** ×40; **(A and C)** H&E, **(B and D)** cytokeratin AE1/AE3, and **(E)** ZEB1.

restricted to characterization or confirmation of a heterologous element, such as myogenin or MYOD1 to confirm a rhabdomyosarcomatous component.

Summary Answer

Immunoreactivity for cytokeratin can be helpful in supporting a malignant spindle or giant cell carcinoma pattern in pleomorphic carcinoma. Immunohistochemistry in rare subtypes can confirm heterologous elements, or in a blastoma, a fetal adenocarcinoma component.

What Is the Role of Immunohistochemistry in the Diagnosis of Large Cell Carcinoma?

As defined in the current 2015 WHO classification, large cell carcinoma is an undifferentiated non-small cell cancer without morphologic (Figure 6-9A) or immunohistochemical features, allowing further subtyping. Therefore, large cell carcinoma is a diagnosis of exclusion

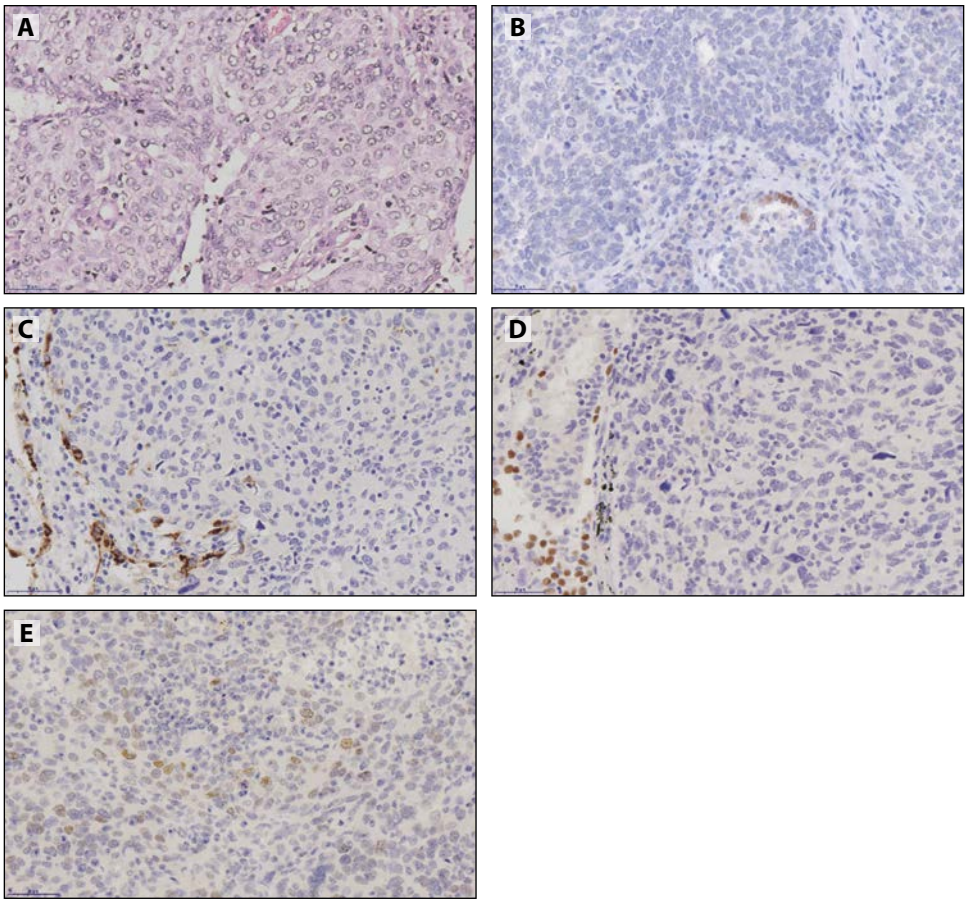


Figure 6-9. Large cell carcinoma. **(A)** Morphologically undifferentiated non-small cell carcinoma **(B)** negative for thyroid transcription factor-1 (TTF1), **(C)** napsin A, **(D)** p40, and **(E)** p63. ×40; **(A)** H&E, **(B)** TTF1, **(C)** napsin A, **(D)** p40, and **(E)** p63.

and thus may only be considered on resection specimens. In addition to negative immunostains, mucin stains need to be negative to further exclude a solid adenocarcinoma.

TTF1 and p40 must be negative in large cell carcinomas. TTF1 may highlight only pneumocytes lining preexistent alveolar walls (Figure 6-9B). Napsin A should also be negative (Figure 6-9C), and p40 highlights only the bronchiolar basal cell layer if present, serving as internal positive control (Figure 6-9D). Faint and focal p63 positivity may be seen in large cell carcinomas or adenocarcinomas and is nonspecific (Figure 6-9E). In particular, it is not sufficient for typing the tumor as squamous cell carcinoma.

This diagnosis requires a resection specimen so the selection of the block for performing immunohistochemistry should be made after reviewing all of the tumor slides to identify the best block that might demonstrate either adenocarcinoma or squamous cell carcinoma by review of the hematoxylin and eosin (H&E) stains.

If an initial work-up of TTF1 and p40 is negative in a solid tumor, additional stains should be performed to confirm the tumor is a carcinoma (cytokeratin stains) rather than a melanoma, lymphoma, or sarcoma, and consideration should be given to a work-up to exclude metastases depending on clinical context and morphologic features (see Chapter 16).

Additionally, not otherwise specified (NOS)/large cell carcinomas could be considered for staining with SMARCA4, depending on the histologic features. Pulmonary adenocarcinomas that were *SMARCA4*-deficient were shown to be TTF1 negative in 80% of the cases (Agaimy et al 2017; Herpel et al 2017). This is, however, not to be confused with *SMARCA4*-deficient thoracic tumor (see Chapter 15).

Summary Answer

In mucicarmine negative undifferentiated carcinomas without TTF1, napsin A, or p40 staining, a diagnosis of large cell carcinoma can be rendered, after consideration of *SMARCA4*-deficient carcinoma.

Conclusions

In daily practice, TTF1 and p40 suffice for subtyping most non-small cell carcinomas without defining morphologic characteristics or neuroendocrine morphology. This panel may be expanded when metastases or rarer variants are suspected.

References

Agaimy A, Fuchs F, Moskalev EA, et al. SMARCA4-deficient pulmonary adenocarcinoma: clinicopathological, immunohistochemical, and molecular characteristics of a novel aggressive neoplasm with consistent TTF1^{neg}/CK7^{pos}/HepPar-1^{pos} immunophenotype. *Virchows Arch*. 2017;471(5):599-609.

Bishop JA, Teruya-Feldstein J, Westra WH, et al. p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. *Mod Pathol*. 2012;25(3):405-415.

Gurda GT, Zhang L, Wang Y, et al. Utility of five commonly used immunohistochemical markers TTF1, napsin A, CK7, CK5/6 and P63 in primary and metastatic adenocarcinoma and squamous cell carcinoma of the lung: a retrospective study of 246 fine needle aspiration cases. *Clin Transl Med*. 2015;4:16.

Haack H, Johnson LA, Fry CJ, et al. Diagnosis of NUT midline carcinoma using a NUT-specific monoclonal antibody. *Am J Surg Pathol*. 2009;33(7):984-991.

Herpel E, Rieker RJ, Dienemann H, et al. SMARCA4 and SMARCA2 deficiency in non-small cell lung cancer: immunohistochemical survey of 316 consecutive specimens. *Ann Diagn Pathol*. 2017;26:47-51.

Johansson L. Histopathologic classification of lung cancer: relevance of cytokeratin and TTF-1 immunophenotyping. *Ann Diagn Pathol*. 2004;8(5):259-267.

Kadota K, Nitadori J, Rekhtman N, et al. Reevaluation and reclassification of resected lung carcinomas originally diagnosed as squamous cell carcinoma using immunohistochemical analysis. *Am J Surg Pathol*. 2015;39(9):1170-1180.

Kashima K, Hashimoto H, Nishida H, et al. Significant expression of thyroid transcription factor-1 in pulmonary squamous cell carcinoma detected by SPT24 monoclonal antibody and CSA-II system. *Appl Immunohistochem Mol Morphol*. 2014;22(2):119-124.

Koh J, Go H, Kim MY, et al. A comprehensive immunohistochemistry algorithm for the histological subtyping of small biopsies obtained from non-small cell lung cancers. *Histopathology*. 2014;65(6):868-878.

Loo PS, Thomas SC, Nicholson MC, et al. Subtyping of undifferentiated non-small cell carcinomas in bronchia biopsy specimens. *J Thorac Oncol*. 2010;5(4):442-447.

Matsubara D, Kishaba Y, Yoshimoto T, et al. Immunohistochemical analysis of the expression of E-cadherin and ZEB1 in non-small cell lung cancer. *Pathol Int*. 2014;64(11):560-568.

Mukhopadhyay S, Katzenstein AL. Comparison of monoclonal napsin A, polyclonal napsin A, and TTF-1 for determining lung origin in metastatic adenocarcinomas. *Am J Clin Pathol*. 2012;138(5):703-711.

Nicholson AG, Gonzalez D, Shah P, et al. Refining the diagnosis and EGFR status of non-small cell lung carcinoma in biopsy and cytologic material, using a panel of mucin staining, TTF-1, cytokeratin 5/6, and P63, and EGFR mutation analysis. *J Thorac Oncol*. 2010;5(4):436-441.

Noh S, Shim H. Optimal combination of immunohistochemical markers for subclassification of non-small cell lung carcinomas: a tissue microarray study of poorly differentiated areas. *Lung Cancer*. 2012;76(1):51-55.

Ordonez NG. Value of thyroid transcription factor-1 immunostaining in tumor diagnosis: a review and update. *Appl Immunohistochem Mol Morphol*. 2012;20(5):429-444.

Rekhtman N, Ang DC, Sima CS, et al. Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. *Mod Pathol*. 2011;24(10):1348-1359.

Righi L, Graziano P, Fornari A, et al. Immunohistochemical subtyping of nonsmall cell lung cancer not otherwise specified in fine-needle aspiration cytology: a retrospective study of 103 cases with surgical correlation. *Cancer*. 2011;117(15):3416-3423.

Travis WD, Brambilla E, Burke AP, et al, eds. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015.

Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol*. 2011;6(2):244-285.

Viswanathan K, Siddiqui MT, Borczuk AC. The diagnostic utility of zinc E-box-1 (ZEB1) transcription factor for identification of pulmonary sarcomatoid carcinoma in cytologic and surgical specimens. *J Am Soc Cytopathol*. 2019;9(1):55-61.

Warth A, Muley T, Herpel E, et al. Large-scale comparative analyses of immunomarkers for diagnostic subtyping of non-small-cell lung cancer biopsies. *Histopathology*. 2012;61(6):1017-1025.

Whithaus K, Fukuoka J, Prihoda TJ, et al. Evaluation of napsin A, cytokeratin 5/6, p63, and thyroid transcription factor 1 in adenocarcinoma versus squamous cell carcinoma of the lung. *Arch Pathol Lab Med*. 2012;136(2):155-162.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practice recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Thyroid Transcription Factor-1

By Prudence A. Russell, Jin-Haeng Chung, and Yasushi Yatabe

7

Introduction

Thyroid transcription factor-1 (TTF1) is a 38 kDa nuclear protein belonging to the *NKX2-1* gene family of homeodomain transcription factors and is encoded by the *NKX2-1* gene on chromosome 14q13 (Ordonez 2012b). It is necessary for the development of the lung and thyroid gland. In the normal lung, TTF1 stimulates the production of club (formerly known as Clara) cell secretory protein and lung-specific surfactant proteins and is expressed in the nuclei of type II pneumocytes and club cells, providing a useful positive internal control (Ordonez 2012b). TTF1 is one of the most useful and widely utilized antibodies in clinical practice. It is usually employed in 2 main clinicopathologic settings, which are the distinction of primary lung adenocarcinoma from carcinomas of other primary sites and the differential diagnosis of lung adenocarcinoma from squamous cell carcinoma in morphologically indeterminate non-small cell carcinoma (NSCC). These 2 settings require specificity of TTF1 staining, which involves a trade-off with sensitivity. Furthermore, determination of lung versus non-lung site of origin and distinction of lung adenocarcinoma from squamous cell carcinoma are of the utmost importance to guide decision-making regarding the management of patients with advanced NSCC in the era of precision medicine. This latter question is examined more broadly in Chapter 5. At the risk of stating the obvious, it is worthwhile for pathologists to remember that TTF1 is widely expressed in thyroid epithelial tumors. TTF1 is also found in up to 90% of small cell lung carcinomas, two-thirds of large cell neuroendocrine carcinomas, and in spindle cell carcinoid tumors (see Chapter 6) (Travis et al 2015a, 2015b).

In this chapter, the critical role of TTF1 in distinguishing lung adenocarcinomas from extrapulmonary adenocarcinoma, particularly when small samples are being examined, and from squamous cell carcinoma, is explored, focusing on staining differences between the most widely used TTF1 clones and the extent of positive reactions required for TTF1 positivity. Pre-analytic issues about TTF1 immunostaining are also examined.

Are There Any Staining Differences in Lung Adenocarcinoma Between TTF1 Clones?

There are a number of different TTF1 clones commercially available, including rabbit and goat polyclonal antibodies; mouse monoclonal antibodies, including 8G7G3/1, SPT24, BGX-397A, SMP150, and 5S143 clones; and rabbit monoclonal antibodies, including SP141, EP15844, C12-I, and G21-G clones (Ordonez 2012b). 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 (Ordonez 2012a, 2012b; Smits et al 2015; Klebe et al 2016; Tran et al 2016) and are focused on here.

Looking at reported sensitivity and specificity of the different clones to detect lung adenocarcinoma first, a recent review found that across 37 published studies, 76.7% of lung adenocarcinoma cases were positive with the 8G7G3/1 clone while 81.3% of lung adenocarcinoma cases across 7 studies were positive with the SPT24 clone (Ordonez 2012b). The only study comparing all 3 widely used TTF1 clones found 89% of lung adenocarcinomas stained positively with 8G7G3/1, 93% stained with SPT24, and 93% stained with SP141, with a cutoff of 1% staining used to indicate a positive reaction (Vidarsdottir et al 2018). These results underscore that the 8G7G3/1 clone is less sensitive for the detection of lung adenocarcinoma than both the SPT24 and SP141 clones (Figure 7-1).

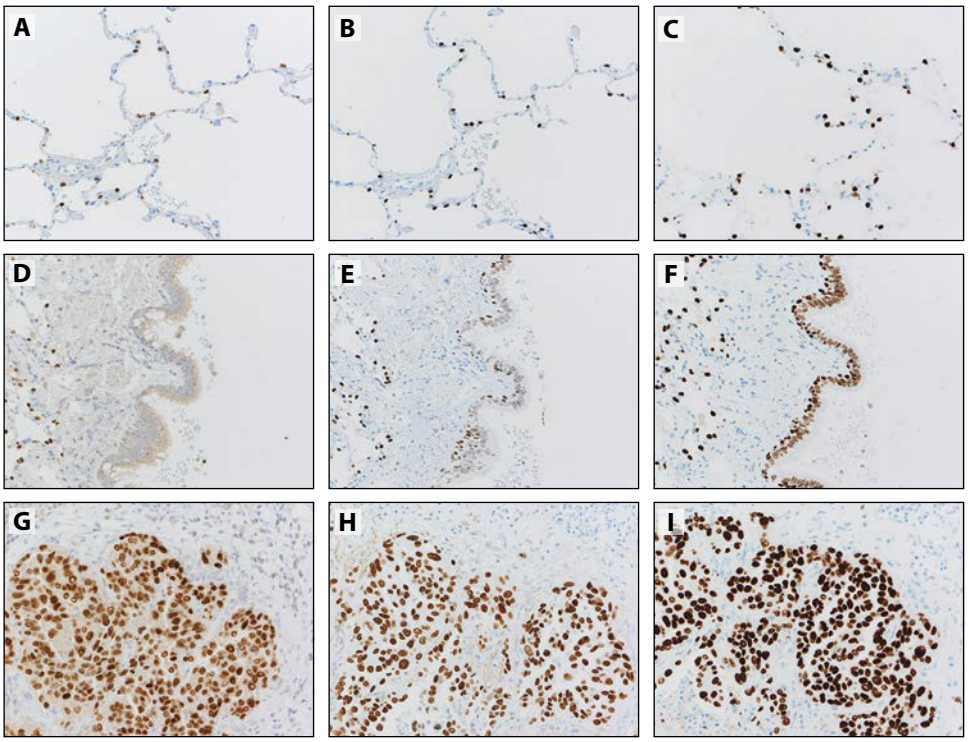


Figure 7-1. Thyroid transcription factor-1 (TTF1) staining in the nonneoplastic lung tissues ([**A-C**] peripheral parenchyma and [**D-F**] bronchiolar epithelium) and (**G-I**) solid adenocarcinoma, using 8G7G3/1 as shown in panels **A, D,** and **G**; SPT24 as show in panels **B, E,** and **H**; and SP141 as shown in panels **C, F,** and **I**. Intensity was different among the clones because the detection system and amplification procedure were different (Dako Flex system for 8G7G3/1; OptiView DAB IHC Detection Kit for SPT24 and SP141). However, positive distribution was identical in peripheral parenchyma and adenocarcinoma, in contrast to different staining extent in the bronchiolar epithelium.

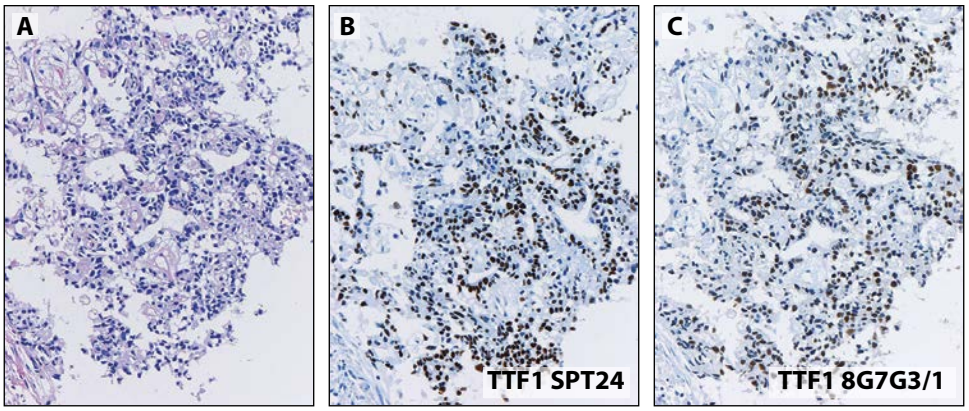


Figure 7-2. Thyroid transcription factor-1 (TTF1) expression of ovarian cancer metastasizing to the lung. **(A)** The tumor cells (H&E) show positive reaction with both **(B)** SPT24 and (C) 8G7G3/1 clones. TTF1 expression in gynecologic tumors can be a pitfall in differential diagnosis between the metastasis and primary lung adenocarcinoma.

Table 7-1. Results of TTF1 Expression in Tumors from Non-Lung Primary Sites Including Female Genital Tract, Breast, Colon, and Stomach in Some Published Studies[a]

	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%)
Uterine cervical squamous carcinoma	7	0 (0%)		
Breast carcinoma	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%)

[a] Data for clones 8G7G3/1 and SPT24 are modified based on data from Ordonez 2012b.

Considering the distinction of lung adenocarcinoma from adenocarcinoma from other primary sites, a recent review comparing the SPT24 and 8G7G3/1 clones found that a small percentage of adenocarcinomas from primary sites other than the lung can be positive for TTF1 (Ordonez 2012b). These primary sites include the female genital tract (Figure 7-2), breast, colon, and stomach, with higher staining percentages reported for the SPT24 clone than for the 8G7G3/1 clone (Table 7-1). Of note, the SPT24 clone is postulated to have stronger binding for the TTF1 protein, resulting in more even nuclear positivity and less unpredictable cytoplasmic staining than the 8G7G3/1 clone, which can show nonspecific staining of the cytoplasm of both hepatocytes and hepatocellular carcinoma (Bae et al 2018; Pan et al 2004; Smits et al 2015). The postulated stronger binding of SPT24 is thought to explain its increased staining in carcinomas arising in non-lung sites, when compared with the 8G7G3/1 clone (Smits et al 2015; Compérat et al 2005). Also, some nonneoplastic basal bronchial epithelial cells can be stained with SPT24; thus the positive staining of tumor cells should be differentiated from any found in nonneoplastic cells when this clone is used, particularly when the tissue is heavily fragmented (Figure 7-3) (see also the further discussion in Chapter 5). There is less literature examining the staining performance of the newer SP141 clone. However, the available studies (Vidarsdottir et al 2018; Klebe et al 2016; Bae et al 2018;

Prabhakaran et al 2019) all appear to indicate that the SP141 clone has similar characteristics to the SPT24 clone, with less specificity for the detection of lung adenocarcinoma compared to the 8G7G3/1 clone (Table 7-2).

In the differential diagnosis of lung adenocarcinoma from squamous cell carcinoma, several recent studies found positive expression of TTF1 in lung squamous cell carcinoma in low percentages overall, but with higher percentages reported with the SPT24 clone and much lower percentages or no staining reported with the 8G7G3/1 clone (Table 7-2; Figure 7-4) (Vidarsdottir et al 2018; Ordonez 2012a; Matoso et al 2010; Kadota et al 2015; Kashima et al 2014). Similar findings have been reported using the SP141 clone (Vidarsdottir et al 2018; Klebe et al 2016), with positivity seen in a small number of squamous cell carcinomas, which were all negative with the 8G7G3/1 clone (Table 7-3). Given the important role that TTF1 immunohistochemistry (IHC) now has in defining characteristics of poorly differentiated

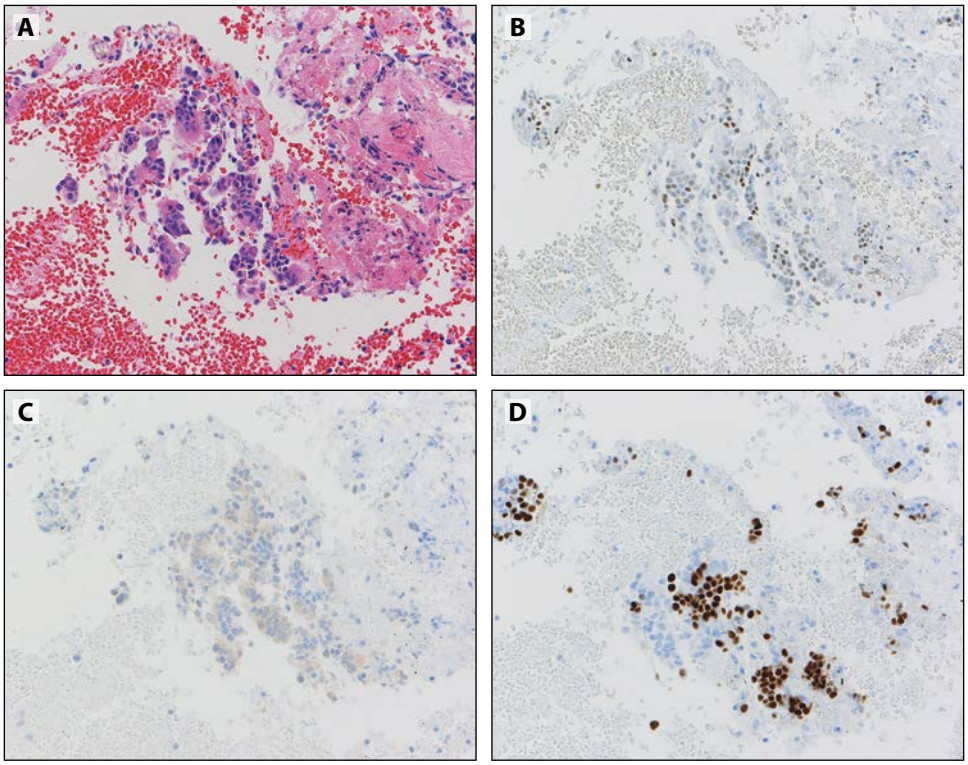


Figure 7-3. (A) Fragments of nonneoplastic bronchial epithelium (H&E), which are positive for **(B)** clone SPT24, but negative for **(C)** clone 8G7G3/1. Positive cells with **(D)** p40 staining overlap with SPT24 positive cells, suggesting bronchial basal cells react with clone SPT24.

Table 7-2. Results of TTF1 Expression from Non-Lung Primary Sites Including Colon and Prostate in 3 Published Studies with All 3 Widely Used TTF1 Clones

		8G7G3/1		SPT24		SP141	
		n	Positive, n (%)	n	Positive, n (%)	n	Positive, n (%)
Vidarsdottir et al 2018	Colon carcinoma	166	3 (2%)	166	7 (4%)	166	7 (4%)
Bae et al 2018	Colon carcinoma	1319	0 (0%)	1319	68 (5%)	1319	68 (5%)
Prabhakaran et al 2019	Colon carcinoma	104	2 (2%)	104	6 (5.7%)	104	6 (5.7%)
Prabhakaran et al 2019	Prostate carcinoma	112	6 (5.3%)	112	31 (28%)	112	26 (23%)

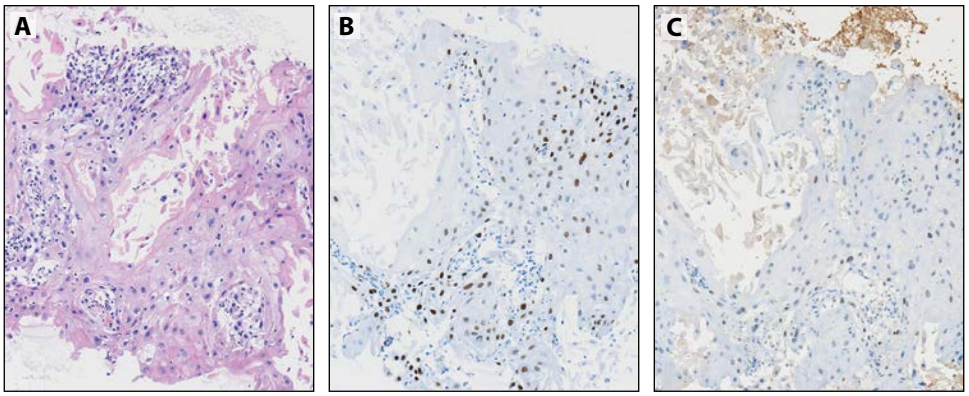


Figure 7-4. (A) Thyroid transcription factor-1 (TTF1) expression according to antibody clones in primary squamous cell carcinoma of the lung. **(A)** H&E staining showed well-differentiated squamous cell carcinoma. **(B)** Positive reactions with clone SPT24 staining is contrasted with **(C)** weak or negative with clone 8G7G3/1.

Table 7-3. TTF1 Expression in Lung Squamous Cell Carcinoma with All 3 Widely Used TTF1 Clones

	8G7G3/1		SPT24		SP141	
Squamous cell carcinoma	**n**	**Positive, n (%)**	**n**	**Positive, n (%)**	**n**	**Positive, n (%)**
Vidarsdottir et al 2018	201	0 (0%)	201	12 (6%)	201	16 (8%)
Klebe et al 2016	12	0 (0%)			12	5 (42%)
Ordonez 2012a	85	0 (0%)				
Matoso et al 2010	97	1 (1%)	97	14 (16.8%)		
Kadota et al 2015	449	0 (0%)	448	27 (6%)		
Kashima et al 2014	38 38	1 (3%) with EnVision[a] 4 (11%) with CSA-II[c]	38 38	5 (13%) with EnVision[b] 20 (53%) with CSA-II[b]		

[a] EnVision is manufactured by Dako.

[b] The study directly compared 8G7G3/1 clone with SPT24 clone in an identical series of cases.

[c] CSA-II is manufactured by Roche Tissue Diagnostics.

adenocarcinoma in the 2015 World Health Organization (WHO) classification (Travis et al 2015a), these differences in antibody clone performance become even more important (see Chapter 6). Parenthetically, a study investigating TTF1 expression in lung squamous cell carcinoma found increased SPT24 positivity in lung squamous cell carcinomas using a signal amplification system (the catalyzed signal amplification [CSA]-II detection system), which the authors suggest has the potential to enable distinction of primary squamous cell carcinomas of the lung from metastases to the lung from primary sites such as the head and neck (Kashima et al 2014). More data are required before this approach can be used in clinical practice.

Overall, the available literature appears to indicate that the 8G7G3/1 clone has increased specificity for the detection of lung adenocarcinoma when compared with the SP141 and SPT24 clones in both common clinicopathologic scenarios in which TTF1 is used, including the distinction of primary lung adenocarcinoma from adenocarcinomas from non-pulmonary sites and the differential diagnosis of lung adenocarcinoma from squamous cell carcinoma.

Summary Answer

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

What Extent of TTF1 Staining Is Considered a Positive Test Result?

In the 2 common clinicopathologic settings just mentioned, it is acknowledged that focal positivity with TTF1 is considered a positive reaction. However, this raises the question of the definition of focal staining (ie, 1% vs. 10% vs. 50%, etc) and whether the different clones have any influence on this. From the available literature, it appears that the amount of staining used to indicate a positive reaction can be influenced by the TTF1 clone used.

Using the 8G7G3/1 and SPT24 clones, Smits and coworkers identified that when the same cutoff value for positivity was used in both clones, there was a significant difference between the clones at all cutoff values, resulting in lower sensitivity of 8G7G3/1 at high cutoff values and lower specificity of SPT24 at low cutoff values (Smits et al 2015). After determining the optimal cutoff value for each clone of greater than 5% staining for 8G7G3/1 and greater than 50% staining for SPT24, there was no significant difference in sensitivity (0.79 for 8G7G3/1 vs. 0.82 for SPT24) or specificity (0.98 for 8G7G3/1 vs. 0.98 for SPT24) (Smits et al 2015) (Figure 7-5).

In a more recent study (Vidarsdottir et al 2018) investigating all 3 widely used clones for the distinction of nonsquamous lung cancer from lung squamous cell carcinoma and lung metastasis, receiver operating characteristic (ROC) curve analysis identified that the best cutoff for both 8G7G3/1 and SPT24 clones is 1% staining (area under curve [AUC]) of 0.92 for 8G7G3/1 vs. AUC of 0.94 for SPT24), while the best cutoff for SP141 clone is 10% staining (AUC of 0.93). Higher cutoffs of 10% staining for SPT24 and 50% staining for SP141 were necessary when the ROC analysis was performed to separate lung adenocarcinoma from other lung carcinomas and lung metastasis (AUC of 0.93 for both SPT24 and SP141 clones), whereas 1% staining remained the best cutoff for the 8G7G3/1 clone (AUC of 0.93).

Therefore, according to these recent studies, the amount of focal staining indicative of a positive reaction with TTF1 seems to vary among the 3 most widely used clones (Figure 7-1). Further investigations to verify these findings are necessary, but it appears that the optimal cutoff indicative of a positive reaction for the more specific 8G7G3/1 clone is a lot less than the optimal cutoffs for the more sensitive clones, SPT24 and SP141. It is recommended that pathologists consider these reported differences in optimal cutoffs when choosing, using, and interpreting a particular TTF1 clone in the laboratory to ensure equal reliability for the detection of lung adenocarcinoma in the proper clinical context. In practical terms, when using the 8G7G3/1 clone, any positive reactions, at any level of intensity, may be considered "positive," particularly when used in small samples.

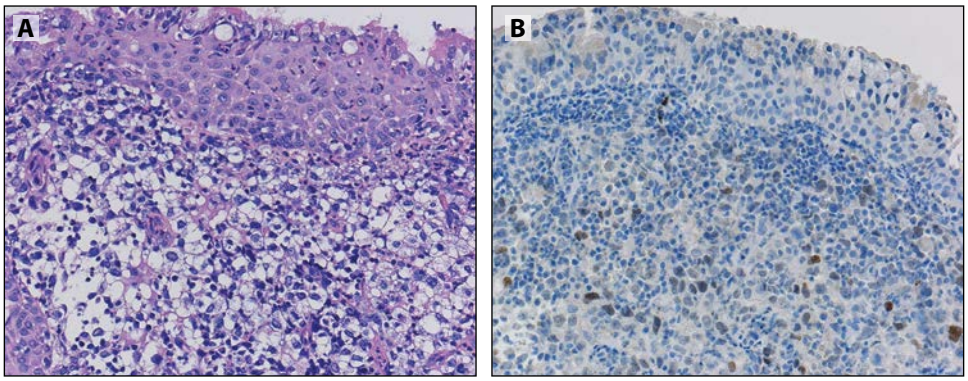


Figure 7-5. (A) Thyroid transcription factor-1 (TTF1) staining in poorly differentiated carcinoma (H&E). **(B)** Even with this positive extent, with clone 8G7G3/1, this specimen should be considered as positive for TTF1.

Summary Answer

Focal positivity for TTF1 is considered a positive reaction indicating pulmonary adenocarcinoma in the proper clinical context; however, the optimal cutoff values vary among the clones.

Are There Any Pre-Analytic Considerations for TTF1 Immunostaining?

There are several pre-analytic considerations to make about TTF1 immunostaining, some of which deserve special mention. A recent study found reduced or absent TTF1 staining in cytology specimens fixed in alcohol-based fixatives, including CytoLyt, and in surgical pathology specimens subjected to decalcifying agents, such as formic or hydrochloric acid (Gruchy et al 2015). This reduction in TTF1 staining was not seen in specimens that were fixed only with routine 10% buffered formalin. It is important to recognize that IHC protocols must be validated on control tissues that undergo the same pre-analytic conditions as the test tissue, including fixation in alcohol-based fixatives and decalcification treatments, even when using gentler ethylenediaminetetraacetic acid (EDTA)-based solutions. IHC protocols developed for tissues fixed in 10% buffered formalin may give suboptimal results when used on alcohol-fixed tissue.

Anecdotally, a staining gradient of TTF1 expression may be observed in surgically resected lung adenocarcinoma specimens, implying that poorer fixation may be related to poorer detection of epitope for a given staining protocol.

Summary Answer

There are some specific pre-analytic considerations regarding TTF1 immunostaining, mostly in relation to reduced or absent staining in specimens fixed in alcohol-based fixatives and subjected to certain decalcifying agents.

Conclusions

The staining performance of TTF1 is different among the antibody clones, so pathologists should be aware of the characteristics, and an informed decision should be made according to which clone is used in the laboratory. This is of critical importance given the pivotal role of this marker in several aspects of lung carcinoma classification, which has major therapeutic implications.

References

Bae JM, Kim JH, Park JH, et al. Clinicopathologic and molecular implications of aberrant thyroid transcription factor-1 expression in colorectal carcinomas: an immunohistochemical analysis of 1319 cases using three different antibody clones. *Histopathology*. 2018;72(3):423-432.

Compérat E, Zhang F, Perrotin C, et al. Variable sensitivity and specificity of TTF-1 antibodies in lung metastatic adenocarcinoma of colorectal origin. *Mod Pathol*. 2005;18(10):1371-1376.

Gruchy JR, Barnes PJ, Dakin Hache KA. CytoLyt fixation and decalcification pretreatments alter antigenicity in normal tissues compared with standard formalin fixation. *Appl Immunohistochem Mol Morphol*. 2015;23(4):297-302.

Kadota K, Nitadori J, Rekhtman N, et al. Reevaluation and reclassification of resected lung carcinomas originally diagnosed as squamous cell carcinoma using immunohistochemical analysis. *Am J Surg Pathol*. 2015;39(9):1170-1180.

Kashima K, Hashimoto H, Nishida H, et al. Significant expression of thyroid transcription factor-1 in pulmonary squamous cell carcinoma detected by SPT24 monoclonal antibody and CSA-II system. *Appl Immunohistochem Mol Morphol*. 2014;22(2):119-124.

Klebe S, Swalling A, Jonavicius L, et al. An immunohistochemical comparison of two TTF-1 monoclonal antibodies in atypical squamous lesions and sarcomatoid carcinoma of the lung, and pleural malignant mesothelioma. *J Clin Pathol*. 2016;69(2):136-141.

Matoso A, Singh K, Jacob R, et al. Comparison of thyroid transcription factor-1 expression by 2 monoclonal antibodies in pulmonary and nonpulmonary primary tumors. *Appl Immunohistochem Mol Morphol*. 2010;18(2):142-149.

Ordonez NG. Thyroid transcription factor-1 is not expressed in squamous cell carcinomas of the lung: an immunohistochemical study with review of the literature. *Appl Immunohistochem Mol Morphol*. 2012;20(6):525-530 (a)

Ordonez NG. Value of thyroid transcription factor-1 immunostaining in tumor diagnosis: a review and update. *Appl Immunohistochem Mol Morphol*. 2012;20(5):429-444. (b)

Pan CC, Chen PC, Tsay SH, et al. Cytoplasmic immunoreactivity for thyroid transcription factor-1 in hepatocellular carcinoma: a comparative immunohistochemical analysis of four commercial antibodies using a tissue array technique. *Am J Clin Path*. 2004;121(3):343-349.

Prabhakaran S, Woo WLW, Xing G, et al. The incidence of labelling of non-lung adenocarcinomas with antibodies against TTF-1 and diagnostic implications. *Appl Immunohistochem Mol Morphol*. Published online May 23, 2019. doi:10.1097/PAI.0000000000000775

Smits AJ, Vink A, Tolenaars G, et al. Different cutoff values for thyroid transcription factor-1 antibodies in the diagnosis of lung adenocarcinoma. *Appl Immunohistochem Mol Morphol*. 2015;23(6):416-421.

Tran L, Mattson JS, Nodin B, et al. Various antibody clones of napsin A, thyroid transcription factor 1, and p40 and comparisons with cytokeratin 5 and p63 in histopathologic diagnostics of non-small cell lung carcinoma. *Appl Immunohistochem Mol Morphol*. 2016;24(9):648-659.

Travis WD, Brambilla E, Burke AP, et al, eds. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015. (a)

Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors: impact of genetic, clinical and radiologic advances since the 2004 WHO classification. *J Thorac Oncol*. 2015;10(9);1243-1260. (b)

Vidarsdottir H, Tran L, Nodin B, et al. Comparison of three different TTF-1 clones in resected primary lung cancer and epithelial pulmonary metastases. *Am J Clin Pathol*. 2018;150(6):533-544.

Immunohistochemistry for p40 and p63 in Lung Cancer

8

By Teh-Ying Chou and Wendy A. Cooper

Introduction

The *p63* gene is a homologue of the *p53* tumor suppressor gene, which is required for the proliferation or differentiation of progenitor cell populations necessary for epithelial development (Nobre et al 2013). The *p63* gene is located on chromosome 3q27-29, contains 15 exons, and exhibits a remarkable sequence and structural homology with *p53*. Like *p53*, the *p63* gene encodes an N-terminal transactivation domain, a core DNA binding domain, and a carboxy-oligomerization domain. p63 is normally expressed in the nuclei of basal and progenitor cells of the stratified epithelia of the skin, esophagus, tonsil, urothelium, exocervix, and vagina, and in the basal cells of the glandular structures of the thymus, prostate, breast, and bronchi (Di Como et al 2002).

p63 isoforms consist of several variants, which fall into 2 major groups (TAp63 and ΔNp63), which differ in structure at the N-terminal domain (Nylander et al 2002). TAp63 isoform contains a transactivation-competent TA domain with homology to p53, which regulates expression of the growth-inhibition genes. The ΔNp63 isoform, however, contains an alternative transcriptionally inactive ΔN domain, which is thought to antagonize the activity of TAp63 and p53, therefore acting as an oncoprotein. The p63 antibody recognizes both TAp63 and ΔNp63 isoforms and is therefore a *pan*-63 marker. By contrast, the p40 antibody is directed against the ΔNp63 isoform and does not recognize the TAp63 isoform.

In What Cases Should p40 Be Used Rather Than p63?

The most widely used p63 antibody clone in pathology laboratories is 4A4, which shows an excellent sensitivity of 94% to 100% for squamous cell carcinomas. However, the main limitation of p63 immunohistochemistry is low specificity because of the unexpected expression in lung adenocarcinoma (16%-65%) and other malignancies, such as large cell lymphomas (up to one-half) (Bishop et al 2012), and rarely in some soft tissue tumors (Jo and Fletcher 2011). Although the expression in non-squamous tumors is usually weak to moderate in a minority of cells, strong and diffuse expression can be observed in rare cases.

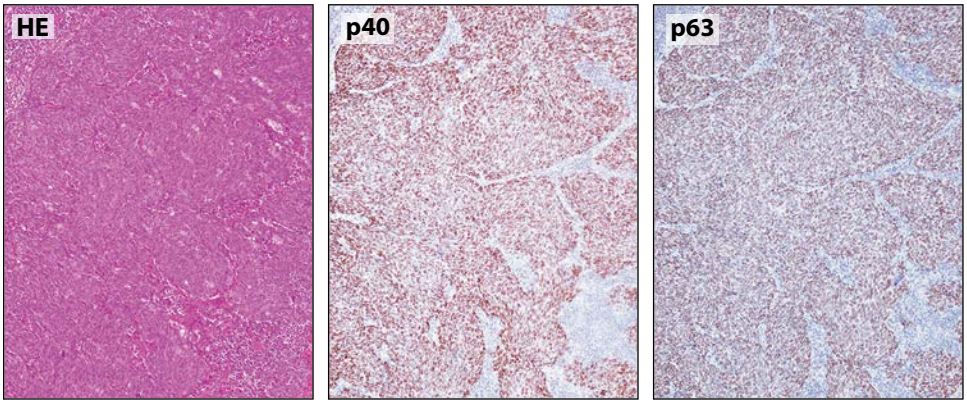


Figure 8-1. Poorly differentiated squamous cell carcinoma showing strong diffuse expression of p40 and p63. HE = hematoxylin-eosin.

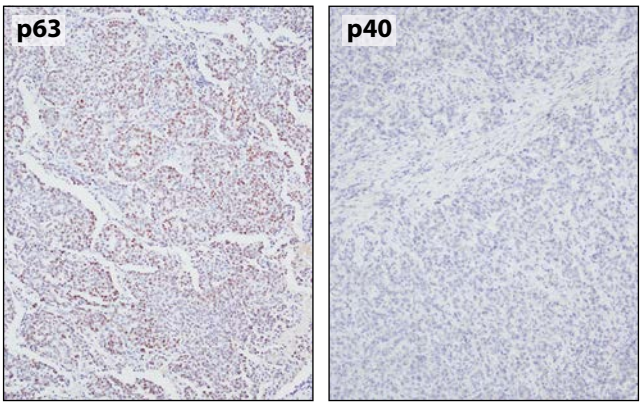


Figure 8-2. p63 is not specific for squamous cell carcinoma. Poorly differentiated lung adenocarcinoma showing fairly diffuse expression of p63 and no expression of p40.

Several studies have shown that p40 has comparable high sensitivity to p63 but higher specificity for squamous cell carcinomas and is a reliable marker for identification of squamous differentiation (Figures 8-1 and 8-2) (Kriegsmann et al 2019). Both polyclonal and monoclonal p40 antibodies (clone BC28) are used in laboratories, and one study showed comparable sensitivity and specificity with high concordance between the polyclonal and monoclonal antibodies (Tran et al 2016). p40 has greater accuracy than p63 for identification of squamous cell carcinomas. However, it should be remembered that if there is morphologic evidence of squamous differentiation in a tumor (keratinization or intercellular bridges), then immunohistochemistry (IHC) is not required for confirmation. In a morphologically undifferentiated non-small cell lung carcinoma in a small biopsy sample, both a squamous marker and an adenocarcinoma marker should be used in the attempt to subtype the tumor (eg, p40 and TTF1). If there is expression of both p63/p40 and TTF1 in the same tumor cells, the TTF1 expression trumps the p63/p40, and the tumor is favored to be an adenocarcinoma (Travis et al 2013). One study found that co-expression of TTF1 and p63 is frequently detected in anaplastic lymphoma kinase (ALK)-positive adenocarcinomas, but no TTF1 and p40 co-expression is observed (Sakai et al 2013).

Summary Answer

p40 should be used for identification of morphologically undifferentiated squamous cell carcinomas as it demonstrates superior accuracy to p63 in this setting.

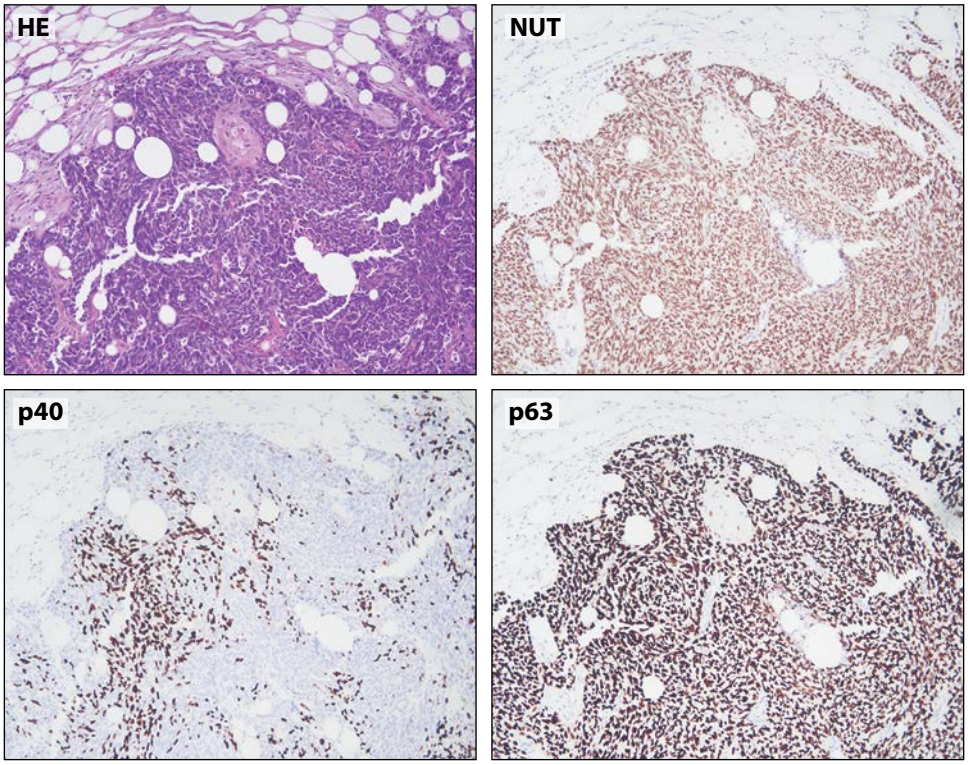


Figure 8-3. Nuclear protein in testis (NUT) carcinoma of lung showing focal p40 and diffuse p63 expression. HE = hematoxylin-eosin.

In What Cases Should p63 Be Used Instead of p40?

Discordant expression between p63 and p40 is seen in nuclear protein in testis (NUT) carcinoma in some instances, with diffuse expression of p63 and few cells expressing p40 (Figure 8-3). This may result in a diagnostic pitfall when using p40, especially in a biopsy setting. Therefore, in cases where NUT carcinoma is included as a differential diagnosis, p63 may be more useful than p40, in addition to assessment of morphology and other IHC markers (see Chapter 14 for more details on NUT carcinomas).

Summary Answer

If NUT carcinoma is considered in the differential diagnosis of a tumor, then p63 may be more useful than p40.

What Extent of p40/p63 Positive Reactions Should Be Considered Positive?

In squamous cell carcinomas, there is usually strong and diffuse positivity for p40 and p63 (Figure 8-1). The cutoff value for p40 and p63 should be positivity in more than 50% of tumor nuclei to be considered specific for squamous cell carcinomas. Positivity in less than 10% of nuclei should not be used for diagnostic classification alone as focal and weak positivity of p40 or p63 can be seen in adenocarcinomas and other types of tumors. A range of 10% to 50% positivity is a matter for consideration and depends on the clinical context

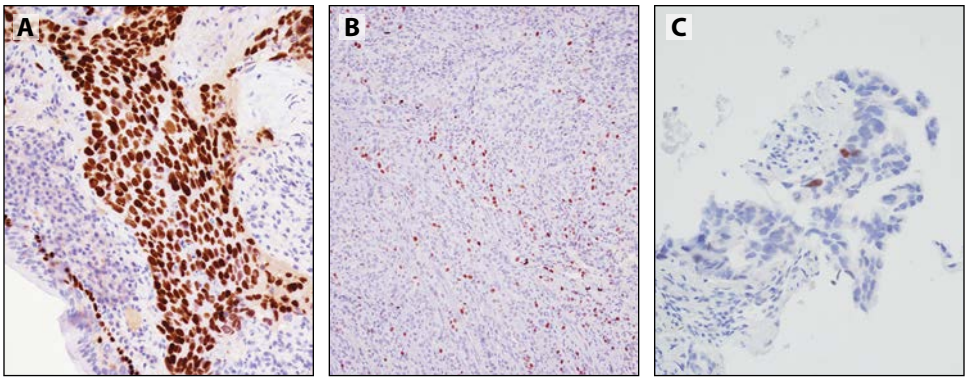


Figure 8-4. Different levels of p40 expression in lung carcinomas. **(A)** Squamous cell carcinoma with strong diffuse p40 staining. **(B)** Adenocarcinoma with patchy staining in approximately 10% of nuclei. **(C)** Adenocarcinoma with only very focal p40 staining (<5% of nuclei).

and intensity of staining along with morphologic features and other immunohistochemical findings (Figure 8-4). Other IHC markers of squamous differentiation such as cytokeratin 5/6 (CK5/6) can be a useful backup in problematic cases (Loo et al 2010) (see Chapter 9). Of note, the keratinizing component of a squamous cell carcinoma is often negative for p40, and therefore negative staining of this component does not exclude the diagnosis of squamous cell carcinoma. However, given that keratinization is a diagnostic criterion for squamous cell carcinoma, p40 immunostaining is not required in this setting.

Summary Answer

There is usually diffuse strong positivity for p40 (and p63) in squamous cell carcinomas, and expression in at least 50% of nuclei should be considered positive.

Conclusions

In daily practice, p40 is preferable to p63 to identify squamous cell carcinoma or squamous differentiation if morphologic features are insufficient (Table 8-1). The positivity cutoff should

Table 8-1. Studies Comparing p40 Against p63 for Squamous Cell Carcinoma (SQCC)

Study	Total, n	SQCC, n	p40			p63		
			Clone	Sensitivity, %	Specificity, %	Clone	Sensitivity, %	Specificity, %
Bishop et al 2012	470	81	5-17	100	98	4A4	100	60
Nonaka 2012	200	50	p40	100	100	4A4	100	82
Pelosi et al 2013	141	27	Poly	100	97	4A4	100	78
Ao et al 2014	154	77	Poly	81	90	4A4	94	80
Koh et al 2014	184	59	Poly	93	98	7JUL	80	98
Tatsumori et al 2014	580	158	5-17	97	97	4A4	97	73
Kadota et al 2015	469	449	5-17	100	85	4A4	100	60
Tran et al 2016	557	167	BC28	94	96	4A4	95	87
Micke et al 2016	656	192	BC28	97	98	4A4	97	74
Affandi et al 2018	70	35	BC28	77	100	DAK-p63	86	63
Kriegsmann et al 2019	1244	569	BC28	94	97	4A4	94	84

be the presence of staining in more than 50% of tumor nuclei, as focal or weak positivity is not diagnostic of squamous cell carcinoma. Additional squamous markers such as CK5/6 should be considered in problematic cases. Distinction of squamous cell carcinomas from other tumor types should also take into account morphologic features and other IHC markers (eg, TTF1 in an undifferentiated non-small cell carcinoma [NSCC]).

References

Affandi KA, Tizen NMS, Mustangin M, et al. p40 immunohistochemistry is an excellent marker in primary lung squamous cell carcinoma. *J Pathol Transl Med*. 2018;52(5):283-289.

Ao MH, Zhang H, Sakowski L, et al. The utility of a novel triple marker (combination of TTF1, napsin A, and p40) in the subclassification of non-small cell lung cancer. *Hum Pathol*. 2014;45(5):926-934.

Bishop JA, Teruya-Feldstein J, Westra WH, et al. p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. *Mod Pathol*. 2012;25(3):405-415.

Di Como CJ, Urist MJ, Babayan I, et al. p63 expression profiles in human normal and tumor tissues. *Clin Cancer Res*. 2002;8(2):494-501.

Jo VY, Fletcher CD. P63 immunohistochemical staining is limited in soft tissue tumors. *Am J Clin Pathol*. 2011;136(5):762-766.

Kadota K, Nitadori J, Rekhtman N, et al. Reevaluation and reclassification of resected lung carcinomas originally diagnosed as squamous cell carcinoma using immunohistochemical analysis. *Am J Surg Pathol*. 2015;39(9):1170-1180.

Koh J, Go H, Kim MY, et al. A comprehensive immunohistochemistry algorithm for the histological subtyping of small biopsies obtained from non-small cell lung cancers. *Histopathology*. 2014;65(6):868-878.

Kriegsmann K, Cremer M, Zgorzelski C, et al. Agreement of CK5/6, p40, and p63 immunoreactivity in non-small cell lung cancer. *Pathology*. 2019;51(3):240-245.

Loo PS, Thomas SC, Nicolson MC, et al. Subtyping of undifferentiated non-small cell carcinomas in bronchial biopsy specimens. *J Thorac Oncol*. 2010;5(4):442-447.

Micke P, Mattsson JS, Djureinovic D, et al. The impact of the fourth edition of the WHO classification of lung tumours on histological classification of resected pulmonary NSCCs. *J Thorac Oncol*. 2016;11(6):862-872.

Nobre AR, Albergaria A, Schmitt F. p40: a p63 isoform useful for lung cancer diagnosis—a review of the physiological and pathological role of p63. *Acta Cytol*. 2013;57(1):1-8.

Nonaka D. A study of ΔNp63 expression in lung non-small cell carcinomas. *Am J Surg Pathol*. 2012;36(6):895-899.

Nylander K, Vojtesek B, Nenutil R, et al. Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J Pathol*. 2002;198(4):417-427.

Pelosi G, Rossi G, Cavazza A, et al. ΔNp63 (p40) distribution inside lung cancer: a driver biomarker approach to tumor characterization. *Int J Surg Pathol*. 2013;21(3):229-239.

Sakai Y, Nakai T, Ohbayashi C, et al. Immunohistochemical profiling of ALK fusion gene-positive adenocarcinomas of the lung. *Int J Surg Pathol*. 2013;21(5):476-482.

Tatsumori T, Tsuta K, Masai K, et al. p40 is the best marker for diagnosis pulmonary squamous cell carcinoma: comparison with p63, cytokeratin 5/6, desmocollin-3, and sox2. *Appl Immunohistochem Mol Morphol*. 2014;22(5):377-382.

Tran L, Mattsson JS, Nodin B, et al. Various antibody clones of napsin A, thyroid transcription factor 1, and p40 and comparisons with cytokeratin 5 and p63 in histopathologic diagnostics of non-small cell lung carcinoma. *Appl Immunohistochem Mol Morphol*. 2016;24(9):648-659.

Travis WD, Brambilla E, Noguchi M, et al. Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. *Arch Pathol Lab Med*. 2013;137(5):668-684.

Cytokeratin Markers

By Deepali Jain, Sylvie Lantuejoul, Ming Sound Tsao, and Alain C. Borczuk

9

Introduction

Cytokeratins are intermediate filaments forming the cytoskeleton of epithelial cells. More than 20 types of keratins are expressed in a set of paired basic/type I (CK1-8) and acidic/type II (CK9-20) keratins (Winter and Schweizer 1981; Eichner et al 1985; Ordonez 2013). Within each category, they are divided into low-molecular-weight (LMW) and high-molecular-weight (HMW) keratins. Some cytokeratins are expressed in specific organs or tissues and their expression is generally maintained after neoplastic transformation. CK1 to CK6 (basic) and CK9-17 (acidic) are HMW keratins expressed in squamous epithelia and basal epithelial cells, whereas LMW keratins are CK7 and CK8 (basic) and CK18-20 (acidic) and are expressed in simple epithelium including glandular epithelia (Winter et al 1980). Because cytokeratin expression profile is used in clinical practice to determine the origin of various tumors, the value of different cytokeratins for the diagnosis of lung tumors is described in this chapter.

What Are Pancytokeratin Stains and What Is Their Role in the Diagnosis of Lung Cancer?

AE1/AE3, KL1, MNF116, and OSCAR are cocktails of antibodies that react with different types of both LMW and HMW keratins. Table 9-1 shows some commonly used clones of monoclonal antibodies that react with different types of cytokeratins (Ordonez 2013). In the lung, they show diffuse cytoplasmic positivity in adenocarcinoma and squamous cell carcinoma (Tan and Zander 2008) (Figure 9-1A and B). Perinuclear and dotlike expression is characteristic of small cell carcinoma (Figure 9-1C and D), but a diffuse cytoplasmic staining can also be found (Thunnissen et al 2017). These pancytokeratin antibody cocktails are useful when the tumor is morphologically undifferentiated and carries a differential diagnosis of carcinoma, melanoma, lymphoma, mesothelioma, and sarcoma (Figure 9-1E and F). Diffuse and strong cytoplasmic positivity of keratin stain practically establishes the diagnosis of carcinoma. A subset of lymphoma, synovial sarcoma, epithelioid sarcoma, angiosarcoma, and smooth muscle tumors show keratin expression. In these instances, further lineage-specific markers should be done.

Table 9-1. Commonly Used Clones of Keratin Antibodies with Their Reactivity to Various Types of Keratins

Antibody clone	Reactivity to keratins
AE1/AE3	CK1-8, 10, 14-16
CAM5.2	CK8, CK7 (lesser extent)
KL1	CK1, K2, CK5-8, CK11, CK14, CK16-K18
Lu5	CK1, CK5, CK6, CK8, CK14, CK18, CK19
MNF116	CK5, 6, 8, 17
OSCAR	CK7, 8, 18, and 19
Pan-CK	CK4-K8, CK10, CK13-CK16, CK18
34βE12	CK1, 5, 10, 14

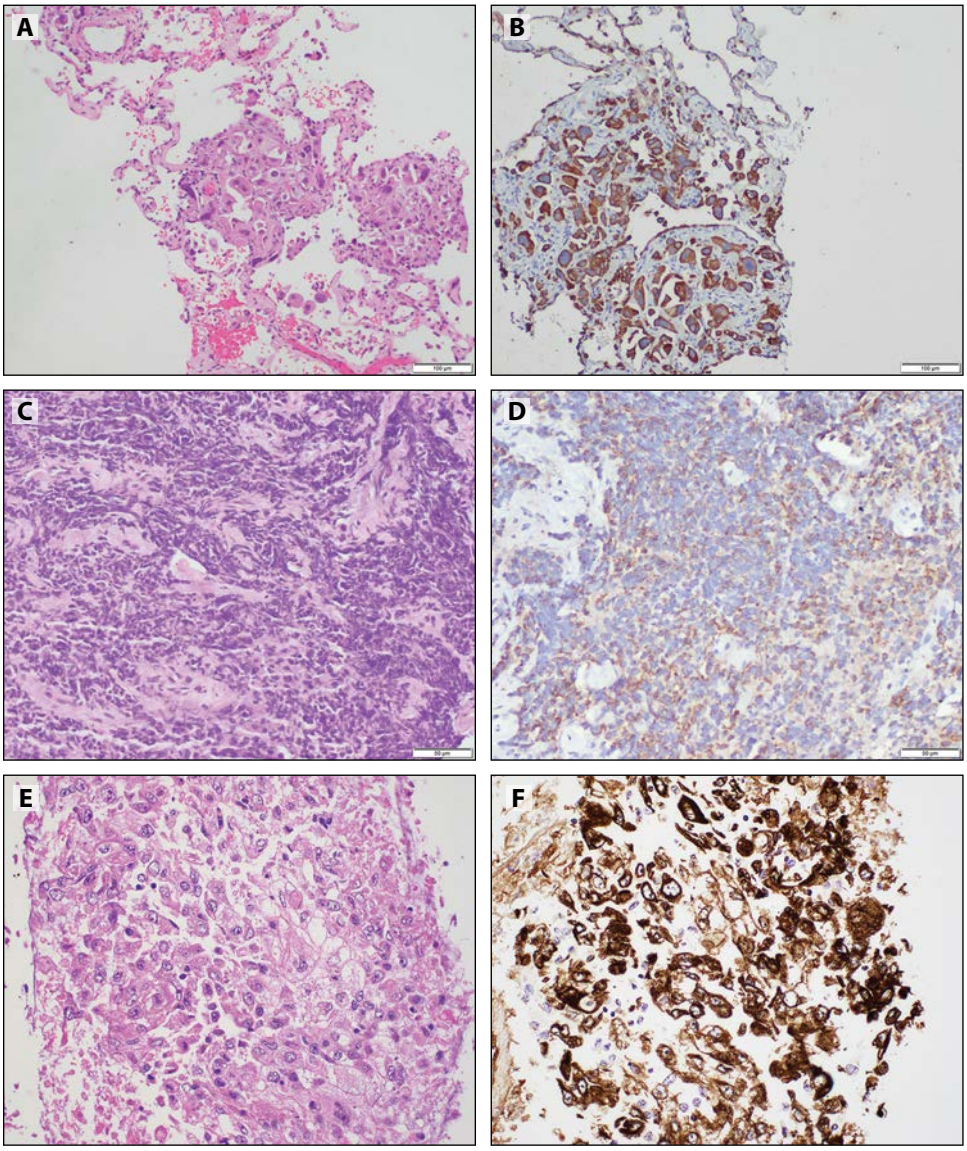


Figure 9-1. Pancytokeratin antibodies help identify morphologically undifferentiated tumors as carcinoma. **(A and B)** Pulmonary adenocarcinoma, **(C and D)** small cell carcinoma, and **(E and F)** undifferentiated non-small cell carcinoma. **(A, C, E)** Hematoxylin-eosin and **(B, D, F)** pankeratin AE1/AE3.

Summary Answer
Pancytokeratin stains can establish a diagnosis of carcinoma when the tumor is morphologically undifferentiated.

Are Cytokeratins 5 or 5/6 Sensitive and Specific Markers for Squamous Cell Carcinoma of Lung?

Cytokeratin 5 and 6 are related proteins; cytokeratin 5/6 (CK5/6) antibody detects both while CK5 is more specific. CK5/6 stains bronchial and bronchiolar basal (reserve) cells in a cytoplasmic and membranous pattern. CK5/6 antibody exhibits global sensitivity and specificity of 98% and 82%, respectively, for the diagnosis of squamous cell carcinoma on surgical samples. However, the sensitivity decreases to 90%, whereas the specificity reaches 97% when the staining is diffuse (Rekhtman et al 2011; Whithaus et al 2012). Notably, CK5/6 can be focal, weak, or absent in up to 25% of resected squamous cell carcinomas and can be expressed in up to 18% of adenocarcinomas (Rekhtman et al 2011). Thus, more sensitive and specific markers, such as p40, should be used instead for diagnosis of squamous cell carcinoma of the lung (Wang et al 2002; Tatsumori et al 2014; Walia et al 2017; Jain et al 2014). In addition, CK5/6 also stains mesothelial cells and mesotheliomas (Ordonez 2013; King et al 2006). Hence in a pleural biopsy, CK5/6 positivity should be supported by other mesothelial stains such as calretinin, Wilms tumor protein (WT1), and negativity of squamous markers (34βE12, p40) to establish correct diagnosis of mesothelioma and squamous cell carcinoma respectively.

Summary Answer
CK5/6 does not have adequate sensitivity and specificity; thus should not be used alone to diagnose lung squamous cell carcinoma.

Should Cytokeratin 7 Be Used to Differentiate Lung Adenocarcinoma from Squamous Cell Carcinoma?

Cytokeratin 7 (CK7) is expressed in bronchial epithelium of conducting airways, bronchiolar epithelium, type I and II pneumocytes, and club (formerly known as Clara) cells. CK7 also stains submucosal seromucinous glands. The expression pattern is cytoplasmic. CK7 stains almost all adenocarcinomas of the lung with more than 90% to 100% sensitivity (Chu et al 2000) (Figure 9-2A-C). Of note, the mucinous variant shows positivity in a smaller number of cases (50%-90%). However, more than a third (Vidarsdottir et al 2019) of squamous cell carcinomas are positive for CK7, thus making it less specific for lineage discrimination within the lung (Figure 9-2D-F). Therefore, CK7 is not recommended in distinguishing adenocarcinoma from squamous cell carcinoma of the lung. In addition, CK7 is not specific for pulmonary origin, as it can also be seen in adenocarcinomas of other organ systems such as the pancreatobiliary tract, stomach, ovary, and breast (Chu et al 2000). Therefore, a more important complete panel inclusive of thyroid transcription factor-1 (TTF1) and p40 is required to classify non-small cell carcinomas, with additional markers such as napsin A as needed. Cytokeratin profile is important in challenging poorly differentiated cases as discussed in the preceding section.

Summary Answer
CK7 should not be used to distinguish between lung adenocarcinoma and squamous cell carcinoma.

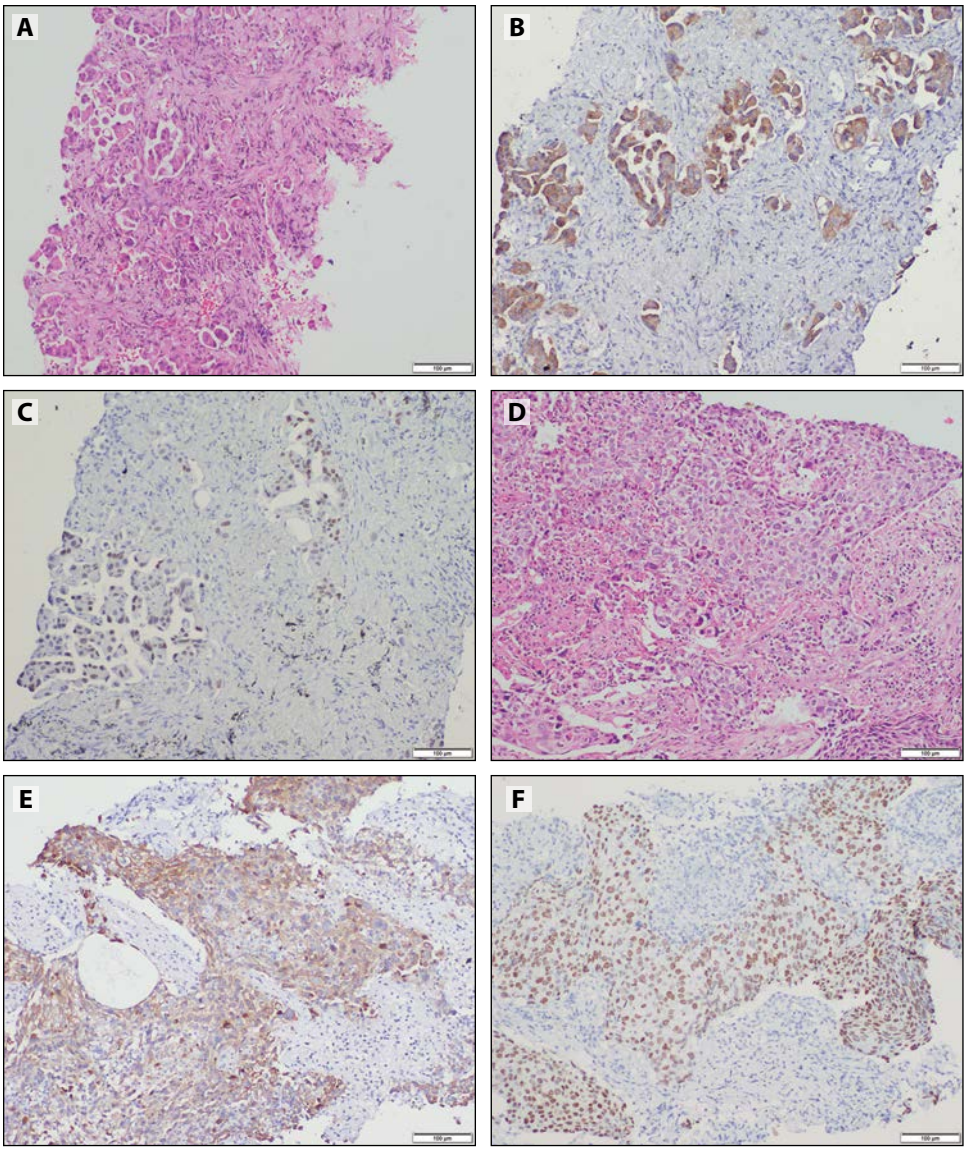


Figure 9-2. Cytokeratin 7 (CK7) is not specific for pulmonary adenocarcinoma. **(A, B, C)** Adenocarcinoma and **(D, E, F)** poorly differentiated squamous cell carcinoma. **(A and D)** Hematoxylin-eosin, **(B and E)** CK7, **(C)** thyroid transcription factor-1 (TTF1), and **(F)** p40.

Should a Non-Small Cell Carcinoma That Is Diffusely Positive for CK7 but Negative for TTF1 and p40 Be Regarded as "Probably Adenocarcinoma"?

CK7 is not specific for adenocarcinoma, as it can also stain positive in squamous cell carcinoma. Therefore, the use of CK7 is discouraged for the subtyping of non-small cell carcinoma (Yatabe et al 2019). Such carcinomas should be better regarded as a non-small cell carcinoma, not otherwise specified (NSCC, NOS).

Summary Answer

CK7 alone is insufficient to establish a diagnosis of lung adenocarcinoma.

Is CK7 a Helpful Stain in Differentiating Pulmonary Adenocarcinoma from Mesothelioma?

CK7 may also be strongly expressed in epithelioid mesotheliomas, thus, it is not useful to differentiate adenocarcinomas from mesotheliomas (Tot 2001). Approximately 90% of mesothelioma cases are CK7 positive (Tot 2001). CK7 is notably absent from most thymus, prostate, hepatocellular, and most colonic adenocarcinomas and clear cell renal cell carcinomas (Chu et al 2000) (see Chapter 16).

Summary Answer

CK7 cannot differentiate lung adenocarcinoma from malignant mesothelioma.

Which Cytokeratin Antibody Is Preferred to Stain Small Cell Lung Carcinoma?

CK7 and CK5/6 are not useful stains in the diagnosis of small cell lung carcinoma (SCLC). CK7 is either negative or focally positive in small cell carcinoma with only about half of SCLC staining (Figure 9-3) (Chu et al 2000). HMW cytokeratin, such as 34βE12, is negative in neuroendocrine tumors including small cell and large cell neuroendocrine carcinomas (Sturm et al 2001; Zhang et al 2005; Lyda and Weiss 2000).

Summary Answer

Pancytokeratin antibodies are preferred when being used to recognize SCLC.

What Is the Utility of CK20 in the Diagnosis of Lung Cancer?

Cytokeratin 20 (CK20) is expressed only in less than 5% of normal bronchial epithelium. Invasive mucinous adenocarcinoma, colloid and enteric adenocarcinoma, and other adenocarcinomas of the lung with mucinous features typically co-express CK20 along with CK7

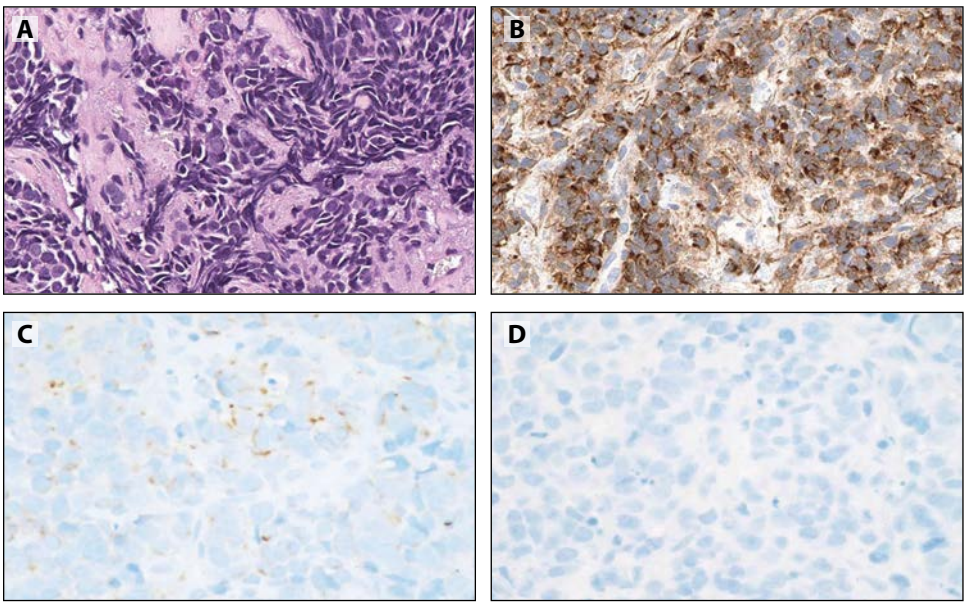


Figure 9-3. Small cell carcinoma is best stained with broad-spectrum pancytokeratin antibodies. **(A)** Hematoxylin-eosin, **(B)** AE1/AE3, **(C)** cytokeratin 7 (CK7), and **(D)** cytokeratin 5/6 (CK5/6).

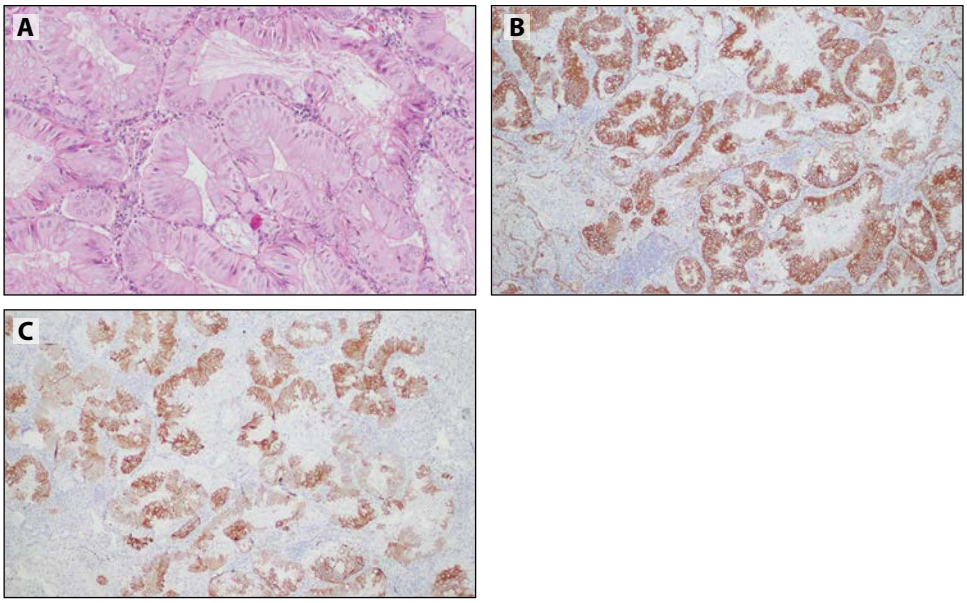


Figure 9-4. Pulmonary invasive mucinous adenocarcinoma can co-express cytokeratin 7 (CK7) and cytokeratin 20 (CK20). **(A)** Hematoxylin-eosin, **(B)** CK7, and **(C)** CK20.

(Figure 9-4). The expression is patchy in more than 90% of cases. CK20 is largely negative in SCLC, with only about 10% cases positive (Chu et al 2000). In these rare CK20-positive SCLC cases, other immunohistochemistry (IHC), such as TTF1 and neurofilament protein, should be done to exclude metastatic Merkel cell carcinoma (Bobos et al 2006).

Summary Answer
CK20 positivity does not exclude mucinous adenocarcinoma of lung origin.

Which Cytokeratin Antibody Should Be Used for Mesothelioma?

AE1/AE3, CAM5.2, and MNF116 are useful to identify virtually all epithelioid and most sarcomatoid mesotheliomas (see also Chapter 17). About 5% to 10% of sarcomatoid mesotheliomas are negative for broad-spectrum cytokeratins (Attanoos et al 2000; Klebe et al 2008). CK5 or CK5/6 is expressed in 75% to 100% of epithelioid mesotheliomas; however, low sensitivity of CK5/6 in sarcomatoid mesothelioma limits its utility in the diagnosis of the latter (Cury et al 2000; Husain et al 2018).

Summary Answer
Pancytokeratin and CK5/6 are useful in diagnosing mesothelioma, in conjunction with other mesothelioma markers.

What Is the Role of Cytokeratins in the Diagnosis of Thymoma?

Cytokeratins play an important role in small biopsies of mediastinal lesions to distinguish type B thymomas from lymphomas and type A thymomas from spindle cell tumors (see Chapter 18). Generally, pancytokeratins and CK19 are expressed by both cortical and medullary epithelial cells. However, CK10 and CK14 are restricted to mature medullary epithelial

cells and Hassall corpuscles. Types A and AB thymomas are consistently positive for all acidic keratins except CK20. Type B thymomas are positive for pancytokeratins (AE1/AE3) with the exception of CK20. CK20 is expressed in rare thymic adenocarcinomas (Kuo 2000).

Summary Answer

Pancytokeratins are very useful in the differential diagnosis of thymomas from other mediastinal lesions.

Conclusions

Lung carcinomas, being epithelial in origin, express all types of cytokeratins, thus IHC of broad-spectrum cytokeratins is encouraged in cases of challenging poorly differentiated tumors to establish their epithelial origin. However, because of the nonspecificity of most of the keratins and in order to save tumor tissue for further molecular testing, routine use in small biopsies of lung carcinoma cases is not advisable (Table 9-2). CK7 is not useful in distinguishing between different subtypes of lung carcinomas and in differentiating lung adenocarcinomas from extrapulmonary metastatic carcinomas. HMW keratins are more specifically expressed by squamous cell carcinomas; however, a small percentage of adenocarcinomas also express HMW cytokeratin. Small cell carcinomas show characteristic perinuclear and dotlike pattern of pancytokeratin, but a diffuse cytoplasmic staining may also be seen. SCLC are commonly negative for CK5/6, CK7, and CK20. Understanding cytokeratin pattern and profiling is important for their judicious use in the diagnosis of lung cancer.

Table 9-2. Summary of Preferred Cytokeratins in the Diagnosis of Pulmonary and Pleural Neoplasms

Tumor	Keratins
Poorly differentiated malignant tumor	Broad-spectrum cytokeratins/pancytokeratins
Squamous cell carcinoma	CK5, CK5/6, 34βE12
Adenocarcinoma	CK7; see text
Invasive mucinous adenocarcinoma	CK7, CK20
Small cell carcinoma	Broad-spectrum cytokeratins/pancytokeratins
Epithelioid mesothelioma	CK5 or CK5/6
Sarcomatoid mesothelioma	Broad-spectrum cytokeratins/pancytokeratins

References

Attanoos RL, Dojcinov SD, Webb R, et al. Antimesothelial markers in sarcomatoid mesothelioma and other spindle cell neoplasms. *Histopathology*. 2000;37(3):224-231.

Bobos M, Hytiroglou P, Kostopoulous I, et al. Immunohistochemical distinction between Merkel cell carcinoma and small cell carcinoma of the lung. *Am J Dermatopathol*. 2006;28(2):99-104.

Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol*. 2000;13(9):962-972.

Cury PM, Butcher DN, Fisher C, et al. Value of the mesothelium-associated antibodies thrombomodulin, cytokeratin 5/6, calretinin, and CD44H in distinguishing epithelioid mesothelioma from adenocarcinoma metastatic to the pleura. *Mod Pathol*. 2000;13(2):107-112.

Eichner R, Rew P, Engel A, et al. Human epidermal keratin filaments: studies on their structure and assembly. *Ann N Y Acad Sci*. 1985;455:381-402.

Husain AN, Colby TV, Ordonez NG, et al. Guidelines for pathologic diagnosis of malignant mesothelioma 2017 update of the consensus statement from the

International Mesothelioma Interest Group. *Arch Pathol Lab Med*. 2018;142(1):89-108.

Jain D, Mathur SR, Guleria R, et al. Utility and pattern of positivity of p40 in the diagnosis of squamous cell carcinoma of the lung by cytology: the first study on fine needle aspiration smears. *Cytopathology*. 2014;25(5):330-335.

King JE, Thatcher N, Pickering CA, et al. Sensitivity and specificity of immunohistochemical markers used in the diagnosis of epithelioid mesothelioma: a detailed systematic analysis using published data. *Histopathology*. 2006;48(3):223-232.

Klebe S, Mahar A, Henderson DW, et al. Malignant mesothelioma with heterologous elements: clinicopathological correlation of 27 cases and literature review. *Mod Pathol*. 2008;21(9):1084-1094.

Kuo Tt. Cytokeratin profiles of the thymus and thymomas: histogenetic correlations and proposal for a histological classification of thymomas. *Histopathology*. 2000;26(5):403-414.

Lyda MH, Weiss LM. Immunoreactivity for epithelial and neuroendocrine antibodies are useful in the differential diagnosis of lung carcinomas. *Hum Pathol*. 2000:31(8):980-987.

Ordonez NG. Broad-spectrum immunohistochemical epithelial markers: a review. *Hum Pathol*. 2013;44(7):1195-1215.

Rekhtman N, Ang DC, Sima CS, et al. Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. *Mod Pathol*. 2011;24(10):1348-1359.

Sturm N, Lantuejoul S, Laverriere MH, et al. Thyroid transcription factor 1 and cytokeratins 1, 5, 10, 14 (34betaE12) expression in basaloid and large-cell neuroendocrine carcinomas of the lung. *Hum Pathol*. 2001;32(9):918-925.

Tan D, Zander DS. Immunohistochemistry for assessment of pulmonary and pleural neoplasms: a review and update. *Int J Clin Exp Pathol*. 2008;1(1):19-31.

Tatsumori T, Tsuta K, Msai K, et al. p40 is the best marker for diagnosis pulmonary squamous cell carcinoma: comparison with p63, cytokeratin 5/6, desmocollin-3, and sox2. *Appl Immunohistochem Mol Morphol*. 2014;22(5):377-382.

Thunnissen E, Borczuk AC, Flieder DB, et al. The use of immunohistochemistry improves the diagnosis of small cell lung cancer and its differential diagnosis. An international reproducibility study in a demanding set of cases. *J Thorac Oncol*. 2017;12(2):334-346.

Tot T. The value of cytokeratins 20 and 7 in discriminating metastatic adenocarcinoma from pleural mesotheliomas. *Cancer*. 2001;92(10):2727-2732.

Vidarsdottir H, Tran L, Nodin B, et al. Immunohistochemical profiles in primary lung cancers and epithelial pulmonary metastases. *Hum Pathol*. 2019;84:221-230.

Walia R, Jain D, Madan K, et al. p40 & thyroid transcription factor-1 immunohistochemistry: a useful panel to characterize non-small cell lung carcinoma-not otherwise specified (NSCLC-NOS) category. *Indian J Med Res*. 2017;146(1):42-48.

Wang BY, Gil J, Kaufman D, et al. P63 in pulmonary epithelium, pulmonary squamous neoplasms, and other pulmonary tumors. *Hum Pathol*. 2002;33(9):921-926.

Whithaus K, Fukuoka J, Prihoda TJ, et al. Evaluation of napsin A, cytokeratin 5/6, p63, and thyroid transcription factor 1 in adenocarcinoma versus squamous cell carcinoma of the lung. *Arch Pathol Lab Med*. 2012;136(2):155-162.

Winter H, Schweizer J. Carcinoma-specific keratin polypeptide patterns in keratinizing epithelia of rodents: independence of species- and tissue-specific variations. *Carcinogenesis*. 1981;2(7):613-621.

Winter H, Schweizer J, Goerttler K. Keratins as markers of malignancy in mouse epidermal tumors. *Carcinogenesis*. 1980;1(5):391-398.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practice recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Zhang H, Liu J, Cagle PT, et al. Distinction of pulmonary small cell carcinoma from poorly differentiated squamous cell carcinoma: an Immunohistochemical approach. *Mod Pathol*. 2005;18(1):111-118.

Neuroendocrine Markers

By Mary Beth Beasley, Mauro Papotti, and Alain C. Borczuk

10

Introduction

Primary neuroendocrine neoplasms (NENs) of the lung include the low- and intermediate-grade typical carcinoid (TC) and atypical carcinoid (AC) and the high-grade neuroendocrine carcinomas (NECs), large cell neuroendocrine carcinoma (LCNEC), and small cell lung carcinoma (SCLC) (Travis et al 2015a). Rarer tumors, which may exhibit neuroendocrine (NE) differentiation, such as nuclear protein in testis (NUT) midline carcinoma (Stathis et al 2016), primitive neuroectodermal tumor (Weissferdt and Moran 2012; Marino-Enriquez and Fletcher 2014), and desmoplastic small round cell tumor (Parkash et al 1995), may also occur in the lung (immunomarkers for rare tumors are discussed in Chapters 14 and 15).

A panel of NE markers including chromogranin A, synaptophysin, and neural cell adhesion molecule 1 (NCAM1, also known as CD56) are the most commonly recommended markers for identification of NEN (Travis et al 2015a, 2015b; Yatabe et al 2019). More recently, insulinoma-associated protein 1 (INSM1) has shown high sensitivity and specificity for labeling the entire spectrum of NENs (Mukhopadhyay et al 2019; Rooper et al 2017; Rosenbaum et al 2015). Additional markers may also have utility as discussed in the following section. There is no clear cutoff for any of the NE markers regarding what constitutes positive immunohistochemistry (IHC) staining, and the interpretation should be made in the context of the morphologic features, sample type, and extent of positive reaction. Similarly, there is no consensus on whether one or multiple markers should be used. In general, the combination of NE morphologic features (organoid nesting, rosette-like structures, palisading patterns) and positive staining for any of these NE markers is suggestive of the diagnosis of an NEN. The extent and/or intensity of positive reactions may vary among histologic subtypes, and approximately 5% to 10% of SCLC may be negative for chromogranin, synaptophysin, and CD56. Conversely, approximately 10% to 20% of non-small cell lung carcinomas (NSCLCs), that is, adenocarcinoma or squamous cell carcinoma, without overt NE morphology exhibit positive staining for one or more markers (Travis et al 2015a; Yatabe et al 2019). Such tumors have been referred to as "non-small cell carcinoma with neuroendocrine differentiation";

however, studies have found no significance to this finding regarding treatment, outcome, or prognosis, and such tumors should be managed and classified as NSCLC (Travis et al 2011). Occasionally, a tumor has morphologic features of NE differentiation but lacks expression of chromogranin, synaptophysin, or CD56, and such tumors are termed "non-small cell carcinoma with NE morphology" (Travis et al 2015a).

What IHC Markers Are Useful to Support Neuroendocrine Morphology in the Classification of Neuroendocrine Neoplasms?

Commonly Used Neuroendocrine Immunostains

See Figures 10-1 and 10-2 for commonly used NE immunostains.

Chromogranin A and Synaptophysin

Chromogranin A and synaptophysin are true markers of NE differentiation. Chromogranin A is contained in dense core granules, and synaptophysin is present in synaptic vesicles. Staining is cytoplasmic for both markers (Loy et al 1995). Chromogranin A is positive in 90% to 100% of TC, 60% to 70% of AC and LCNEC but only approximately 25% of SCLC. Synaptophysin is positive in approximately 70% to 80% of pulmonary NEN regardless of grade but is generally weaker and less diffuse in high-grade NEC (Yatabe et al 2019).

CD56 (NCAM)

CD56 is more sensitive than chromogranin or synaptophysin in high-grade NEC in particular, but is less specific for NE differentiation than chromogranin, synaptophysin, or INSM1 (Rooper et al 2017; Pelosi et al 2017; Lantuejoul et al 1998). Positive staining is typically

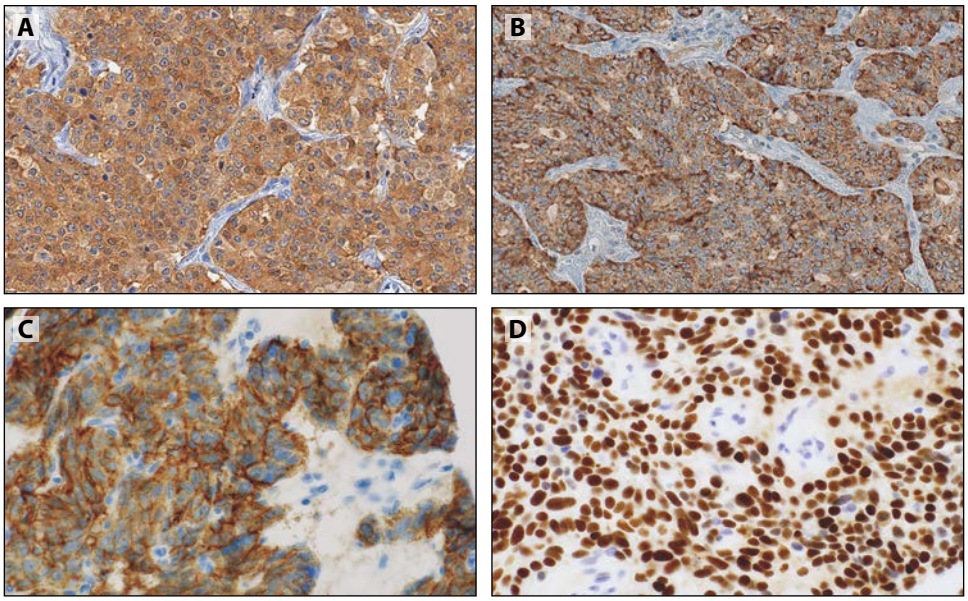


Figure 10-1. Neuroendocrine markers in carcinoid tumors. **(A)** Diffuse and intense cytoplasmic immunoreactivity is seen for synaptophysin, **(B)** chromogranin, and **(C)** CD56/NCAM. **(D)** Intense and diffuse nuclear staining is seen for insulinoma-associated protein 1 (INSM1).

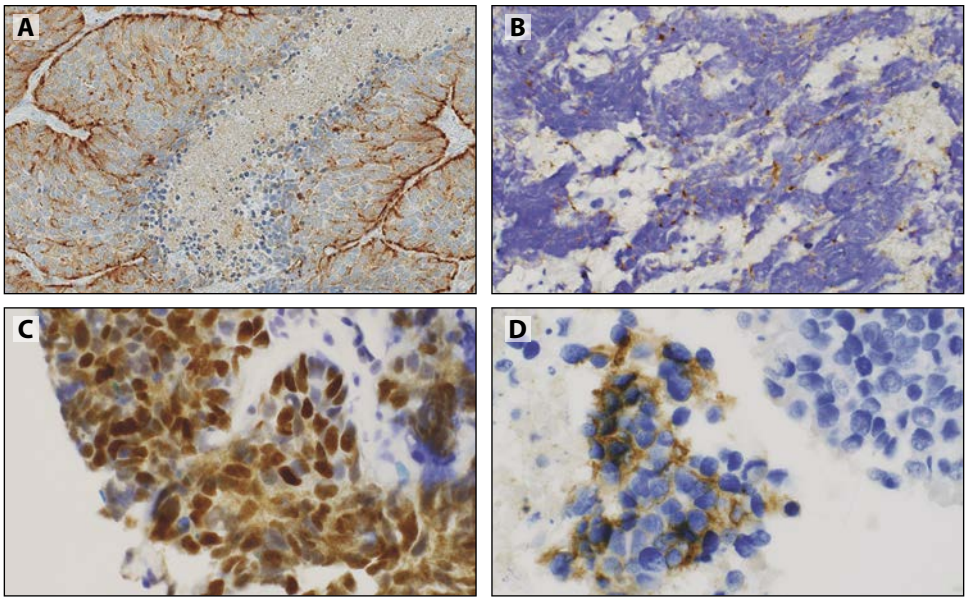


Figure 10-2. Neuroendocrine markers in small cell carcinoma. Immunoreactivity in small cell carcinoma for neuroendocrine markers can be weaker and less uniform than in carcinoid tumors. **(A)** Chromogranin is patchy and weak to moderate. **(B)** Synaptophysin is weakly perceptible and "dot and rim-like" rather than diffuse. **(C)** Insulinoma-associated protein 1 (INSM1) shows irregular but moderate nuclear staining. **(D)** Cytoplasmic and membranous staining for CD56.

membranous. CD56 may be expressed on a variety of normal cells including neurons, glia, various hematopoietic cells (natural killer cells, γδ-T cells, activated CD8+ T cells, and dendritic cells), as well as skeletal muscle. Additionally, CD56 is expressed in up to 30% of NSCLC as well as some mesenchymal tumors, multiple myeloma, sarcomas, and in some mesotheliomas (Lantuejoul et al 2000; Agaimy and Wunsch 2008; Olsen et al 2006). As such, CD56 positivity should be interpreted in the context of epithelial differentiation and NE morphologic features.

INSM1

INSM1 has generally been shown to be a highly sensitive and specific marker for the entire spectrum of NENs. Studies have shown variable results about whether INSM1 is more or less sensitive and/or specific than chromogranin, synaptophysin, and/or CD56 or a combination thereof (Kriegsmann et al 2020). Thus far, INSM1 has proven to be a consistently reliable marker, particularly in the setting of SCLC where markers such as chromogranin may be negative, and the nuclear staining may provide greater ease in interpretation. In addition, given the expression of CD56 across many tumor types, INSM1 may be more specific than CD56 (Mukhopadhyay et al 2019; Nakra et al 2019; Rooper et al 2017; Rosenbaum et al 2015; Roy et al 2019).

Other Neuroendocrine Markers

Human achaete-scute homolog-1 (hASH1) is considered a lineage marker of NE cells as it is an embryonic nuclear determinant of NE differentiation and, hence, is highly specific for NENs. The overall sensitivity of the marker is low as hASH1 is often lost or poorly expressed

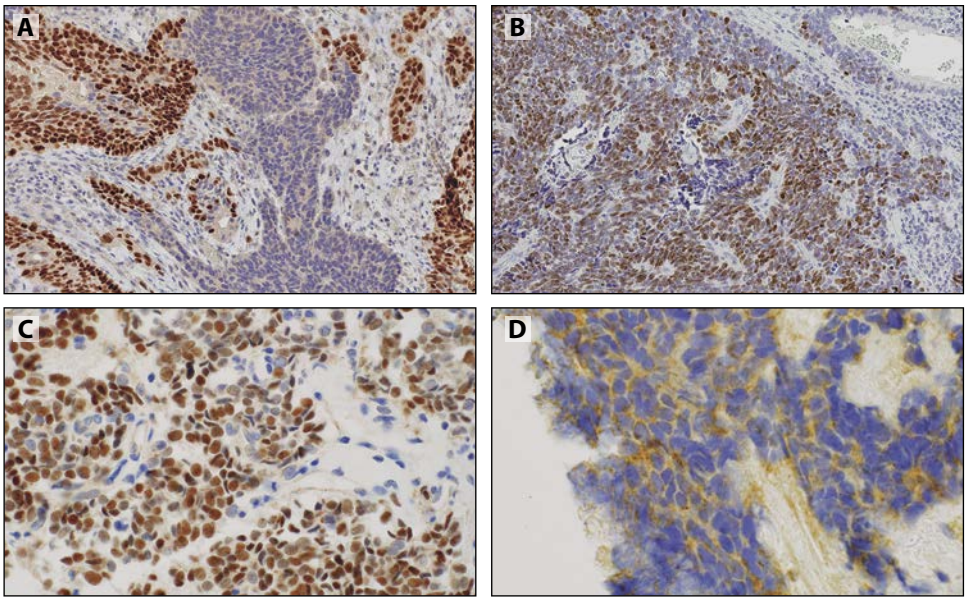


Figure 10-3. Other markers in neuroendocrine tumors. **(A)** Nuclear immunoreactivity for p40 highlights the squamous component of a combined small cell carcinoma with squamous carcinoma. **(B)** Human achaete-scute homolog-1 (hASH1) shows nuclear immunoreactivity in the small cell carcinoma. **(C)** Orthopedia homeobox protein (OTP) shows specific nuclear immunoreactivity in a carcinoid tumor, while **(D)** a small cell carcinoma shows only nonspecific cytoplasmic reactivity.

in carcinoid tumors; however, it is retained in high-grade tumors, particularly SCLC (Miki et al 2012; Ye et al 2016) (Figure 10-3).

Orthopedia homeobox protein (OTP) is believed to play a role in the development of the hypothalamic NE system. Cytoplasmic staining is generally nonspecific, but nuclear staining is considered highly specific for NE differentiation (Nonaka et al 2016). OTP is preferentially expressed in carcinoid tumors and is only rarely expressed in SCLC (Hanley et al 2018; Viswanathan et al 2019). While most extensively studied in lung carcinoids, OTP expression has also been reported in NE tumors of other sites including the prostate and ovary (Roy et al 2019), although data are conflicting and other studies have shown positive OTP staining in pulmonary NE tumors only (Nonaka et al 2016) (Figure 10-3).

Leu7 (CD57) has some utility as an NE marker but has low sensitivity and is not specific for NE differentiation. Similar to CD56, Leu7 is positive in a variety of hematopoietic cells and is additionally positive in a variety of nerve sheath tumors, spindle cell thymoma, synovial sarcoma, and some papillary thyroid carcinomas (Uherova et al 2003; Miettinen 1993).

Neuron-specific enolase (NSE) is highly sensitive but has very low specificity and, as such, is generally not recommended as a marker for evaluation of pulmonary NECs (Travis et al 2015a).

Summary Answer

Chromogranin, synaptophysin, CD56, and INSM1 are useful NE markers in support of NE morphology.

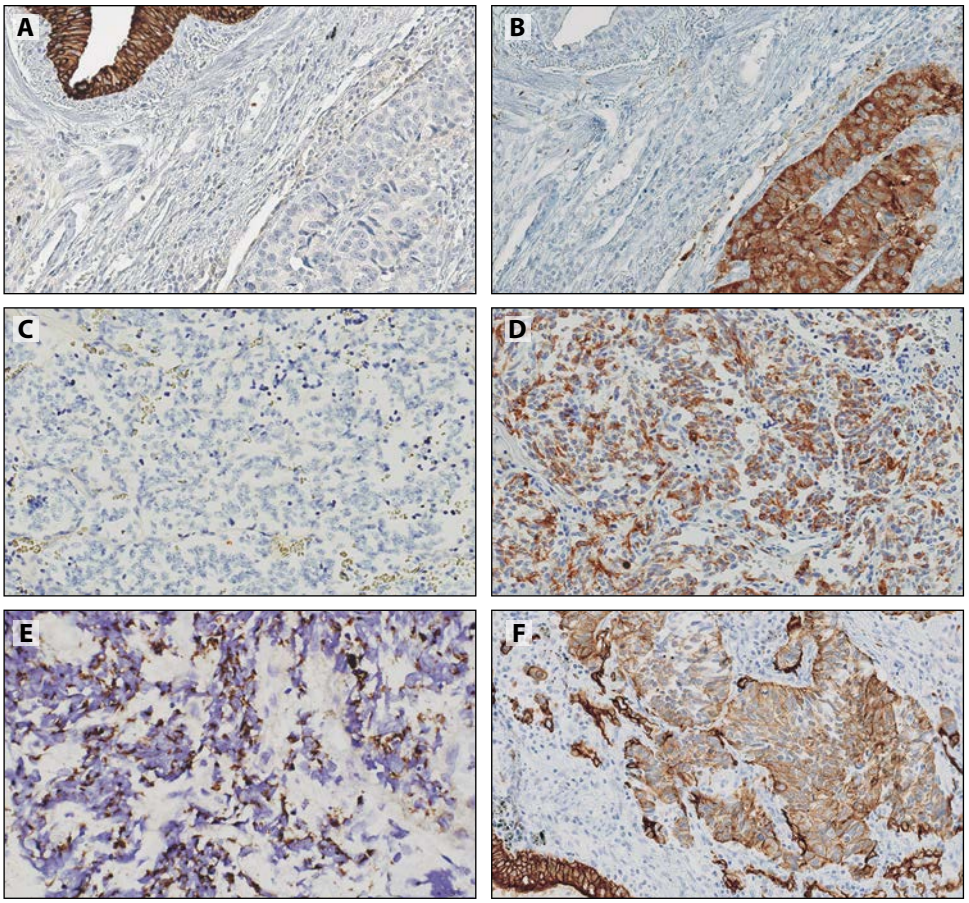


Figure 10-4. Cytokeratin in neuroendocrine neoplasms. **(A and B)** High-molecular-weight cytokeratin **(A)** is negative in this large cell neuroendocrine carcinoma, with positive airway epithelium, while **(B)** synaptophysin highlights the tumor. **(C and D)** This carcinoid tumor is negative for cytokeratin cocktail AE1/AE3 **(C)**. If diagnostically needed, CAM5.2 can be attempted, and a subset of AE1/AE3 negative tumors are CAM5.2 positive as shown in **(D)**. Cytokeratin stains in small cell carcinoma can be punctate and dot-like, as seen in **(E)**, but more diffuse cytoplasmic/membranous staining can also be seen in **(F)**.

What Non-Neuroendocrine Markers Can Assist in the Classification of Tumors in the Differential Diagnosis of NEN?

Broad cytokeratin cocktails, such as AE1/AE3, and low-molecular-weight keratin CAM5.2 (cytokeratin 8 [CK8] and some cytokeratin 7 [CK7]) stain most NENs regardless of grade (Figure 10-4). Approximately 10% of NENs are reportedly negative (Rekhtman 2010), particularly for AE1/AE3, although these cases may stain for other cytokeratins such as CAM5.2. SCLC classically shows a "rim and dot"–type pattern of staining with cytokeratins, particularly CAM5.2 (Travis et al 2015a). Although CK7 may be positive in a variable number of pulmonary NEN, a positive CK20 stain should raise the possibility of Merkel cell carcinoma (Cheuk et al 2001). High-molecular-weight cytokeratins, such as CK34βE12, are generally negative in pulmonary NEN, and a positive stain in the presence of negative NE markers is usually indicative of basaloid squamous cell carcinoma, the morphology of which may closely mimic the high-grade NECs (Sturm et al 2001, 2003). Diffuse positive staining with

either p40 or p63 supports a diagnosis of squamous cell carcinoma in this scenario. A small percentage of high-grade NEC may show focal staining for p63 and rarely for p40, but diffuse staining should not be present (Rekhtman et al 2016) (Figure 10-4).

Summary Answer

Pankeratin and low-molecular-weight keratins rather than high-molecular-weight keratins should be positive in NENs.

When Should NE Markers Be Applied to a Non-Small Cell Carcinoma?

NE IHC markers should only be applied when morphologic features of NE differentiation are present. As stated earlier, a variable percentage of NSCLC may be positive for NE markers in the absence of NE morphology (non-small cell carcinoma with NE differentiation) (Figure 10-5). As such, because of the lack of data supporting the clinical relevance of positive NE markers in the absence of NE morphology, it is generally not recommended that NE markers be performed on tumors lacking NE morphology. As also noted, occasional tumors may show NE morphology but lack staining with chromogranin, synaptophysin, or CD56 (non-small cell carcinoma with NE morphology) (Travis et al 2015a, 2015b; Yatabe et al 2019; Rekhtman 2010; Zacharias et al 2003). The addition of INSM1 may resolve this problem in at least some of these tumors, but its addition to difficult cases requires further study (Figure 10-6).

In the setting of small biopsy or cytology specimens, NE morphologic features may be more difficult to discern, and making a definitive diagnosis of LCNEC on a small biopsy can be particularly problematic. Currently, it is recommended that if positive staining for NE

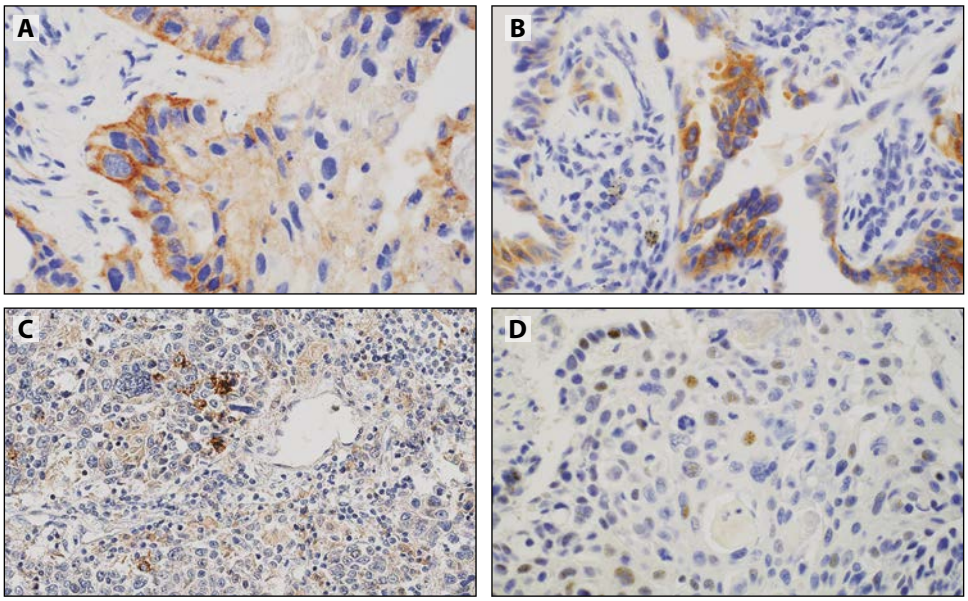


Figure 10-5. Neuroendocrine markers in non-neuroendocrine, non-small cell carcinoma (non-small cell carcinoma with NE differentiation). **(A)** A squamous cell carcinoma is moderately and multifocally immunoreactive for CD56. **(B)** An adenocarcinoma with multifocal synaptophysin reactivity. **(C)** Focal chromogranin reactivity is seen in a large cell carcinoma. **(D)** Insulinoma-associated protein 1 (INSM1) shows weak and focal staining in a squamous cell carcinoma.

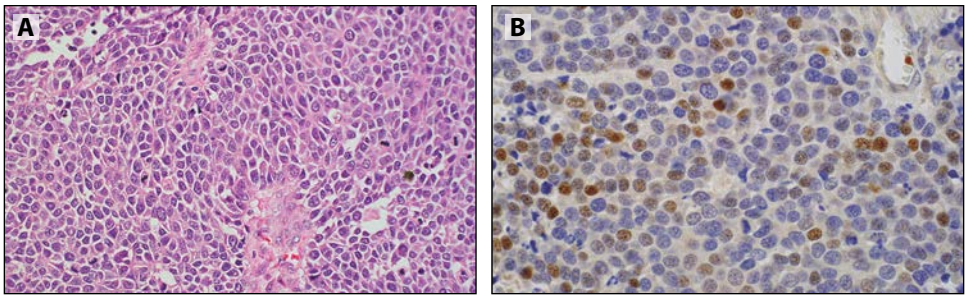


Figure 10-6. Non-small cell carcinoma with neuroendocrine morphology. **(A)** The tumor was previously diagnosed as non-small cell carcinoma with neuroendocrine morphology after negative staining for CD56, chromogranin, and synaptophysin. More recently, **(B)** use of insulinoma-associated protein 1 (INSM1) allowed for reclassification as a large cell neuroendocrine carcinoma.

markers is detected in an NSCLC and NE morphology is present, a diagnosis of "non-small cell carcinoma (NSCC), favor LCNEC" is provided (Travis et al 2015a). If the tumor lacks NE morphology, a diagnosis of NSCC is recommended with a comment regarding the positive NE staining. Given that most lung cancers are diagnosed on small biopsy or cytology, the difficulty in correctly identifying LCNEC on small biopsy is problematic given potential differences in treatment strategies compared to other NSCLCs (Travis et al 2015a). Overt NE morphology may be absent in small biopsies from LCNEC, and its presence has been shown to correlate with the size of the biopsy. Positive staining with 2 or 3 NE markers (chromogranin, synaptophysin, CD56) has been shown to have a sensitivity of 80% and a specificity of 99% in a study by Derks and colleagues (2019). Further verification of this is needed, and the impact of adding INSM1 to the algorithm similarly warrants study.

The diagnosis of carcinoid tumors is generally straightforward on small biopsies; however, mitotic figures and necrosis-discriminating TC and AC may not be present, and subtyping is therefore preferentially done on a resected specimen. The role of the proliferative marker Ki-67 is discussed in detail in Chapter 11, but at present, Ki-67 does not have a role in discriminating TC from AC. Ki-67 is useful, however, in small biopsies in discriminating high-grade NECs from carcinoid tumors, especially when morphology is suboptimal (Travis et al 2015a; Pelosi et al 2014, 2017).

Summary Answer

NE markers should only be used when morphologic features of NE differentiation are present. In small samples where NE morphology may be difficult to assess, there may be greater specificity when 2 or more markers are positive.

Conclusions

In tumors with NE morphology, a combination of chromogranin, synaptophysin, CD56, and INSM1 are useful IHC markers to confirm NE differentiation. In high-grade tumors, markers may be more focal or absent, so a combination of markers may be needed. In small samples where NE morphology can be hard to assess, 2 positive markers may be more specific than single markers. In difficult cases, a combination of high-molecular-weight cytokeratin, low-molecular-weight keratin, and p63/p40 may help in the differential diagnosis with non-NE morphologic mimics.

References

Agaimy A, Wunsch PH. Distribution of neural cell adhesion molecule (NCAM/CD56) in gastrointestinal stromal tumors and their intra-abdominal mesenchymal mimics. *J Clin Pathol*. 2008;61(4):499-503.

Cheuk W, Kwan MY, Suster S, et al. Immunostaining for thyroid transcription factor 1 and cytokeratin 20 aids the distinction of small cell carcinoma from Merkel cell carcinoma, but not pulmonary from extrapulmonary small cell carcinomas. *Arch Pathol Lab Med*. 2001;125(2):228-231.

Derks JL, Dingemans AC, van Suylen RJ, et al. IS the sum of positive neuroendocrine immunohistochemical stains useful for diagnosis of large cell neuroendocrine carcinoma (LCNEC) on biopsy specimens? *Histopathology*. 2019;74(4):555-566.

Hanley KZ, Dureau ZJ, Cohen C, et al. Orthopedia homeobox is preferentially expressed in typical carcinoids of the lung. *Cancer Cytopathol*. 2018;126(4):236-242.

Kriegsmann K, Zgorzelski C, Kazdal D, et al. Insulinoma-associated protein 1 (INSM1) in thoracic tumors is less sensitive but more specific compared with synaptophysin, chromogranin A, and CD56. *Appl Immunohistochem Mol Morphol*. 2020;28(3):237-242.

Lantuejoul S, Laverriere MH, Strum N, et al. NCAM (neural cell adhesion molecules) expression in malignant mesotheliomas. *Hum Pathol*. 2000;31(4):415-421.

Lantuejoul S, Moro D, Michalides RJ, et al. Neural cell adhesion molecules (NCAM) and NCAM-PSA expression in neuroendocrine lung tumors. *Am J Surg Pathol*. 1998;22(10):1267-1276.

Loy TS, Darkow GV, Quesenberry JT. Immunostaining in the diagnosis of pulmonary neuroendocrine carcinomas. An immunohistochemical study with ultrastructural correlations. *Am J Surg Pathol*. 1995;19(2):173-182.

Marino-Enriquez A, Fletcher CD. Round cell sarcomas—biologically important refinements in subclassification. *Int J Biochem Cell Biol*. 2014;53:493-504.

Miettinen M. Immunohistochemistry in tumour diagnosis. *Ann Med*. 1993;25(3):221-233.

Miki M, Ball DW, Linnoila RI. Insights into the achaete-scute homolog-1 gene (hASH1) in normal and neoplastic human lung. *Lung Cancer*. 2012;75(1):58-65.

Mukhopadhyay S, Dermawan JK, Lanigan CP, et al. Insulinoma-associated protein 1 (INSM1) 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):100-109.

Nakra T, Nambirajan A, Guleria P, et al. Insuloma-associated protein 1 is a robust nuclear immunostain for the diagnosis of small cell lung carcinoma in cytology smears. *Cancer Cytopathol*. 2019;127(8):539-548.

Nonaka D, Papaxoinis G, Mansoor W. Diagnostic utility or orthopedia homeobox (OTP) in pulmonary carcinoid tumors. *Am J Surg Pathol*. 2016;40(6):738-744.

Olsen SH, Thomas DG, Lucas DR. Cluster analysis of immunohistochemical profiles in synovial sarcoma, malignant peripheral nerve sheath tumor, and Ewing sarcoma. *Mod Pathol*. 2006;19(5):659-668.

Parkash V, Gerald WL, Parma A, et al. Desmoplastic small round cell tumor of the pleura. *Am J Surg Pathol*. 1995;19(6):659-665.

Pelosi G, Rindi G, Travis WD, et al. Ki-67 antigen in lung neuroendocrine tumors: unraveling a role in clinical practice. *J Thorac Oncol*. 2014;9(3):273-284.

Pelosi G, Sonzogni A, Harari S, et al. Classification of pulmonary neuroendocrine tumors: new insights. *Transl Lung Cancer Res*. 2017;6(5):513-529.

Rekhtman N. Neuroendocrine tumors of the lung: an update. *Arch Pathol Lab Med*. 2010; 134(11):1628-1638.

Rekhtman N, Pietanza MC, Hellmann MD, et al. Next-generation sequencing of pulmonary large cell neuroendocrine carcinoma reveals small cell carcinoma-like non-small cell carcinoma-like subsets. *Clin Cancer Res*. 2016;22(14):3618-3629.

Rooper LM, Sharma R, Li QK, et al. INSM1 demonstrates superior performance to the individual combined use of synaptophysin, chromogranin and CD56 for diagnosing neuroendocrine tumors of the thoracic cavity. *Am J Surg Pathol*. 2017;41(11):1561-1569.

Rosenbaum JN, Guo Z, Baus RM, et al. INSM1: a novel immunohistochemical and molecular marker for neuroendocrine and neuroepithelial neoplasms. *Am J Clin Pathol*. 2015;144(4):579-591.

Roy M, Buehler DG, Zhang R, et al. Expression of insulinoma-associated protein 1 (INSM1) and orthopedia homeobox (OTP) in tumors with neuroendocrine differentiation at rate sites. *Endocr Pathol*. 2019;30(1):35-42.

Stathis A, Zucca E, Bekradda M, et al. Clinical response of carcinomas harboring the BRD4-NUT oncoprotein to the targeted bromodomain inhibitor OTX015/MK-8628. *Cancer Discov*. 2016;6(5):492-500.

Sturm N, Lantuejoul S, Laverriere MH, et al. Thyroid transcription factor 1 and cytokeratins 1, 5, 10, 14 (34betaE12) expression in basaloid and large-cell

neuroendocrine carcinomas of the lung. *Hum Pathol*. 2001;32(9):918-925.

Sturm N, Rossi G, Lantuejoul S, et al. 34BetaE12 expression along the whole spectrum of neuroendocrine proliferations of the lung, from neuroendocrine cell hyperplasia to small cell carcinoma. *Histopathology*. 2003;42(2):156-166.

Travis WD, Brambilla, E, Burke, AP, et al. *WHO Classification of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015. (a)

Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification. *J Thorac Oncol*. 2015;10(9):1243-1260. (b)

Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol*. 2011;6(2):244-285.

Uherova P, Valdez R, Ross CW, et al. Nodular lymphocyte predominant Hodgkin lymphoma. An immunophenotypic reappraisal based on a single-institution experience. *Am J Clin Pathol*. 2003;119(2):192-198.

Viswanathan K, Borczuk AC, Siddiqui MT. Orthopedia homeobox protein (OPT) is a sensitive and specific marker for primary pulmonary carcinoid tumors in cytologic and surgical specimens. *J Am Soc Cytopathol*. 2019;8(1):39-46.

Weissferdt A, Moran CA. Primary pulmonary primitive neuroectodermal tumor (PNET): a clinicopathological and immunohistochemical study of six cases. *Lung*. 2012;190(6):677-683.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Ye B, Cappel J, Findeis-Hosey J, et al. hASH1 is a specific immunohistochemical marker for lung neuroendocrine tumors. *Hum Pathol*. 2016;48:142-147.

Zacharias J, Nicholson AG, Ladas GP, et al. Large cell neuroendocrine carcinoma and large cell carcinomas with neuroendocrine morphology of the lung: prognosis after complete resection and systematic nodal dissection. *Ann Thorac Surg*. 2003;75(2):348-352.

Proliferation Markers

By Fernando Lopez-Rios, Masayuki Noguchi, and Wendy A. Cooper

11

Introduction

There are many proliferation-associated or cell-cycle regulating markers such as Ki-67, proliferating cell nuclear antigen (PCNA), topoisomerase, MCM, and epithelial cell transforming 2 (ECT2). Among them, immunohistochemical staining for Ki-67 is a widely used method to estimate the proliferative activity of tumors. Ki-67 is a DNA-binding protein that is encoded by the *MKI67* gene located on chromosome 10 in humans. Ki-67 is expressed in all active phases of the cell cycle but not in G_0 (Chirieac 2016; Rekhtman et al 2019a).

When Should a Proliferation Marker Be Used in Diagnosis?

Although assessment of the Ki-67 proliferation rate can assist in confirming a highly proliferative tumor, there are no primary thoracic tumors that require Ki-67 for diagnostic criteria assessment. Mitotic count assessment is required for categorizing and grading pulmonary neuroendocrine tumors, but this can sometimes be difficult to assess, particularly in small crushed biopsies or suboptimal cytology specimens. In these instances, a Ki-67 proliferative marker can be helpful to assist in distinguishing carcinoid tumors from high-grade neuroendocrine carcinomas (large cell neuroendocrine carcinomas and small cell carcinomas) (Yatabe et al 2019) (Figure 11-1). A Ki-67 proliferative index threshold of 20% has been suggested as the upper limit for atypical carcinoid tumors (Travis et al 2015) and 40% to 50% as the lower limit of high-grade neuroendocrine carcinomas (Yatabe et al 2019). However, from a practical standpoint, small cell carcinomas usually exhibit very high Ki-67 proliferation rates of 50% to 100% (Travis et al 2015; Rekhtman 2010) (Figure 11-2).

Summary Answer

A proliferation marker such as Ki-67 can be useful in small crushed biopsies or cytology samples to assist in the distinction of carcinoid tumors from high-grade neuroendocrine carcinomas as crushed poorly preserved cells can mimic high-grade tumors. Proliferative

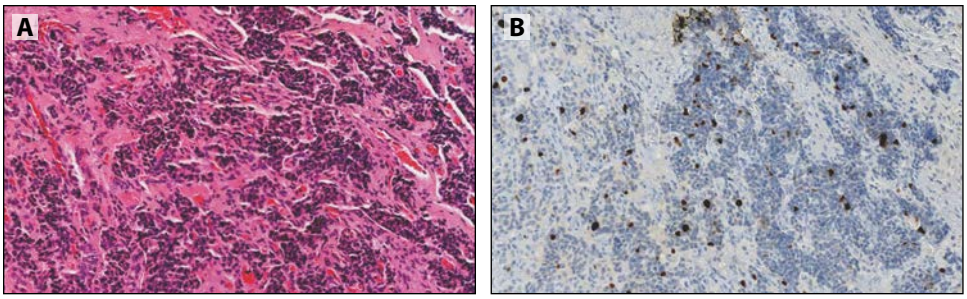


Figure 11-1. Ki-67 staining showing a low proliferative index can help identify carcinoid tumors in crushed biopsies that can morphologically mimic high-grade neuroendocrine tumors: **(A)** H&E, ×400; **(B)** Ki-67, ×400.

markers are not required in routine diagnostic assessment of primary thoracic neuroendocrine tumors or any other thoracic tumors.

What Is the Role of Ki-67 in Distinguishing Typical and Atypical Carcinoid Tumors?

In pulmonary (and thymic) carcinoid tumors, assessment of mitotic rate and presence or absence of necrosis is used to distinguish typical from atypical carcinoid tumors using the 2015 World Health Organization (WHO) classification (Travis et al 2015). This classification does not require Ki-67 to diagnose or grade thoracic neuroendocrine tumors. Although this is somewhat controversial because Ki-67 is used for routine assessment of enteropancreatic neuroendocrine tumors (Marchevsky et al 2018; Naheed et al 2019), the biology of neuroendocrine tumors arising in different anatomic locations is not necessarily the same (Pelosi et al 2014), and there is a relative lack of data to support Ki-67 in pulmonary neuroendocrine tumors. Carcinoid tumors can display a range of Ki-67 staining (and there is some data suggesting a Ki-67 proliferative index range of 2.3%-4.15% is seen in typical carcinoid tumors and 9%-17.8% in atypical carcinoid tumors) (Pelosi et al 2014). Recently, several tripartite divisions of pulmonary carcinoid tumors using the Ki-67 labeling index alone (<10%, between 10% and 19%, and ≥20%) or combined with histology (typical carcinoids with Ki-67 <5%, typical carcinoids with Ki-67 ≥5%, and atypical carcinoids) are linked to prognosis (Marchevsky et al 2018; Pelosi et al 2019). A recent study of 165 carcinoid tumors found that the Ki-67 index (assessed using a digital algorithm) was significantly increased in atypical versus typical carcinoids and was the only significant predictor of disease recurrence in the cohort (Dermawan and Farver 2020).

Summary Answer

There is currently no established role for routine assessment of Ki-67 in distinguishing typical and atypical carcinoid tumors.

What Level of Concordance Is There Between Proliferative Index in Biopsy Samples and Surgical Specimens?

Most of the available evidence in comparing proliferative index in different specimen types relates to carcinoid tumors. The proliferative index in carcinoid tumors may differ between small biopsy and resection specimens (Figure 11-3). The level of concordance is controversial, partly relating to the lack of standardized scoring approaches (see the "How Is Ki-67

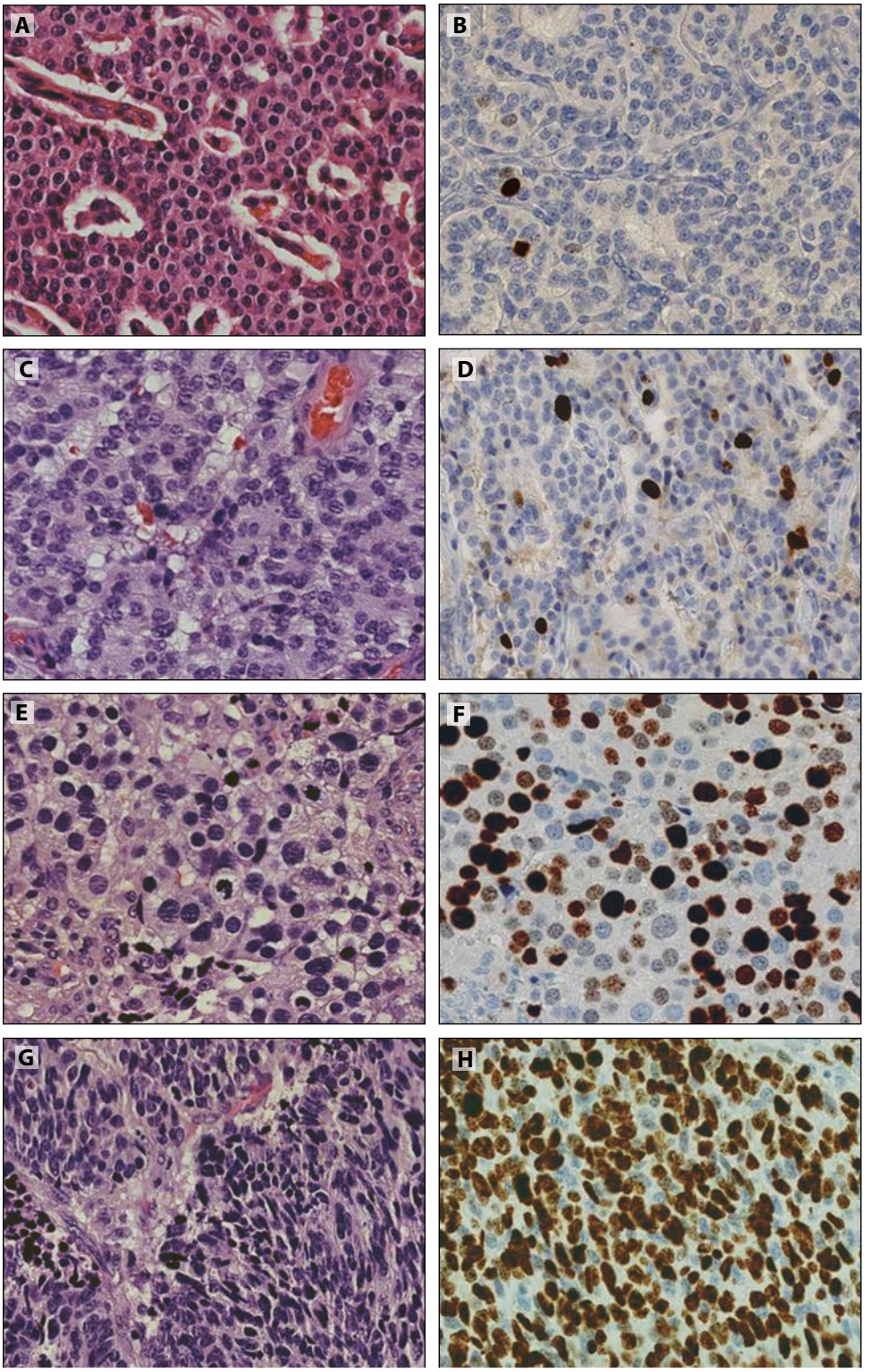


Figure 11-2. The spectrum of lung neuroendocrine tumors includes **(A and B)** typical carcinoid tumors, **(C and D)** atypical carcinoid tumors, **(E and F)** large cell neuroendocrine carcinoma, and **(G and H)** small cell carcinoma; (*left column*) H&E, ×400 and (*right column*) Ki-67 (MIB-1 clone), ×400.

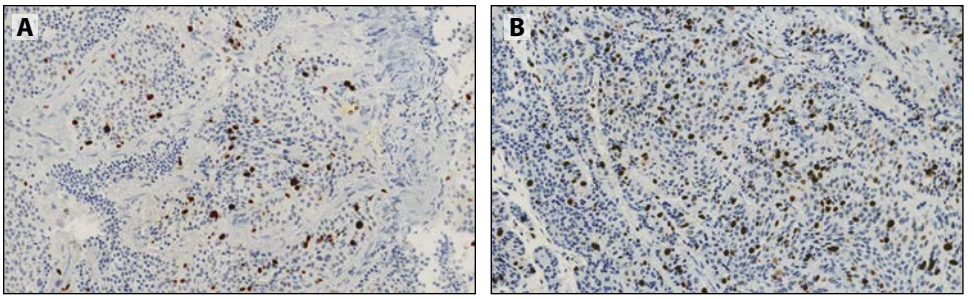


Figure 11-3. The labeling index of this carcinoid tumor was **(A)** 8.96% in the initial bronchial biopsy and **(B)** 17.25% in the surgical resection specimen. Ki-67 (MIB-1 clone), ×400.

Evaluated" section) (Fabbri et al 2017; Boland et al 2020), heterogeneity of Ki-67, and the biology of these tumors with higher Ki-67 and mitotic rates often seen in metastatic samples compared to primary tumors (Rekhtman et al 2019b).

Summary Answer

The concordance of Ki-67 proliferative index between small biopsy and resection specimens has not been well characterized.

What Is the Prognostic Role of Ki-67 in Non-Small Cell Lung Carcinomas?

Several studies and meta-analyses have suggested that a high Ki-67 level is a negative prognostic factor for patients with non-small cell carcinoma (Wei et al 2018; Yatabe et al 2019; Chirieac 2016). The lack of good quality data based on a standardized assessment of Ki-67 along with the lack of clear clinical utility has prevented its use in routine clinical practice.

Summary Answer

There is no established clinical role for assessment of Ki-67 as a prognostic marker in non-small cell carcinomas.

Does the Ki-67 Immunohistochemical Antibody Matter?

There are several Ki-67 antibodies commercially available (Biocompare, n.d.) and the MIB-1 clone is the most frequently used, although there are no good quality data available comparing different antibody clones in lung tumors (Pelosi et al 2014). The use of CytoLyt fixation inhibits MIB-1 immunoreactivity, so the Ki-67 30-9 clone has recently been recommended for CytoLyt-fixed cell blocks (Buonocore et al 2019). Alternatively, fixation in neutral buffered formalin may avoid this problem (Figure 11-4). If antigen activation (heat-induced epitope retrieval) is correctly performed, under-fixation (<6 hours) may be more detrimental to MIB-1 staining rather than over-fixation.

Summary Answer

The MIB-1 clone is the most frequently used antibody to assess Ki-67, although there are little data comparing different clones in lung tumors.

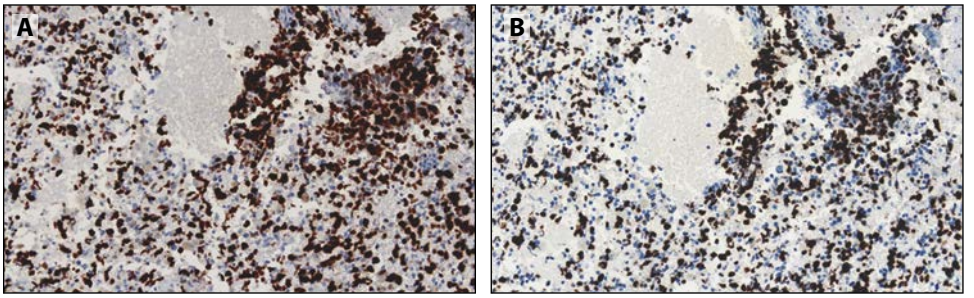


Figure 11-4. This cell block from a metastatic small cell carcinoma was fixed in buffered formalin. No significant differences can be seen in the expression of Ki-67 when **(A)** the MIB-1 clone was compared with **(B)** the 30-9 clone. Ki-67 (MIB-1 clone), ×400.

How Is Ki-67 Evaluated?

A standard scoring method has not been established for pulmonary neuroendocrine tumors (or any thoracic tumors), and reproducibility studies comparing different approaches are lacking. Most studies have focused on carcinoid tumors (Rekhtman et al 2019b; Marchevsky et al 2018; Boland et al 2020) and have used the hot spot method and manual counting of positive cells (Marchevsky et al 2018). In tumors with homogeneous expression, a single value for the Ki-67 proliferative index can be calculated based on the percentage of cells showing nuclear Ki-67 staining in (1) a ×20 field or 2 mm^2 area, or (2) 500 to 2000 cells (Rekhtman et al 2019b; Pelosi et al 2019). In cases of heterogeneity, both hot spot and average Ki-67 values have been used (Rekhtman et al 2019b). Automated quantification with validated nuclear algorithms may contribute to standardization (Boland et al 2020), but this approach is not widely available.

Summary Answer

There is no established standardized approach for evaluating Ki-67 in thoracic tumors.

Conclusions

There is currently no role for routine use of immunohistochemical proliferation markers in the diagnostic assessment of pulmonary neuroendocrine tumors (or other thoracic tumors). In the setting of small crushed biopsies or suboptimal cytology specimens of neuroendocrine tumors, Ki-67 can be useful to assist in the distinction of carcinoid tumors from high-grade neuroendocrine carcinomas and to avoid the diagnostic pitfall of over-diagnosing high-grade neuroendocrine tumors.

References

Biocompare. Anti-Ki-67 antibody products. Accessed March 30, 2020. https://www.biocompare.com/pfu/110447/soids/3219/Antibodies/Ki-67

Boland JM, Kroneman TN, Jenkins SM, et al. Ki-67 labeling index in pulmonary carcinoid tumors: comparison between small biopsy and resection using tumor tracing and hot spot methods. *Arch Pathol Lab Med*. Published online January 16, 2020. doi:10.5858/arpa.2019-0374-OA

Buonocore DJ, Konno F, Jungbluth AA, et al. CytoLyt fixation significantly inhibits MIB1 immunoreactivity whereas alternative Ki-67 clone 30-9 is not susceptible to the inhibition: critical diagnostic implications. *Cancer Cytopathol*. 2019;127(10):643-649.

Chirieac LR. Ki-67 expression in pulmonary tumors. *Transl Lung Cancer Res*. 2016;5(5):547-551.

Dermawan JKT, Farver CF. The role of histologic grading and Ki-67 index in predicting outcomes in pulmonary carcinoid tumors. *Am J Sug Pathol*. 2020;44(2):224-231.

Fabbri A, Cossa M, Sonzogni A, et al. Ki-67 labeling index of neuroendocrine tumors of the lung has a high level of correspondence between biopsy samples and surgical specimens when strict counting guidelines are applied. *Virchows Arch*. 2017;470(2):153-164.

Marchevsky AM, Hendifar A, Walts AE. The use of Ki-67 labeling index to grade pulmonary well-differentiated neuroendocrine neoplasms: current best evidence. *Mod Pathol*. 2018;31(10):1523-1531.

Naheed S, Holden C, Tanno L, et al. The utility of Ki-67 as a prognostic biomarker in pulmonary neuroendocrine tumours: protocol for a systematic review and meta-analysis. *BMJ Open*. 2019;9(8):e031531.

Pelosi G, Massa F, Gatti G, et al. Ki-67 evaluation for clinical decision in metastatic lung carcinoids: a proof of concept. *Clin Pathol*. 2019;12:2632010X19829259.

Pelosi G, Rindi G, Travis WD, et al. Ki-67 antigen in lung neuroendocrine tumors: unraveling a role in clinical practice. *J Thorac Oncol*. 2014;9(3):273-284.

Rekhtman N. Neuroendocrine tumors of the lung: an update. *Arch Pathol Lab Med*. 2010;134(11):1628-1638.

Rekhtman N, Baine MK, Bishop JA. *Quick Reference Handbook for Surgical Pathologists*. 2nd ed. Springer; 2019. (a)

Rekhtman N, Desmeules P, Litvak AM, et al. Stage IV lung carcinoids: spectrum and evolution of proliferation rate, focusing on variants with elevated proliferation indices. *Mod Pathol*. 2019;32(8):1106-1122. (b)

Travis WD, Brambilla E, Burke AP, et al. eds. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015.

Wei DM, Chen WJ, Meng RM, et al. Augmented expression of Ki-67 is correlated with clinicopathological characteristics and prognosis for lung cancer patients: an up-dated systematic review and meta-analysis with 108 studies and 14,732 patients. *Respir Res*. 2018;19(1):150.

Yatabe Y, Dacic S, Borcuk AC, et al. Best practice recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Immunohistochemistry in Cytology

12

By Lukas Bubendorf, Yuchen Han, and Andre L. Moreira

Introduction

The ability to perform highly accurate immunostaining in cytologic specimens is crucial because up to 40% of all lung cancer diagnoses are made by cytology alone. Cytology is not only equivalent to histology for subtyping non-small cell carcinoma (NSCC), but also an attractive, minimally invasive method to collect tumor material for repetitive biomarker analysis on recurrent or metastatic disease (Sigel et al 2011). Transbronchial endoscopic ultrasound-guided fine-needle aspiration (EBUS-FNA) is among the most rewarding cytologic techniques. However, other modalities, such as transthoracic FNA, bronchial secretions or brushes, bronchoalveolar lavage and pleural effusions, or FNA from distant metastatic sites are also important. The major difference and challenge in cytology relates to the greater variability of pre-analytical conditions and the lack of tissue contexture as compared to histology. With an escalating number of predictive biomarkers emerging in NSCC, immunohistochemistry (IHC) has been used as a rapid, cost-effective alternative to fluorescence in situ hybridization (FISH) and molecular testing in the screening of several of these alterations (Jain et al 2019). The current situation and challenges of IHC testing in cytology have recently been addressed in several publications (Zhou and Moreira 2016; Bubendorf et al 2017; Yatabe et al 2019; Jain et al 2019). The necessity to perform immunohistochemical testing on cytologic lung cancer specimens is undisputed.

What Portion of the Cytology Sample Is Best for Immunostaining: Cell Block or Air-Dried or Ethanol-Fixed Smears?

Cell Block Specimens

In principle, one can group cytologic preparations into *cell block* cytology and *non-cell block* cytology. Cell blocks are the most easily accessible cytology format for immunostaining because they can be handled in the same way as formalin-fixed paraffin-embedded (FFPE) histologic specimens for which immunostaining protocols are optimized

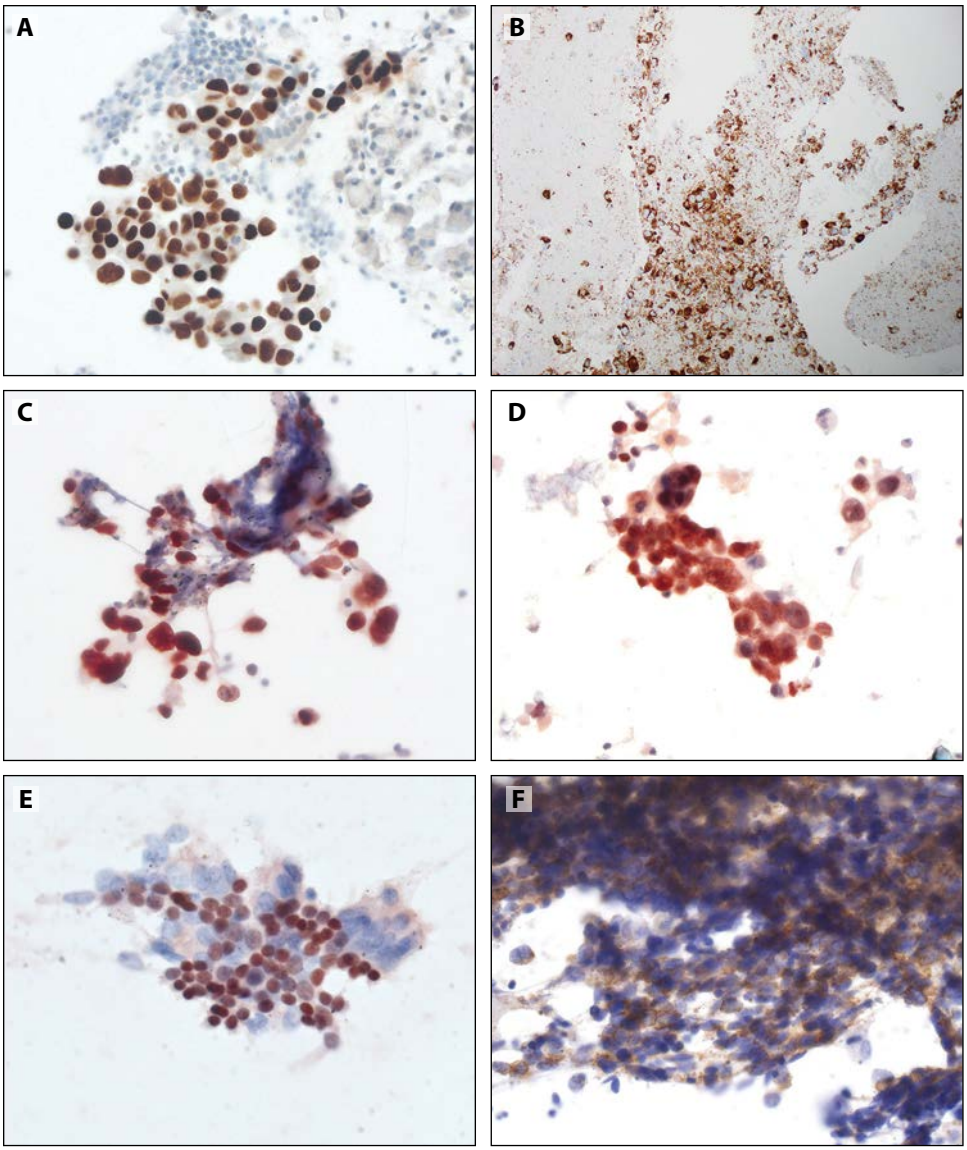


Figure 12-1. (A) Thyroid transcription factor-1 (TTF1)-positive non-small cell carcinoma (NSCC) on cell block from endoscopic ultrasound-guided (EBUS)-transbronchial needle aspiration (TBNA) using 3,3′-diaminobenzidine (DAB) chromogen (BenchMark ULTRA, ×400). **(B)** Napsin A–positive NSCC on cell block from EBUS-TBNA using DAB chromogen (BenchMark ULTRA, ×400). **(C-F)** IHC on previously ethanol-fixed and Papanicolaou-stained smears using 3-amino-9-ethylcarbazole (AEC) chromogen (Leica Bond). **(C)** TTF1-positive NSCC in EBUS-TBNA (×400). **(D)** p40-positive squamous cell carcinoma in brush cytology (×630). **(E)** p40-positive benign basal cells underlying ciliated respiratory cells (×400). **(F)** CD56 expression in small cell carcinoma with membranous accentuation (TBNA, ×400).

(Figure 12-1A and B). This is supported by studies showing highly concordant results for different markers between cell blocks and matched histologic specimens (Yatabe et al 2019). However, lack of international standards for pre-fixation methods and preparation protocol remains a major issue in cell blocks (Jain et al 2014; Saqi 2016). Currently, more than 10 methods for cell block preparations are in use, the most common ones in the United States being plasma thrombin, HistoGel (Thermo Fisher Scientific), Cellient automated cell block

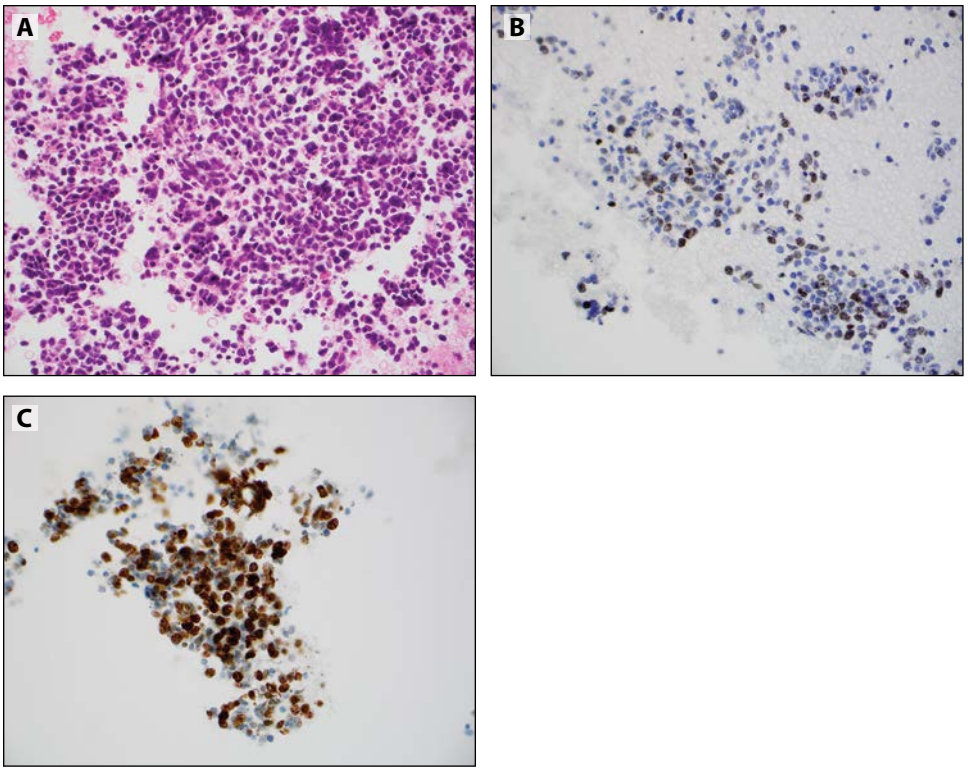


Figure 12-2. (A) H&E stained section of a cell block for a neuroendocrine tumor, stain for chromogranin and synaptophysin were positive. The specimen was fixed with ethanol **(B)** and formalin **(C)**, and immunostained. **(B)** IHC stain for Ki-67 (clone MIB-1) showing low positivity for the marker, which is suggestive of a low-grade carcinoid tumor. **(C)** IHC stain for Ki-67 (clone 30.9) is positive in more than 50% of tumor cells, which is suggestive of a high-grade neuroendocrine (NE) tumor. (Images provided by Dr. N. Rekhtman, Memorial Sloan Kettering Cancer Center)

system (Hologic) (Crapanzano et al 2014), and modifications of these (Rekhtman et al 2018). 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 fixation ranges from fixing the cell material in 10% buffered formalin, pre-fixation in ethanol or methanol-based solution before formalin fixation, or even pure fixation in 95% ethanol.

Although the large variety of transport media, pre-fixatives, and cell block protocols appears not to cause systematic problems on immunostaining according to a previous survey (Fischer et al 2014), recent analyses pinpoint specific challenges related to pre-analytical factors in cell blocks, especially with ethanol or methanol pre-fixation (Zhou and Moreira 2016). In addition to absent or near absent expression of thyroid transcription factor-1 (TTF1) with CytoLyt fixative (Gruchy et al 2015), nearly half (43%) of 30 antibodies tested on the Cellient (Hologic) cell block system failed initial validation using conditions established for FFPE tissue specimens on the BenchMark XT Automated IHC/ISH staining instrument (Sauter et al 2016). In addition, awareness of the impact of antibody clone and pre-fixation conditions can prevent significant problems when evaluating diagnostic IHC. Figure 12-2 highlights the effect of alcohol-based fixation in a commonly used clone of Ki-67 (Buonocore et al 2019).

Although FFPE cell blocks have become the predominant type of cytology preparation for IHC analysis, they have some limitations. Cell blocks are more time consuming,

costlier, and more technically challenging than non-cell block preparations, and they are not uniformly available in every patient and across all laboratories (Nambirajan and Jain 2018; Hendry et al 2020).

Non-Cell Block Specimens

Non-cell block cytology specimens consist of a variety of preparations, which include air-dried and alcohol-fixed smears, cytospin specimens (Cytospin, Thermo Fisher Scientific), ThinPrep (Hologic), or SurePath (Becton Dickinson) liquid-based preparations. The large variety of pre-analytical conditions and preparation methods makes standardization of immunostaining on non-cell block specimens even more challenging than in cell blocks. The most widely used fixatives in non-cell block cytology include the ethanol-based Saccomanno (50% ethanol and 2% polyethylene glycol), Delaunay (equal parts of ethanol and acetone admixed with 0.5 mL of 1 *M* trichloroacetic acid), and commercial spray fixatives, while the methanol-based CytoLyt or PreservCyt solutions (the latter being optimized for the ThinPrep liquid-based cytology slide preparation system) (Hologic) serve as preservatives and transport media. The ethanol-based hemolytic CytoRich Red collection fluid (Thermo Fisher Scientific) also contains formalin. All of these fixatives and transport media can be problematic for immunostaining, and frequent discordant results with histology have been obtained for some antibodies (Gong et al 2003; Skoog and Tani 2011; Sauter et al 2016; Gorman et al 2012; Buonocore et al 2019).

Nevertheless, in the U.K. National External Quality Assessment Service (UK NEQAS) for immunohistochemistry, nearly all non-formalin fixatives including Delaunay, methanol- and ethanol-based solutions, and the ethanol- and formalin-based CytoRich Red yielded similar quality of immunostaining as formalin alone (Kirbis et al 2011). Thus, a laboratory may use any of these fixatives as suited to their needs, the only caveat being the need to rigorously revalidate their immunohistochemistry procedure prior to clinical application. In fact, there is good evidence that with appropriate modifications in analytical factors, IHC on non-cell block preparations can be equivalent to IHC on FFPE tissue or cell block sections (Abendroth and Dabbs 1995; Leung and Bedard 1996; Denda et al 2012; Kalhor et al 2006; Savic et al 2013; Russell-Goldman et al 2018; Lozano et al 2019). Many laboratories that apply immunostaining to non-cell block specimens use the diagnostic Papanicolaou-stained slides (Fischer et al 2014; Schmitt et al 2011) (Figure 12-1C-F).

Prior Papanicolaou staining, which does not negatively interfere with the immunostaining reaction, allows triaging the available slides for immunostaining and marking areas of special interest. Variable results have been obtained with air-dried direct smears and cytospin specimens with some authors reporting complete lack of staining (Fischer et al 2014; Liu and Farhood 2004) while others report successful IHC on unstained slides post-fixed in formalin (Fulciniti et al 2008; Roh et al 2012) and/or alcohol/methanol-acetone (Skoog and Tani 2011). The counterstains of Papanicolaou stain are usually bleached out during endogenous peroxidase blocking and/or during antigen retrieval (Denda et al 2013). Prior to IHC, the pre-stained slides of non-cell block cytology need to be soaked in xylene to dissolve the permanent mounting media and remove the coverslip. This process takes a few hours for fresh specimens but may require up to several days in retrospective studies depending on the length of archival time. The epitopes in previously stained alcohol-fixed cytology slides

remain intact during an archiving period of at least 1 to 2 years, if properly sealed by a coverslip (Vlajnic et al 2018). Heat-induced antigen retrieval (HIAR) was found to be essential for uncovering epitope reactivity for all nuclear antigens and a subset of cytoplasmic and membranous antigens in alcohol-fixed Papanicolaou-stained smears (Denda et al 2012). HIAR has also been found to improve IHC staining for certain antigens on ThinPrep specimens (Zhang et al 2012) and for air-dried smears post-fixed in formalin (Roh et al 2012). A shorter duration of HIAR is usually sufficient for cytology smears as compared to FFPE tissue. HIAR should be optimized separately for each antibody.

Use of automated staining platforms would improve standardization and reproducibility of IHC results, but published data on non-cell block preparations are still scarce. Some laboratories work with the Leica Bond autostainer (Vlajnic et al 2018), the BenchMark XT/ULTRA platforms (Jain et al 2018; Martinez et al 2013; Lozano et al 2019), or the Dako autostainers (Noll et al 2018). External quality assessment is also important to maintain a high immunostaining quality. In fact, UK NEQAS has an external quality assessment program in place to help standardize and improve the quality of immunostaining in cytology (Kirbis et al 2011).

Summary Answer

All cytology preparations including cell blocks, ethanol-fixed, and air-dried slides can principally be used for immunostaining. Formalin-fixed cell blocks are most straightforward and most commonly used. Rigorous protocol optimization, validation, and quality control are required in immunostaining cytology specimens, particularly in non-cell block preparations.

How Reliable Is Predictive Immunohistochemical Biomarker Testing in Cytologic Lung Cancer Specimens?

Interest in IHC of cytology has steeply gained ground in the era of predictive biomarker testing, either as selective biomarker for treatment (eg, programmed death ligand-1 [PD-L1] and anaplastic lymphoma kinase [ALK]) or as a prescreening method for subsequent molecular testing (eg, *c-ros oncogene 1* [*ROS1*] and *neurotrophic tyrosine receptor kinase* [*NTRK*]). PD-L1 has clearly been the main driver of this development as evidenced by the greatly increasing number of publications on PD-L1 testing in cytology over the past few years (Gosney et al 2020). Validated predictive IHC assays were tailored to histologic specimens, and cytologic specimens had not been analyzed in related clinical trials with targeted agents or immune checkpoint inhibitors (Thunnissen et al 2018). Nevertheless, with advances in minimally invasive diagnostic procedures that yield predominantly cytology samples, there is increasing demand for predictive biomarker testing on cytology samples in clinical practice (Jain et al 2019). Assay revalidation is required when a validated IHC assay is performed on cytology specimens because of differences in their processing techniques (Fitzgibbons et al 2014). As discussed earlier, the greater variability in pre-analytical factors of cytology samples makes standardization of immunohistochemistry in cytology challenging.

PD-L1 IHC

PD-L1 testing on cell blocks has become a common diagnostic practice using the assays and protocols that were developed for histologic specimens. In fact, this practice has been

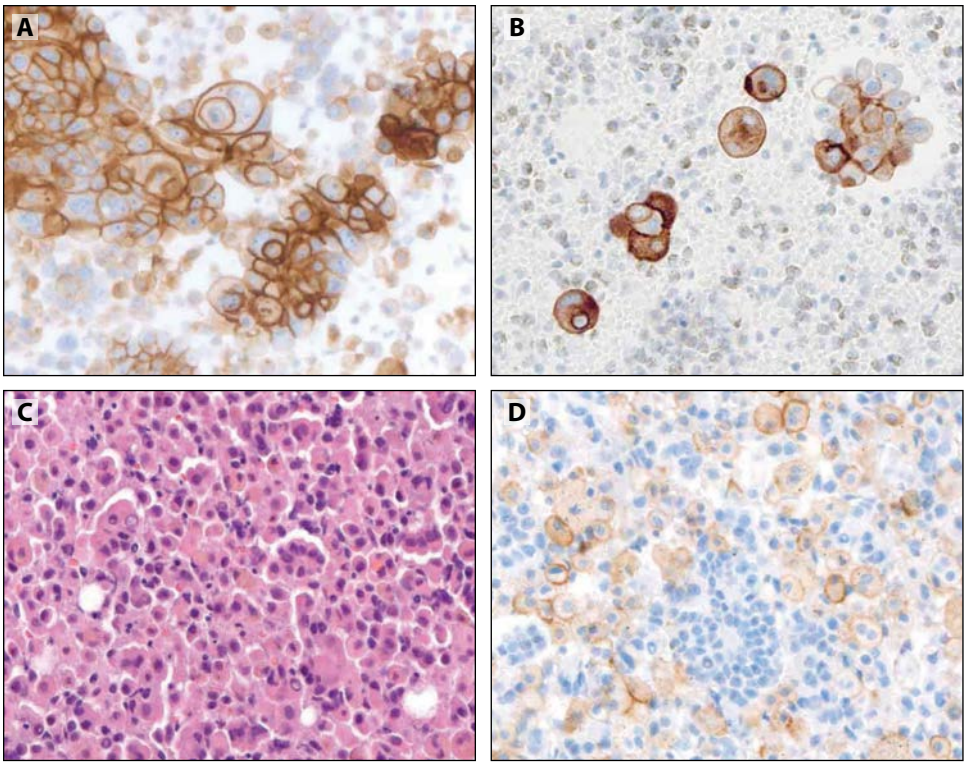


Figure 12-3. Programmed death ligand-1 (PD-L1) IHC on cell block non-small cell carcinoma (NSCC) specimens using VENTANA PD-L1 (SP263) assay on BenchMark ULTRA. Pronounced membranous staining in **(A)** all tumor cells or **(B)** most tumor cells (malignant effusions; ×400, each). **(C-D)** PD-L1 negative aggregates of adenocarcinoma cells and admixed focally pigmented histiocytes, most of which are weakly PD-L1 positive (H&E and PD-L1, FNA: ×400, each).

justified by the accumulated post hoc evidence showing a high success rate of PD-L1 IHC and a high concordance between cell blocks and matched histology as shown in a recent review of 9 eligible studies (Gosney et al 2020). In contrast, data on non-cell block specimens are only emerging but point in the same direction (Noll et al 2018; Jain et al 2018; Capizzi et al 2018; Lozano et al 2019; Munari et al 2019).

In histologic specimens, PD-L1 staining positivity is defined as complete circumferential or partial linear cytoplasmic membrane staining of tumor cells of any intensity. Only cytoplasmic staining in tumor cells is not considered positive for scoring purposes. This is also true for cell block sections (Figure 12-3A and B). In non-cell block cytologic specimens, however, membranous staining is less distinct because the cell membranes are intact and not cut as in FFPE tissue sections (Bubendorf et al 2017). Thus, PD-L1 staining of the horizontally oriented cell membrane can appear as a diffuse surface staining mimicking cytoplasmic staining (Figure 12-4). Overestimation of PD-L1 positivity caused by nonspecific cytoplasmic staining of background macrophages and inflammatory cells can occur in both cell block and non-cell block specimens and can be particularly challenging in effusion samples with predominantly singly lying tumor cells admixed with inflammatory cells and mesothelial cells (Figures 12-3C and D and 12-4D). A confirmatory immunostain such as TTF1 and/or a pan-leukocyte marker (eg, CD45) performed on a corresponding section can aid in confirmation of tumor cells for scoring of PD-L1 positivity. Considering these inherent difficulties in

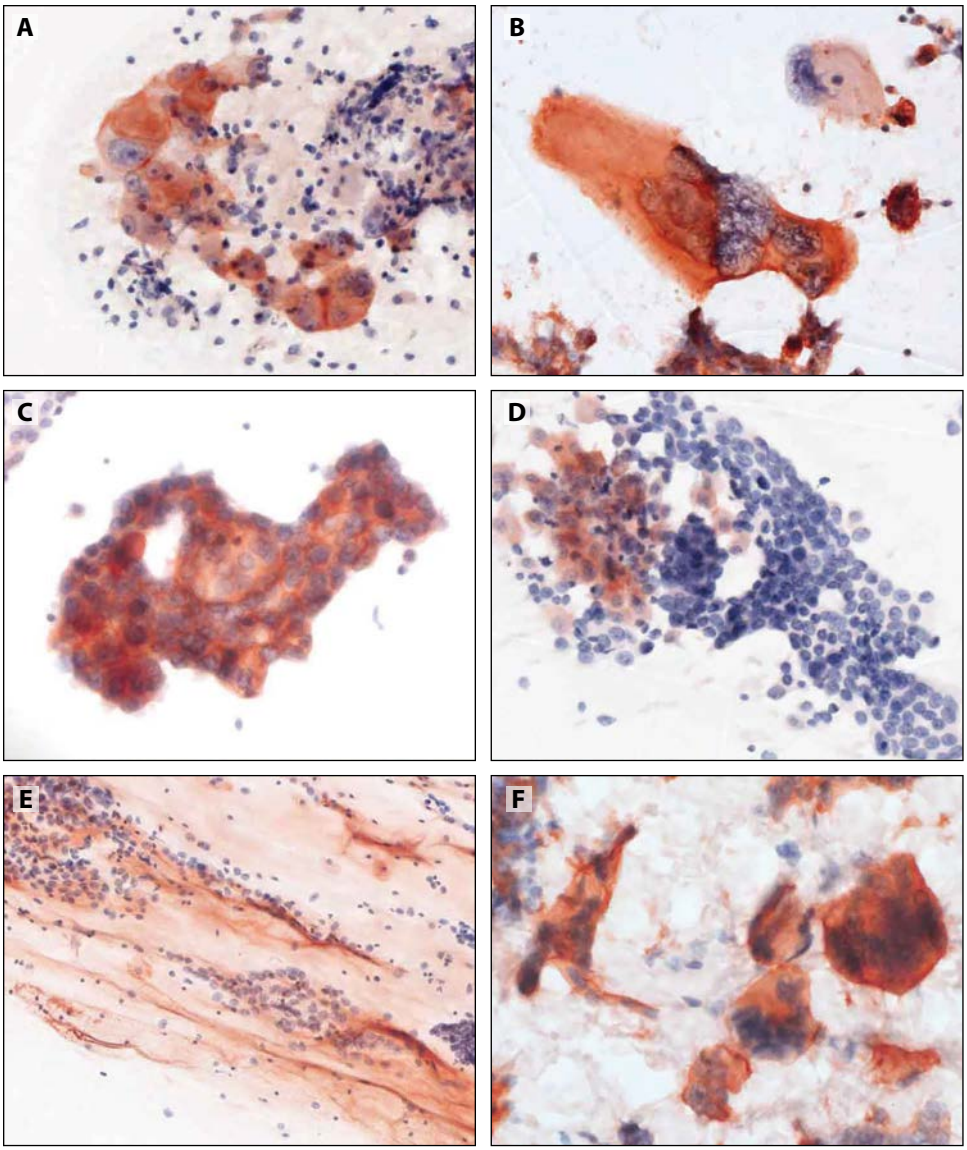


Figure 12-4. Programmed death ligand-1 (PD-L1) IHC of ethanol-fixed and Papanicolaou-stained NSCC cytology specimens using PD-L1 laboratory-developed test (LDTs) on Leica Bond. **(A)** PD-L1 positive tumor cells with focal membranous accentuation (VENTANA PD-L1 [SP263] assay, ×400). **(B)** Pleomorphic tumor cell with diffuse cytoplasmic-like staining (VENTANA PD-L1 [SP142] assay, ×630). **(C)** Tumor cells with cytoplasmic-like staining and focal membranous accentuation (Leica 73-10, ×400). **(D)** PD-L1 negative tumor cells with macrophages serving as internal positive staining control (VENTANA PD-L1 [SP263] assay, ×400). **(E)** Unspecific weak staining of mucin and tumor cells are PD-L1 negative (VENTANA PD-L1 [SP263] assay, ×200). **(F)** Dissociated placental trophoblastic cells serving as PD-L1 positive controls (VENTANA PD-L1 [SP263] assay, ×400).

PD-L1 IHC interpretation and the known heterogeneity of PD-L1 expression, testing should be avoided in cytologic samples with less than 100 tumor cells (Hendry et al 2020), while some others even suggest at least 400 tumor cells (Dong et al 2020). In contrast to tumor cell scoring, reliable immune cell (IC) scoring of cytologic specimens is almost impossible because of the lack of tissue architectural context and should therefore not be practiced.

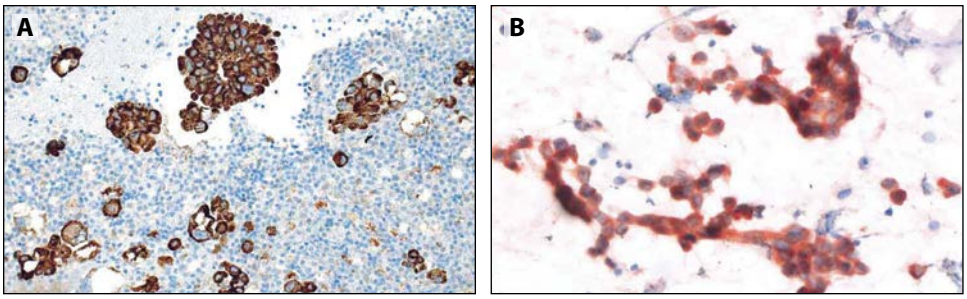


Figure 12-5. Two IHC of anaplastic lymphoma kinase (ALK) rearranged lung adenocarcinomas. Laboratory-developed test (LDTs) with 5A4 antibody (Novocastra) on **(A)** cell block from malignant effusion of lung adenocarcinoma (BenchMark ULTRA, 3,3'-diaminobenzidine [DAB], ×200), and **(B)** previously ethanol-fixed and Papanicolaou-stained transbronchial needle aspiration (TBNA) specimen (Leica Bond, 3-amino-9-ethylcarbazole [AEC], ×400).

ALK, ROS1, and pan-TRK IHC

IHC to detect overexpression of the ALK or ROS1 protein is a well-established method to screen NSCC for subsequent FISH or for further evaluation of uncertain FISH findings (as described in detail in Chapter 19). As with histologic specimens, ALK IHC is equally well applicable to cytologic specimens (Figure 12-5). Most studies on ALK IHC in cytology have been performed on FFPE cell blocks without modification of the IHC procedure, using 5A4 or D5F3 clones, on various automated staining platforms (Jain et al 2019). These studies have uniformly demonstrated 100% sensitivities, albeit with variable specificities (83%-100%), for the presence of ALK-rearrangement by FISH. Ethanol-fixed non-cell block preparations have also been tested by ALK IHC. Except for 2 studies that report 100% sensitivity on alcohol-fixed smears, other studies report relatively poor sensitivities by IHC, ranging from 66% to 86% (Jain et al 2019.) None of the latter studies used the highly sensitive VENTANA ALK (D5F3) CDx Assay that has been used in most cell block studies.

ROS1 IHC is highly accurate for prescreening of *ROS1*-rearranged lung cancers in histologic specimens and cell blocks as outlined in Chapter 19. This appears also to be the case for cytology smears and Cytospin specimens when using the D4D6 rabbit monoclonal antibody (Cell Signaling Technology, Inc.) on the Leica Bond automated immunostainer (Vlajnic et al 2018) (Figure 12-6). Data on a new VENTANA ROS1 (SP384) Rabbit Monoclonal Primary Antibody in cytologic specimens are not yet available. ROS1 IHC is highly sensitive but lacks specificity (Hung and Sholl 2018). The antibody stains macrophages and reactive pneumocytes and shows some reactivity in non-*ROS1* rearranged adenocarcinomas, therefore confirmation of the results by FISH or next-generation sequencing is recommended.

Testing for *NTRK1-3* rearrangements, a tumor-agnostic biomarker to select patients for treatment with NTRK inhibitors, has become another necessity in NSCCs and other solid tumors. Because of the very low prevalence of *NTRK* rearrangements in NSCC (<0.5%), prescreening by IHC has been recommended. Pan-TRK IHC using the anti-pan-TRK antibody EPR173 has been shown to be sensitive and specific in histologic specimens. A commercial Ventana in vitro diagnostics (IVD) assay for FFPE neoplastic tissue (Hechtman et al 2017) is available. It is foreseeable that pan-TRK will also work on cytology specimens, but such data are not yet available (Figure 12-7).

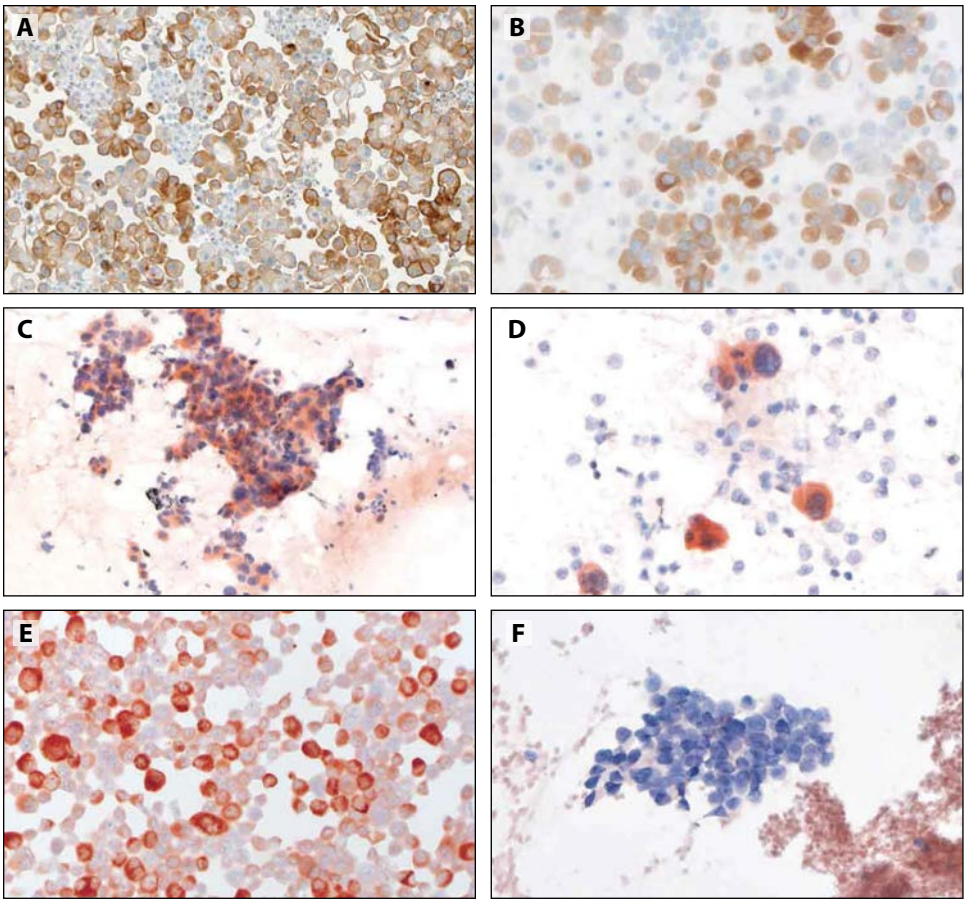


Figure 12-6. ROS1 IHC. **(A-B)** Cell blocks on BenchMark ULTRA using the Optiview DAB IHC detection kit) and **(C-F)** previously ethanol-fixed Papanicolaou-stained specimens using D4D6 antibody (Cell Signaling Technology, Inc.) on Leica Bond (AEC as chromogen). **(A)** Malignant effusion with c-ros oncogene 1 (*ROS1*) re-rearranged lung adenocarcinomas using D4D6 antibody (Cell Signaling Technology, Inc., ×200). **(B)** Unspecific ROS1 staining of a non-*ROS1* rearranged, Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutated lung adenocarcinoma using VENTANA ROS1 (SP384) Rabbit Monoclonal Primary Antibody assay (×400). **(C-D)** *ROS1*-rearranged lung adenocarcinomas showing homogenous cytoplasmic staining (×400, each). **(E)** *ROS1*-rearranged non-small cell carcinoma (NSCC) cell line HCC78 serving as positive staining control (×200). **(F)** ROS1-negative NSCC with hemorrhagic background (×400).

Summary Answer

Cytologic specimens can be used for predictive PD-L1, ALK, and ROS1 IHC, and cell blocks are currently the recommended preparations. The limited data on these predictive IHC on non-cell block slides are promising but need further confirmation.

Conclusions

It is undisputed that cytologic specimens serve as useful and indispensable resources for ancillary testing in lung cancer for both diagnostic and

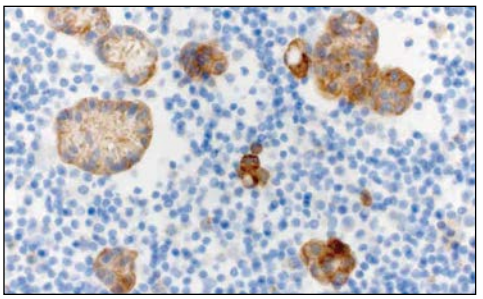


Figure 12-7. Cells of lung adenocarcinoma with confirmed *CLIP1-NTRK1* gene fusion. Positive immunohistochemistry with the Abcam pan-TRK antibody EPR17341 (cell block, ×400). (Image courtesy of Joachim Diebold, Lucerne)

predictive purposes including IHC. FFPE cell blocks are the preferred and most commonly used format that can be integrated in the existing technical workflow using tissue-based IHC protocols. Although IHC testing also works on non-cell block cytology specimens in experienced laboratories, further work is needed.

References

Abendroth CS, Dabbs DJ. Immunocytochemical staining of unstained versus previously stained cytologic preparations. *Acta Cytol*. 1995;39(3):379-386.

Bubendorf L, Lantuejoul S, de Langen AJ, et al. Nonsmall cell lung carcinoma: diagnostic difficulties in small biopsies and cytological specimens. In: Dorfmuller P, Cavazza A, eds. *Pathology for the Clinician*, vol. 2. *Eur Respir Rev*. 2017;26(144):170007.

Buonocore DJ, Konno F, Jungbluth AA, et al. CytoLyt fixation significantly inhibits MIB1 immunoreactivity whereas alternative Ki-67 clone 30-9 is not susceptible to the inhibition: critical diagnostic implications. *Cancer Cytopathol*. 2019;127(10):643-649.

Capizzi E, Ricci C, Giunchi F, et al. Validation of the immunohistochemical expression of programmed death ligand 1 (PD-L1) on cytological smears in advanced non small cell lung cancer. *Lung Cancer*. 2018;126:9-14.

Crapanzano JP, Heymann JJ, Monaco S, et al. The state of cell block variation and satisfaction in the era of molecular diagnostics and personalized medicine. *Cytojournal*. 2014;11:7.

Denda T, Kamoshida S, Kawamura J, et al. Optimal antigen retrieval for ethanol-fixed cytologic smears. *Cancer Cytoplathol*. 2012;120(3):167-176.

Denda T, Kamoshida S, Kwamura J, et al. Rapid immunocytochemistry with simple heat-induced antigen retrieval technique for improvement in the quality of cytological diagnosis. *J Histochem Cytochem*. 2013;61(12):920-930.

Dong Z, Liu Y, Jiang T, et al. Cell block as a surrogate for programmed death-ligand 1 staining testing in patients of non-small cell lung cancer. *J Cancer*. 2020;11(3):551-558.

Fischer AH, Schwartz MR, Moriarty AT, et al. Immunohistochemistry practices of cytopathology laboratories: a survey of participants in the College of American Pathologists Nongynecologic Cytopathology Education Program. *Arch Pathol Lab Med*. 2014;138(9):1167-1172.

Fitzgibbons PL, Bradley LA, Fatheree LA, et al. Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med*. 2014;138(11):1432-1443.

Fulciniti F, Frangella C, Staiano M, et al. Air-dried smears for optimal diagnostic immunocytochemistry. *Acta Cytol*. 2008;52(2):178-186.

Gong Y, Sun X, Michael CW, et al. Immunocytochemistry of serous effusion specimens: a comparison of ThinPrep vs cell block. *Diagn Cytopathol*. 2003;28(1):1-5.

Gorman BK, Kosarac O, Chakraborty S, et al. Comparison of breast carcinoma prognostic/predictive biomarkers on cell blocks obtained by various methods: cellient, formalin and thrombin. *Acta Cytol*. 2012;56(3):289-296.

Gosney JR, Boothman AM, Ratcliffe M, et al. Cytology for PD-L1 testing: a systematic review. *Lung Cancer*. 2020;141:101-106.

Gruchy JR, Barnes PJ, Dakin Hache KA. CytoLyt fixation and decalcification pretreatments alter antigenicity in normal tissues compares with standard formalin fixation. *Appl Immunohistochem Mol Morphol*. 2015;23(4):297-302.

Hechtman JF, Benayed R, Hyman DM, et al. Pan-TRK immunohistochemistry is an efficient and reliable screen for the detection of NTRK fusions. *Am J Surg Pathol*. 2017;41(11):1547-1551.

Hendry S, Byrne DJ, Christie M, et al. Adequate tumour cellularity is essential for accurate PD-L1 immunohistochemistry assessment on cytology cell-block specimens. *Cytopathology*. 2020;31(2):90-95.

Hung YP, Sholl LM. Diagnostic and predictive immunohistochemistry for non-small cell lung carcinomas. *Adv Anat Pathol*. 2018;25(6):374-386.

Jain D, Mathur SR, Iyer VK. Cell blocks in cytopathology: a review of preparative methods, utility in diagnosis and role in ancillary studies. *Cytopathology*. 2014;25(6):356-371.

Jain D, Nambirajan A, Borczuk A, et al. Immunocytochemistry for predictive biomarker testing in lung cancer cytology. *Cancer Cytopathol*. 2019;127(5):325-339.

Jain D, Sukumar S, Mohan A, et al. Programmed death-ligand 1 immunoexpression in matched biopsy and liquid-based cytology samples of advanced stage non-small cell lung carcinomas. *Cytopathology*. 2018;29(6):550-557.

Kalhor N, Zander DS, Liu J. TTF-1 and p63 distinguishing pulmonary small-cell carcinoma from poorly differentiated squamous cell carcinoma in

previously pan-stained cytologic material. *Mod Pathol*. 2006;19(8):1117-1123.

Kirbis IS, Maxwell P, Flezar MS, et al. External quality control for immunocytochemistry on cytology samples: a review UK NEQAS ICC (cytology module) results. *Cytopathology*. 2011;22(4):230-237.

Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. *Mod Pathol*. 1996;9(3):304-306.

Liu J, Farhood A. Immunostaining for thyroid transcription factor-1 on fine-needle aspiration specimens of lung tumors: a comparison of direct smears and cell block preparations. *Cancer*. 2004;102(2):109-114.

Lozano MD, Abengozar-Muela M, Echeveste JI, et al. Programmed death-ligand 1 expression on direct Pap-stained cytology smears from non-small cell lung cancer: comparison with cell blocks and surgical resection specimens. *Cancer Cytopathol*. 2019;127(7):470-480.

Martinez P, Hernandez-Losa J, Montero MA, et al. Fluorescence in situ hybridization and immunohistochemistry as diagnostic methods for ALK positive non-small cell lung cancer patients. *PLoS One*. 2013;8(1):e52261.

Munari E, Zamboni G, Sighele G, et al. Expression of programmed cell death ligand 1 in non-small cell lung cancer: comparison between cytologic smears, core biopsies, and whole sections using the SP263 assay. *Cancer Cytopathol*. 2019;127(1):52-61.

Nambirajan A, Jain D. Cell blocks in cytopathology: an update. *Cytopathology*. 2018;29(6):505-524.

Noll B, Wang WL, Gong Y, et al. Programmed death ligand 1 testing in non-small cell lung carcinoma cytology cell block and aspirate smear preparations. *Cancer Cytopathol*. 2018;126(5):342-352.

Rekhtman N, Buonocore DJ, Rudomina D, et al. Novel modifications of HistoGel-based cell block preparation method: improved sufficiency for molecular studies. *Arch Pathol Lab Med*. 2018;142(4):529-535.

Roh MH, Schmidt L, Placido J, et al. The application and diagnostic utility of immunocytochemistry on direct smears in the diagnosis of pulmonary adenocarcinoma and squamous cell carcinoma. *Diagn Cytopathol*. 2012;40(11):949-955.

Russell-Goldman E, Kravets S, Dahlberg SE, et al. Cytologic-histologic correlation of programmed death-ligand 1 immunohistochemistry in lung carcinomas. *Cancer Cytopathol*. 2018;126(4):253-263.

Saqi A. The state of cell blocks and ancillary testing: past, present, and future. *Arch Pathol Lab Med*. 2016;140(12):1318-1322.

Sauter JL, Grogg KL, Vrana JA, et al. Young investigator challenge: validation and optimization of immunohistochemistry protocols for use on cellient cell block specimens. *Cancer Cytopathol*. 2016;124(2):89-100.

Savic S, Bode B, Diebold J, et al. Detection of ALK-positive non-small-cell lung cancers on cytological specimens: high accuracy of immunocytochemistry with the 5A4 clone. *J Thorac Oncol*. 2013;8(8):1004-1011.

Schmitt F, Cochand-Priollet B, Toetsch M, et al. Immunocytochemistry in Europe: results of the European Federation of Cytology Societies (EFCS) inquiry. *Cytopathology*. 2011;22(4):238-242.

Sigel CS, Moreira AL, Travis WD, et al. Subtyping of non-small cell lung carcinoma: a comparison of small biopsy and cytology specimens. *J Thorac Oncol*. 2011;6(11):1849-1856.

Skoog L, Tani E. Immunocytochemistry: an indispensable technique in routine cytology. *Cytopathology*. 2011;22(4):215-229.

Thunnissen E, Allen TC, Adam J, et al. Immunohistochemistry of pulmonary biomarkers: a perspective from the members of the Pulmonary Pathology Society. *Arch Pathol Lab Med*. 2018;142(3):408-419.

Vlajnic T, Savic S, Barascud A, et al. Detection of ROS1-positive non-small cell cancer on cytological specimens using immunocytochemistry. *Cancer Cytopathol*. 2018;126(6):421-429.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practice recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Zhang Z, Zhao L, Guo H, et al. Diagnostic significance of immunocytochemistry on fine needle aspiration biopsies processed by thin-layer cytology. *Diagn Cytopathol*. 2012;40(12):1071-1076.

Zhou F, Moreira AL. Lung carcinoma predictive biomarker testing by immunoperoxidase stains in cytology and small biopsy specimens: advantages and limitations. *Arch Pathol Lab Med*. 2016;140(12):1331-1337.

Immunomarkers for Lung Adenocarcinoma Variants

13

By Mari Mino-Kenudson and Sanja Dacic

Introduction

Lung adenocarcinoma variants comprise invasive mucinous adenocarcinoma (IMA), colloid adenocarcinoma, enteric adenocarcinoma, and fetal adenocarcinoma. They exhibit characteristic cytomorphologic features, and immunohistochemistry (IHC) is usually not required for the diagnosis. Metastases from extrapulmonary sites, however, may share the same histologic features. Thus, IHC may be helpful in differentiating between primary and metastatic tumors, and clinicopathologic correlation is often necessary to establish a correct diagnosis. In addition, IHC may be useful in excluding non-malignant mimics to confirm the diagnosis of IMA.

Invasive Mucinous Adenocarcinoma

The diagnosis of IMA is typically made based on characteristic cytomorphology: tall columnar cells with abundant intracytoplasmic mucin and/or goblet cells comprising a significant fraction of tumor cells showing a lepidic pattern of growth. Thus, IHC is usually not required to render the diagnosis, but there are some circumstances in which IHC may be useful in confirming the diagnosis. These are (1) to support the diagnosis in a small tissue sample and (2) to differentiate from non-malignant processes including peribronchiolar metaplasia with mucinous metaplasia and ciliated muconodular papillary tumor (bronchiolar adenoma), among others. In addition, conventional adenocarcinoma rarely produces exuberant mucin and may be confused with IMA. As for the differentiation of IMA from a mucin-producing tumor of an extrapulmonary site, it can be extremely challenging given that the immunoprofile of IMA is shared with pancreatobiliary and gastrointestinal (GI) tract primary adenocarcinomas.

What Is the Immunoprofile of Invasive Mucinous Adenocarcinoma?

Most IMAs are either negative or only focally immunoreactive to thyroid transcription factor-1 (TTF1) and napsin A, while almost all lesions express cytokeratin 7 (CK7)

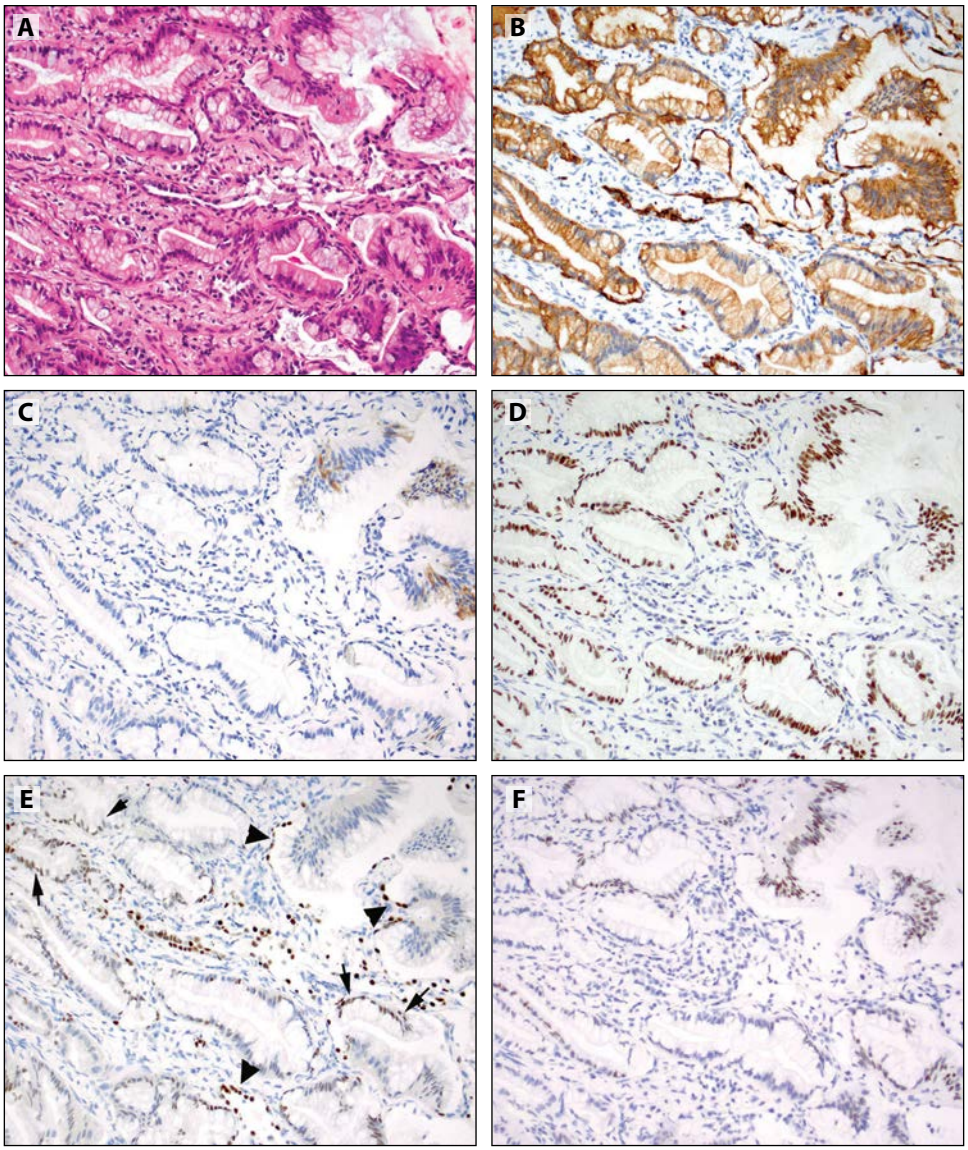


Figure 13-1. (A) An example of invasive mucinous adenocarcinoma demonstrating tall columnar cells with abundant cytoplasmic mucin and scattered goblet cells in acinar and lepidic patterns. **(B)** The lesional cells are diffusely positive for cytokeratin 7 (CK7) and **(C)** focally positive for CK20. **(D)** A hepatocyte nuclear factor 4 alpha (HNF4α) immunostain highlights almost all tumor cell nuclei, while **(E,** *arrows***)** nuclear expressions of thyroid transcription factor-1 (TTF1) and **(F)** CDX2 are limited to subsets of tumor cells. CK20 and CDX2 tend to co-locate. Of note, **(E,** *arrowheads***)** the background pneumocytes exhibit strong nuclear expression of TTF1.

(Figure 13-1). Concurrently, CK20 and an intestinal transcription factor, CDX2, are positive in 40% to 50% of IMAs (Yatabe et al 2019). Interestingly, most IMAs, in particular those that lack TTF1 expression, react to hepatocyte nuclear factor 4 alpha (HNF4α), which is another nuclear transcription factor found in the hepatobiliary and GI tracts (Sugano et al 2013).

Are Immunostains Useful in the Diagnosis of Invasive Mucinous Adenocarcinoma?

Mucinous adenocarcinomas of extrapulmonary sites may exhibit a lepidic pattern of growth when metastatic to the lung; thus, the differentiation of primary lung IMA from these tumors

can be extremely challenging based on the morphology alone. Unfortunately, IHC may not be useful in this context, given that IMA often expresses intestinal differentiation markers including CDX2 and HNF4α along with CK7, which are markers that are shared with pancreatobiliary and upper GI tract primaries. Significant TTF1 expression however, is supportive for a lung primary tumor.

Conversely, IHC can be useful in differentiating IMA from non-malignant entities. For example, IMA comprises a significant fraction of lung cancers that develop in patients with usual interstitial pneumonia (UIP) (Masai et al 2016; Calio et al 2017), and mucinous metaplasia, which is often seen in association with peribronchiolar metaplasia/traction bronchiolectasis found in UIP, may mimic IMA. Given its often well-differentiated morphology, diagnosing IMA in a background of interstitial fibrosis and/or in a small biopsy specimen can be challenging. In this context, a panel of TTF1 (± napsin A), p40, and HNF4α stains can be useful. Nonneoplastic proliferations retain p40 expressing basal cells and do not express HNF4α. Ciliated muconodular papillary tumor is another important differential diagnosis because of its abundant mucin pooling, presence of mucinous cells, and diverse growth patterns, which can closely resemble IMA (Lu and Yeh 2019). The absence of p40+ basal cells along with the lack of cilia in a group of mucinous cells lining alveolar walls or forming glands supports the diagnosis of IMA (Chang et al 2018).

Figures 13-2E and F show another example of IMA from a patient with interstitial fibrosis. There are mucinous glands with mildly irregular contour but no overt cytologic atypia in the background of fibrous stroma and a few foci of peribronchiolar metaplasia with or without mucin (Figure 13-2E, *arrows*). A p40 immunostain reveals positive nuclei in the bronchioles and alveolar parenchyma with peribronchiolar metaplasia (Figure 13-2F), while the groups of mucinous glands are completely negative for p40 (the absence of basal cells) supporting the diagnosis of IMA.

Summary Answer

IMA often expresses intestinal differentiation markers including CDX2 and HNF4α along with CK7, while expression of TTF1 and napsin A are limited; thus, the differentiation between IMA and metastasis from an extrapulmonary primary tumor, in particular, an upper GI or pancreatobiliary primary tumor may be extremely challenging without clinicopathologic correlation. Conversely, IHC can be useful in differentiating IMA from non-malignant entities and non-IMA lung adenocarcinoma.

Colloid Adenocarcinoma

Colloid adenocarcinoma of the lung, characterized by pools of mucin with scant epithelium, is an extremely rare tumor and shares similar histologic features with mucinous adenocarcinomas of the GI tract, ovary, and breast. Thus, IHC may be required to support the diagnosis of a lung primary tumor.

What Is the Immunoprofile of Colloid Adenocarcinoma of the Lung?

Colloid adenocarcinomas of the lung often exhibit reactivity to both lung and intestinal adenocarcinoma markers (Figure 13-3). TTF1, napsin A, and CK7 expression is seen in 60%, 50%, and 90% of the tumors, respectively, while CDX2 and CK20 expression is present in 70%

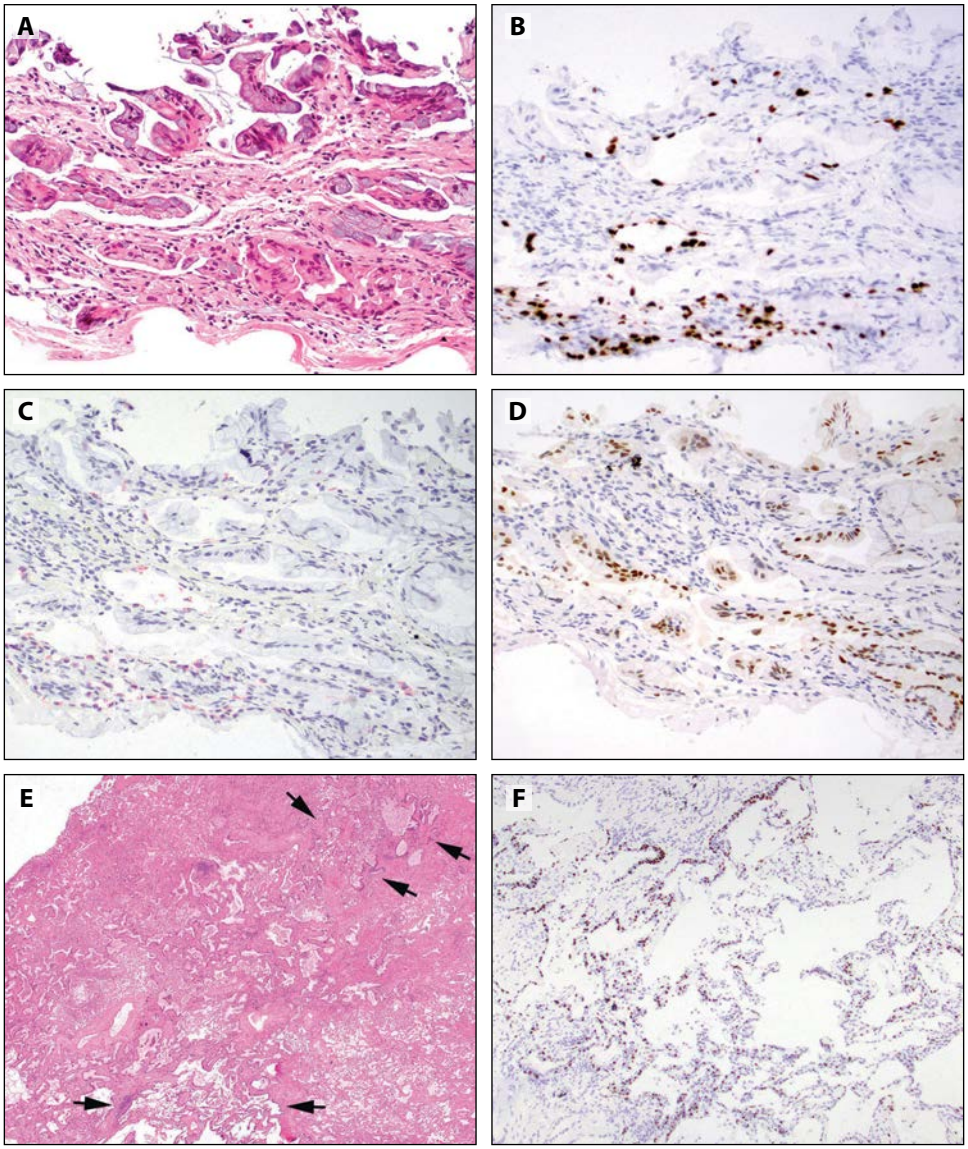


Figure 13-2. (A-D) A core biopsy with invasive mucinous adenocarcinoma demonstrating clusters of mucinous cells with mild cytologic atypia lining alveolar walls or in **(A)** the background of mildly fibrous stroma. A thyroid transcription factor-1 (TTF1) immunostain highlights pneumocytes, while the mucinous cells are not reactive to **(B)** TTF1 or **(C)** p40. Conversely, the vast majority of the mucinous cells show **(D)** nuclear expression of hepatocyte nuclear factor 4 alpha (HNF4α), confirming the diagnosis of invasive mucinous adenocarcinoma. **(E and F)** Another example of invasive mucinous adenocarcinoma from a patient with interstitial fibrosis. A 1.5-cm, ill-defined subpleural nodule consists of mucinous glands with mildly irregular contour but no overt cytologic atypia in the background of fibrous stroma and a few foci of peribronchiolar metaplasia with or without mucin **(E,** *arrows***)**. A p40 immunostain reveals positive nuclei in the bronchioles and alveolar parenchyma with peribronchiolar metaplasia **(F)**, while the groups of mucinous glands are completely negative for p40 (the absence of basal cells) confirming the diagnosis of invasive mucinous adenocarcinoma.

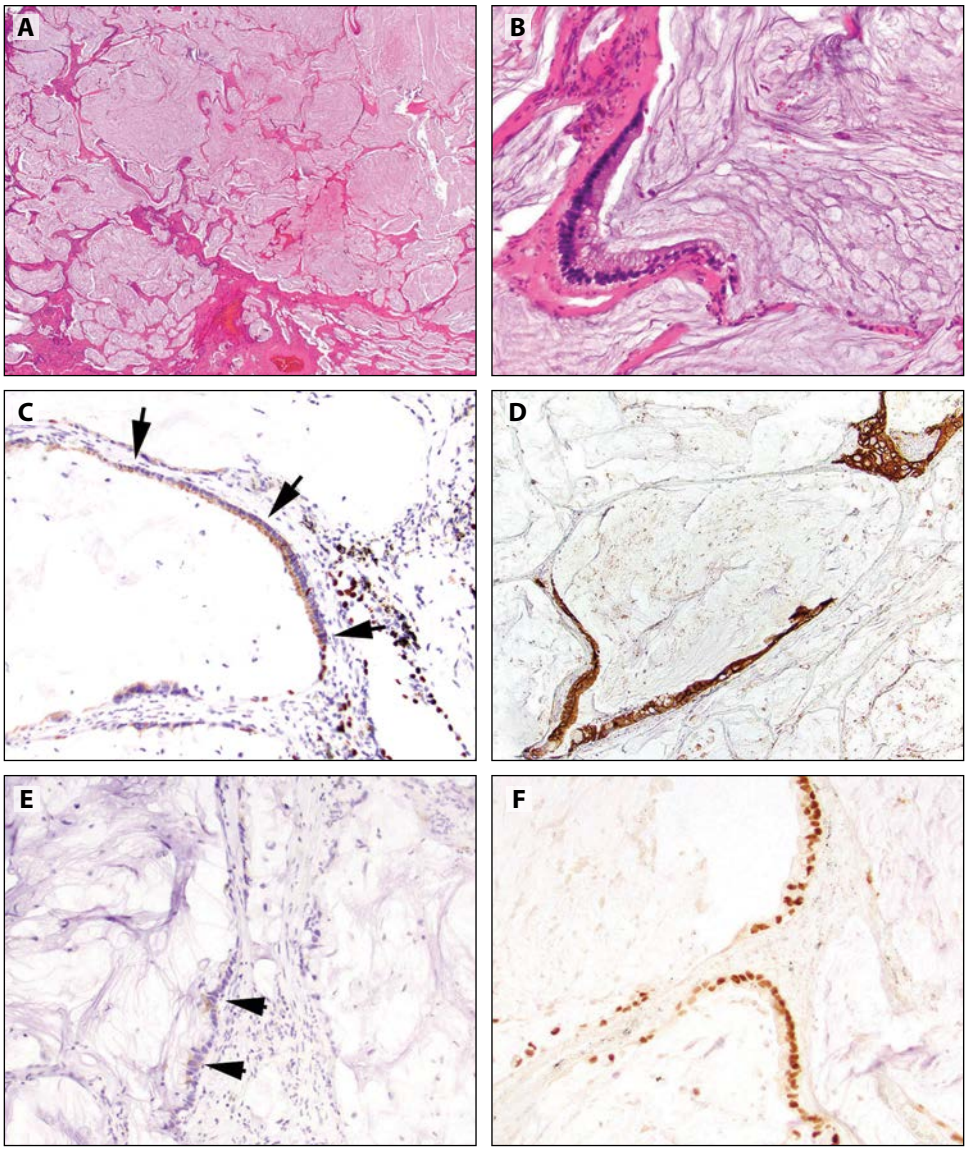


Figure 13-3. (A) A colloid adenocarcinoma of the lung consists of a mucin pool expanding and destroying alveoli with **(B)** rare scattered foci of mucinous cells focally lining alveolar septae. **(C,** *arrows***)** Immunohistochemistry shows negative nuclear expression for thyroid transcription factor-1 (TTF1) in tumor cells**,** while there is weak cytoplasmic expression. The latter does not help in differential diagnosis. **(D)** The tumor exhibits diffuse CK7 expression and **(E,** *arrowheads***)** very focal CK20 expression. **(F)** CDX2 also highlights most tumor nuclei. This mixed pattern of lung and intestinal marker expression is characteristic of colloid adenocarcinoma of the lung.

and 90%, respectively (Rossi et al 2004; Zenali et al 2015; Yatabe et al 2019). A panel including these markers along with GATA3 and estrogen receptor (ER) (for breast colloid carcinoma) and PAX8 (for ovarian mucinous carcinoma) may be useful in differentiating between a lung primary tumor and metastasis from another site. Unfortunately, IHC will not always provide a clear-cut conclusion in this situation because not all metastatic tumors replicate the expression pattern of the primary site. Thus, clinicopathologic correlation is always important.

Summary Answer
A panel of IHC (CK7, CK20, TTF1, napsin A, CDX2, and other extrapulmonary site specific markers) can be useful in differentiating colloid adenocarcinoma of the lung from metastatic mucinous adenocarcinoma of an extrapulmonary site.

Enteric Adenocarcinoma

Enteric adenocarcinoma is a rare variant of lung adenocarcinoma that resembles adenocarcinoma arising in the colorectum. Thus, the diagnosis requires the exclusion of metastasis from a colorectal primary site (Travis et al 2015). Careful clinicopathologic correlation, together with IHC work-up, is helpful.

What Is the Best Panel of IHC for the Differentiation of Enteric Adenocarcinoma of the Lung from Metastatic Colorectal Adenocarcinoma?
As expected, pulmonary enteric adenocarcinoma often expresses intestinal markers. Half of the tumors express CK20 and CDX2, while TTF1 and napsin A expression is limited to a third of cases. In this context, inclusion of CK7 and SATB2 in an IHC panel may be helpful, although the data suggest power to discriminate is probably weak. Whereas CK7 is usually strong and diffuse in about 85% of lung adenocarcinomas but weak and focal in up to 27% of colorectal adenocarcinomas, the reverse is usually seen with SATB2; strong and diffuse in 85% of colorectal cancer (CRC), weak and focal in approximately 14% of lung adenocarcinomas (Lin et al 2013; Jurmeister et al 2019; Gu et al 2019; Zhang et al 2019; Bian et al 2017; Matsushima et al 2017).

Summary Answer
A panel of IHC (including CK7 and SATB2) may help in differentiating pulmonary enteric adenocarcinoma from metastatic colorectal adenocarcinoma (Figure 13-4).

Fetal Adenocarcinoma

Fetal adenocarcinoma of the lung resembles the pseudo-glandular phase of the fetal lung and is characterized by complex glandular structures composed of glycogen-rich non-ciliated cells. Low-grade and high-grade fetal adenocarcinomas have been described. The low-grade form exhibits low nuclear atypia and morule formation in a background of loose fibromyxoid stroma, while the high-grade form shows more prominent nuclear atypia, a lack of morules, necrosis, and transition to a minor component of conventional adenocarcinoma (Travis et al 2015). IHC may be required to differentiate fetal adenocarcinoma of the lung from metastatic endometrial adenocarcinoma.

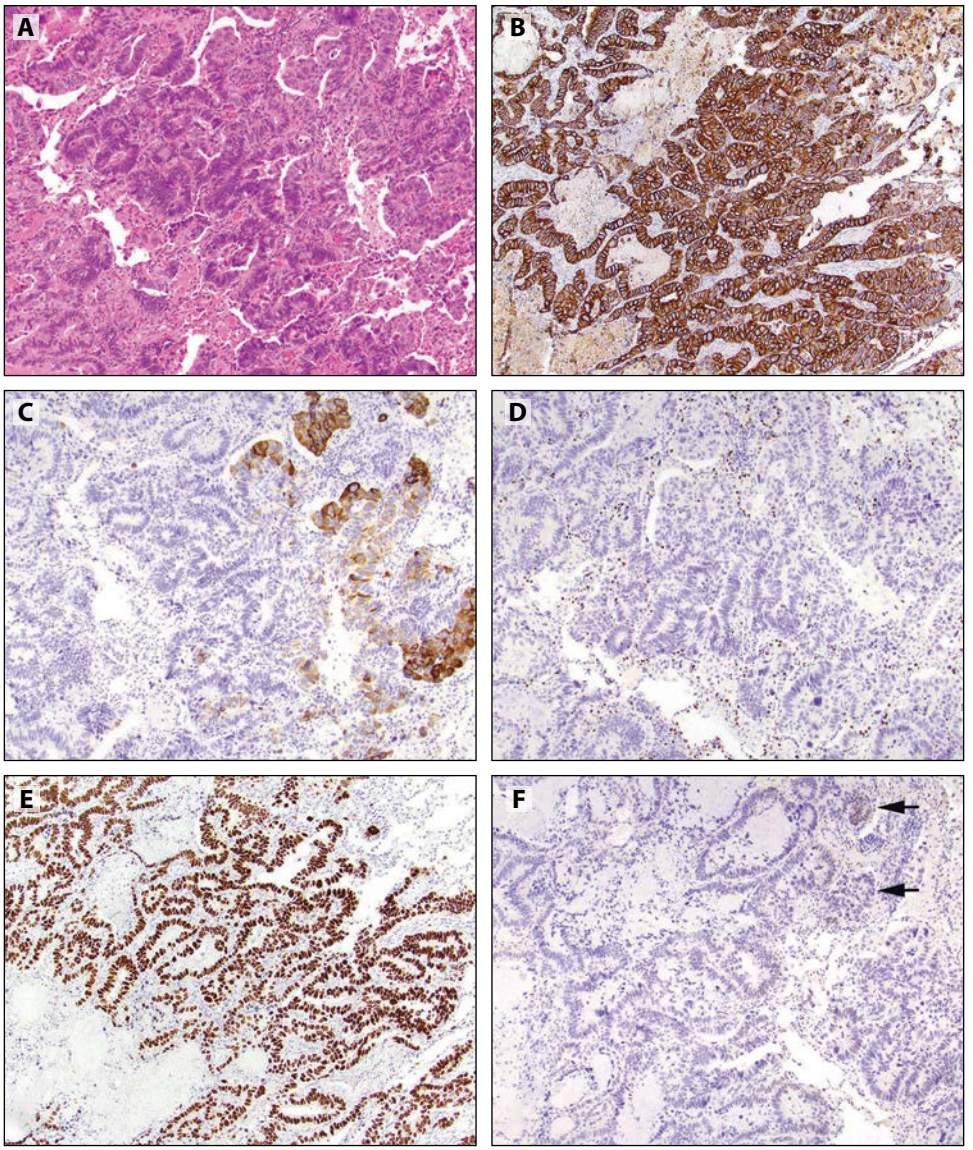


Figure 13-4. An example of enteric adenocarcinoma. **(A)** The tumor consists of tall columnar cells with hyperchromatic nuclei forming glands. Luminal necrosis is also evident. **(B)** Almost all tumor cells are reactive to cytokeratin 7 (CK7), while **(C)** a fraction of tumor cells exhibit CK20 expression, and **(D)** thyroid transcription factor-1 (TTF1) only highlights entrapped pneumocytes but no tumor cells. **(E)** The vast majority of tumor cells are also reactive to CDX2, but **(F,** *arrows***)** only a small fraction shows weak expression of SATB2. This expression pattern of CK7 and SATB2 is consistent with a lung primary tumor.

What Are the Immunoprofiles of Low- and High-Grade Fetal Adenocarcinomas of the Lung?

Low-grade fetal adenocarcinomas express TTF1 and show aberrant nuclear localization of β-catenin, typically in the morules, while cytoplasmic membrane staining of β-catenin is preserved in the high-grade form, and TTF1 expression is seen in only about 50% of high-grade tumors (Figures 13-5 and 13-6). More than 90% of low-grade tumors harbor neuroendocrine cells that are immunoreactive to synaptophysin and/or chromogranin, while only about 50% of high-grade tumors contain such cells (Nakatani et al 2002, 2004; Morita et al 2013; Suzuki

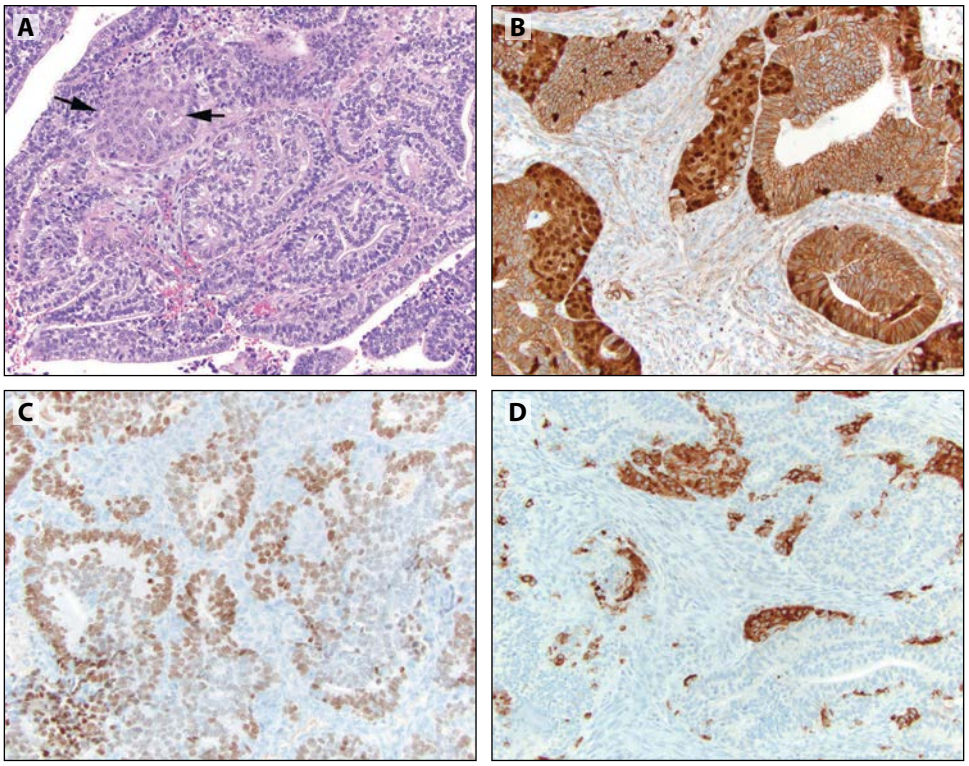


Figure 13-5. (A, *arrows***)** An example of low-grade fetal adenocarcinoma of the lung characterized by complex glandular structures composed of glycogen-rich non-ciliated cells and morule formation that is associated with **(B)** nuclear expression of β-catenin. **(C)** Most tumor cells exhibit thyroid transcription factor-1 (TTF1) nuclear staining of various intensities, and **(D)** synaptophysin highlights scattered clusters of neuroendocrine cells in the tumor. Tumor cells were negative for estrogen receptor (ER), progesterone receptor (PR), and PAX8 (*not shown*).

et al 2015). Interestingly, high-grade fetal adenocarcinomas often express oncofetal proteins, for example, α-fetoprotein, glypican 3, and/or sal-like protein 4 (SALL4) (Morita et al 2013; Suzuki et al 2015). Neither the low-grade nor the high-grade form is immunoreactive to ER, progesterone receptor (PR), or PAX8, the expressions of which are typically seen in endometrial adenocarcinoma.

Summary Answer

Low-grade fetal adenocarcinoma is characterized by aberrant nuclear localization of β-catenin, typically in the morules, while high-grade fetal adenocarcinoma often expresses oncofetal proteins, such as α-fetoprotein, glypican 3, and/or SALL4. Further, a panel of IHC, including TTF1 and PAX8, may be required to differentiate fetal adenocarcinoma of the lung from metastatic endometrial adenocarcinoma.

Conclusions

A panel of IHC can be useful in the diagnosis of colloid adenocarcinoma of the lung or pulmonary enteric adenocarcinoma (CK7, CK20, TTF1, napsin A, CDX2, and SATB2) and in the differentiation of fetal adenocarcinoma from metastatic endometrial adenocarcinoma (TTF1 and PAX8), while the differentiation between IMA and metastasis from a pancreatobiliary

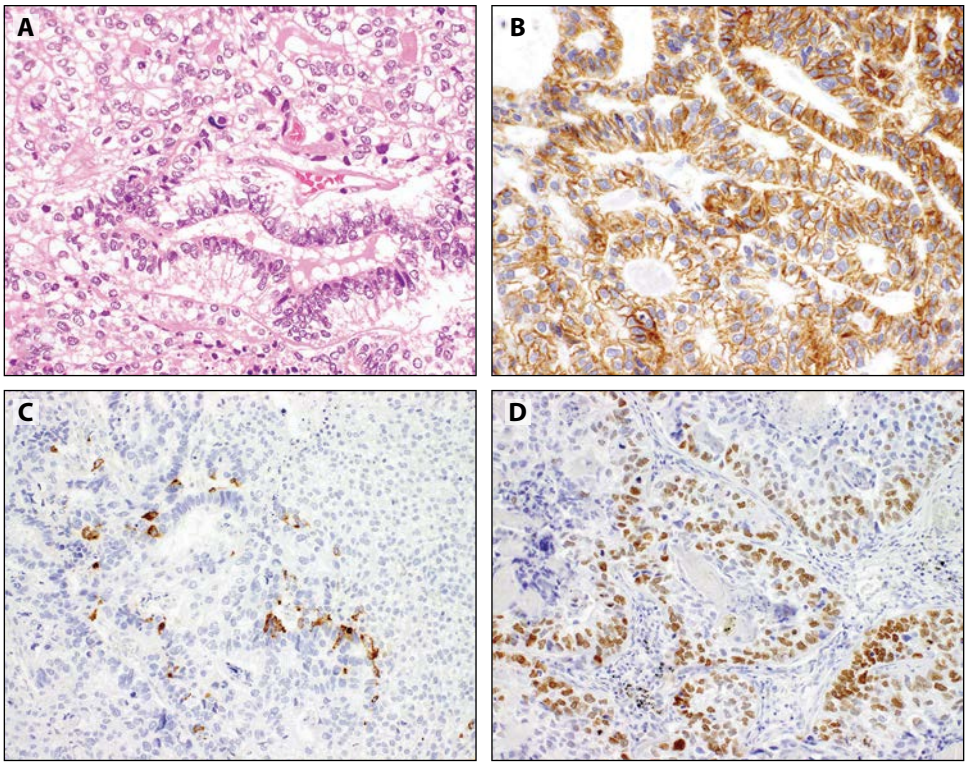


Figure 13-6. (A) High-grade fetal adenocarcinoma of the lung demonstrating glycogen-rich non-ciliated cells with prominent nuclear atypia forming complex glandular structures without morules. **(B)** β-catenin expression is preserved in a cytoplasmic membranous pattern. **(C)** Chromogranin A highlights scattered neuroendocrine cells, and **(D)** SALL4 expression is also seen in a fraction of tumor cells, while the expression of estrogen receptor (ER), progesterone receptor (PR), and PAX8 (*not shown*) is absent. (Images courtesy of Dr. Yukio Nakatani)

or upper GI tract site can be extremely challenging based on the morphology and IHC alone. Conversely, IHC with TTF1, p40, and/or HNF4α may be useful in the diagnosis of IMA when the lesional tissue is limited, or non-malignant entities are in the differential diagnosis.

References

Bian T, Zhao J, Feng J, et al. Combination of cadeherin-17 and SATB homeobox 2 serves as potential optimal makers for the differential diagnosis of pulmonary enteric adenocarcinoma and metastatic colorectal cancer. *Oncotarget*. 2017;8(38):63442-63452.

Calio A, Lever V, Rossi A, et al. Increased frequency of bronchiolar histotypes in lung carcinomas associated with idiopathic pulmonary fibrosis. *Histopathology*. 2017;71(5):725-735.

Chang JC, Montecalvo J, Borsu L, 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):1010-1026.

Gu L, Wang XZ, Wen W, et al. Clinical analysis of 23 patients pathologically diagnosed with primary and secondary pulmonary enteric adenocarcinoma. *Chin Med J (Engl)*. 2019;132(11):1368-1369.

Jurmeister P, Scholer A, Arnold A, et al. DNA methylation profiling reliably distinguishes pulmonary enteric adenocarcinoma from metastatic colorectal cancer. *Mod Pathol*. 2019;32(6):855-865.

Lin D, Zhao Y, Xing X. Pulmonary enteric adenocarcinoma with villin brush border immunoreactivity: a case report and literature review. *J Thorac Dis*. 2013;5(1):E17-20.

Lu YW, Yeh YC. Ciliated muconodular papillary tumors of the lung. *Arch Pathol Lab Med*. 2019;143(1):135-139.

Masai K, Tsuta K, Motoi N, et al. Clinicopathological, immunohistochemical, and genetic features of primary lung adenocarcinoma occurring in the setting of usual interstitial pneumonia pattern. *J Thorac Oncol*. 2016;11(12):2141-2149.

Matsushima J, Yazawa T, Suzuki M, et al. Clinicopathological, immunohistochemical, and mutational analysis of pulmonary enteric adenocarcinoma: usefulness of SATB2 and β-catenin immunostaining for differentiation from metastatic colorectal carcinoma. *Hum Pathol*. 2017;64:179-185.

Morita S, Yoshida A, Goto A, et al. High-grade lung adenocarcinoma with fetal lung-like morphology clinicopathologic, immunohistochemical, and molecular analysis of 17 cases. *Am J Surg Pathol*. 2013;37(6):924-932.

Nakatani Y, Masudo K, Miyagi Y, et al. Aberrant nuclear localization and gene mutation of beta-catenin in low-grade adenocarcinoma of fetal lung type: up-regulation of the Wnt signaling pathway may be a common denominator for the development of tumors that form morules. *Mod Pathol*. 2002;15(6):617-624.

Nakatani Y, Miyagi Y, Takemura T, et al. Aberrant nuclear/cytoplasmic localization and gene mutation of beta-catenin in classic pulmonary blastoma: beta-catenin immunostaining is useful for distinguishing between classic pulmonary blastoma and a blastomatoid variant of carcinosarcoma. *Am J Surg Pathol*. 2004;28(7):921-927.

Rossi G, Murer B, Cavazza A, et al. Primary mucinous (so-called colloid) carcinomas of the lung: a clinicopathologic and immunohistochemical study with special reference to CDX-2 homeobox gene and MUC2 expression. *Am J Surg Pathol*. 2004;28(4):442-452.

Sugano M, Nagasaka T, Sasaki E, et al. HNF4α as a marker for invasive mucinous adenocarcinoma of the lung. *Am J Surg Pathol*. 2013;37(2):211-218.

Suzuki M, Yazawa T, Ota S, et al. High-grade fetal adenocarcinoma of the lung is a tumour with a fetal phenotype that shows diverse differentiation, including high-grade neuroendocrine carcinoma: a clinicopathological, immunohistochemical and mutational study of 20 cases. *Histopathology*. 2015;67(6):806-816.

Travis WD, Brambilla E, Burke AP, et al. *WHO Classification of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Zenali MJ, Weissferdt A, Solis LM, et al. An update on clinicopathological, immunohistochemical, and molecular profiles of colloid carcinoma of the lung. *Hum Pathol*. 2015;46(6):836-842.

Zhang J, Xiang C, Han Y, et al. Differential diagnosis of pulmonary enteric adenocarcinoma and metastatic colorectal carcinoma with the assistance of next-generation sequencing and immunohistochemistry. *J Cancer Res Clin Oncol*. 2019;145(1):269-279.

Immunomarkers for Other Rare Tumors

14

By Anja C. Roden and Yuko Minami

Introduction

Apart from a number of tumors already discussed in preceding chapters, other rare primary tumors occur in the thorax, ranging from benign lesions, such as bronchial adenomas, to malignant neoplasms such as nuclear protein in testis (NUT) carcinoma and salivary-type tumors. Their timely diagnosis is important for treatment and management of the patient and, in a subset of these tumors, for early enrollment in clinical trials (eg, bromodomain and extra-terminal [BET] domain family inhibitors for treatment of NUT carcinomas [Salati et al 2019]). Some of these neoplasms, such as pulmonary adenomas or sclerosing pneumocytomas, are largely diagnosed on hematoxylin and eosin (H&E), but others require immunostains to establish the diagnosis or to confirm the H&E impression, especially in smaller biopsies. In addition, some tumors may be difficult to distinguish from morphologic mimics; adenocarcinoma is often in the differential diagnosis for alveolar adenoma, sclerosing pneumocytoma, or bronchiolar adenoma/ciliated muconodular papillary tumor (CMPT). Some of these tumors are defined by genetic alterations (Table 14-1). Immunohistochemistry (IHC) does, however, have a role to play in some circumstances.

Does IHC Aid in the Diagnosis of Alveolar Adenoma?

Thyroid transcription factor-1 (TTF1), pankeratin, epithelial membrane antigen (EMA), and surfactant protein A (SP-A) highlight the single layer of bland, cuboidal, or flattened type II pneumocytes (Figure 14-1B, *arrow*) that line cysts and overlay the spindle-rich stroma. These stromal cells (Figure 14-B, *arrowhead*) may express CD34 and possibly S100 and smooth muscle actin (SMA). Alveolar adenomas are generally an H&E diagnosis, but IHC might be used to confirm the diagnosis or to distinguish this tumor from lymphangiomas, which lack keratin-positive cyst-lining cells, and from sclerosing pneumocytoma. In contrast to alveolar adenoma in which TTF1 only stains the cyst-lining cells, in sclerosing pneumocytoma, both the luminal and the stromal cells express TTF1 (see the section "Are Immunomarkers

Table 14-1. Genetic Alterations That Are Highly Specific for Rare Thoracic Tumors

Neoplasm	Genetic alteration	Tumors with genetic alteration, %	Antibody directed against the oncogene
Bronchopulmonary mucoepidermoid carcinoma	t(11;19)(q21;p13) CRCT1-MAML2[a]	67-100	None
NUT carcinoma	t(15;19)(q14;p13.1) *BRD4-NUT*[b] *BRD3-NUT* NUT-variant fusions with other non-BRD containing genes (eg, *NDSD3, ZNF532*)[c]	70-86 (BRD4-NUT)	NUT (clone C52B1)
Adenoid cystic carcinoma[d]	t(6;9)(q22-23;p23-24) MYB-NFIB	41-50	MYB (59%)[e]

Abbreviations: BRD = bromodomain and extra-terminal (BET) domain containing protein; MYB = myeloblastosis proto-oncogene, transcription factor; NUT = nuclear protein in testis.
[a] Roden et al 2014; Achcar Rde et al 2009; Huo et al 2015.
[b] French et al 2003; Chau et al 2016.
[c] French et al 2014; Alekseyenko et al 2017.
[d] Roden et al 2015; Brill et al 2011.
[e] Vallonthaiel et al 2017; Poling et al 2017.

Useful in Distinguishing Sclerosing Pneumocytoma from Adenocarcinoma?") (Sak et al 2007; Burke et al 1999; De Rosa et al 2012).

Summary Answer
Although primarily an H&E diagnosis, IHC can be valuable to highlight the neoplastic pneumocytes and mesenchymal stroma of alveolar adenoma (Figure 14-1).

Does p40 IHC Have a Role in the Diagnosis of CMPT, Distinguishing It from Adenocarcinoma?

Basal cell markers associated with squamous differentiation, including p40, p63, and cytokeratin 5/6 (CK5/6), highlight an intact layer of basal cells (Figure 14-2B, *arrow*) beneath the lesional columnar cells, which helps distinguish CMPTs from adenocarcinomas that lack a basal cell layer. Ki-67 shows a low proliferative index with reported values of less than 1% to less than 5% of tumor cell nuclei staining in CMPT and a single case of 10% (Lu and Yeh 2019; Shao et al 2019, Kataoka et al 2018). MUC5AC and EMA are variably expressed in the ciliated cells. CK7, CK20, TTF1, napsin A, and CDX2 are generally not helpful in that distinction because similar to lung adenocarcinomas, the ciliated columnar cells (Figure 14-2B, *arrowhead*) and goblet cells that are luminal to the basal cells in CMPT express CK7 and most are also positive for TTF1; whereas CK20, CDX2, and napsin A are generally negative (Shao et al 2019; Kashima et al 2019; Lu and Yeh 2019).

Summary Answer
Basal cell markers, such as p40, p63, and CK5/6, help identify the double epithelial cell layer in CMPT (Figure 14-2).

What Is the Immunoprofile of NUT Carcinomas?

A subset of NUT carcinomas can be negative for keratins, markers of squamous differentiation, and other markers (Figure 14-3A-D). However, many of these tumors show at least focal expression of various keratins, including pankeratin (77%), OSCAR keratin, and CK7, and

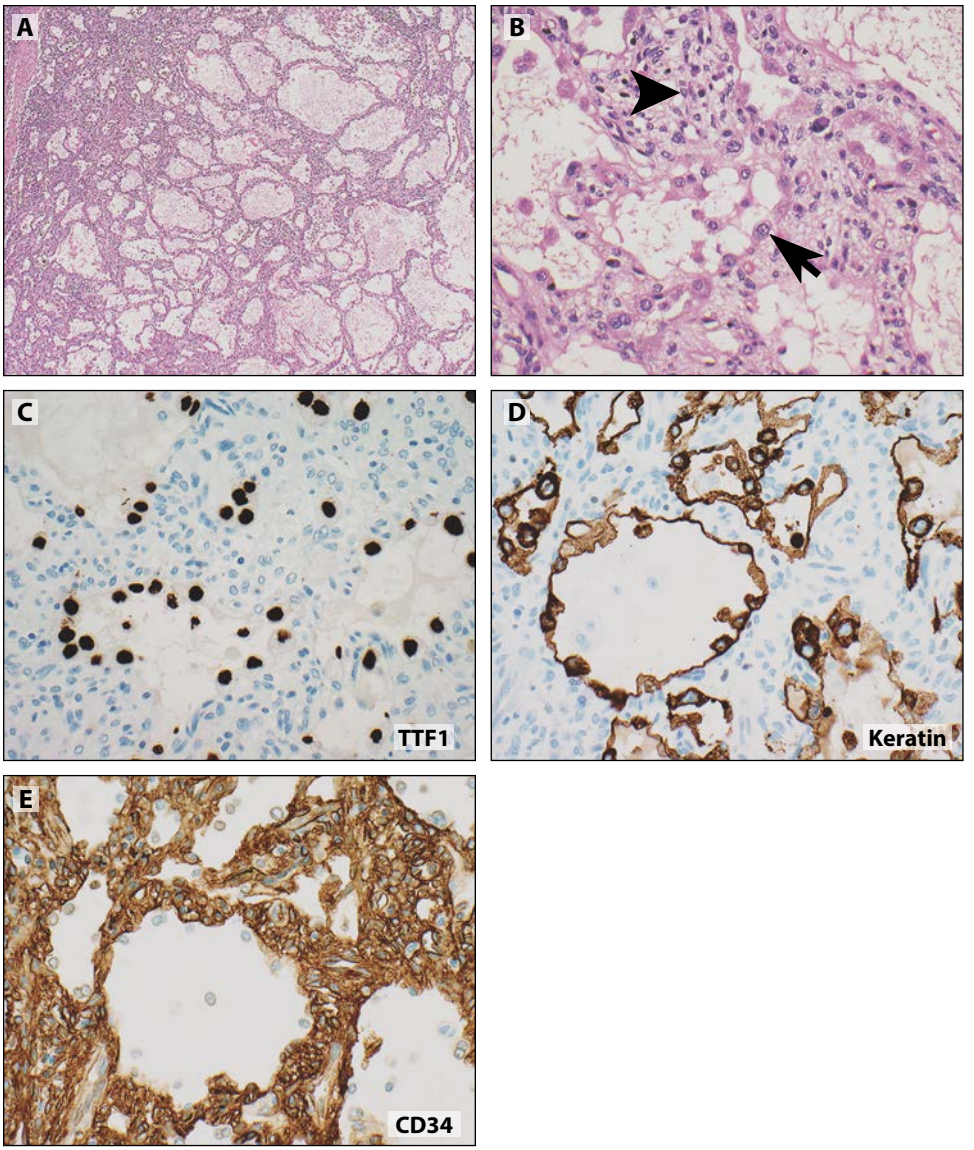


Figure 14-1. Alveolar adenoma. **(A)** The cystic appearance of an alveolar adenoma at low power, **(B)** coupled with the hobnail cells (*arrow*) lining the spaces, and despite the histology of the stroma (*arrowhead*), may raise the differential with lymphangioma or sclerosing pneumocytoma. **(C)** Staining for thyroid transcription factor-1 (TTF1) and **(D)** cytokeratin highlight the pneumocyte differentiation of the lining cells. **(E)** The interstitial cells are TTF1 negative while often CD34 positive. Magnification **(A)** H&E, ×40; **(B)** H&E, ×400; **(C)** TTF1 (clone SPT24), ×400; **(D)** pankeratin (clones AE1/AE3), ×400; and **(E)** CD34, ×400.

markers associated with squamous differentiation such as p40, p63 (90%), and CK5/6. They might express TTF1, sometimes in the same neoplastic cells that express p63 and so forth, a constellation that might hint at a NUT carcinoma, although this can also be seen in poorly differentiated adenocarcinomas (see Chapter 8). Some NUT carcinomas also express EMA (75%), CD34, and/or CD99 (Evans et al 2012). Positive NUT IHC is enough to make the diagnosis, but *NUT* rearrangement can be confirmed (Figure 14-4) by fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR) (French 2012).

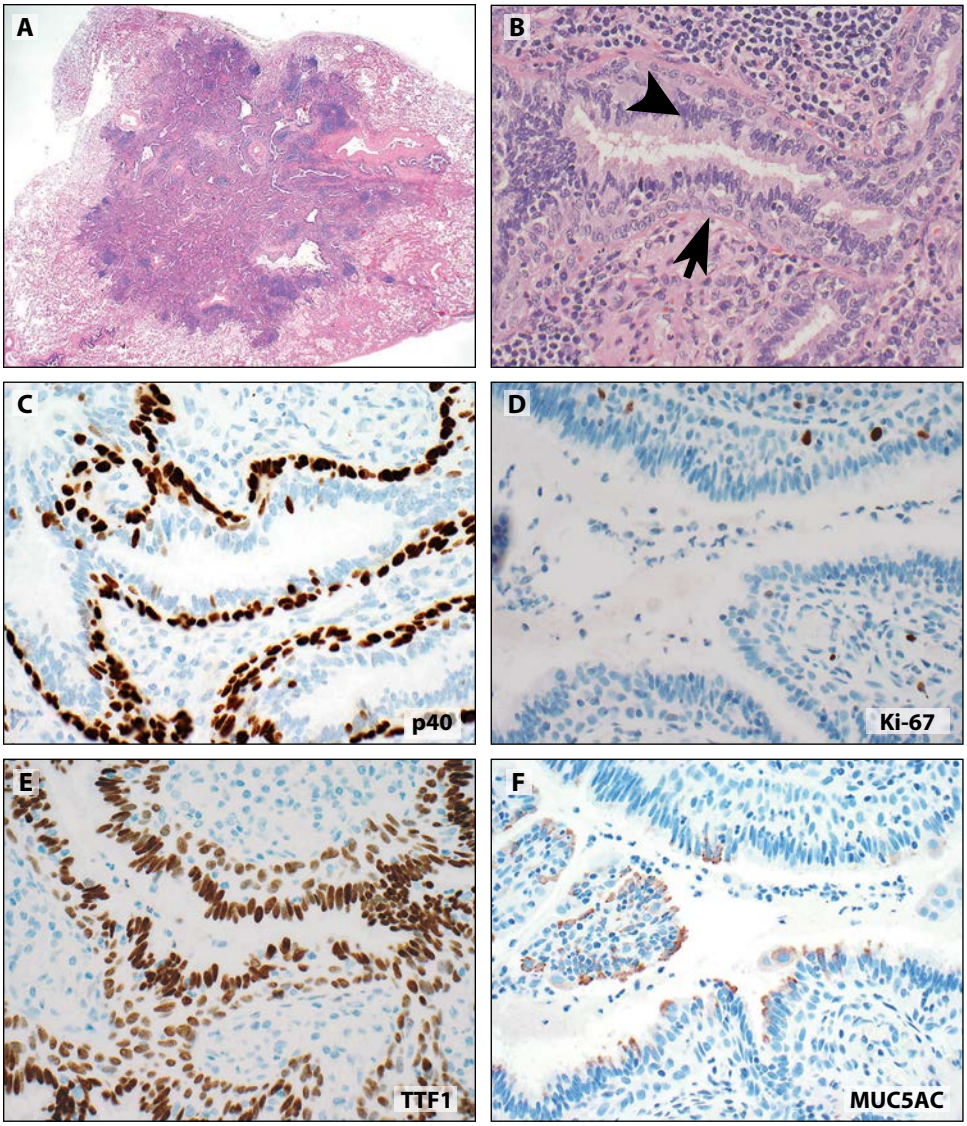


Figure 14-2. Ciliated muconodular papillary tumor. **(A)** These tumors appear vaguely circumscribed but not encapsulated at low magnification and **(B)** contain epithelial-lined spaces (*arrowhead*), which have a double layer (*arrow*). **(C)** This can be highlighted by staining for p40. The lining cells should have a **(C)** low Ki-67 index, and **(D)** are positive for thyroid transcription factor-1 (TTF1). Ciliated cells may be MUC5AC positive. Magnification **(A)** H&E, ×12.5; **(B)** H&E, ×400; **(C)** p40, ×400; **(D)** Ki-67, ×400; **(E)** TTF1 (clone SPT24), ×400; and **(F)** MUC5AC, ×400.

Summary Answer

Apart from NUT expression, keratins and basal cell markers are often expressed, but TTF1 and EMA may also be found.

What Is the Pattern of NUT Expression in NUT Carcinomas?

Summary Answer

A nuclear speckled pattern in more than 50% of tumor cells is characteristic and diagnostic for NUT carcinoma (Haack et al 2009; French 2018) (Figure 14-4). NUT immunostain is sensitive (87%) and specific (100% after exclusion of seminoma [French 2018]).

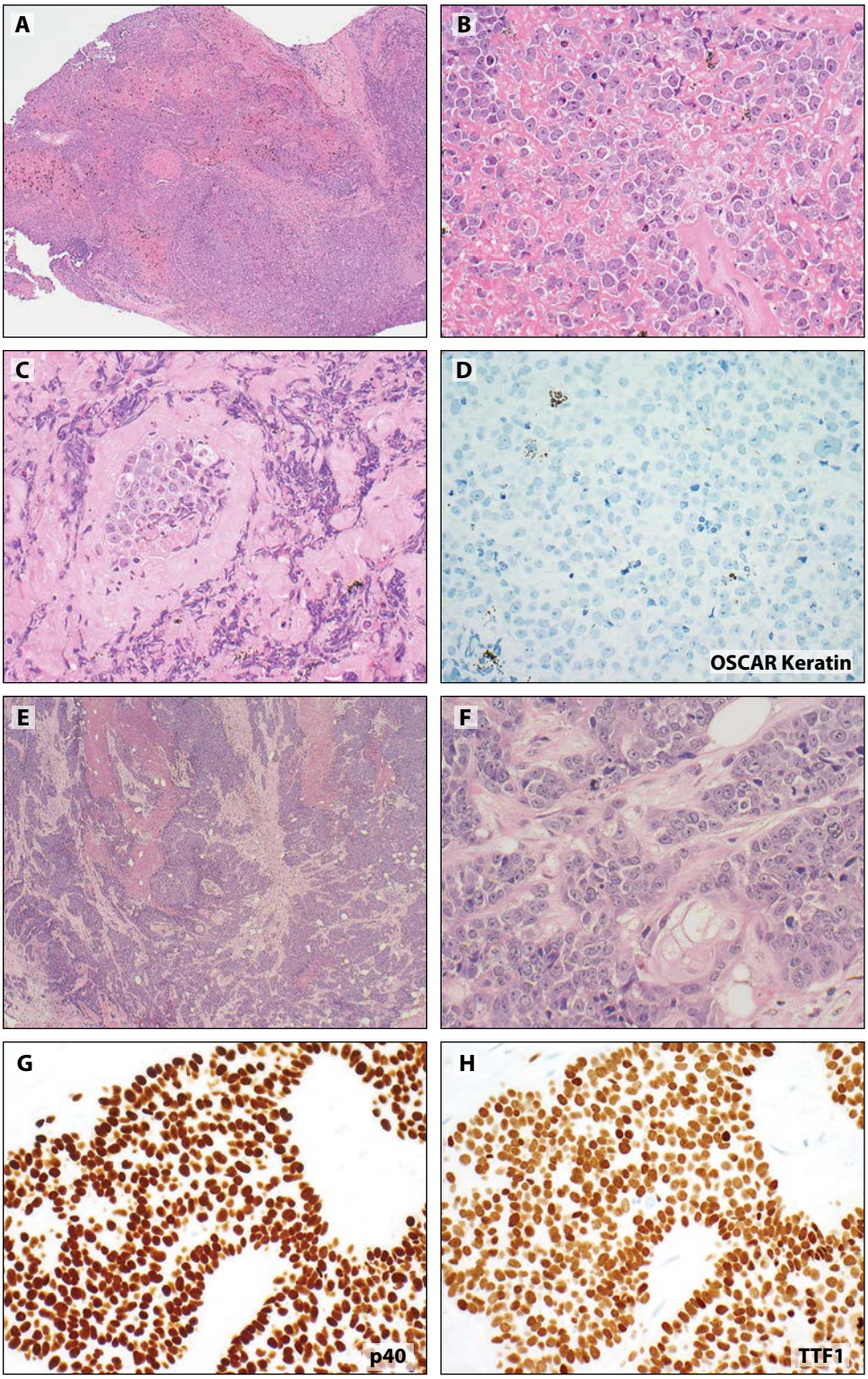


Figure 14-3. Nuclear protein in testis (NUT) carcinoma. **(A)** This cellular tumor is characterized by **(E and F)** dyscohesive epithelioid cells with areas of hyalinized stroma, necrosis, and focal squamous differentiation. **(C)** Immunohistochemistry (IHC) for cytokeratin can be focal or negative, but **(G and H)** p40 and thyroid transcription factor-1 (TTF1) can be double positive in the same cells. Magnification **(A and E)** H&E, ×40; **(B, C,** and **F)** H&E, ×400; **(D)** keratin (clone OSCAR), ×400; **(G)** p40, ×400; and **(H)** TTF1 (clone SPT24), ×400.

Are Immunomarkers Useful in Distinguishing Sclerosing Pneumocytoma from Adenocarcinoma?

The recognition of 2 distinct cell populations on H&E, surface cuboidal cells (Figure 14-5B, *arrow*), which morphologically and immunophenotypically resemble type II pneumocytes and stromal round cells (Figure 14-5B, *arrowhead*), is important for the diagnosis and to distinguish that tumor from adenocarcinoma. Markers that differentially highlight these 2 cell populations and therefore also help in the distinction from adenocarcinoma include pankeratin (AE1/AE3), napsin A, and if available, surfactant protein (SP-A and SP-B) markers that almost selectively highlight the surface cell component (Table 14-2). While progesterone receptor has been shown to highlight only the round cell component in most cases in a study by Rodriguez-Soto et al (2000), this has not been validated. TTF1 is expressed in both components and while it might be overall useful for the diagnosis of sclerosing pneumocytoma, it does not

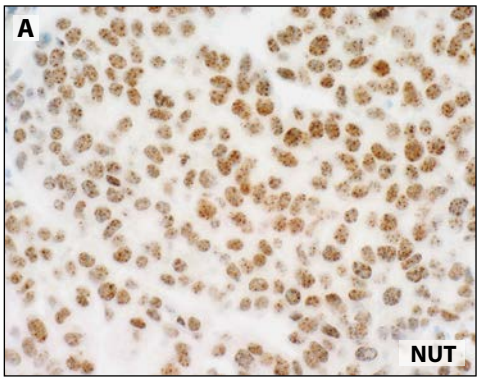


Figure 14-4. Nuclear protein in testis (NUT) immunohistochemistry. Nuclear staining in more than 50% of cells, often in a speckled pattern. **(A)** Magnification ×600.

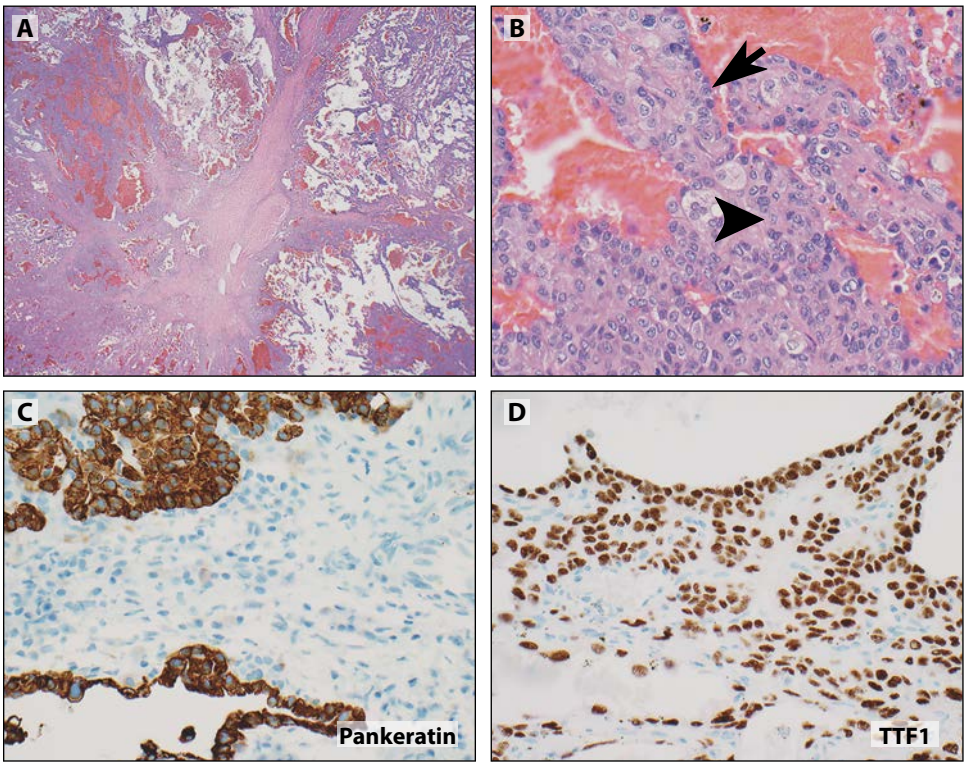


Figure 14-5. Sclerosing pneumocytoma. **(A)** The lower power view shows sclerosis and papillary architecture, which at high magnification **(B)** shows an interstitial round cell population (*arrowhead*) and an epithelial lining (*arrow*). **(C)** Staining for cytokeratin is positive in the lining cells only, while **(D)** thyroid transcription factor-1 (TTF1) highlights both the round cell and lining cells. Magnification **(A)** H&E, ×40; **(B)** H&E, ×400; **(C)** pankeratin (clones AE1/AE3), ×400; and **(D)** TTF1 (clone SPT24), ×400.

Table 14-2. Antigens Expressed by Cellular Components of Sclerosing Pneumocytoma

Antibody	Surface cuboidal cells	Stromal round cells
	% Cases with ≥ 10% or 1% of cells staining[a]	
TTF1	97[a]-100[b,c]	92[a]-100[b,c]
Pankeratin	100	1
CAM5.2	73	17
Keratin 903	0	0
CK7	100	31
Pro SP-A, p	92	0
Pro SP-B, p	100	0
Napsin A	100[b]	17[d]

Abbreviations: CK7 = cytokeratin 7; SP-A = surfactant protein A; SP-B = surfactant protein B; TTF1 = thyroid transcription factor-1.
[a] Clone not provided.
[b] 1% of cells staining.
[c] Clone 8G7/G3/1.
[d] In 1%-25% of cells.
Source: Devouassoux-Shisheboran et al 2000; Schmidt et al 2012.

help in the distinction from lung adenocarcinoma (Devouassoux-Shisheboran et al 2000; Schmidt et al 2012).

Summary Answer
Immunostains may help in the diagnosis of sclerosing pneumocytoma by assisting in the identification of the 2 cellular compartments with different immunoprofiles; keratins and TTF1 are key elements in this distinction (Figure 14-5).

Salivary Gland-Type Tumors

Are Immunomarkers Helpful in Diagnosing Pulmonary Mucoepidermoid Carcinomas?
The diagnosis of low-grade mucoepidermoid carcinoma is normally based on an H&E stain showing the presence of 3 cell types—mucous (Figure 14-6B, *arrow*), epidermoid (Figure 14-6B, *arrowhead*), and intermediate cells—and lack of keratinization. If necessary, the diagnosis might be confirmed by *MAML2* rearrangement studies (Table 14-1). On small biopsies or in high-grade mucoepidermoid carcinomas, the diagnosis can be challenging. In these cases, immunostains can aid as p63, p40, and CK5/6 highlight the epidermoid subset of cells, and TTF1 and napsin are negative (Roden et al 2014; Huo et al 2015). A mucin stain, such as mucicarmine or Alcian blue/periodic acid–Schiff (PAS), can be added to highlight cytoplasmic mucin in the mucous cells. The sensitivity of p40 might be lower in pulmonary mucoepidermoid carcinomas than p63 (Roden et al 2014). Again, if in doubt, *MAML2* rearrangement studies should be performed to establish the diagnosis (Roden et al 2014; Huo et al 2015).

Summary Answer
Of only limited use, IHC for p63, p40, or CK5/6 may highlight the epidermoid cell component (Figure 14-6). TTF1 and napsin should be negative.

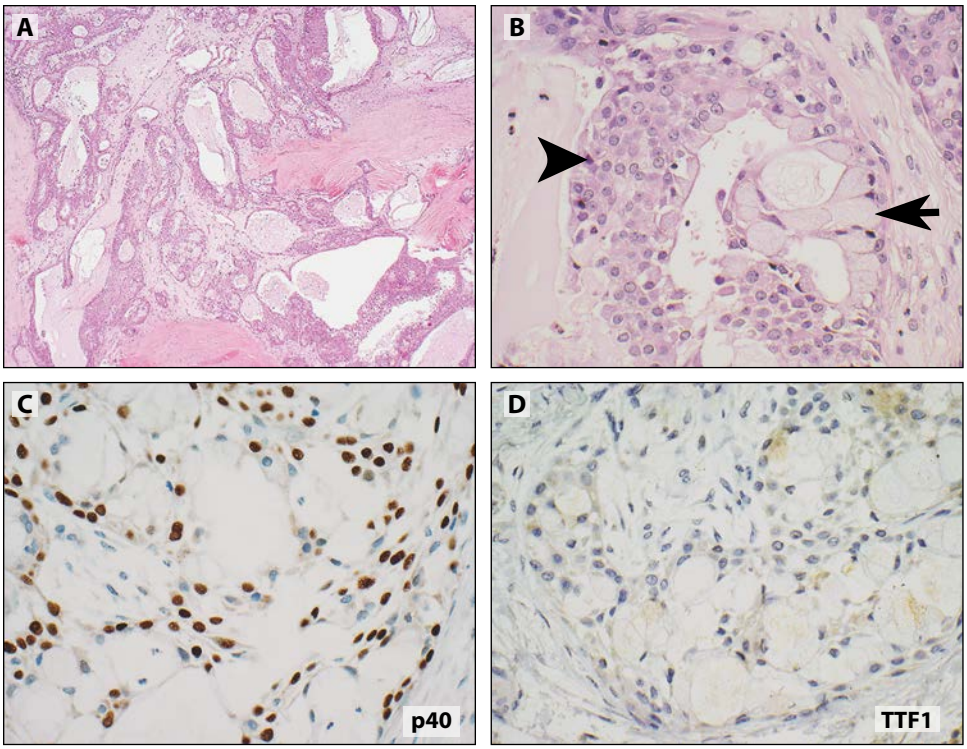


Figure 14-6. Mucoepidermoid carcinoma. The lower power varying cystic and solid nested tumor **(A)** shows at high magnification **(B)** both a uniform epithelioid population (*arrowhead*) and mucus positive cells (*arrow*). **(C)** Immunohistochemistry (IHC) for p40 is positive in a subpopulation of cells, highlighted a dual population, but **(D)** thyroid transcription factor-1 (TTF1) is negative in this tumor. Magnification **(A)** H&E, ×40; **(B)** H&E, ×400; **(C)** p40, ×400; and **(D)** TTF1, ×400.

Do Immunostains Aid in the Diagnosis of Pulmonary Adenoid Cystic Carcinomas and Their Distinction from Both Non-Small Cell and Small Cell Carcinoma?

In general, the diagnosis of adenoid cystic carcinoma can be achieved by H&E stain (Figure 14-7). To help confirm the diagnosis, immunostains might be performed with ductal/luminal cells usually expressing CD117 and EMA while myoepithelial/peripheral cells are positive for p63, S100, SMA, muscle specific actin (MSA), and calponin (Roden et al 2015; Namboodiripad 2014). Keratin is not helpful in the distinction between these 2 cell components as it is expressed in both. CD117, p63, and S100 might be helpful in establishing the diagnosis of challenging cases, such as solid pattern or in small biopsies, as these stains differentially highlight the 2 distinct cell populations. However, other lung carcinomas that are in the differential diagnosis of adenoid cystic carcinoma might, at least focally, express these markers as well. *MYB* gene rearrangement studies and/or an MYB protein immunostain might be helpful in a subset of cases although the latter still needs to be validated in larger studies (Table 14-1) (Roden et al 2015; Brill et al 2011; Vallonthaiel et al 2017; Poling et al 2017).

Summary Answer

Just as with mucoepidermoid carcinoma, the diagnosis of adenoid cystic carcinoma is normally based on an H&E stain. IHC may, however, help in identifying the dual cell population, especially in morphologically challenging samples, when CD117 can highlight ductal/luminal cells while p63 and S100 stains the myoepithelial/peripheral cells.

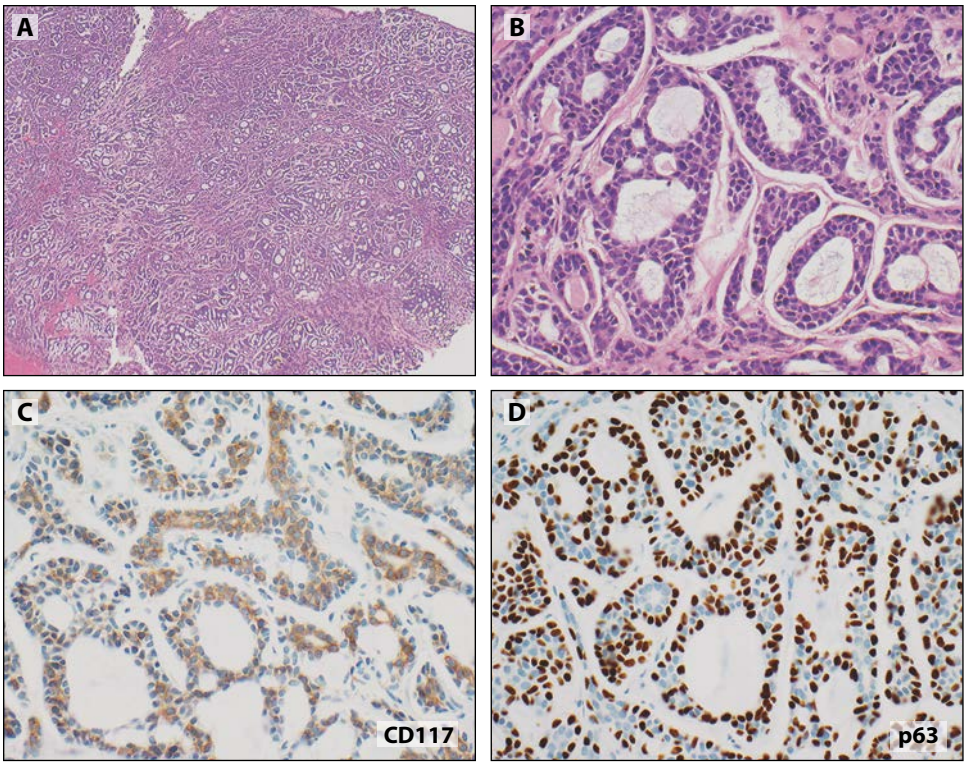


Figure 14-7. Adenoid cystic carcinoma. **(A)** This cellular neoplasm has the classical well-formed glandular structures that have **(B)** dual lining, **(C and D)** highlighted by CD117 and p63 staining. Magnification **(A)** H&E, ×40; **(B)** H&E, ×400; **(C)** CD117, ×400; and **(D)** p63, ×400.

Can Immunostains Aid in the Diagnosis of Epithelial-Myoepithelial Carcinoma in the Lung?

Epithelial-myoepithelial carcinomas are comprised of 2 cell populations, epithelial cells (Figure 14-8B-E, *arrow*) that are positive for keratin, and myoepithelial cells (Figure 14-8B-E, *arrowhead*) that are positive for p63, p40, S100, SMA, MSA, and weakly positive for keratin, CD117, and glial fibrillary acidic protein (GFAP) (Dimitrijevic et al 2015; Guleria et al 2019). Rarely, the epithelial cells express TTF1 and SP-A (so-called pneumocytic adenomyoepithelioma [Chang et al 2007]). p63, S100, keratin, CD117, and mucin can be helpful to distinguish this tumor from mucoepidermoid carcinomas; however, the distinction from adenoid cystic carcinomas can be challenging as immunoprofiles are similar (Figure 14-8).

Summary Answer

As with other salivary-type tumors, IHC only assists in identifying the different cell populations defining the lesions in the correct morphologic context of the H&E stained section.

Conclusions

In rare thoracic tumors, IHC is most useful in their differential diagnosis from more common neoplasms. Inevitably, because most pathologists are unfamiliar with such rare entities, help and supporting evidence is sought with IHC. Some of the rare thoracic tumors are comprised of 2 or more cellular components, and although they usually can be identified on H&E in resection specimens, this can be difficult in small biopsies, high-grade tumors, or when

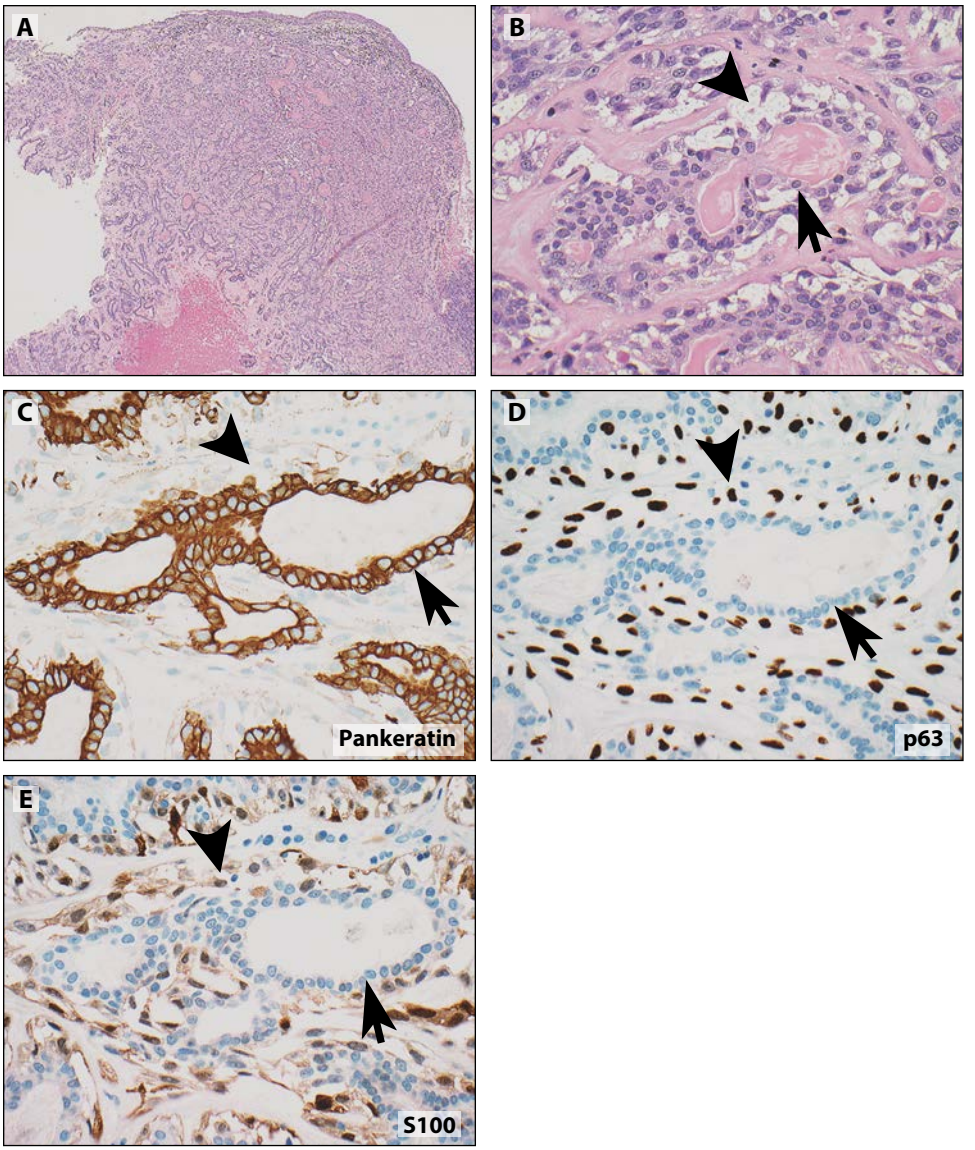


Figure 14-8. Epithelial-myoepithelial carcinoma. **(A)** This diffusely gland-forming tumor shows **(B)** a dual layer with the outside layer showing a dual-layer population (*arrow*), **(B)** with the outer layer (*arrowhead*) showing clear cells consistent with myoepithelial cells. **(C)** The pankeratin stain the luminal population (*arrow*) but not the basal population (*arrowhead*), while the **(D)** p63 and **(E)** S100 highlight the basal population (*arrowhead*) but not the luminal population (*arrow*). Magnification **(A)** H&E, ×40; **(B)** H&E, ×400; **(C)** pankeratin, ×400 (clones AE1/AE3); **(D)** p63, ×400; and **(E)** S100, ×400.

there is a solid growth pattern. Furthermore, many of these tumors, such as salivary gland-type tumors or NUT carcinomas, are not specific to the thorax and can be seen elsewhere in the body. The immunophenotype of these tumors is identical to those arising in other locations, so that metastatic disease must be excluded by clinicoradiologic correlation. There are no lung or thorax specific markers that can help in that distinction.

References

Achcar Rde O, Nikiforova MN, Dacic S, et al. Mammalian mastermind like 2 11q21 gene rearrangement in bronchopulmonary mucoepidermoid carcinoma. *Hum Pathol*. 2009;40(6):854-860.

Alekseyenko AA, Walsh EM, Zee BM, et al. Ectopic protein interactions within BRD4-chromatin complex drives oncogene megadomain formation in NUT midline carcinoma. *Proc Natl Acad Sci U S A*. 2017;114(21):E4184-E4192.

Brill LB 2nd, Kanner WA, Fehr A, et al. Analysis of MYB expression and MYB-NFIB gene fusions in adenoid cystic carcinoma and other salivary neoplasms. *Mod Pathol*. 2011;24(9):1169-1176.

Burke LM, Rush WI, Khoor A, et al. Alveolar adenoma: a histochemical, immunohistochemical, and ultrastructural analysis of 17 cases. *Hum Pathol*. 1999;30(2):158-167.

Chang T, Husain AN, Colby T, et al. Pneumocytic adenomyoepithelioma: a distinctive lung tumor with epithelial, myoepithelial, and pneumocytic differentiation. *Am J Surg Pathol*. 2007;31(4):562-568.

Chau NG, Hurwitz S, Mitchell CM, et al. Intensive treatment and survival outcomes in NUT midline carcinoma of the head and neck. *Cancer*. 2016;122(23):3632-3640.

De Rosa N, Maiorino A, De Rosa I, et al. CD34 expression in the stromal cells of alveolar adenoma. *Case Rep Med*. 2012;913517.

Devouassoux-Shisheboran M, Hayashi T, Linnoila RI, et al. A clinicopathologic study of 100 cases of pulmonary sclerosing hemangioma with immunohistochemical studies: TTF1 is expressed in both round and surface cells, suggesting an origin from primitive respiratory epithelium. *Am J Surg Pathol*. 2000;24(7):906-916.

Dimitrijevic MV, Tomanovic NR, Jesic SD, et al. Epithelial-myoepithelial carcinoma–a review of clinicopathological and immunohistochemical features. *Arch Iran Med*. 2015;18(4):218-222.

Evans AG, French CA, Cameron MJ, et al. Pathologic characteristics of NUT midline carcinoma arising in the mediastinum. *Am J Surg Pathol*. 2012;36(8):1222-1227.

French CA. NUT carcinoma: clinicopathological features, pathogenesis, and treatment. *Pathol Int*. 2018;68(11):583-595.

French CA. Pathogenesis of NUT midline carcinoma. *Annu Rev Pathol*. 2012;7:247-265.

French CA, Miyoshi I, Kubonishi I, et al. BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. *Cancer Res*. 2003;63(2):304-307.

French CA, Rahman S, Walsh EM, et al. NSD3-NUT fusion oncoprotein in NUT midline carcinoma: implications for a novel oncogenic mechanism. *Cancer Discov*. 2014;4(8):928-941.

Guleria P, Madan K, Kumar S, et al. Pulmonary epithelial myoepithelial carcinoma with papillary architecture: an uncommon morphology of a rare tumor. *Pathology*. 2019;51(4):443-445.

Haack H, Johnson LA, Fry CJ, et al. Diagnosis of NUT midline carcinoma using a NUT-specific monoclonal antibody. *Am J Surg Pathol*. 2009;33(7):984-991.

Huo Z, Wu H, Li J, et al. Primary pulmonary mucoepidermoid carcinoma: histopathological and molecular genetic studies of 26 cases. *PLoS One*. 2015;10(11):e0143169.

Kashima J, Hishima T, Tonooka A, et al. Genetic and immunohistochemical analysis of ciliated muconodular papillary tumors of the lung: a report of five cases. *SAGE Open Med Case Rep*. 2019;7:e2050313X19830483.

Kataoka T, Okudela K, Matsumura M, et al. A molecular pathological study of four cases of ciliated muconodular papillary tumors of the lung. *Pathol Int*. 2018;68(6):353-358.

Lu YW, Yeh YC. Ciliated muconodular papillary tumors of the lung. *Arch Pathol Lab Med*. 2019;143(1):135-139.

Namboodiripad PC. A review: immunological markers for malignant salivary gland tumors. *J Oral Biol Craniofac Res*. 2014;4(2):127-134.

Poling JS, Yonescu R, Subhawong AP, et al. MYB labeling by immunohistochemistry is more sensitive and specific for breast adenoid cystic carcinoma than MYB labeling by FISH. *Am J Surg Pathol*. 2017;41(7):973-979.

Roden AC, Garcia JJ, Wehrs RN, et al. Histopathologic, immunophenotypic and cytogenetic features of pulmonary mucoepidermoid carcinoma. *Mod Pathol*. 2014;27(11):1479-1488.

Roden AC, Greipp PT, Knutson DL. Histopathologic and cytogenic features of pulmonary adenoid cystic carcinoma. *J Thorac Oncol*. 2015;10(11):1570-1575.

Rodriguez-Soto J, Colby TV, Rouse RV. A critical examination of the immunophenotype of pulmonary sclerosing hemangioma. *Am J Surg Pathol*. 2000;24(3):442-450.

Sak SD, Koseoglu RD, Demirag F, et al. Alveolar adenoma of the lung. Immunohistochemical and flow cytometric characteristics of two new cases and a review of the literature. *APMIS*. 2007;115(12):1443-1449.

Salati M, Baldessari C, Bonetti LR, et al. NUT midline carcinoma: current concepts and future perspectives of a novel tumour entity. *Crit Rev Oncol Hematol*. 2019;144:102826.

Schmidt LA, Meyers JL, McHugh JB. Napsin A is differentially expressed in sclerosing hemangiomas of the lung. *Arch Pathol Lab Med*. 2012;136(12):1580-1584.

Shao K, Wang Y, Xue Q, et al. Clinicopathological features and prognosis of ciliated muconodular papillary tumor. *J Cardiothorac Surg*. 2019;14(1):143.

Vallonthaiel AG, Jain D, Singh V, et al. c-Myb overexpression in cytology smears of tracheobronchial and pulmonary adenoid cystic carcinomas. *Acta Cytol*. 2017;61(1):77-83.

Immunomarkers for Thoracic Sarcoma

15

By Akihiko Yoshida and Anja C. Roden

Introduction

The thoracic cavity harbors a variety of tumors with sarcomatous phenotype, including sarcomatoid carcinoma, sarcomatoid mesothelioma, and true sarcoma. The diagnostic work-up begins with conventional histology in the clinicoradiologic context; however, in most instances, immunohistochemistry (IHC) is necessary for diagnostic assessment. Molecular analysis is also helpful and sometimes necessary for the diagnosis of tumors that harbor specific genetic abnormalities, such as synovial sarcomas (*SS18-SSX* fusion) and Ewing sarcomas (*EWSR1-ETS* fusion). Sarcomatoid neoplasms arising from the thoracic cavity are most likely either sarcomatoid carcinoma or sarcomatoid mesothelioma. Therefore, a diagnosis of sarcoma should be made with great caution, particularly when the morphology and immunophenotype is not compatible with a specific type of sarcoma in elderly patients. Sarcomatoid carcinoma and mesothelioma are in general positive for cytokeratin, and a wide-spectrum anti-keratin, such as AE1/AE3, should be used for detection. However, some sarcomas can also be positive for cytokeratin, and therefore, more specific markers are required for their differentiation. Some sarcomatoid carcinomas and sarcomatoid mesotheliomas are so poorly differentiated that they may fail to disclose, through immunohistochemical investigation, even a hint of their derivation and may become indistinguishable from true sarcoma if taken out of context.

Which Immunomarkers Are Useful in Diagnosing So-Called *SMARCA4*-Deficient Thoracic Malignant Tumors?

Although the originally proposed term *SMARCA4*-deficient thoracic sarcoma has been widely used in the literature (Le Loarer et al 2015; Perret et al 2019; Sauter et al 2017; Yoshida et al 2017), recent data suggest epithelial derivation in many of these cases (Rekhtman et al 2020), which raises controversy regarding appropriate terminology. The 2020 World Health Organization (WHO) classification uses the term thoracic *SMARCA4*-deficient undifferentiated tumor.

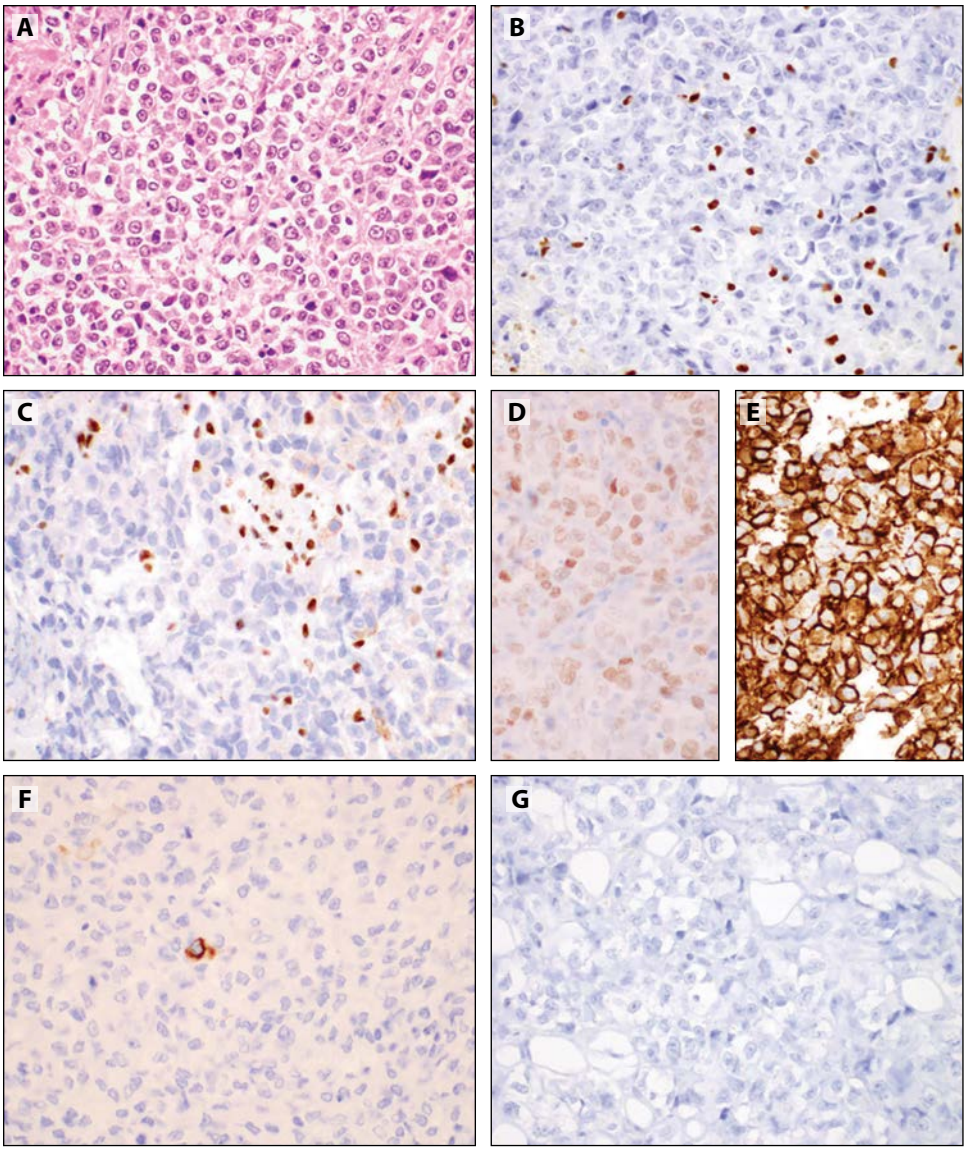


Figure 15-1. (A) Thoracic *SMARCA4*-deficient undifferentiated tumor. **(B)** SMARCA4 expression is typically entirely lost against the intact positive controls such as endothelial cells or inflammatory cells. **(C)** SMARCA2 (BRM) staining is lost in the great majority of cases. SMARCB1 (integrase interactor 1 [INI1]) expression is always retained. Many cases express **(D)** spalt-like transcription factor 4 (sal-like protein 4 [SALL4]) and **(E)** CD34. **(F)** Cytokeratin should be focal or negative, and **(G)** claudin-4 should be negative.

The defining feature of this entity is the *SMARCA4* (BRG1) deficiency in addition to classic histology. SMARCA4 expression is typically entirely lost against the internal positive controls such as endothelial cells or inflammatory cells (Perret et al 2019; Sauter et al 2017; Yoshida et al 2017). In addition, SMARCA2 (BRM) staining is lost in the great majority of cases (Perret et al 2019; Yoshida et al 2017), but SMARCB1 (integrase interactor 1 [INI1]) expression is always retained. Cases can express spalt-like transcription factor 4 (sal-like protein 4 [SALL4]), CD34, or SOX2 (Perret et al 2019; Yoshida et al 2017), and SALL4 expression may lead to misdiagnosis of germ cell tumor. Cytokeratin is often expressed typically in a focal

manner; diffuse strong solid cytoplasmic cytokeratin staining is highly unusual and should suggest carcinoma or mesothelioma. Claudin-4 is negative in most cases. Exceptional cases may express thyroid transcription factor-1 (TTF1). They are uniformly negative for nuclear protein in testis (NUT), desmin, myogenin, and S100. Loss of SMARCA4 expression is not specific for thoracic *SMARCA4*-deficient undifferentiated tumors as it can be seen in carcinomas of various organs including the lung, small cell carcinoma of the ovary, hypercalcemic type, and a subset of atypical teratoid rhabdoid tumors of the central nervous system (Figure 15-1A-G).

In a small subset of cases, SMARCA4 staining can be markedly reduced as compared with control cells but still visible (Yoshida et al 2017; Rekhtman et al 2020), as is shown in Figure 15-2.

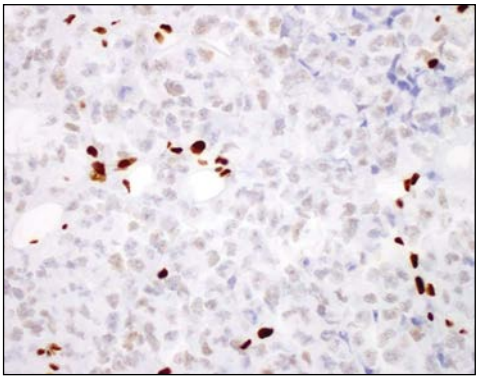


Figure 15-2. SMARCA4 staining can be reduced but visible, as compared to internal control.

Summary Answer

Thoracic *SMARCA4*-deficient undifferentiated tumor shows absence or marked reduction of immunostaining for SMARCA4 (BRG1).

When Should SMARCA4 Staining Be Considered in the Assessment of Thoracic Tumors?

A minor subset of pulmonary adenocarcinoma, large cell carcinoma, and pleomorphic carcinoma lack SMARCA4 immunoreactivity (Matsubara et al 2013; Oike et al 2013; Yoshimoto et al 2015). Such a subset of carcinoma tends to display poorly differentiated histology and is typically negative for TTF1. These tumors do not meet the histologic criteria of thoracic *SMARCA4*-deficient undifferentiated tumor, as they lack undifferentiated histology consisting of relatively monotonous mildly discohesive round cells with or without rhabdoid elements, which mimic malignant rhabdoid tumors or the proximal variant of epithelioid sarcoma (Figure 15-3). When there is clear epithelial differentiation, such as cohesive sheets, gland formation, papillary structure, or keratinization, SMARCA4 staining is not routinely

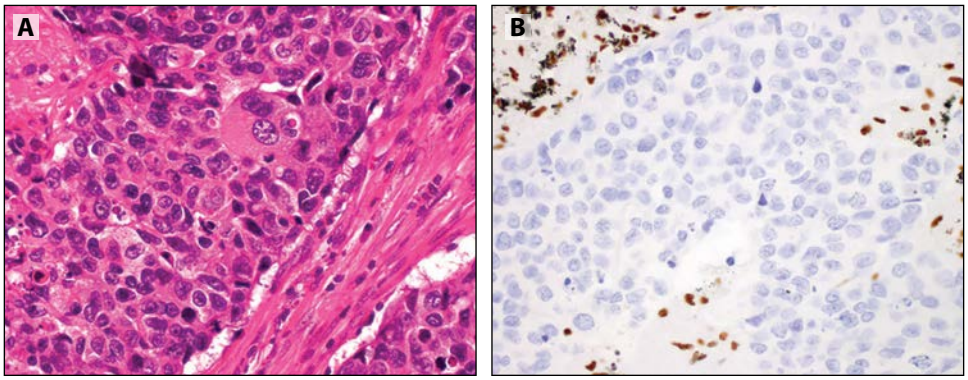


Figure 15-3. (A and B) An unequivocal case of carcinoma showing absence of SMARCA4 staining.

indicated for diagnosis. A recent study also identified a small number of composite tumors including a juxtaposing clear-cut carcinoma component (Rekhtman et al 2020).

Summary Answer

Staining for SMARCA4 should be performed in morphologically undifferentiated, relatively monotonous, discohesive, or rhabdoid pattern tumors, and not more generally in morphologically undifferentiated carcinomas.

Which Immunomarkers Are Useful to Prove Vascular Endothelial Differentiation?

The reliable combination of vascular endothelial markers is ERG and CD31, which stains more than 95% of vascular endothelial neoplasms (Miettinen et al 2011). Nuclear ERG expression in endothelial tumors is usually diffuse and strong, in contrast to being negative or at most, focally weakly expressed in some carcinomas. CD31 expression is usually diffuse and membranous in endothelial cells (Figure 15-4). Other endothelial markers include CD34, FLI1, and factor VIII-related antigen, but their sensitivity and specificity are not sufficient, and therefore not recommended as definitive markers of endothelial differentiation.

CD31 is also expressed in macrophages/histiocytes (Figure 15-5). Strong CD31 expression of tumor-infiltrating histiocytes should not be misconstrued as tumor reactivity and

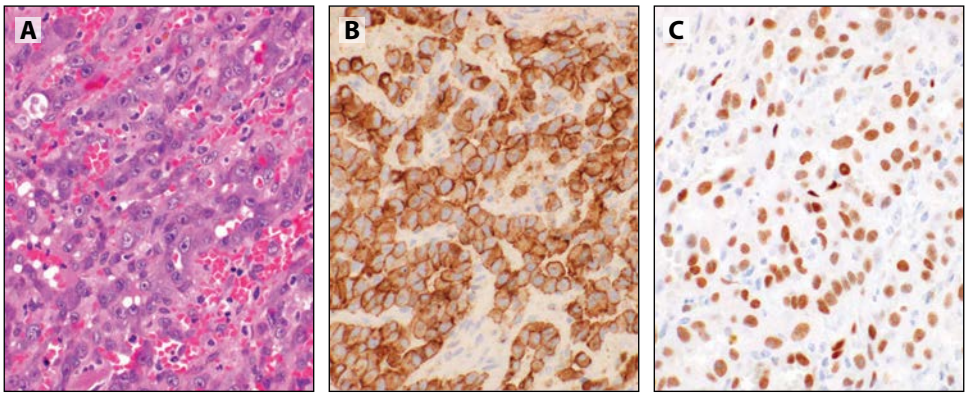


Figure 15-4. An epithelioid angiosarcoma **(A)** with immunohistochemistry for **(B)** CD31 with diffuse membranous staining and **(C)** ERG with strong nuclear staining.

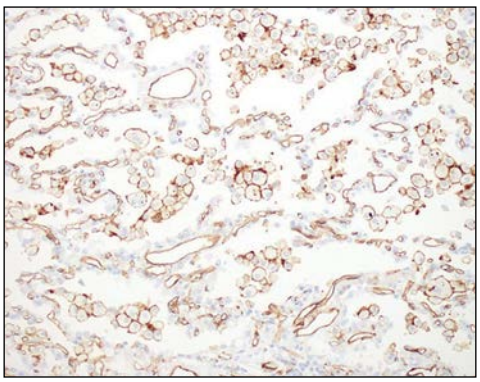


Figure 15-5. Immunohistochemistry for CD31 in lung tissue staining alveolar macrophages.

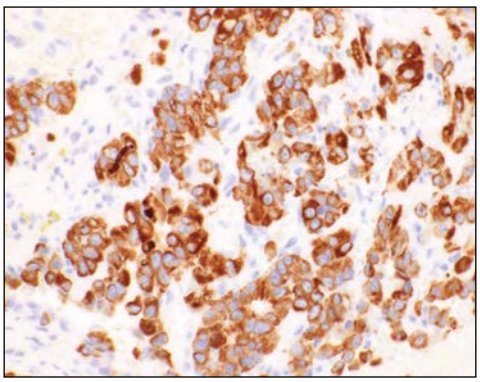


Figure 15-6. Immunohistochemistry for cytokeratin is positive in an epithelioid angiosarcoma.

evidence of endothelial differentiation (McKenney et al 2001). ERG expression is also observed in hematolymphoid cells, cartilaginous cells, epithelioid sarcoma, a subset of prostatic adenocarcinoma, and Ewing sarcoma.

Cytokeratin expression is relatively common in epithelioid vascular endothelial neoplasms and should not be taken as evidence of epithelial differentiation (Figure 15-6).

Summary Answer

ERG and CD31 are useful endothelial markers. Other markers such as CD34 and FLI1 are potentially useful but less definitive.

Which Immunomarkers Are Useful in Subtyping Malignant Vascular Endothelial Tumors?

Angiosarcomas and epithelioid hemangioendotheliomas (EHEs) are 2 distinct malignant vascular endothelial neoplasms that may arise from the lung and pleura. Often, morphology allows distinction between these 2 entities. Apart from endothelial markers found in both of these tumors, there are no other markers specific for angiosarcoma.

More than 95% of EHE harbors *WWTR1-CAMTA1* fusion and as a result, calmodulin-binding transcription activator 1 (CAMTA1) IHC is diffusely expressed in the nucleus of the neoplastic cells (Shibuya et al 2015; Doyle et al 2016) (Figure 15-7).

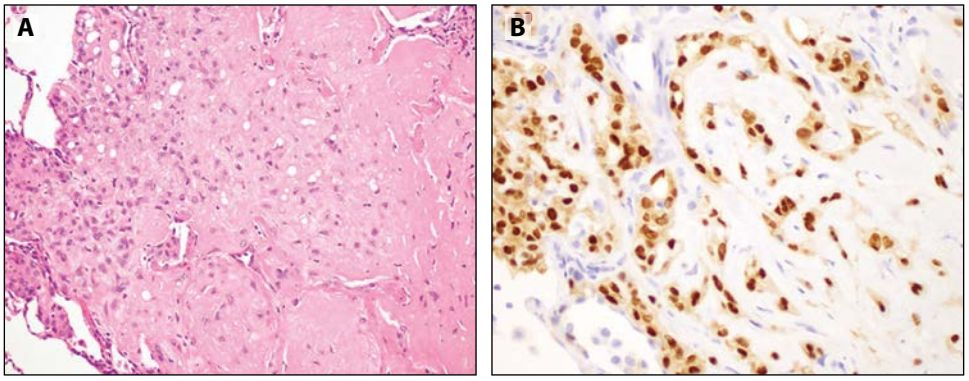


Figure 15-7. (A) An epithelioid hemangioendothelioma (EHE), showing **(B)** strong nuclear expression of calmodulin-binding transcription activator 1 (CAMTA1).

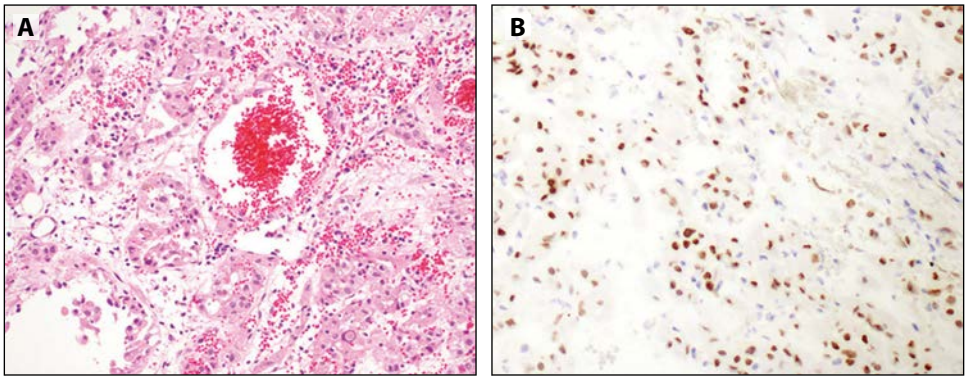


Figure 15-8. (A) *TFE3*-rearranged epithelioid hemangioendothelioma (EHE), showing **(B)** transcription factor E3 (TFE3) nuclear reactivity.

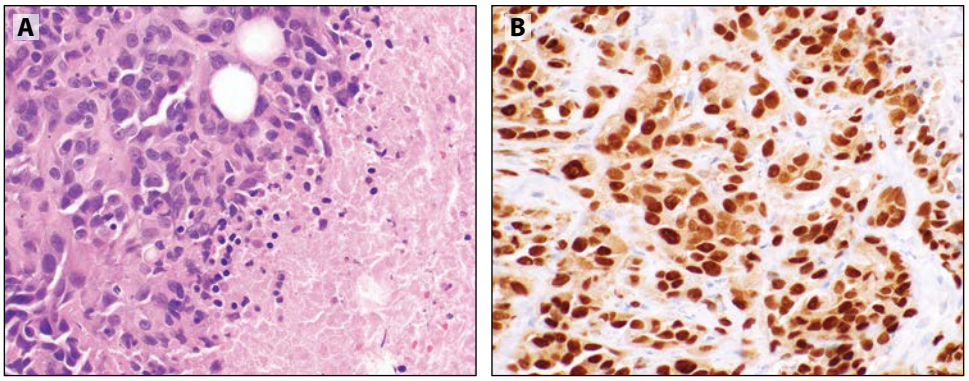


Figure 15-9. (A) This epithelioid hemangioendothelioma (EHE) with an aggressive morphology showed **(B)** a positive calmodulin-binding transcription activator 1 (CAMTA1) staining. This confirms the diagnosis.

The remaining less than 5% of EHE harbors *YAP1-TFE3* fusion, and accordingly, they are diffusely positive for nuclear transcription factor E3 (TFE3) reactivity (Figure 15-8) (Antonescu et al 2013).

Some EHEs show aggressive histology, and its CAMAT1 reactivity, which is lacking in epithelioid angiosarcoma, helps to resolve the differential diagnosis (Figure 15-9).

Summary Answer

IHC for CAMTA1 and TFE3 is a surrogate for translocation and can be useful in vascular tumor subclassification.

Which Immunomarkers Are Useful for Distinguishing Synovial Sarcoma from Its Mimics?

Synovial sarcomas are positive for cytokeratin and/or epithelial membrane antigen (EMA) in 80% to 90% of cases, with EMA staining often more widespread than cytokeratin (Pelmus et al 2002) (Figure 15-10). Conversely, many other tumors with sarcomatoid histology (eg, sarcomatoid carcinomas) may express EMA and cytokeratin, and therefore, it is imperative not to overly rely on these markers for a diagnosis of synovial sarcoma. In contrast to solitary fibrous tumors, synovial sarcomas are usually negative for CD34 (Pelmus et al 2002), and they lack signal transducer and activator of transcription 6 (STAT6) staining. p40 helps to distinguish synovial sarcoma from a type A thymoma. Moderate to strong nuclear expression of transducin-like enhancer protein 1 (TLE1) is observed in 90% of synovial sarcomas (Figure 15-10C); however, this is not entirely specific (Terry et al 2007; Kosemehmetoglu et al 2009; Foo et al 2011). Reduced expression of SMARCB1 (INI1) when compared to intact intensity of in-background endothelial cells and inflammatory cells is seen in 90% of cases, which is thought to be specific for synovial sarcoma among spindle cell tumors (Ito et al 2016; Arnold et al 2013) (Figure 15-10D).

Many synovial sarcomas express CD56 (Hartel et al 2007), with a minority of cases positive for synaptophysin (Satoh et al 2015), which is a phenotype that might pose a pitfall for a misdiagnosis as neuroendocrine carcinoma, especially on small biopsies (Figure 15-11).

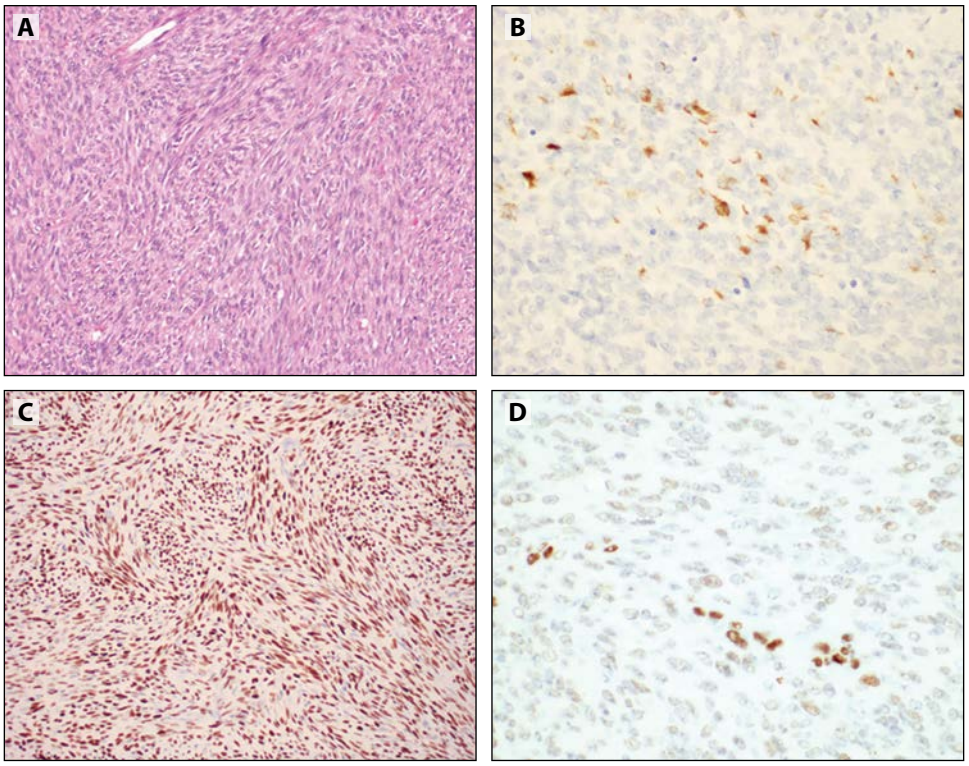


Figure 15-10. Synovial sarcoma immunohistochemistry. The monophasic synovial sarcoma in **(A)** is focally positive for **(B)** cytokeratin but shows **(C)** diffuse transducin-like enhancer of split 1 (TLE1) nuclear expression. Reduction in SMARCB1 (integrase interactor 1 [INI1]) is a known staining pattern, as compared to endothelial cells, shown in **(D)**.

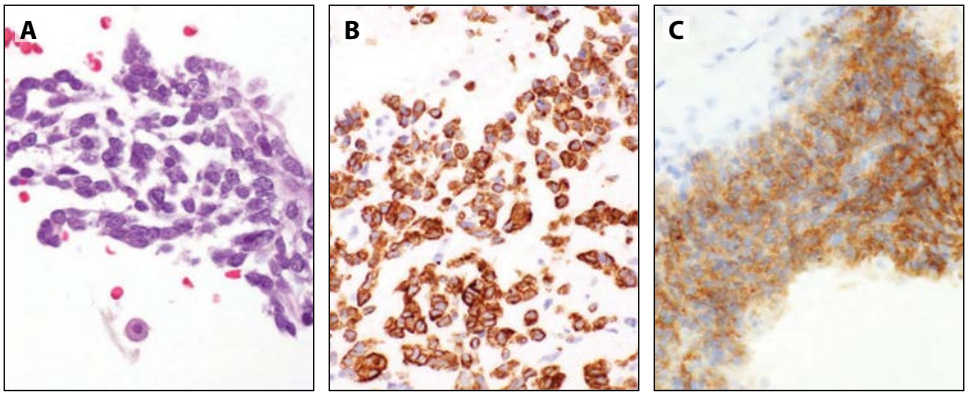


Figure 15-11. (A) A synovial sarcoma with small cell morphology, where **(B)** the positivity for keratin and **(C)** synaptophysin in such cases can mimic small cell carcinoma.

Summary Answer

IHC for cytokeratin and TLE1, with relevant negatives, can be helpful in the diagnosis of synovial sarcoma. However, difficult cases can be confirmed with fluorescence in situ hybridization (FISH) or molecular testing.

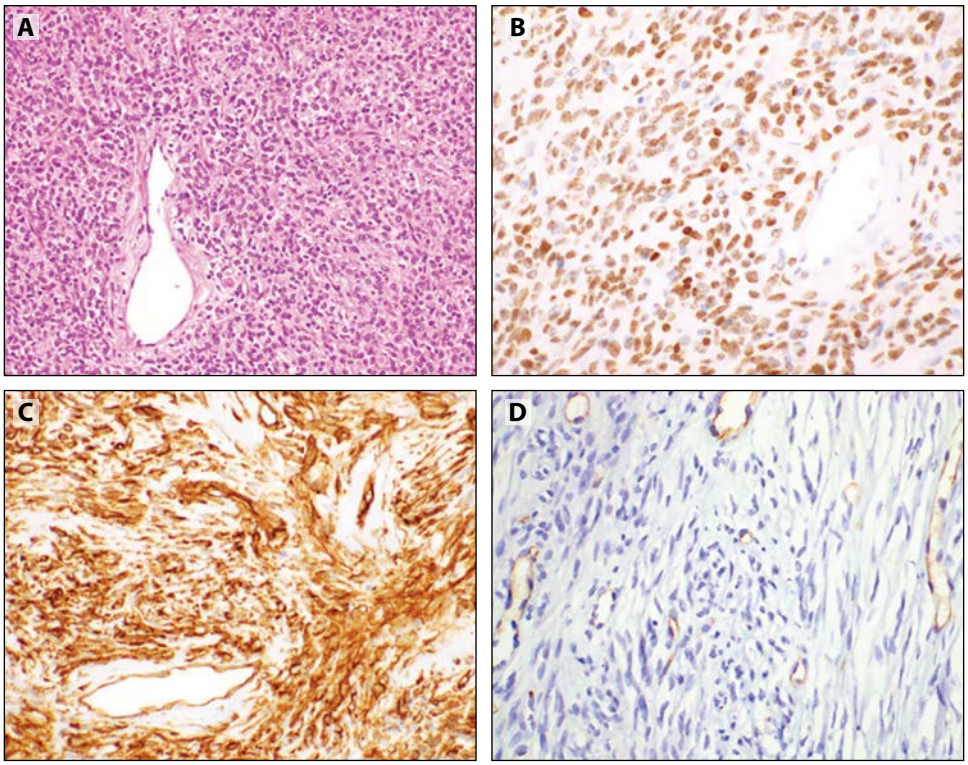


Figure 15-12. (A) Solitary fibrous tumor with **(B)** nuclear signal transducer and activator of transcription 6 (STAT6) and **(C)** positive CD34. However, CD34 is negative **(D)** in some cases, especially malignant tumors.

Which Immunomarkers Are Useful for Assessing Solitary Fibrous Tumors?

Virtually all solitary fibrous tumors (SFTs) harbor the specific fusion *NAB2-STAT6*. This fusion leads to abnormal accumulation of STAT6 protein in the nucleus, which is readily detected as strong nuclear STAT6 immunostaining (Figure 15-12A and B) The reactivity is observed across a wide histologic spectrum of SFTs and is accepted as a highly sensitive and specific marker for that diagnosis (Doyle et al 2014b; Yoshida et al 2014). CD34 is positive in more than 90% of SFTs, but staining can be focal or even absent, particularly in malignant SFTs. Although bcl-2 is often positive, this marker is not specific (Figure 15-12C and D).

The performance of STAT6 IHC can be markedly influenced by fixation. For example, large resection specimens often show reactivity only at the periphery with the center of the sample negative (Figure 15-13A), requiring careful searching for positive areas (Yoshida et al 2014).

Although monoclonal STAT6 antibody often enables unequivocal interpretation (Cheah et al 2014), a polyclonal STAT6 antibody sometimes gives a blurry reaction both in the cytoplasm and nuclei in non-SFT tumors, which should be interpreted with caution (Figure 15-13B). A small subset (~10%) of dedifferentiated liposarcomas shows STAT6 immunoreactivity because of *STAT6* amplification (Doyle et al 2014a) (Figure 15-13C).

Summary Answer

STAT6, using a monoclonal antibody, is a sensitive and relatively specific marker for SFTs.

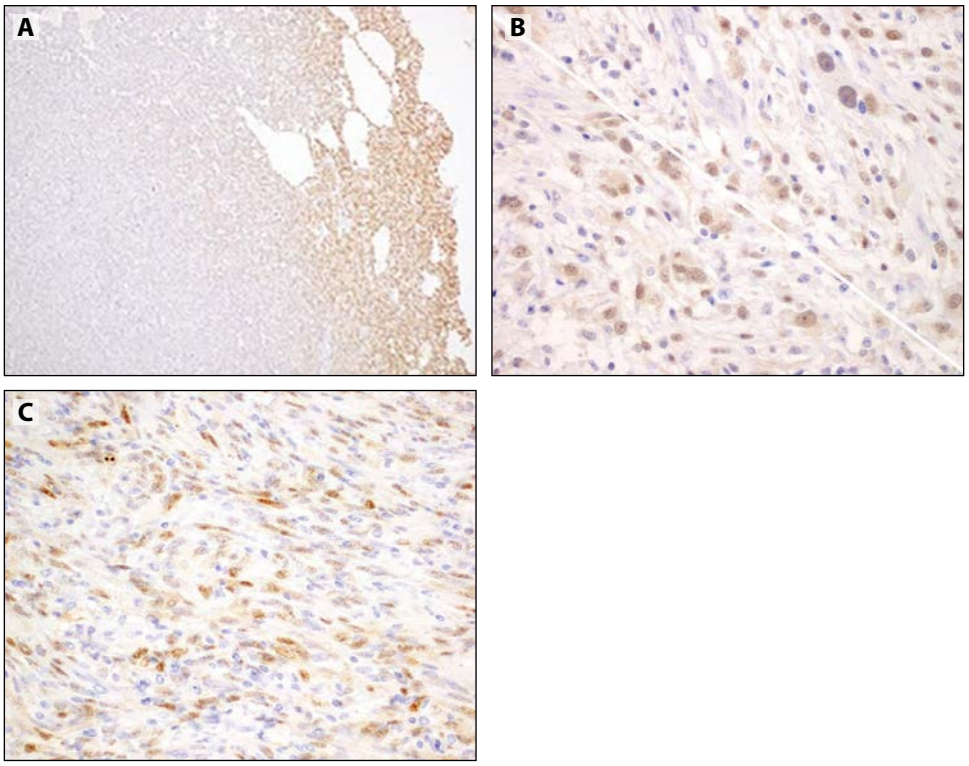


Figure 15-13. Pitfalls of signal transducer and activator of transcription 6 (STAT6) immunohistochemistry. **(A)** Fixation can result in a peripheral staining pattern in the tumor tissue. **(B)** Undifferentiated pleomorphic sarcoma showing cytoplasmic/nuclear STAT6 reactivity with a polyclonal reagent, which should not be interpreted as diagnostically positive with respect to solitary fibrous tumor (SFT). **(C)** A de-differentiated liposarcoma with multifocal staining for STAT6 with both cytoplasmic and nuclear distribution.

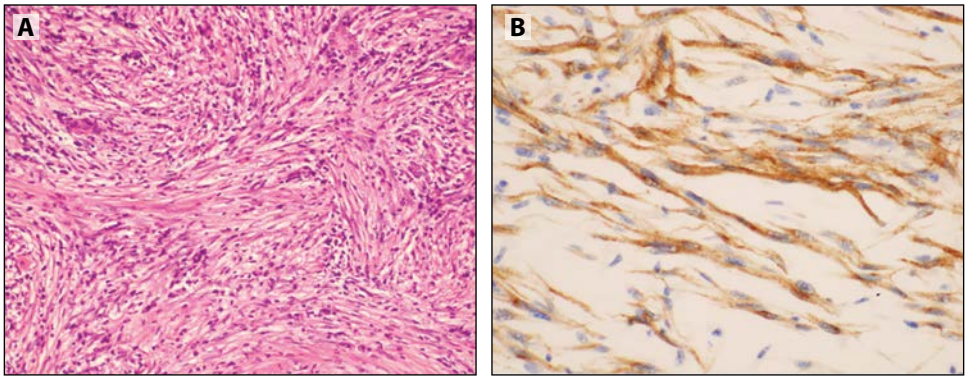


Figure 15-14. (A) Inflammatory myofibroblastic tumors are **(B)** immunopositive for anaplastic lymphoma kinase (ALK). This is seen in *ALK* translocation positive cases (60%-70%).

Are Immunomarkers Helpful in Diagnosing Inflammatory Myofibroblastic Tumors?

Approximately 60% to 70% of inflammatory myofibroblastic tumors (IMTs) harbor *ALK* fusions, with a number of different partner genes (Figure 15-14). Some fusions (such as *PPFIBP1-ALK*) express low level of anaplastic lymphoma kinase (ALK) protein, and therefore

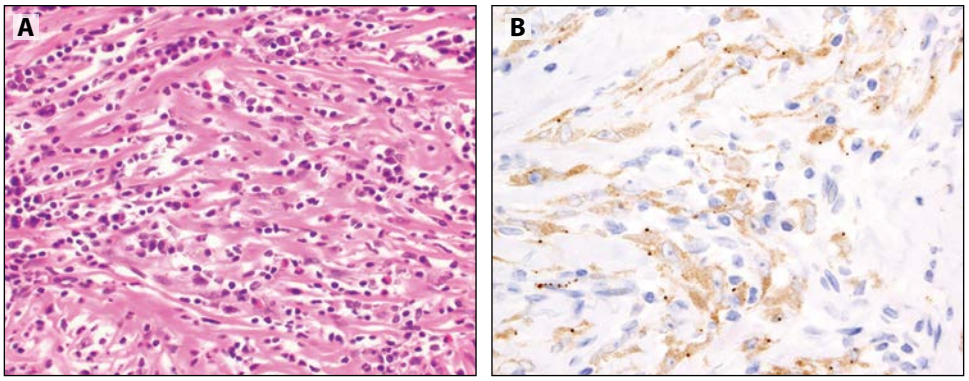


Figure 15-15. (A) This inflammatory myofibroblastic tumor (IMT) with c-ros oncogene 1 **(B)** (*ROS1*) protein translocation shows ROS1 immunoreactivity.

to achieve an optimal detection, a highly sensitive ALK clone such as D5F3, 1A4, or 5A4, is recommended (Takeuchi et al 2011). ALK expression is not specific for IMT in the setting of a spindle cell tumor in the lung. In addition to sarcomatoid differentiation of *ALK*-rearranged non-small cell lung carcinoma (NSCLC) (Mason et al 2016), other tumors such as rhabdomyosarcoma and angiomatoid fibrous histiocytoma may express ALK despite the lack of fusion gene (Cheah et al 2019; Yoshida et al 2013). IMTs express smooth muscle actin (SMA) and/or desmin at variable degrees reflecting myofibroblastic differentiation. Co-expression of SMA and desmin does not necessarily indicate smooth muscle differentiation. IMTs can be positive for cytokeratin, which should not lead to a misdiagnosis of epithelial tumors (Coffin et al 1995).

A small subset of ALK-negative IMTs harbors alternative c-ros oncogene 1 (*ROS1*) or neurotrophic tyrosine receptor kinase (*NTRK*) rearrangements, and these subsets are detectable by ROS1 (Figure 15-15) and pan-TRK IHC, respectively (Hornick et al 2015; Yamamoto et al 2019).

Summary Answer

In the correct morphologic setting, IHC for ALK or ROS1 can be helpful in the diagnosis of IMT.

Which Immunomarkers Are Useful for Assessing Pleomorphic Spindle Cell Sarcomas?

Virtually all dedifferentiated liposarcomas harbor high-level amplification of the *MDM2* gene, with co-amplification of *CDK4* in 80% of cases. This is reflected by immunohistochemical co-expression of mouse double minute 2 homolog/E3 ubiquitin-protein ligase (MDM2) and cyclin-dependent kinase 4 (CDK4) in more than 90% of cases (Figure 15-16) (Binh et al 2005). The expression of either MDM2 or CDK4 alone is not specific for gene amplification, and MDM2 reactivity itself can be seen in 20% of other sarcoma types (Binh et al 2005). In addition, sarcomatoid carcinomas or mesotheliomas may show MDM2 overexpression and therefore, correlation with morphology and other markers is necessary.

Approximately 70% of intimal sarcomas (including cardiac intimal sarcoma [Neuville et al 2014]) harbor *MDM2* amplification, and as a result, MDM2 immunostaining is positive

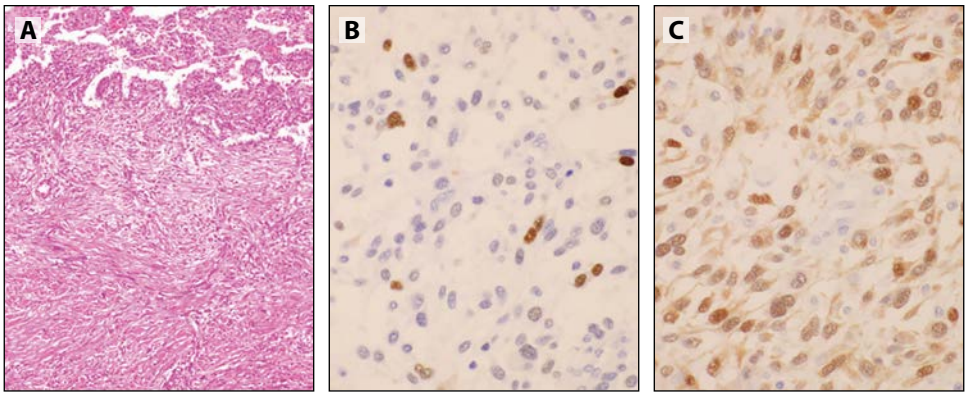


Figure 15-16. (A) Dedifferentiated liposarcoma involving the lung showing **(B)** immunoreactivity for MDM2 and **(C)** CDK4.

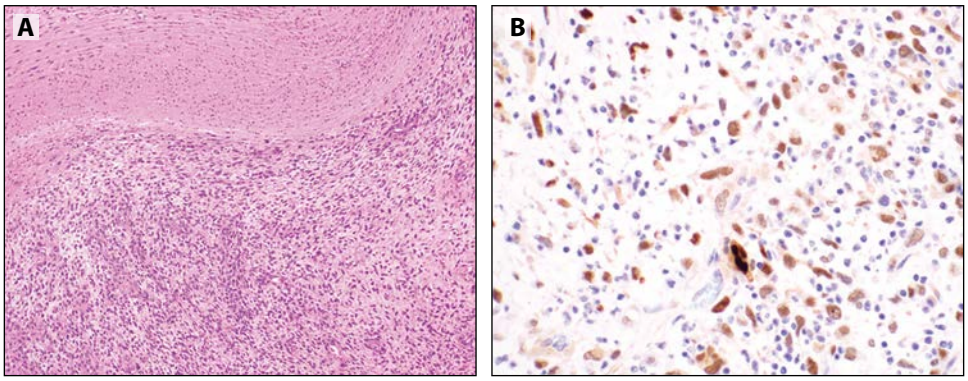


Figure 15-17. (A) Intimal sarcoma of the pulmonary artery, with arterial media lying superiorly in this image, is **(B)** positive for nuclear MDM2.

(Bode-Lesniewska et al 2001) (Figure 15-17). However, lack of *MDM2* amplification or MDM2 overexpression does not exclude the diagnosis. Intimal sarcoma typically shows undifferentiated pleomorphic sarcoma histology; however, a subset of cases display heterologous differentiation, both morphologically and immunophenotypically.

Leiomyosarcomas show smooth muscle differentiation, exemplified by reactivity to SMA, desmin, and h-caldesmon. At least 2 of these markers are positive in addition to classic histology in most cases, and h-caldesmon is the most specific of them (Figure 15-18).

Only half of malignant peripheral nerve sheath tumors (MPNSTs) shows immunoreactivity to S100 protein and/or SOX10, and staining is often weak and focal. Diffuse strong S100 or SOX10 staining therefore suggests an alternative diagnosis, such as cellular schwannoma or sarcomatoid malignant melanoma. MPNST often harbors inactivating alterations of the genes encoding SUZ12 or EED, key components of polycomb repressive complex 2, and consequently, trimethylation of histone H3 at lysine 27 is lost. This phenomenon is visualized as loss of expression of H3K27me3 (Figure 15-19), which is observed in approximately 50% to 60% of MPNSTs, unlike its mimics, such as synovial sarcoma, which retains H3K27me3 (Schaefer et al 2016; Asano et al 2017; Prieto-Granada et al 2016).

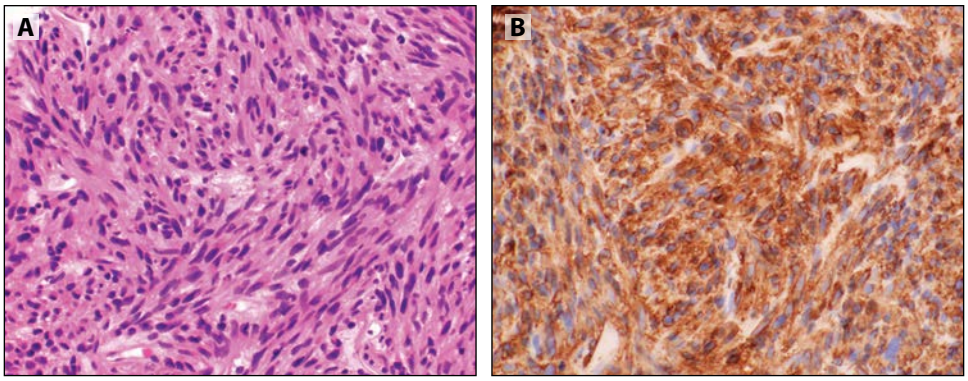


Figure 15-18. (A) A leiomyosarcoma **(B)** positive for h-caldesmon.

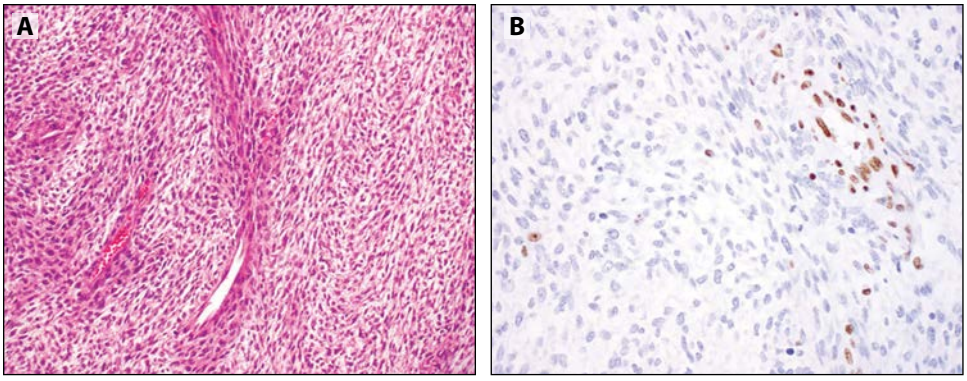


Figure 15-19. (A) Malignant peripheral nerve sheath tumors (MPNSTs) with **(B)** nuclear loss of H3K27me3.

Summary Answer

Various markers can help classify spindle cell sarcoma, including MDM2, muscle markers, and H3K27me3, but all can have reactivity in more than one tumor type.

Which Immunomarkers Are Useful in Assessing Round Cell Sarcoma?

Most Ewing sarcomas display CD99 expression. Although CD99 positivity itself can be seen in many tumors, diffuse strong membranous CD99 staining can still be a helpful finding to trigger further work-up for Ewing sarcoma. These tumors may also express CD56, synaptophysin, and/or insulinoma-associated protein 1 (INSM1), and are positive for cytokeratin in approximately 20% of cases, making a distinction from small cell carcinoma even more difficult. More specific Ewing sarcoma markers include NKX2-2 and PAX7 (Toki et al 2018) (Figure 15-20).

However, NKX2-2 can be positive in a proportion of small cell carcinomas and neuroendocrine tumors of pancreatic and gastrointestinal tract origin, and it should be used with caution in light of clinical histologic context (Hung et al 2016; Yoshida et al 2012) (Figure 15-21).

CIC-rearranged sarcomas show nuclear Wilms tumor 1 (WT1) and ETS variant transcription factor 4 (ETV4) expression in 80% to 90% of cases, unlike Ewing sarcoma (Yoshida et al 2016; Hung et al 2016). *CIC*-rearranged sarcomas sometimes show epithelioid morphology together with scattered cytokeratin expression, and many cases express

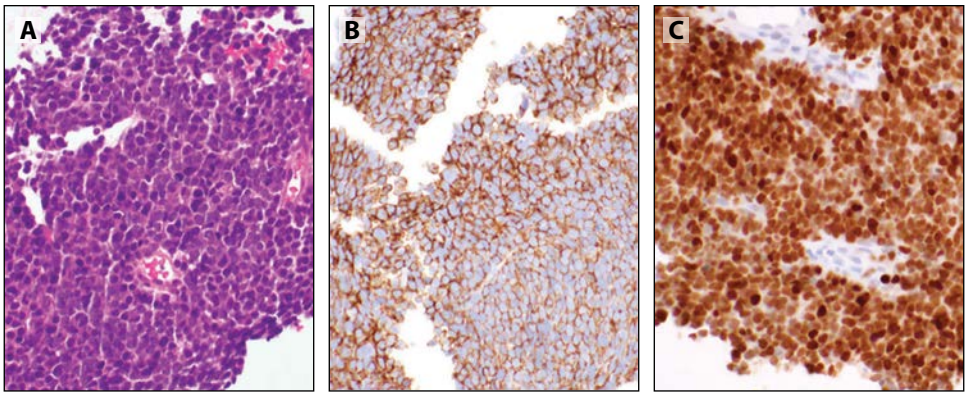


Figure 15-20. (A) Ewing sarcoma with **(B)** strong membranous CD99 and **(C)** nuclear NKX2-2 expression.

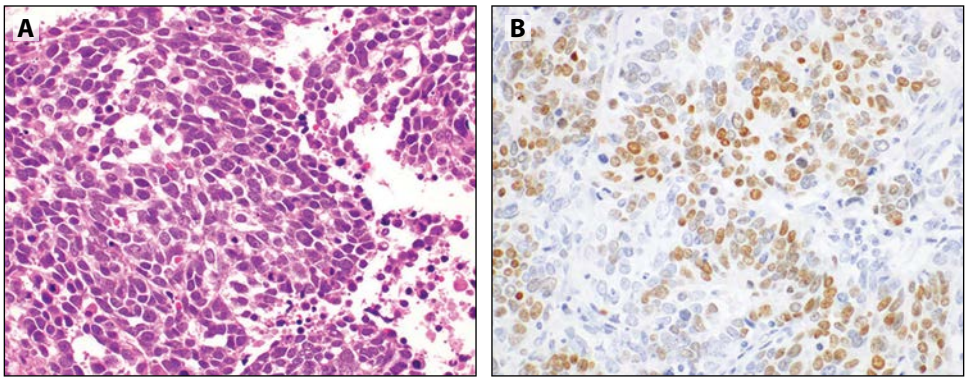


Figure 15-21. (A) A small cell carcinoma is **(B)** positive for NKX2-2, a diagnostic pitfall with respect to Ewing sarcoma.

calretinin in addition to WT1, leading to a potential misdiagnosis of mesothelioma (Yoshida et al 2016) (Figure 15-22).

Desmoplastic small round cell tumors rarely occur in the pleura. They show co-expression of cytokeratin and desmin. These tumors are characterized by nuclear expression of WT1 only when WT1 (C-terminus) antibody is used, whereas WT1 (N-terminus) staining is negative (Barnoud et al 2000) (Figure 15-23).

It is rare for rhabdomyosarcomas to primarily involve the thoracic cavity. Rhabdomyosarcoma is typically positive for myogenin and/or MYOD1, in addition to desmin (Figure 15-24).

Alveolar rhabdomyosarcoma can be positive for cytokeratin (Figure 15-25A), CD56 (Figure 15-25B), and synaptophysin (Figure 15-25C), which may lead to a misdiagnosis of small cell carcinoma (Bahrami et al 2008; Wallace et al 2019).

Summary Answer

Markers of round cell sarcoma may be helpful in the diagnosis, but some overlap remains with more common entities such as small cell carcinoma. Epidemiologic considerations (age) and tumor location can be helpful, as well as FISH and molecular testing, as needed.

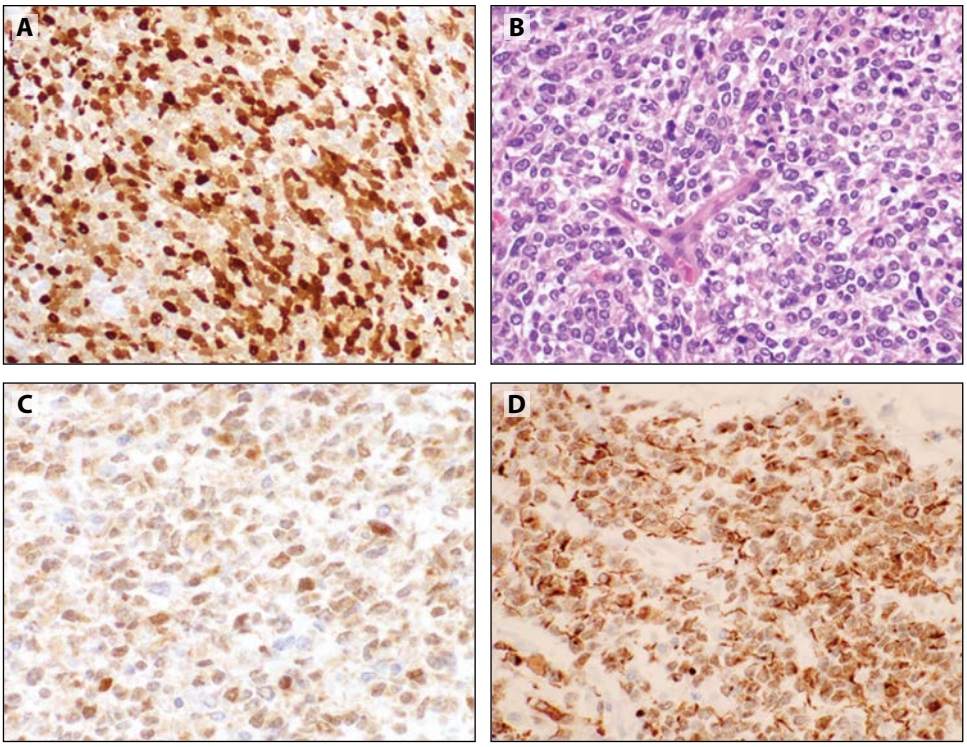


Figure 15-22. (A) *CIC*-rearranged sarcoma with **(B)** membranous ETS translocation variant 4 (ETV4), **(C)** nuclear Wilms tumor 1 (WT1), and **(D)** positive calretinin expression.

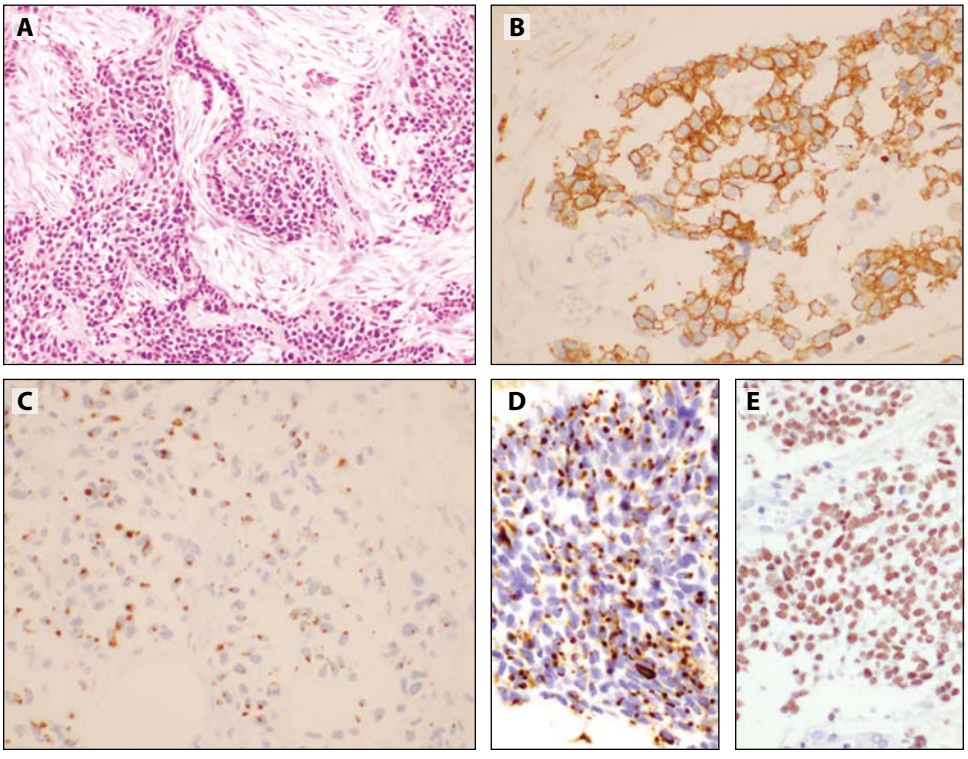


Figure 15-23. (A) Desmoplastic small round cell tumors, **(B)** positive for cytokeratin AE1/AE3, **(C)** desmin, **(D)** Wilms tumor 1 (WT1) N-terminus showing only cytoplasmic reactivity with negative nuclear staining, **(E)** WT1 C-terminus showing positive nuclear staining.

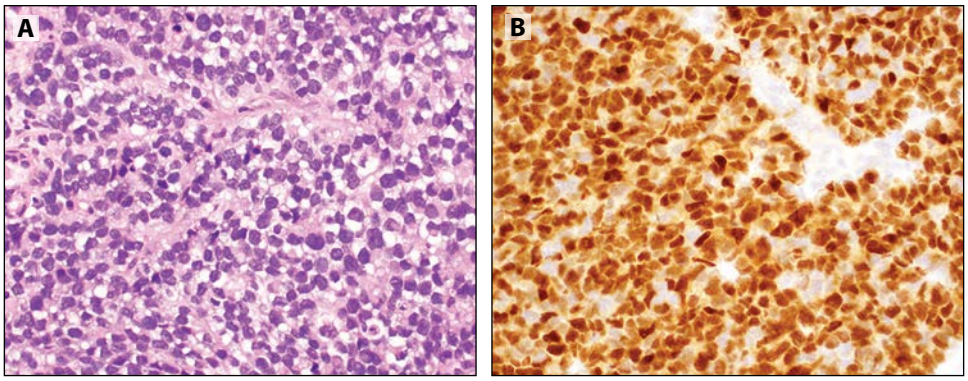


Figure 15-24. (A) An alveolar rhabdomyosarcoma, **(B)** positive for myogenin.

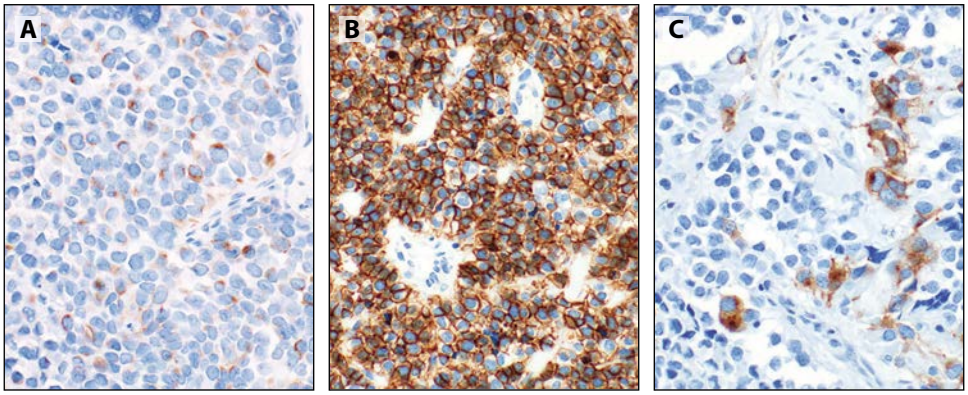


Figure 15-25. (A) An alveolar rhabdomyosarcoma, positive for cytokeratin, **(B)** CD56, and **(C)** synaptophysin.

Which Immunomarkers Help Distinguish PEComa from Its Mimics?

PEComas (synonyms for perivascular epithelioid cell tumors are clear cell tumors or sugar tumors) usually express melanocytic markers including HMB45 (focal granular cytoplasmic staining pattern), melan A, tyrosinase A, CD68 (clone KP1), and cathepsin K; and a subset expresses S100 and/or microphthalmia transcription factor (MiTF). In general, these tumors are negative for keratin and TTF1. These stains help to distinguish this tumor from carcinomas that might show clear cell features such as squamous cell carcinoma, solid type adenocarcinoma, or metastatic clear cell renal cell carcinoma. In contrast to PEComas, lesional cells in lymphangioleiomyomatosis more extensively express SMA and sometimes desmin and are negative for S100 (Calio et al 2018; Thway and Fisher 2015; Lantuejoul et al 1997) (Figure 15-26).

Summary Answer

PEComas are generally negative for cytokeratin and positive for HMB45, melan A, and tyrosinase A.

Conclusions

Although primary thoracic mesenchymal tumors and sarcomas are rare, they can mimic many other tumors in the lung and mediastinum and therefore need to be considered in the work-up of other neoplasms. Moreover, sarcomas frequently metastasize to the thorax,

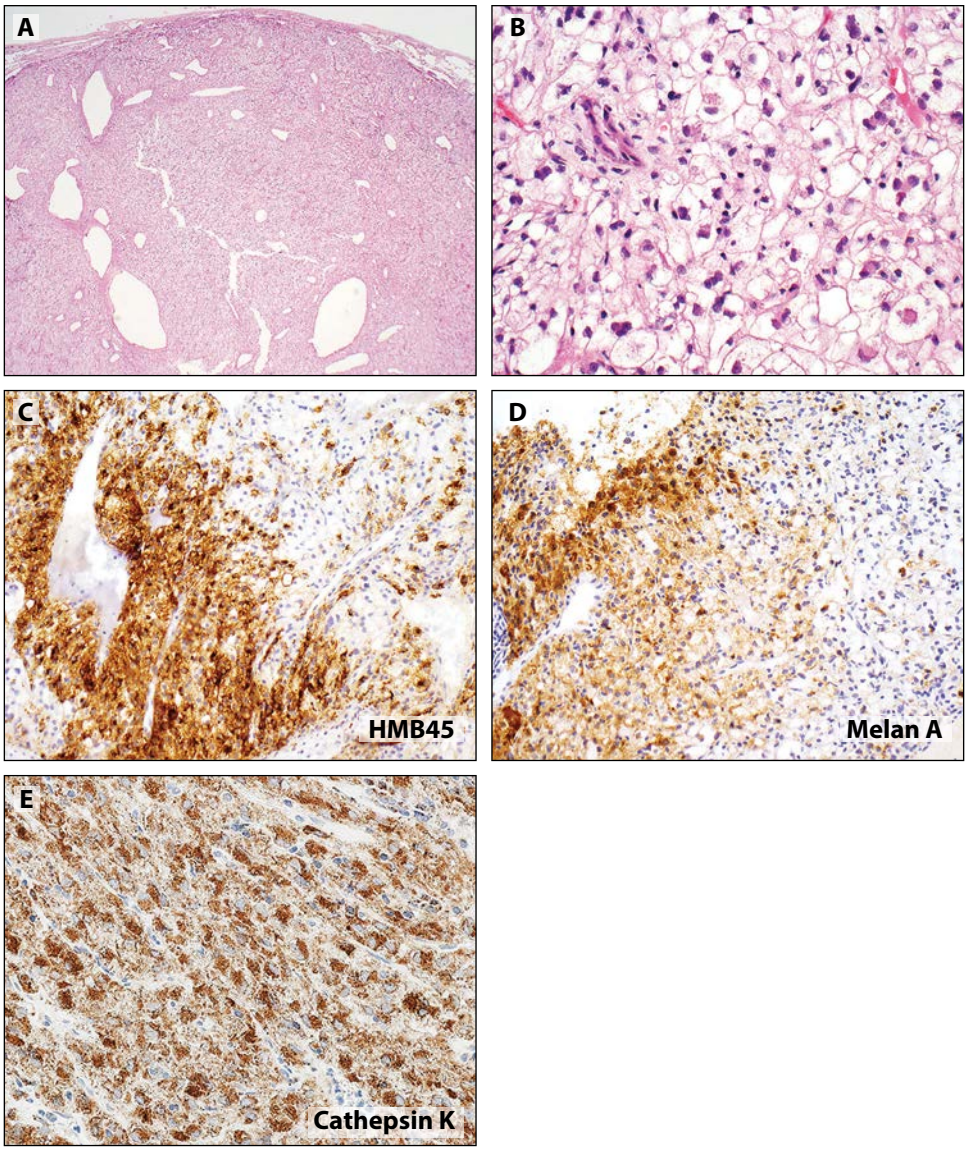


Figure 15-26. (A) PEComa at low magnification is circumscribed and **(B)** at higher magnification shows clear cells. **(C)** Immunohistochemistry (IHC) for HMB45, **(D)** melan A, and **(E)** cathepsin K are positive. (**E** is courtesy of Dr. H. Ninomiya, Division of Pathology, the Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan)

sometimes many years after the primary tumor has been treated. IHC can be valuable in the distinction of many of these tumors, and usually a panel of immunostains has to be applied because most of the markers are not specific for a single tumor entity. Furthermore, in some tumors, although IHC can help in the diagnostic work-up, a final diagnosis may eventually require molecular studies (eg, synovial sarcomas or Ewing sarcomas).

References

Antonescu CR, Le Loarer F, Mosquera JM, et al. Novel YAP1-TFE3 fusion defines a distinct subset of epithelioid hemangioendothelioma. *Genes Chromosomes Cancer*. 2013;52(8):775-784.

Arnold MA, Arnold CA, Li G, et al. A unique pattern of INI1 immunohistochemistry distinguishes synovial sarcoma from its histologic mimics. *Hum Pathol*. 2013;44(5):881-887.

Asano N, Yoshida A, Ichikawa H, et al. Immunohistochemistry for trimethylated H3K27 in the diagnosis of malignant peripheral nerve sheath tumours. *Histopathology*. 2017;70(3):385-393.

Bahrami A, Gown AM, Baird GS, et al. Aberrant expression of epithelial and neuroendocrine markers in alveolar rhabdomyosarcoma: a potentially serious diagnostic pitfall. *Mod Pathol*. 2008;21(7):795-806.

Barnoud R, Sabourin JC, Pasquier D, et al. Immunohistochemical expression of WT1 by desmoplastic small cell tumor: a comparative study with other small round cell tumors. *Am J Surg Pathol*. 2000;24(6):830-836.

Binh MB, Sastre-Garau X, Guillou L, et al. MDM2 and CDK4 immunostainings are useful adjuncts in diagnosing well-differentiated and dedifferentiated liposarcoma subtypes: a comparative analysis of 559 soft tissue neoplasms with genetic data. *Am J Surg Pathol*. 2005;29(10):1340-1347.

Bode-Lesniewska B, Zhao J, Speel EJ, et al. Gains of 12q13-14 and overexpression of mdm2 are frequent findings in intimal sarcomas of the pulmonary artery. *Virchows Arch*. 2001;438(1):57-65.

Calio A, Mengoli MC, Cavazza A, et al. Cathepsin K expression in clear cell "sugar" tumor (PEComa) of the lung. *Virchows Arch*. 2018;473(1):55-59.

Cheah AL, Billings SD, Goldblum JR, et al. STAT6 rabbit monoclonal antibody is a robust diagnostic tool for the distinction of solitary fibrous tumour from its mimics. *Pathology*. 2014;46(5):389-395.

Cheah AL, Zou Y, Lanigan C, et al. ALK expression in angiomatoid fibrous histiocytoma: a potential diagnostic pitfall. *Am J Surg Pathol*. 2019;43(1):93-101.

Coffin CM, Watterson J, Priest JR, et al. Extrapulmonary inflammatory myofibroblastic tumor (inflammatory pseudotumor). A clinicopathologic and immunohistochemical study of 84 cases. *Am J Surg Pathol*. 1995;19(8):859-872.

Doyle LA, Fletcher CD, Hornick JL. Nuclear expression of CAMTA1 distinguishes epithelioid hemangioendothelioma from histologic mimics. *Am J Surg Pathol*. 2016;40(1):94-102.

Doyle LA, Tao D, Marino-Enriquez A. STAT6 is amplified in a subset of dedifferentiated liposarcoma. *Mod Pathol*. 2014;27(9):1231-1237. (a)

Doyle LA, Vivero M, Fletcher CD, et al. Nuclear expression of STAT6 distinguishes solitary fibrous tumor from histologic mimics. *Mod Pathol*. 2014;27(3):390-395. (b)

Foo WC, Cruise MW, Wick MR, et al. Immunohistochemical staining for TLE1 distinguishes synovial sarcoma from histological mimics. *Am J Clin Pathol*. 2011;135(6):839-844.

Hartel PH, Fanburg-Smith JC, Frazier AA, et al. Primary pulmonary and mediastinal synovial sarcoma: a clinicopathologic study of 60 cases and comparison with five prior series. *Mod Pathol*. 2007;20(7):760-769.

Hornick JL, Sholl LM, Dai Cin P, et al. Expression of ROS1 predicts ROS1 gene rearrangement in inflammatory myofibroblastic tumors. *Mod Pathol*. 2015;28(5):732-739.

Hung YP, Fletcher CD, Hornick JL. Evaluation of NKX2-2 expression in round cell sarcomas and other tumors with EWSR1 rearrangement: imperfect specificity for Ewing sarcoma. *Mod Pathol*. 2016;29(4):370-380.

Ito J, Asano N, Kawai A, et al. The diagnostic utility of reduced immunohistochemical expression of SMARCB1 in synovial sarcomas: a validation study. *Hum Pathol*. 2016;47(1):32-37.

Kosemehmetoglu K, Vrana JA, Folpe AL. TLE1 expression is not specific for synovial sarcoma: a whole section study of 163 soft tissue and bone neoplasms. *Mod Pathol*. 2009;22(7):872-878.

Lantuejoul S, Issac S, Pinel N, et al. Clear cell tumor of the lung: an immunohistochemical and ultrastructural study supporting a pericytic differentiation. *Mod Pathol*. 1997;10(10):1001-1008.

Le Loarer F, Watson S, Pierron G, et al. SMARCA4 inactivation defines a group of undifferentiated thoracic malignancies transcriptionally related to BAF-deficient sarcomas. *Nat Genet*. 2015;47(10):1200-1205.

Mason EF, Fletcher CD, Sholl LM. 'Inflammatory myofibroblastic tumour'-like dedifferentiation of anaplastic lymphoma kinase-rearranged lung adenocarcinoma. *Histopathology*. 2016;69(3):510-515.

Matsubara D, Kishaba Y, Ishikawa S, et al. Lung cancer with loss of BRG1/BRM, shows epithelial mesenchymal transition phenotype and distinct histologic and genetic features. *Cancer Sci*. 2013;104(2):266-273.

McKenney JK, Weiss SW, Folpe AL. CD31 expression in intratumoral macrophages: a potential diagnostic pitfall. *Am J Surg Pathol*. 2001;25(9):1167-1173.

Miettinen M, Wang ZF, Paetau A, et al. ERG transcription factor as an immunohistochemical marker for vascular endothelial tumors and prostatic carcinoma. *Am J Surg Pathol*. 2011;35(3):432-441.

Neuville A, Collin F, Bruneval P, et al. Intimal sarcoma is the most frequent primary cardiac sarcoma: clinicopathologic and molecular retrospective analysis of 100 primary cardiac sarcomas. *Am J Surg Pathol*. 2014;38(4):461-469.

Oike T, Oglwara H, Tominaga Y, et al. A synthetic lethality-based strategy to treat cancers harboring a genetic deficiency in the chromatin remodeling factor BRG1. *Cancer Res*. 2013;73(17):5508-5018.

Pelmus M, Gillou L, Hostien I, et al. Monophasic fibrous and poorly differentiated synovial sarcoma: immunohistochemical reassessment of 60 t(X;18)(SYT-SSX)-positive cases. *Am J Surg Pathol*. 2002;26(11):1434-1440.

Perret R, Chalabreysse L, Watson S, et al. SMARCA4-deficient thoracic sarcomas: clinicopathologic study of 30 cases with an emphasis on their nosology and differential diagnoses. *Am J Surg Pathol*. 2019;43(4):455-465.

Prieto-Granada CN, Wiesner T, Messina JL, et al. Loss of H3K27me3 expression is a highly sensitive marker for sporadic radiation-induced MPNST. *Am J Surg Pathol*. 2016;40(4):479-489.

Rekhtman N, Montealvo J, Chang JC, et al. SMARCA4-deficient thoracic sarcomatoid tumors represent primarily smoking-related undifferentiated carcinomas rather than primary thoracic sarcomas. *J Thorac Oncol*. 2020;15(2):231-247.

Satoh H, Takayashiki N, Shiozawa T, et al. Recurrent pulmonary synovial sarcoma effectively treated with amrubicin: a case report. *Exp Ther Med*. 2015;9(5):1947-1949.

Sauter JL, Graham RP, Larsen BT, et al. SMARCA4-deficient thoracic sarcoma: a distinct clinicopathological entity with undifferentiated rhabdoid morphology and aggressive behavior. *Mod Pathol*. 2017;30(10):1422-1432.

Schaefer IM, Fletcher CD, Hornick JL. Loss of H3K27 trimethylation distinguishes malignant peripheral nerve sheath tumors from histologic mimics. *Mod Pathol*. 2016;29(1):4-13.

Shibuya R, Matsuyama A, Shiba E, et al. CAMTA1 is a useful immunohistochemical marker for diagnosis epithelioid haemangioendothelioma. *Histopathology*. 2015;67(6):827-835.

Takeuchi K, Soda M, Togashi Y, et al. Pulmonary inflammatory myofibroblastic tumor expression a novel fusion, PPFIBP1-ALK: reappraisal of anti-ALK immunohistochemistry as a tool for novel ALK fusion identification. *Clin Cancer Res*. 2011;17(10):3341-3348.

Terry J, Saito T, Subramanian S, et al. TLE1 as a diagnostic immunohistochemical marker for synovial sarcoma emerging from gene expression profiling studies. *Am J Surg Pathol*. 2007;31(2):240-246.

Thway K, Fisher C. PEComa: morphology and genetics of a complex tumor family. *Ann Diagn Pathol*. 2015;19(5):359-368.

Toki S, Wakai S, Sekimizu M, et al. PAX7 immunohistochemical evaluation of Ewing sarcoma and other small round cell tumors. *Histopathology*. 2018;73(4):645-652.

Wallace WA, Dorward DA, Salter DM. Immunohistochemistry for small-cell carcinoma: a potential diagnostic pitfall. *Histopathology*. 2019;74(5):792-794.

Yamamoto H, Nozaki Y, Kohashi K, et al. Diagnostic utility of pan-TRK immunohistochemistry for inflammatory myofibroblastic tumours. *Histopathology*. Published online October 3, 2019. doi:10.1111/his.14010

Yoshida A, Goto K, Kodaira M, et al. CIC-rearranged sarcomas: a study of 20 cases and comparisons with Ewing sarcomas. *Am J Surg Pathol*. 2016;40(3):313-323.

Yoshida A, Kobayashi E, Kubo T, et al. Clinicopathological and molecular characterization of SMARCA4-deficient thoracic sarcomas with comparison to potentially related entities. *Mod Pathol*. 2017;30(6):797-809.

Yoshida A, Sekine, S, Tsuta K, et al. NKX2.2 is a useful immunohistochemical marker for Ewing sarcoma. *Am J Surg Pathol*. 2012;36(7):993-999.

Yoshida A, Shibata T, Wakai S, et al. Anaplastic lymphoma kinase status in rhabdomyosarcomas. *Mod Pathol*. 2013;26(6):772-781.

Yoshida A, Tsuta K, Ohno M, et al. STAT6 immunohistochemistry is helpful in the diagnosis of solitary fibrous tumors. *Am J Surg Pathol*. 2014;38(4):552-559.

Yoshimoto T, Matsubara D, Nakano T, et al. Frequent loss of the expression of multiple subunits of the SWI/SNF complex in large cell carcinoma and pleomorphic carcinoma of the lung. *Pathol Int*. 2015;65(11):595-602.

Immunomarkers for Differentiation from Metastatic Tumors

16

By Yasushi Yatabe, Phillipe Joubert, Sabina Berezowska, Daisuke Matsubara, and Wendy A. Cooper

Introduction

Differentiating primary lung carcinoma from an extrapulmonary metastasis is an important task in diagnostic pathology practice. Clinical history and morphologic comparison with any known prior tumors are important; however, immunohistochemistry (IHC) provides strong support for interpretation, particularly when previous materials are unavailable for review or when morphologic assessment results in equivocal findings. In this chapter, common types and sites of metastatic tumors to the lung are reviewed with a focus on distinction from primary lung tumors using IHC.

Is IHC Useful for Distinguishing Metastatic *Squamous Cell Carcinoma* and Primary Lung Squamous Cell Carcinoma?

Distinguishing primary lung squamous cell carcinoma (SQCC) from a metastatic SQCC originating in another organ is very challenging because SQCCs from different sites lack distinct morphologic features. In addition, tumor growth pattern and the degree of keratinization may change, particularly after chemotherapy and/or radiation therapy. There is no IHC marker that can reliably assist in the distinction of a primary from a metastatic SQCC in the lung, and clinicopathologic correlation is required. Genetic testing to compare the molecular profile of lung and extrapulmonary tumors can assist in distinction, but this is often not feasible in routine clinical practice. Detection of high-risk human papilloma virus (HPV) is helpful when the differential diagnosis is of metastatic SQCC from the oropharynx, endocervix, vulva, anus, and penis, as tumors from these sites are often HPV+ (Plummer et al 2016). Detecting HPV in tumor tissue (eg, using HPV RNA or DNA in situ hybridization [ISH] or other molecular techniques) strongly favors metastasis from these sites, because HPV infection is considered exceptional in primary lung SQCC. p16 IHC is a surrogate marker for HPV; however, approximately 20% of primary lung non-small cell carcinomas (NSCCs) have diffuse and intense p16 expression despite the lack of HPV infection (Bishop et al 2012; Chang et al 2015), so this approach is not entirely reliable. Figure 16-1 is an example of a lung

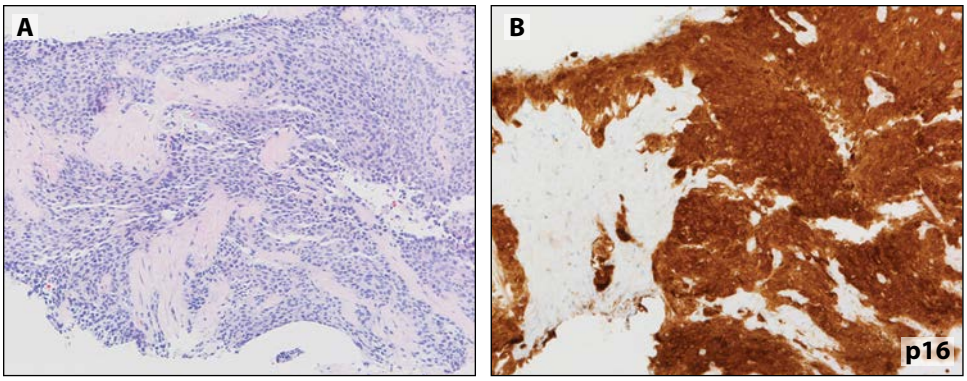


Figure 16-1. Needle biopsy specimen of a lung lesion in a patient with a history of human papilloma virus (HPV+) oropharyngeal carcinoma. **(A)** Morphologic features cannot distinguish between a metastasis or primary lung squamous cell carcinoma (SQCC). **(B)** Diffuse and intense p16 immunohistochemistry (IHC) staining suggests metastatic oropharyngeal carcinoma. However, p16 may also be overexpressed in primary lung cancer without HPV infection, and further molecular testing to confirm presence of HPV is required for more definitive diagnosis.

metastasis of HPV+ oropharyngeal cancer demonstrating positive p16 IHC. Strict diagnostic criteria should be used when assessing p16, and according to guidelines from the College of American Pathologists for assessment of oropharyngeal SQCC (Lewis et al 2018), positive 16 should be considered when there is at least 70% nuclear and cytoplasmic expression of at least moderate intensity is seen.

Summary Answer

There is no IHC marker that can reliably assist in distinction of metastatic from primary SQCC in the lung. Clinicopathologic correlation is required.

What IHC Markers Are Useful in Distinguishing Metastatic Tumors of *Gastrointestinal Tract* Origin from Primary Lung Tumors?

The gastrointestinal (GI) tract is the most common source of pulmonary metastases, accounting for more than 35% of all lung lesions of extrapulmonary origin (Casiraghi et al 2011). Morphologic review of the pathology from any prior GI tract tumors is strongly encouraged to compare the morphologic features when assessing any potential lung metastases. In general, IHC for cytokeratin 7 (CK7) and 20 (CK20) is useful as most pulmonary adenocarcinomas show a CK7+/CK20− profile, whereas lower GI tract carcinomas show a consistent CK7−/CK20+ immunophenotype (Jagirdar 2008; Bahrami et al 2008). However, in pancreaticobiliary and upper GI tract (stomach and esophagus) carcinomas, the labeling for CK7/CK20 is variable, and one can see mixed phenotypes, including CK7−/CK20+, CK7+/CK20−, and CK7+/CK20+ profiles (Selves et al 2018). The addition of pulmonary (thyroid transcription factor-1 [TTF1] and/or napsin A) and GI tract (CDX2) specific IHC stains is useful to confirm a pulmonary versus GI tract origin (Bahrami et al 2008; Jagirdar 2008). TTF1 and monoclonal napsin A stains are positive in about 80% of pulmonary adenocarcinomas and are rarely expressed in GI tract carcinomas (Turner et al 2012; Bishop et al 2010; Rekhtman and Kazi 2015; Ordonez 2012b; Ye et al 2011). Conversely, CDX2 is expressed in a low percentage of pulmonary adenocarcinomas but is strongly and diffusely positive

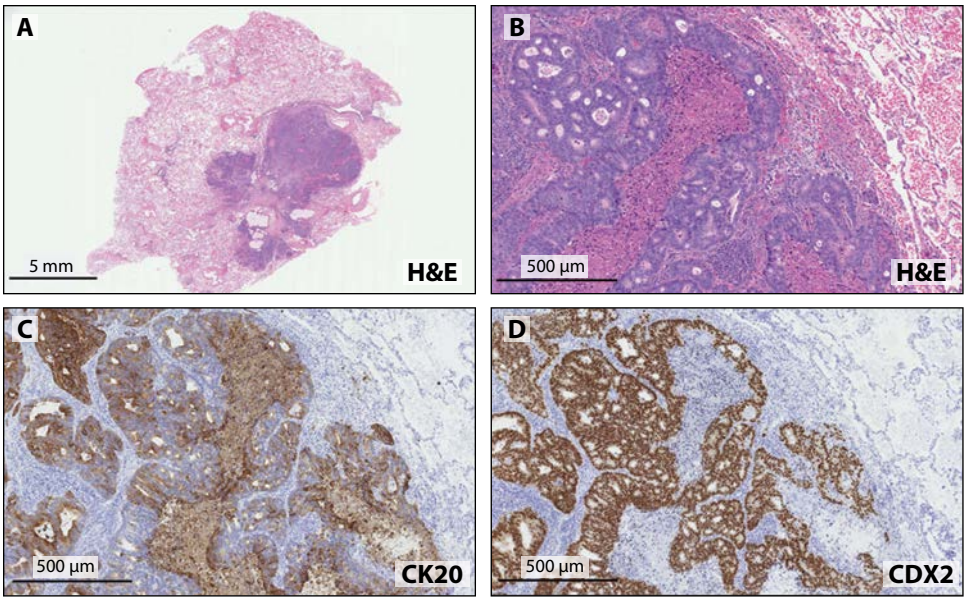


Figure 16-2. (A and B) Lung metastasis from a colon adenocarcinoma, which is characterized by cribriform glands and dirty necrosis (H&E). As expected, the tumor shows **(C and D)** a strong positivity for both cytokeratin 20 (CK20) and CDX2.

in the vast majority of lower GI tract carcinomas, while showing variable immunoreactivity in pancreaticobiliary and upper GI tract carcinomas (Cowan et al 2016; Werling et al 2003; Kaimaktchiev et al 2004) (Figure 16-2). Of note, CDX2 can be expressed in the enteric variant of pulmonary adenocarcinoma (Nottegar et al 2018). In these cases, both TTF1 and napsin A stains are frequently negative, but CK7 labeling is seen in most cases, which may help to differentiate from a colonic metastasis. In this situation, a primary GI tract tumor must be clinically excluded. CDX2, TTF1, and CK7/CK20 markers can also be expressed at different levels in primary lung invasive mucinous adenocarcinoma (CK7+ and often CK20+, CDX2+, and TTF1–), which emphasizes the importance of clinical and radiologic correlation (Selves et al 2018). A newer IHC marker hepatocyte nuclear factor 4α (HNF4α) is expressed in most primary lung invasive mucinous adenocarcinomas, but as it is a primary gut differentiation transcription factor, it is also expressed in GI and pancreatic adenocarcinomas so is not helpful in their distinction (Sugano et al 2013).

Expression of either TTF1 or CDX2 is also helpful to confirm the origin of a well-differentiated neuroendocrine neoplasm given their high specificity (Kyriakopoulous et al 2018). CDX2 immunostaining has been demonstrated in 91% and 83% of the metastatic jejunoileal and appendiceal neuroendocrine neoplasms, but less frequently in duodenal (31%), gastric (14%), pancreatic (16%), and rectal (29%) tumors (Erickson et al 2004; Srivastava and Hornick 2009). TTF1 also show a high specificity for pulmonary carcinoids although the sensitivity is limited in these tumors, ranging between 33% and 46% (Srivastava and Hornick 2009; Chan et al 2012; Zhang et al 2014). Of note, both TTF1 and CDX2 markers are not useful in small cell carcinoma, given their limited specificity and sensitivity in this tumor type (Kaufmann and Dietel 2000; Agoff et al 2000).

Summary Answer

A combination of IHC for cytokeratins (CK7/CK20), lung (TTF1/ napsin A), and GI tract (CDX2) markers is useful to confirm a metastasis from a GI tract origin. In TTF1/ napsin A–negative tumors, positivity for CDX2 points toward a metastasis from the GI tract. In TTF1/ napsin A/CDX2–negative tumors, the CK7/CK20 profile may be helpful, but clinical and radiologic correlation is usually required to confirm the origin.

What IHC Markers Are Useful to Distinguish Metastatic Carcinomas of *Breast* Origin from Primary Lung Carcinoma?

The distinction between a primary lung adenocarcinoma and a breast cancer metastatic to the lung is a commonly encountered clinical situation given the high incidence of breast cancer and that breast cancer patients show a higher risk of developing a second non-breast malignancy, including lung cancer (Mellemkjaer et al 2006). Breast carcinomas are the third most frequent type of epithelial metastases seen in the lung (Casiraghi et al 2011), and the importance of the clinicopathologic correlation in distinguishing a primary from metastatic tumor cannot be overemphasized. When available, review of the prior breast lesion pathology for comparison is extremely useful. However, histopathologic distinction between a breast metastasis and a primary non-small cell lung carcinoma can be challenging, particularly for poorly differentiated lesions.

The vast majority of both lung and breast carcinomas share a similar cytokeratin expression profile (CK7+/CK20–), and additional immunostains are usually required to confirm the histogenesis (Chu et al 2000). In addition to estrogen and progesterone receptors, useful stains to favor breast metastasis are positive staining for GATA3 (sensitivity: 32%-100%; specificity: 71%-93%), mammaglobin (sensitivity: 26%-84%; specificity: 85%-100%), and gross cystic disease fluid protein 15 (GCDFP-15) (sensitivity: 15%-74%; specificity: 93%-100%) (Hsu et al 2016). In well-differentiated carcinomas, a combination of negative TTF1 and/or napsin A stains with positive estrogen receptor/progesterone receptor (ER/PR) can distinguish a breast metastasis with an acceptable sensitivity and specificity (Yang and Nonaka 2010; Yatabe et al 2019; Provenzano et al 2016). In this setting, the addition of napsin A only slightly increases the sensitivity and specificity of TTF1 alone and is helpful in only a limited number of cases (Yang and Nonaka 2010).

In general, GATA3 staining is useful to identify a breast origin as it shows superior sensitivity compared to ER, PR, mammaglobin, or GCDFP-15 (Sangoi et al 2016; Gown et al 2016; Ni et al 2018). However, a word of caution is required regarding the lower specificity of GATA3, as it is expressed in other non-mammary carcinomas, in particular urothelial carcinoma and to a lesser extent, pancreatic carcinomas, some cutaneous carcinomas, and others (Miettinen et al 2014). GATA3 (Vidarsdottir et al 2019) and ER (Yang and Nonaka 2010) are only rarely expressed in lung adenocarcinomas, whereas a small percentage of breast carcinomas may express TTF1 (Ordonez 2012b). Therefore, combining GATA3 with an IHC stain that has a higher specificity for breast carcinomas, such as mammaglobin, is recommended in patients with a lung lesion and no prior history of breast cancer (Yang and Nonaka 2010).

More recently, SOX10 has emerged as a useful marker to confirm metastasis from a triple-negative breast carcinoma (TNBC) as it significantly improves the specificity of GATA3

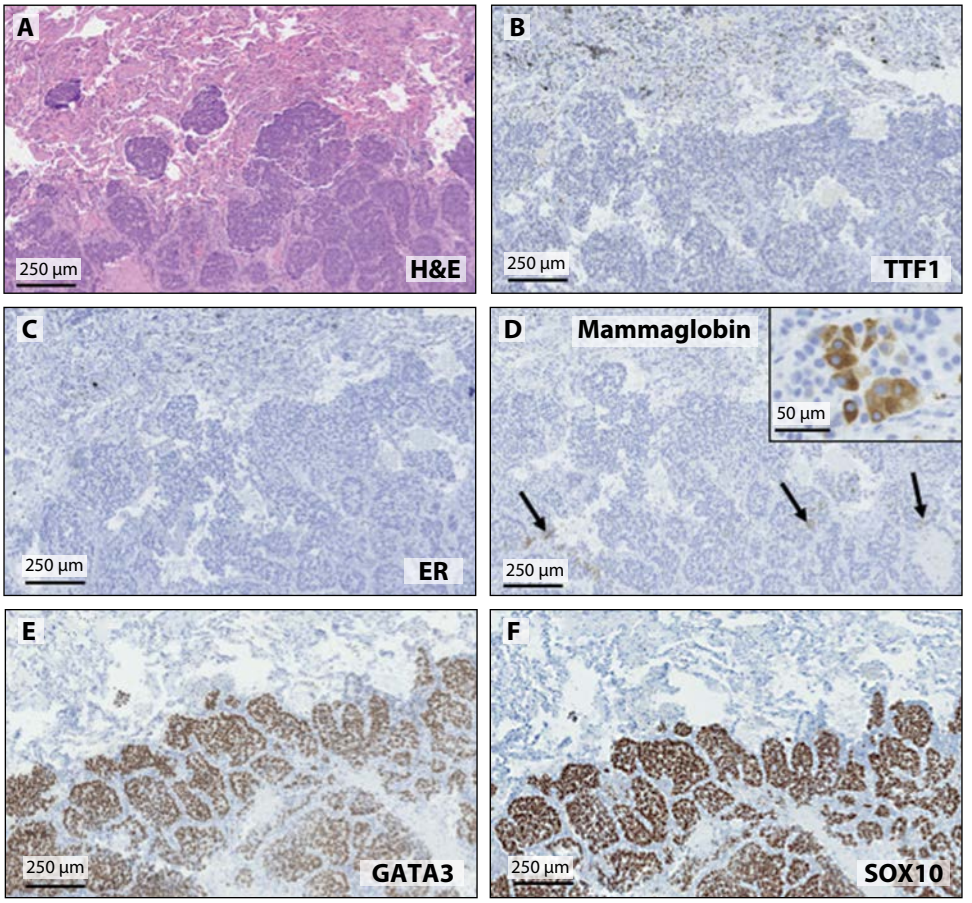


Figure 16-3. (A-F) Lung metastasis from a triple-negative breast carcinoma. **(B)** Thyroid transcription factor-1 (TTF1) and **(C)** estrogen receptor (ER) are negative. **(D)** Focal positivity for mammaglobin (*arrows* and *inset*) is shown along with strong and diffuse positivity for **(E)** GATA3 and **(F)** SOX10.

alone (Figure 16-3). These tumors represent about 10% to 20% of breast cancers and are characterized by a higher risk of lung metastasis as well as absence of ER/PR and human epidermal growth factor receptor 2 (HER2) (Foulkes et al 2010). A combination of TTF1/ napsin A (negative), GATA3 (sensitivity of 30.4%), and SOX10 (sensitivity of 62.3%) immunopositivity has been shown to identify the vast majority of TNBCs (Tozbikian and Zynger 2019; Laurent et al 2019).

Summary Answer

In any patient with a history of a breast carcinoma, comparison with histologic features of the primary breast tumor is recommended where possible. A combination of lung and breast markers that include TTF1 and/or napsin A, and ER/PR or GATA3 will provide a definite answer in most cases. When clinically relevant, GATA3 should be combined with mammaglobin to rule out an extramammary malignancy such as bladder carcinoma. In TNBC, a combination of lung markers with both GATA3 and SOX10 is recommended to distinguish metastatic breast carcinoma from primary lung carcinoma.

What IHC Markers Are Useful to Distinguish Metastatic Carcinoma of *Female Genital Tract* Origin from Primary Lung Carcinoma?

Diagnosis of metastatic carcinoma from the female genital tract in the lung can be challenging, as 10% to 20% of adenocarcinomas from the uterine cervix, endometrium, and ovary show TTF1 expression, even with the more specific clone of the antibody (Kubba et al 2008; Siami et al 2007) (Figure 16-4). Positive TTF1 is particularly high in mesonephric and mesonephric-like adenocarcinomas of the uterine cervix and endometrium (Pors et al 2018; McFarland et al 2016). In addition, all high-grade neuroendocrine carcinomas can be positive for TTF1 regardless of the origin, including the female genital tract. To differentiate metastatic female genital tract carcinomas from primary lung cancer, PAX8 is useful because most female genital tract carcinomas (excluding cervical squamous cervical carcinomas) are positive whereas lung carcinomas are almost always negative (Laury et al 2011; McHugh et al 2019). PAX8 expression can also be seen in tumors from other sites including kidney, thymus, and thyroid (Ordonez 2012a).

Summary Answer

When the differential diagnosis of a lung tumor includes a metastatic female genital tract carcinoma, TTF1 should be used with caution because as with primary lung adenocarcinomas, these tumors may also express TTF1. PAX8 staining is useful to help identify metastatic tumors of female genital tract origin.

What IHC Markers Are Useful to Distinguish Metastatic Carcinomas of *Urothelial* Origin from Primary Lung Carcinoma?

The lungs are one of the most common sites for metastases from the bladder (Goldman et al 1979; Wallmeroth et al 1999; Babaian et al 1980), and urothelial carcinoma is the most common type of bladder cancer accounting for more than 90% of tumors (Moch et al 2016). Assessment of morphologic features and comparison with any known primary tumor may not be sufficient to identify the primary site as some pulmonary non-keratinizing SQCCs morphologically resemble conventional urothelial carcinoma (Travis et al 2015). In addition, up to 60% of urothelial carcinomas exhibit squamous differentiation (Amin 2009). Distinction of metastatic urothelial carcinomas from primary lung carcinoma can be difficult, especially

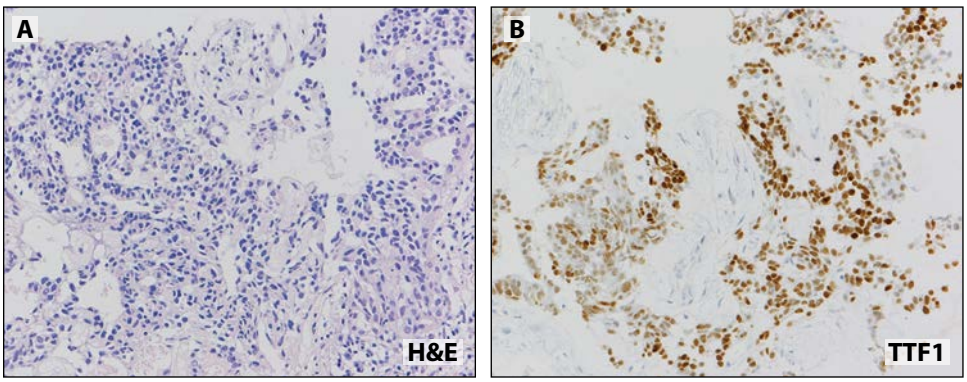


Figure 16-4. (A and B) Metastatic adenocarcinoma from uterine cervix, which shows thyroid transcription factor-1 (TTF1) expression.

in poorly differentiated tumors, but several studies have shown that immunohistochemical markers can be a useful adjunct.

CK7, CK20, and GATA3 are more likely to be positive in urothelial carcinoma than pulmonary SQCC (100% vs. 33%, 54% vs. 7%, and 78% vs. 23%, respectively) (Figure 16-5), and in contrast, CK14 and desmoglein 3 are more likely to be positive in pulmonary SQCC than urothelial carcinoma (77% vs. 32% and 87% vs. 11%, respectively) (Gruver et al 2012).

Uroplakin III is specific for urothelial carcinoma, but the sensitivity is not very high (only 14% positivity) (Gruver et al 2012). Uroplakin II has been reported to be a more sensitive marker than uroplakin III in urothelial carcinoma (Li et al 2014; Hoang et al 2015), but the role for this marker has not been fully established. In the majority of both urothelial carcinomas and pulmonary SQCCs, the squamous markers p40 and p63 are positive (Gruver et al 2012; Gailey and Bellizzi 2013) while PAX8 is negative (Laury et al 2011), making these IHC markers unhelpful in the distinction of these tumor types.

Summary Answer

A combination of CK7, CK20, and GATA3 is most useful in the distinction of metastatic urothelial carcinoma from pulmonary SQCC.

What IHC Markers Are Useful to Distinguish Metastatic Carcinomas of *Renal* Origin from Primary Lung Carcinoma?

Metastatic renal carcinomas can mimic primary non-small cell lung carcinomas and in addition to morphologic comparison with any known primary tumors, IHC can assist in the distinction. Although napsin A can be positive in renal cell carcinomas (approximately 80% in papillary carcinoma and about 40% in conventional clear cell carcinoma), renal cell carcinomas express PAX8 in most cases (>90%) (Figure 16-6), in contrast to rare expression

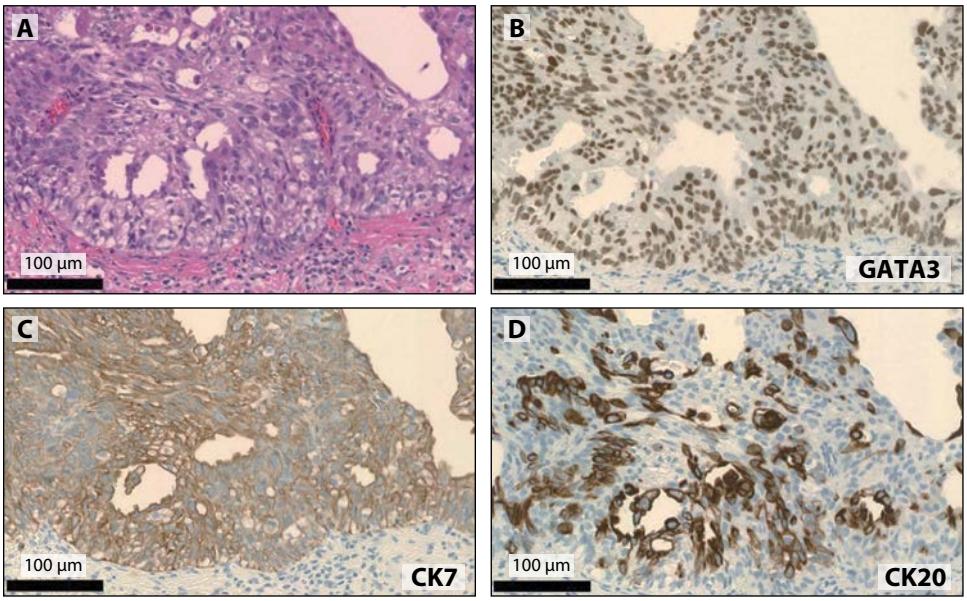


Figure 16-5. (A-D) Metastatic urothelial carcinoma (**[A]** H&E) is more frequently positive for **(B)** GATA3, **(C)** cytokeratin 7 (CK7), and **(D)** cytokeratin 20 (CK20) than pulmonary squamous cell carcinoma.

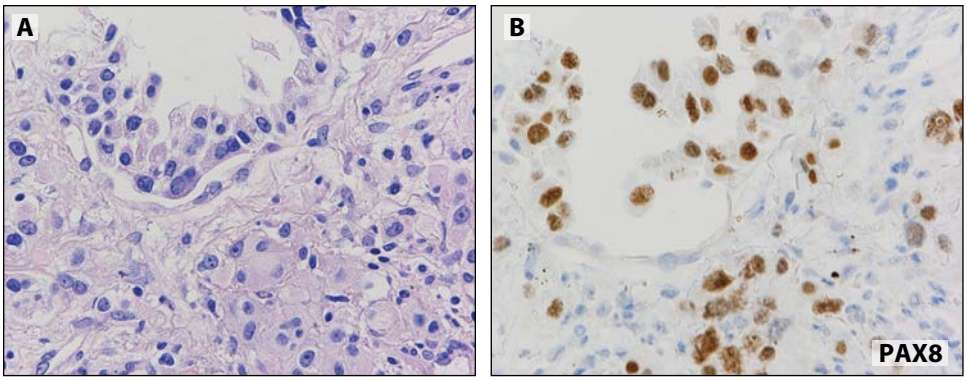


Figure 16-6. (A and B) Renal cell carcinoma metastasis (**[A]** H&E). The tumor mimics lung adenocarcinoma with an acinar and solid growth pattern. However, the patient has a history of prior renal clear cell carcinoma. **(B)** PAX8 immunohistochemistry (IHC) showed strong nuclear staining, suggesting metastatic renal cell carcinoma.

(0%–2%) in lung adenocarcinoma. Furthermore, nearly all clear cell renal cell carcinomas, which is the most common subtype to metastasize to the lung, are positive for carbonic anhydrase IX (CA IX). TTF1 is expressed in most lung adenocarcinomas but is negative in renal cell carcinomas (Bishop et al 2010). CD10 is not useful to identify metastatic renal cell carcinomas as it is also expressed in a variety of cancers, including lung adenocarcinoma (Gurel et al 2012).

Summary Answer

PAX8 and TTF1 are useful to distinguish metastatic renal cell carcinoma (PAX8+/TTF1–) from primary lung adenocarcinoma (PAX8–/TTF1±). Napsin A is not useful in this setting as it is expressed in a variable proportion of renal cell carcinomas.

What IHC Markers Are Useful to Distinguish Metastatic Carcinomas of *Prostate* Origin from Primary Lung Carcinoma?

Metastatic prostate cancer is a relatively common entity and, in a study of 1589 autopsy cases, lungs are the second most common metastatic site (46%) following bone (Bubendorf et al 2000). Morphologic features may aid in identifying the prostatic origin of tumors, which typically display microacinar or tubulopapillary patterns in pulmonary metastatic lesions (Copeland et al 2002) (Figure 16-7).

There are several useful IHC markers to identify the prostatic origin of pulmonary metastatic tumors. CK7 and CK20, low-molecular-weight cytokeratins, are helpful as prostate adenocarcinoma is typically negative for both CK7 and CK20 (>80%) (Bassily et al 2000) (Figure 16-8). In addition, TTF1 is almost always negative in prostatic adenocarcinoma (Goldstein 2002).

Prostatic-specific antigen (PSA), prostatic-specific acid phosphatase (PSAP), and prostate-specific membrane antigen (PSMA) have been used for many years as sensitive and specific cytoplasmic markers for prostate adenocarcinoma, although the intensity of staining is often weaker in poorly differentiated tumors (Chuang et al 2007; Varma and Jasani 2005). The

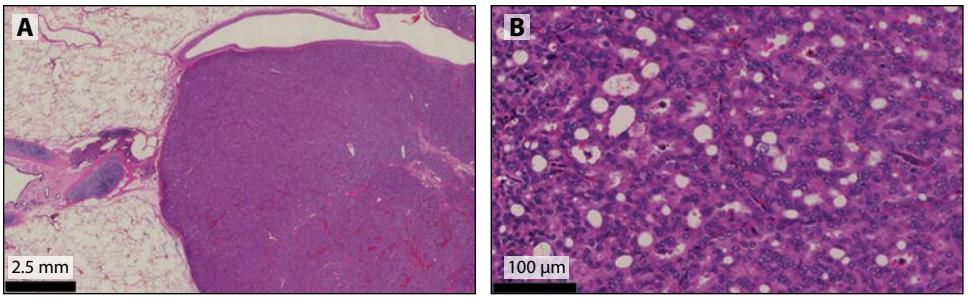


Figure 16-7. (A and B) Typical pattern of metastatic prostate adenocarcinoma in the lung. **(A)** Well-circumscribed tumor with lymphatic invasion. **(B)** Microacinar pattern with cribriform gland formation.

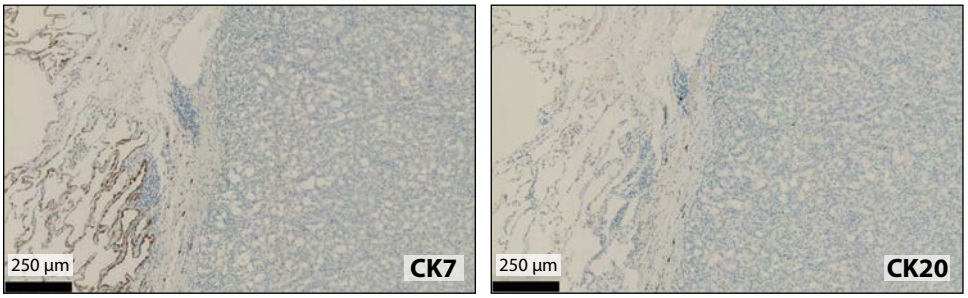


Figure 16-8. (A and B) Metastatic prostate adenocarcinoma is negative for both cytokeratin 7 (CK7) and cytokeratin 20 (CK20) in most cases.

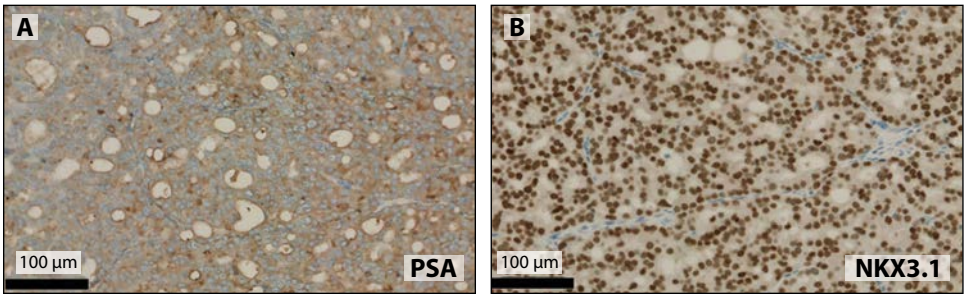


Figure 16-9. Metastatic prostate adenocarcinoma is **(A)** positive for prostatic-specific antigen (PSA) in cytoplasm and **(B)** strongly positive for NKX3.1 in nuclei.

detection rate is about 85% for PSMA, 80% for PSA, and 60% for PSAP in metastatic prostate adenocarcinomas (Kristiansen et al 2017; Steffens et al 1985).

NK3 homeobox 1 (*NKX3.1*) is an androgen-regulated homeodomain gene, which is characteristically expressed in prostate epithelium (Abate-Shen et al 2008). NKX3.1 IHC is a highly sensitive nuclear marker for prostate adenocarcinoma (>98% positive), and several studies have reported that NKX3.1 is a more sensitive and more specific marker than PSA and PSAP (Gurel et al 2010; Gan et al 2019) (Figure 16-9).

Summary Answer

A combination of negative CK7, CK20, and TTF1 together with positive staining for a prostate marker such as NKX3.1 can be used to identify metastatic prostatic carcinoma.

What IHC Markers Are Useful to Distinguish Metastatic Carcinoma of *Hepatic* Origin from Primary Lung Carcinoma?

Diagnosis of metastatic hepatocellular carcinoma (HCC) in the lung can be challenging in some instances as HCC may mimic a poorly differentiated non-small cell lung carcinoma. p40, CK7, and neuroendocrine markers are always negative in HCC, while TTF1 can show aberrant cytoplasmic reaction rather than nuclear staining. Hepatocyte paraffin 1 (HepPar1), arginase-1, and glypican-3 are useful IHC markers to identify HCC (Figures 16-10 and 16-11) with arginase-1 and HepPar1 most sensitive for well-differentiated tumors and arginase-1 and glypican-3 most sensitive for poorly differentiated tumors (Nguyen et al 2015). Recently, however, primary hepatoid adenocarcinoma of the lung has been reported (Haninger et al 2014; Chandan et al 2016) that resembles HCC, and is positive for HepPar1, arginase-1, and α-fetoprotein (AFP). Careful clinicopathologic correlation and positive CK7 staining are required to differentiate pulmonary hepatoid adenocarcinoma from metastasis of HCC.

Summary Answer

A combination of hepatocellular markers such as arginase-1 and HepPar1 together with negative CK7 and pulmonary markers can be used to identify metastatic HCC.

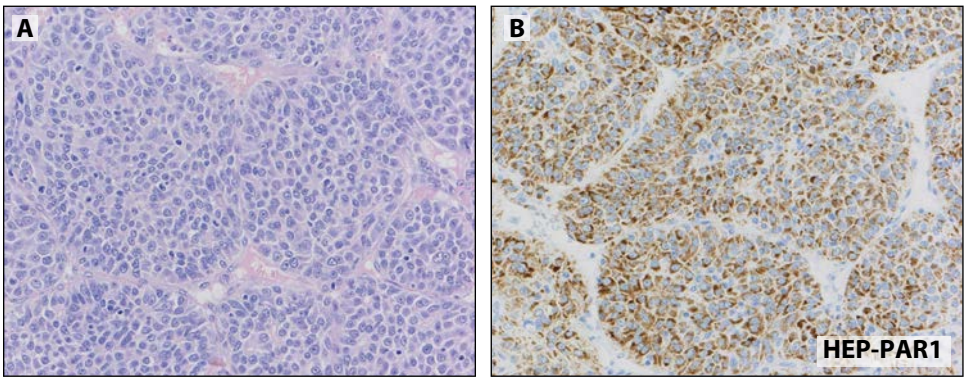


Figure 16-10. (A and B) Lung metastasis of hepatocellular carcinoma (**[A]** H&E). Solid growth of tumor cells mimics a high-grade non-small cell carcinoma (NSCC). However, this tumor was negative for p40, thyroid transcription factor-1 (TTF1), cytokeratin (CK7), and neuroendocrine markers. **(B)** Positive hepatocyte paraffin 1 (HepPar1) (immunohistochemistry [IHC]) led to the diagnosis of metastatic hepatocellular carcinoma (HCC). The patient was positive for serum hepatitis B surface antigen (HB_S) antibody.

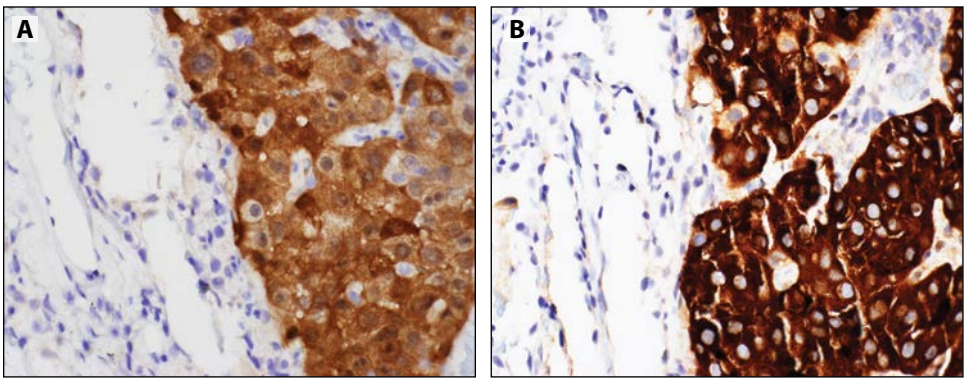


Figure 16-11. Hepatocellular carcinoma showing **(A)** strong staining for arginase-1 and **(B)** glypican-3.

What IHC Markers Are Useful to Distinguish Metastatic Carcinoma of *Thyroid* Origin from Primary Lung Carcinoma?

Metastatic thyroid carcinomas may have overlapping morphologic features with non-small cell lung carcinoma (NSCLC). Although TTF1 is expressed in most thyroid carcinomas (apart from anaplastic carcinomas, which are mostly negative) and most lung adenocarcinomas, napsin A is usually negative in thyroid neoplasms (Nonaka et al 2008; Bishop et al 2010). PAX8 is a nuclear marker expressed in tumors of thyroid, parathyroid, thymus, renal, and female genital tract origin but is negative in lung adenocarcinomas (Ordonez 2012a) (Figures 16-12 and 16-13).

Summary Answer

A combination of PAX8 and TTF1 positivity together with absence of napsin A is useful to identify metastatic thyroid carcinomas.

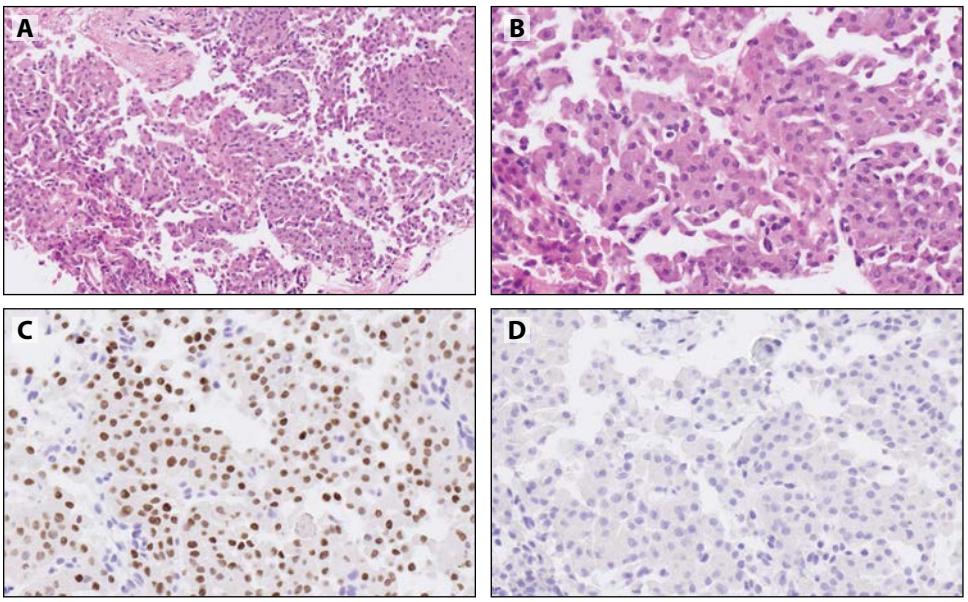


Figure 16-12. Rarely, thyroid transcription factor-1 (TTF1)-positive tumors may need additional immunohistochemical work-up, depending on history. **(A and B)** This cytologically bland oncocytic tumor (**[A]** H&E) showed **(C)** strong TTF1 expression. **(D)** Napsin A (monoclonal) is negative.

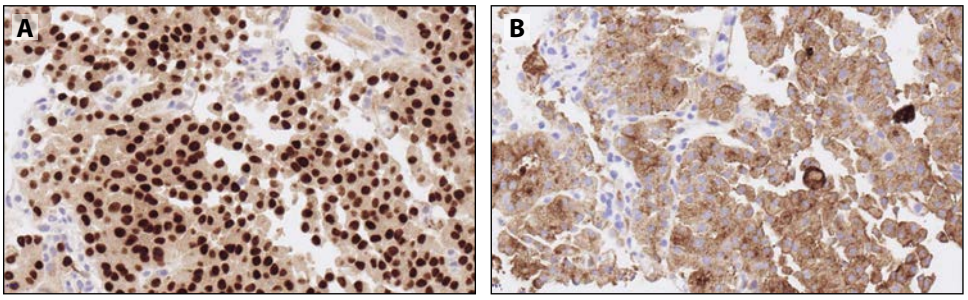


Figure 16-13. In the case shown in Figure 16-12, additional immunostains were performed in a focused approach after clinical information of a thyroid mass was provided. **(A)** PAX8 and **(B)** thyroglobulin immunohistochemistry (IHC) positivity proved this was a metastasis from a thyroid tumor.

Conclusions

Although thorough morphologic assessment of any tumor in the lung together with access to accurate clinical history and comparison with the pathology of any tumors from other sites is crucial in the distinction of primary tumors from metastases, this approach may not be sufficient to reach a definitive diagnosis. Accurate distinction of primary from metastatic tumors in the lung is critical for appropriate patient management and in many instances, IHC is required to confirm or exclude pulmonary metastases. There are no IHC markers with perfect accuracy to determine the origin of tumors, and pathologists need to be aware of the strengths, limitations, and pitfalls of different IHC markers that can be used to reach a more accurate diagnosis.

References

Abate-Shen C, Shen MM, Gelmann E. Integrating differentiation and cancer: the Nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis. *Differentiation*. 2008;76(6):717-727.

Agoff SN, Lamps LW, Philip AT, et al. Thyroid transcription factor-1 is expressed in extrapulmonary small cell carcinomas but not in other extrapulmonary neuroendocrine tumors. *Mod Pathol*. 2000;13(3):238-242.

Amin MB. Histological variants of urothelial carcinoma: diagnostic, therapeutic and prognostic implications. *Mod Pathol*. 2009;22(suppl 2):S96-S118.

Babaian RJ, Johnson DE, Llamas L, et al. Metastases from transitional cell carcinoma of the urinary bladder. *Urology*. 1980;16(2):142-144.

Bahrami A, Truong LD, Ro JY. Undifferentiated tumor: true identity by immunohistochemistry. *Arch Pathol Lab Med*. 2008;132(3):326-348.

Bassily NH, Vallorosi CJ, Akdas G, et al. Coordinate expression of cytokeratins 7 and 20 in prostate adenocarcinoma and bladder urothelial carcinoma. *Am J Clin Pathol*. 2000;113(3):383-388.

Bishop JA, Ogawa T, Chang X, et al. HPV analysis in distinguishing second primary tumors from lung metastases in patients with head and neck squamous cell carcinoma. *Am J Surg Pathol*. 2012;36(1):142-148.

Bishop JA, Sharma R, Illei PB. Napsin A and thyroid transcription factor-1 expression in carcinomas of the lung, breast, pancreas, colon, kidney, thyroid, and malignant mesothelioma. *Hum Pathol*. 2010;41(1):20-25.

Bubendorf L, Schopfer A, Wagner U, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum Pathol*. 2000;31(5):578-583.

Casiraghi M, De Pas T, Maisonneuve P, et al. A 10-year single-center experience on 708 lung metastasectomies: the evidence of the "International Registry of Lung Metastases." *J Thorac Oncol*. 2011;6(8):1373-1378.

Chan ES, Alexander J, Swanson PE, et al. PDX-1, CDX-2, TTF-1, and CK7: a reliable immunohistochemical panel for pancreatic neuroendocrine neoplasms. *Am J Surg Pathol*. 2012;36(5):737-743.

Chandan VS, Shah SS, Torbenson MS, et al. Arginase-1 is frequently positive in hepatoid adenocarcinomas. *Hum Pathol*. 2016;55:11-16.

Chang SY, Keeney M, Law M, et al. Detection of human papillomavirus in non-small cell carcinoma of the lung. *Hum Pathol*. 2015;46(11):1592-1597.

Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol*. 2000;13(9):962-972.

Chuang AY, DeMarzo AM, Veltri RW, et al. Immunohistochemical differentiation of high-grade prostate carcinoma from urothelial carcinoma. *Am J Surg Pathol*. 2007;31(8):1246-1255.

Copeland JN, Amin MD, Humphrey PA, et al. The morphologic spectrum of metastatic prostatic adenocarcinoma to the lung: special emphasis on histologic features overlapping with other pulmonary neoplasms. *Am J Clin Pathol*. 2002;117(4):552-557.

Cowan ML, Li QK, Illei PB. CDX-2 expression in primary lung adenocarcinoma. *Appl Immunohistochem Mol Morphol*. 2016;24(1):16-19.

Erickson LA, Papouchado B, Dimashkieh H, et al. CDX2 as a marker for neuroendocrine tumors of unknown primary sites. *Endocr Pathol*. 2004;15(3):247-252.

Foulkes WD, Smit IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med*. 2010;363(20):1938-1948.

Gailey MP, Bellizzi AM. Immunohistochemistry for the novel markers glypican 3, PAX8, and p40 (ΔNp63) in squamous cell and urothelial carcinoma. *Am J Clin Pathol*. 2013;140(6):872-880.

Gan Q, Joseph CT, Guo M, et al. Utility of NKX3.1 immunostaining in the detection of metastatic prostatic carcinoma on fine-needle aspiration smears. *Am J Clin Pathol*. 2019;152(4):495-501.

Goldman SM, Fajardo AA, Naraval RC, et al. Metastatic transitional cell carcinoma from the bladder: radiographic manifestations. *AJR Am J Roentgenol*. 1979;132(3):419-425.

Goldstein NS. Immunophenotypic characterization of 225 prostate adenocarcinomas with intermediate or high Gleason scores. *Am J Clin Pathol*. 2002;117(3):471-477.

Gown AM, Fulton RS, Kandalaft PL. Markers of metastatic carcinoma of breast origin. *Histopathology*. 2016;68(1):86-95.

Gruver AM, Amin MB, Luthringer DJ, 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):1339-1446.

Gurel B, Ail TZ, Montgomery EA, et al. NKX3.1 as a marker of prostatic origin in metastatic tumors. *Am J Surg Pathol*. 2010;34(8):1097-1105.

Gurel D, Kargi A, Karaman I, et al. CD10 expression in epithelial and stromal cells of non-small cell lung carcinoma (NSCLC): a clinic and pathologic correlation. *Pathol Oncol Res*. 2012;18(2):153-160.

Haninger DM, Kloecker GH, Bousamra Ii M, et al. Hepatoid adenocarcinoma of the lung: report of five cases and review of the literature. *Mod Pathol*. 2014;27(4):535-542.

Hoang LL, Tacha D, Bremer RE, et al. 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):711-716.

Hsu C, Chiang AW, Hwang MJ, et al. Proteins with highly evolvable domain architectures are nonessential but highly retained. *Mol Biol Evol*. 2016;33(5):1219-1230.

Jagirdar J. Application of immunohistochemistry to the diagnostic of primary and metastatic carcinoma to the lung. *Arch Pathol Lab Med*. 2008;132(3):384-396.

Kaimaktchiev V, Terracciano L, Tornillo L, et al. The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. *Mod Pathol*. 2004;17(11):1392-1399.

Kaufmann O, Dietel M. Expression of thyroid transcription factor-1 in pulmonary and extra pulmonary small cell carcinomas and other neuroendocrine carcinomas of various primary sites. *Histopathology*. 2000;36(5):415-420.

Kristiansen I, Stephan C, Jung K, et al. Sensitivity of HOXB13 as a diagnostic immunohistochemical marker of prostatic origin in prostate cancer metastases: comparison to PSA, prostein, androgen receptor, ERG, NKX3.1, PSAP, and PSMA. *Int J Mol Sci*. 2017;18(6):E1151.

Kubba LA, McCluggage WG, Liu J, et al. Thyroid transcription factor-1 expression in ovarian epithelial neoplasms. *Mod Pathol*. 2008;21(4):485-490.

Kyriakopoulous G, Mavroeidi V, Chatzellis E, et al. Histopathological, immunohistochemical, and genetic and molecular markers of neuroendocrine neoplasms. *Ann Transl Med*. 2018;6(12):252.

Laurent E, Begueret H, Bonhomme B, et al. SOX10, GATA3, GCDFP15, androgen receptor, and mammaglobin for the differential diagnosis between triple-negative breast cancer and TTF1-negative lung adenocarcinoma. *Am J Surg Pathol*. 2019;43(3):293-302.

Laury AR, Perets R, Piao H, et al. A comprehensive analysis of PAX8 expression in human epithelial tumors. *Am J Surg Pathol*. 2011;35(6):816-826.

Lewis JS Jr, Beadle B, Bishop JA, et al. Human papillomavirus testing in head and neck carcinomas: guideline from the College of American Pathologists. *Arch Pathol Lab Med*. 2018;142(5):559-597.

Li W, Liang Y, Deavers MT, et al. Uroplakin II is a more sensitive immunohistochemical marker than uroplakin III in urothelial carcinoma and its variants. *Am J Clin Pathol*. 2014;142(6):864-871.

McFarland M, Quick CM, McCluggage WG. Hormone receptor-negative, thyroid transcription factor 1-positive uterine and ovarian adenocarcinomas: report of a series of mesonephric-like adenocarcinomas. *Histopathology*. 2016;68(7):1013-1020.

McHugh KE, Arrossi AV, Farver CF, et al. Does strong and diffuse PAX-8 positivity occur in primary lung carcinoma? An immunohistochemical study of 418 cases and review of the literature. *Appl Immunohistochem Mol Morphol*. 2019;27(2):140-146.

Mellemkjaer L, Friis S, Olsen JH, et al. Risk of second cancer among women with breast cancer. *Int J Cancer*. 2006;118(9):2285-2292.

Miettinen M, McCue PA, Sarlomo-Rikala M, 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.

Moch H, Humphrey PA, Ulbright TM, et al. *WHO Classification of Tumours of the Urinary System and Male Genital Organs*. 4th ed. World Health Organization; 2016.

Nguyen T, Phillips D, Jain D, et al. Comparison of 5 immunohistochemical markers of hepatocellular differentiation for the diagnosis of hepatocellular carcinoma. *Arch Pathol Lab Med*. 2015;139(8):1028-1034.

Ni YB, Tsang JYS, Shao MM, et al. GATA-3 is superior to GCDFP-15 and mammaglobin to identify primary and metastatic breast cancer. *Breast Cancer Res Treat*. 2018;169(1):25-32.

Nonaka D, Tang Y, Chiriboga L, et al. Diagnostic utility of thyroid transcription factors Pax8 and TTF-2 (FoxE1) in thyroid epithelial neoplasms. *Mod Pathol*. 2008;21(2):192-200.

Nottegar A, Tabbo F, Luchini C, et al. Pulmonary adenocarcinoma with enteric differentiation: immunohistochemistry and molecular morphology. *Appl Immunohistochem Mol Morphol*. 2018;26(6):383-387.

Ordonez NG. Value of PAX8 immunostaining in tumor diagnosis: a review and update. *Adv Anat Pathol*. 2012;19(3):140-151. (a)

Ordonez NG. Value of thyroid transcription factor-1 immunostaining in tumor diagnosis: a review and update. *Appl Immunohistochem Mol Morphol*. 2012;20(5):429-444. (b)

Plummer M, de Martel C, Vignat J, et al. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health*. 2016;4(9):e609-616.

Pors J, Cheng A, Leo JM, et al. A comparison of GATA3, TTF1, CD10, and calretinin in identifying mesonephric and mesonephric-like carcinomas of the gynecologic tract. *Am J Surg Pathol*. 2018;42(12):1596-1606.

Provenzano E, Byrne DJ, Russell PA, et al. Differential expression of immunohistochemical markers in primary lung and breast cancers enriched for triple-negative tumours. *Histopathology*. 2016;68(3):367-377.

Rekhtman N, Kazi S. Nonspecific reactivity of polyclonal napsin A antibody in mucinous adenocarcinomas of various sites: a word of caution. *Arch Pathol Lab Med*. 2015;139(4):434-436.

Sangoi AR, Shrestha B, Yang G, et al. The novel marker GATA3 is significantly more sensitive than traditional markers mammaglobin and GCDFP15 for identifying breast cancer in surgical and cytology specimens of metastatic and matched primary tumors. *Appl Immunohistochem Mol Morphol*. 2016;24(4):229-237.

Selves J, Long-Mira E, Mathieu MC, et al. Immunohistochemistry for diagnosis of metastatic carcinomas of unknown primary site. *Cancers (Basel)*. 2018;10(4):E108.

Siami K, McCluggage WG, Ordonez NG, et al. Thyroid transcription factor-1 expression in endometrial and endocervical adenocarcinomas. *Am J Surg Pathol*. 2007;31(7):1759-1763.

Srivastava A, Hornick JL. Immunohistochemical staining for CDX-2, PDX-1, NESP-55, and TTF-1 can help distinguish gastrointestinal carcinoid tumors from pancreatic endocrine and pulmonary carcinoid tumors. *Am J Surg Pathol*. 2009;33(4):626-632.

Steffens J, Friedmann W, Lobeck H. Immunohistochemical diagnosis of the metastasizing prostatic carcinoma. *Eur Urol*. 1985;11(2):91-94.

Sugano M, Nagasaka T, Sasaki E, et al. HNF4α as a marker for invasive mucinous adenocarcinoma of the lung. *Am J Surg Pathol*. 2013;37(2):211-218.

Tozbikian GH, Zynger DL. A combination of GATA3 and SOX10 is useful for the diagnosis of metastatic triple-negative breast cancer. *Hum Pathol*. 2019;85:221-227.

Travis WD, Brambilla E, Burke AP, et al. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. 4th ed. International Agency for Research on Cancer (IARC) Press; 2015.

Turner BM, Cagel PT, Sainz IM, et al. Napsin A, a new marker for lung adenocarcinoma, is complementary and more sensitive and specific than thyroid transcription factor 1 in the differential diagnosis of primary pulmonary carcinoma: evaluation of 1674 cases by tissue microarray. *Arch Pathol Lab Med*. 2012;136(2):163-171.

Varma M, Jasani B. Diagnostic utility of immunohistochemistry in morphologically difficult prostate cancer: review of current literature. *Histopathology*. 2005;47(1):1-16.

Vidarsdottir H, Tran L, Nodin B, et al. Immunohistochemical profiles in primary lung cancers and epithelial pulmonary metastases. *Hum Pathol*. 2019;84:221-230.

Wallmeroth A, Wagner U, Moch H, et al. Patterns of metastasis in muscle-invasive bladder cancer (pT2-4): an autopsy study on 367 patients. *Urol Int*. 1999;62(2):69-75.

Werling RW, Yaziji H, Bacchi CE, et al. CDX2, a highlight sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol*. 2003;27(3):303-310.

Yang M, Nonaka D. A study of immunohistochemical differential expression in pulmonary and mammary carcinomas. *Mod Pathol*. 2010;23(5):654-661.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Ye J, Findeis-Hosey JJ, Yang Q, et al. Combination of napsin A and TTF-1 immunohistochemistry helps in differentiating primary lung adenocarcinoma from metastatic carcinoma in the lung. *Appl Immunohistochem Mol Morphol*. 2011;19(4):313-317.

Zhang C, Schmidt LA, Hatanaka K, et al. Evaluation of napsin A, TTF-1, p63, p40, and CK5/6 immunohistochemical stains in pulmonary neuroendocrine tumors. *Am J Clin Pathol*. 2014;142(3):320-324.

Mesothelioma and Immunohistochemistry

17

By Mauro Papotti, Andrew G. Nicholson, and Sanja Dacic

Introduction

Malignant pleural mesothelioma (MPM) is a highly malignant primary pleural neoplasm that presents with heterogenous morphology, classified into epithelioid, sarcomatoid, and biphasic subtypes. The diagnosis should be confirmed by immunophenotypic marker assessment because of morphologic overlap with other tumors, especially carcinomas that often metastasize to this site. International guidelines recommend that at least 2 positive mesothelioma markers and 2 non-mesothelioma markers should be assessed by immunohistochemistry (IHC). The ideal marker panel for this purpose may vary depending on epithelioid or sarcomatoid morphology of a malignant proliferation (Galateau-Salle et al 2016; Churg et al 2018; Chapel et al 2019; Nicholson et al 2020). The selection of marker panel is not affected by the type of sample (pleural biopsy *vs.* surgical specimen *vs.* effusion cytology) because all perform well in both formalin-fixed and alcohol-fixed samples.

What Are the Best Markers to Distinguish Epithelioid MPM from Carcinoma?

A large number of immunohistochemical markers that could be used in the differential diagnosis of epithelioid MPM versus adenocarcinoma is commercially available. Epithelioid MPM diffusely and frequently strongly expresses most "mesothelioma-associated" markers. One of the best combination of positive mesothelioma markers is calretinin and Wilms tumor protein 1 (WT1) with a reported diagnostic accuracy up to 87%, followed by cytokeratin 5/6 (CK5/6) and D2-40. The most commonly used markers to diagnose adenocarcinoma are claudin 4, MOC31, monoclonal carcinoembryonic antigen (CEA), B72.3, Ber-EP4, and BG8. Thyroid transcription factor-1 (TTF1) and napsin A are helpful to diagnose lung adenocarcinomas. Other markers can be considered based on morphology and clinical history, such as CDX2 (gastrointestinal), PAX8 (renal cell, thymic carcinoma, ovarian), NKX3.1 (prostate), or breast markers such as gross cystic disease fluid protein (GCDFP) and mammaglobin. For squamous cell carcinoma, a strong and diffuse nuclear positivity of p40 or p63 is very useful, as mesotheliomas are infrequently focally positive.

Calretinin staining in epithelioid MPM is often strong and diffuse and is both nuclear and cytoplasmic (Figure 17-1), with a reported sensitivity of 97% and specificity of 87% (Galateau-Salle et al 2016; Le Stang et al 2020). About 5% to 10% of adenocarcinomas can express calretinin, but the staining is usually weak and focal, and tends to be cytoplasmic.

WT1 shows a diffuse nuclear expression in epithelioid MPM, with a reported sensitivity of 88% and specificity of 94% (Le Stang et al 2020). WT1 antibodies may cross-react with cytoplasmic proteins, including those of endothelial cells, and this reactivity should not be misinterpreted as MPM-related. Rather, only nuclear reactivity of any intensity is regarded

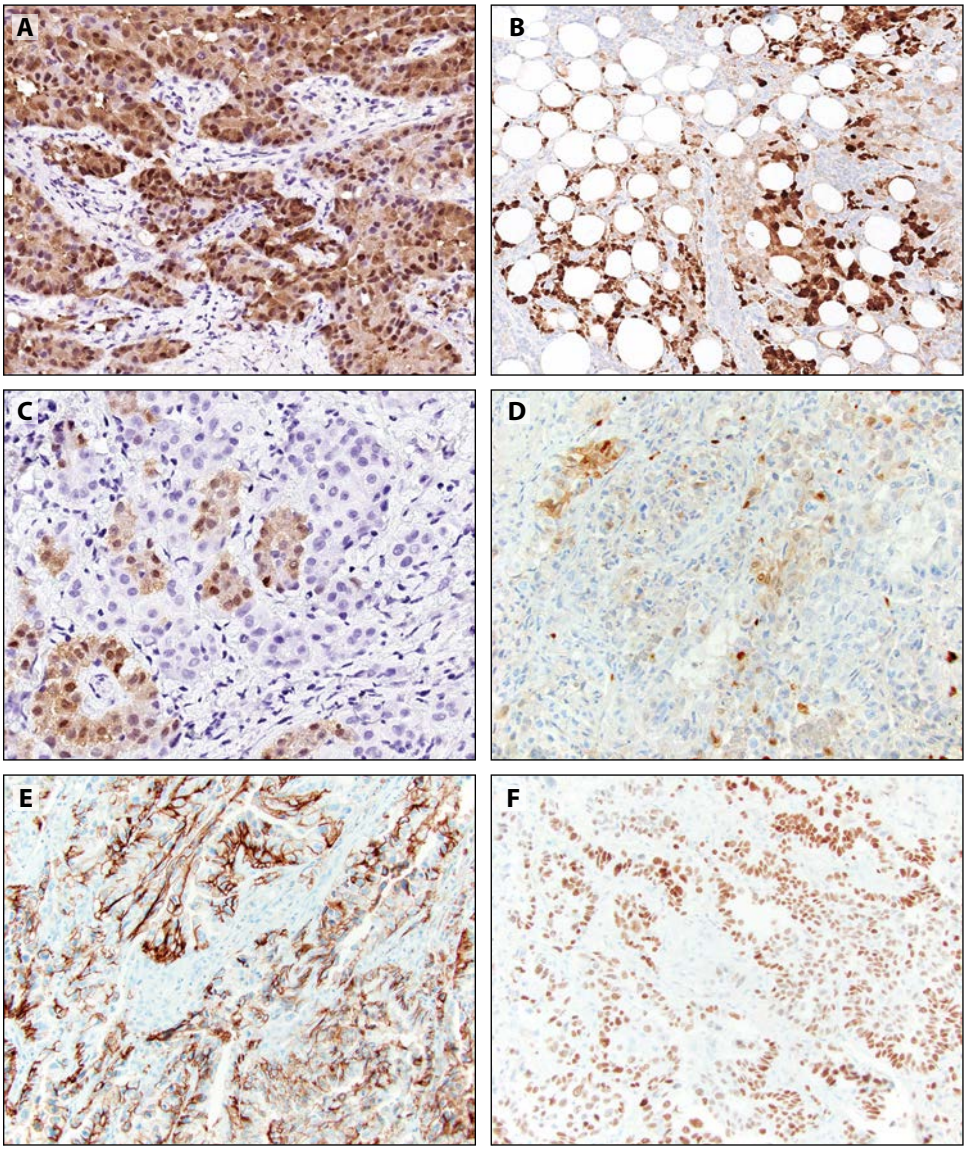


Figure 17-1. (A) Diffuse cytoplasmic and nuclear calretinin expression in an epithelioid mesothelioma, with **(B)** extensive adipose tissue infiltration by single cells or small clusters. **(C)** In another case of epithelioid mesothelioma, calretinin is only weakly and focally expressed. **(D)** An example of adenocarcinoma with a focal and weak expression of calretinin; however, "carcinoma" markers **(E)** BerEP4 and **(F)** thyroid transcription factor-1 (TTF1) confirm the diagnosis of adenocarcinoma.

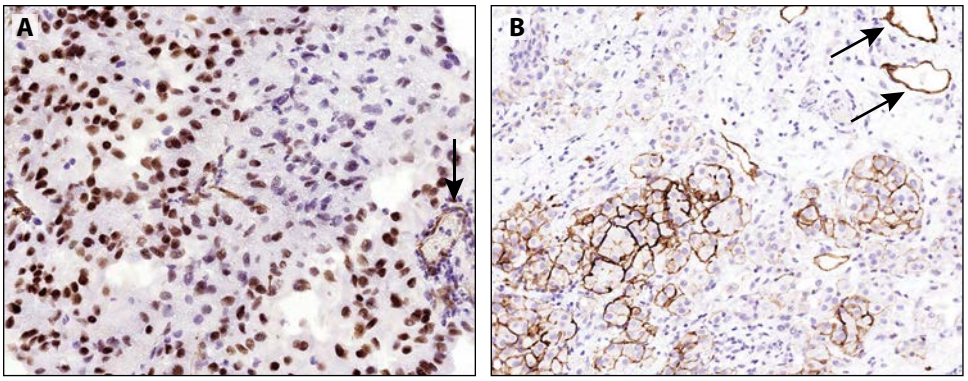


Figure 17-2. (A) Wilms tumor protein 1 (WT1) nuclear expression of variable intensity in epithelioid mesotheliomas may be diffuse or focal. Of note, endothelial cells of small vessels also express WT1 (*arrows*). **(B)** D2-40 often shows a strong membrane staining in most neoplastic cells of epithelioid mesothelioma. Similar to WT1, this marker stains endothelial cells, mostly in lymphatics (*arrow*).

as indicative of mesothelioma (Husain et al 2018) (Figure 17-2). WT1 is negative in lung adenocarcinoma, whereas some carcinomas, particularly ovarian, can be positive.

D2-40 (podoplanin) shows membranous, often diffuse, staining in 90% to 100% of epithelioid MPM with 94% sensitivity and 68% specificity (Le Stang et al 2020; He et al 2017) (Figure 17-2). It also stains lymphatics. Approximately 15% of lung adenocarcinomas can be focally positive.

Summary Answer

Calretinin and WT1 are the best positive mesothelial markers for diagnosis of epithelioid MPM. Claudin 4, MOC31, monoclonal CEA, B72.3, and Ber-EP4 in combination with site-specific markers are best in differentiating carcinoma from epithelioid MPM.

What Are the Best Markers to Distinguish Sarcomatoid MPM from Sarcomatoid Carcinoma?

The work-up of sarcomatoid tumors of the pleura should include, in addition to cytokeratins and mesothelioma markers, a panel of mesenchymal markers such as desmin, S100 protein, myogenin, signal transducer and activator of transcription 6 (STAT6), CD34, ERG, CD31, FLI1, and also melanoma markers (HMB45 and melan A) (Galateau-Salle et al 2016). Carcinoma markers, such as claudin 4, MOC31, Ber-EP4, and CEA, are not very helpful in the differential diagnosis of sarcomatoid tumors and do not need to be included in the panel, particularly if tissue is limited (Husain et al 2018). In the differential diagnosis from sarcomatoid carcinomas, organ site and differentiation-specific markers, such as TTF1 and p40, can be helpful.

Sarcomatoid mesotheliomas almost invariably stain at least focally with cytokeratins including AE1/AE3, CAM5.2, and pancytokeratin antibodies OSCAR and KL1 (Figure 17-3A). Pancytokeratin may be negative in up to 7% of sarcomatoid MPM (Klebe et al 2010). Positive cytokeratin expression alone does not differentiate from sarcomatoid carcinomas or some sarcomas but should be considered as the first step in the work-up and should be interpreted along with other mesothelial and non-mesothelial markers. (Marchevsky et al 2017). In some cases, multiple blocks, if available, should be stained to demonstrate cytokeratin expression.

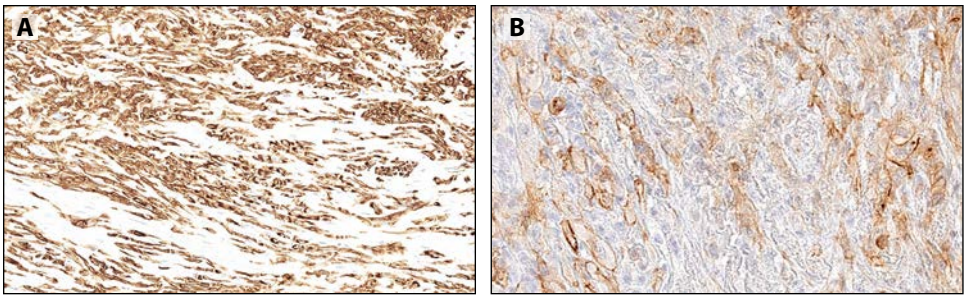


Figure 17-3. (A) Broad-spectrum cytokeratins (eg, clone AE1/AE3) are strongly expressed in neoplastic spindle cells; the **(B)** D2-40 is focally expressed in this sarcomatoid mesothelioma with a typical membrane staining of some spindle or oval tumor cells.

D2-40 (podoplanin) represents a reliable alternative to support a sarcomatoid MPM diagnosis, because of its higher sensitivity in highlighting the neoplastic mesothelioma cell membranes (Churg et al 2018; Chapel et al 2019; Nicholson et al 2020) (Figure 17-3B). This mesothelial marker is the most useful to establish the diagnosis of sarcomatoid MPM and is positive in approximately 74% of cases (Marchevsky et al 2017). The distinction from lymphatic vessels may be challenging in sarcomatoid MPM, especially in the case of rare spindle cells scattered in a desmoplastic stroma. Endothelial reactivity should not be misinterpreted as tumor-related in sarcomatoid MPM cases. This distinction is of utmost importance because D2-40 may be the only positive mesothelioma marker (though focally expressed) in the very rare cases lacking calretinin, WT1, and even cytokeratin reactivity.

Calretinin is more irregularly expressed in approximately 54% of sarcomatoid MPM, where it can be focal or even absent (Figure 17-4A and B). Nuclear staining is viewed as more specific for mesothelioma. WT1 is expressed in about 45% of sarcomatoid MPM (Marchevsky et al 2017). Nuclear reactivity, can be weak in some cases, but is accepted as specific (Galateau-Salle et al 2016) (Figure 17-4C).

Recently, GATA3 IHC was suggested as a marker for distinguishing sarcomatoid and desmoplastic MPM from sarcomatoid lung carcinoma (Berg and Churg 2017). Strong and diffuse GATA3 expression is observed in mesotheliomas, whereas sarcomatoid carcinomas are largely negative or show weak and patchy staining.

Summary Answer

Sarcomatoid MPM can be diagnosed with positive cytokeratin and mesothelial markers most frequently D2-40 and calretinin, while carcinoma markers are negative. If cytokeratins are negative, the differential diagnosis should include sarcomas, and a work-up for specific gene fusions/rearrangements should be considered.

What Is the Role of Cytokeratins in the Diagnosis of MPM?

Cytokeratin is generally expressed in all histologic subtypes of MPM with a sensitivity of 100% for epithelioid MPM (Le Stang et al 2020). It is useful in excluding sarcomas, with 2 caveats: The first is that rare sarcomas may express cytokeratin (including angiosarcoma and synovial sarcoma), and the second is that there are rare sarcomatoid MPM cases that lack cytokeratin expression (7%) (Klebe et al 2010).

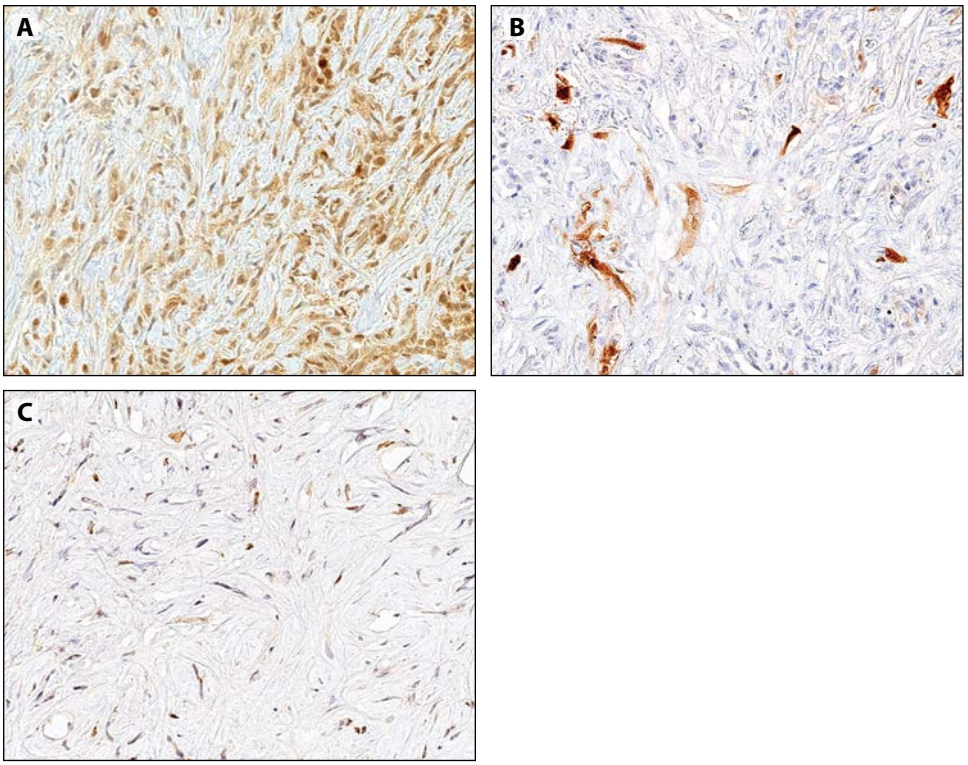


Figure 17-4. Calretinin is variably expressed in these 2 examples of sarcomatoid mesotheliomas showing **(A)** diffuse and **(B)** focal reactivity. In some cases, only rare single cells express this marker. Occasionally, the staining can be weak **(A)** and can be interpreted as nonspecific or artifact, but the associated nuclear positivity may be a helpful pitfall that the staining is real. **(C)** An example of sarcomatoid mesothelioma with strong and weak intensity of Wilms tumor protein 1 (WT1) expression in spindle neoplastic nuclei.

Among cytokeratin subtypes, CK5/6 is reported to specifically stain MPM when assessed against most adenocarcinomas (Figure 17-5A). It is, however, essential to keep in mind that this same cytokeratin subtype is also expressed by squamous cell carcinomas of various anatomic sites, thus its use is of limited value in the differential with squamous cell carcinoma metastases.

Cytokeratin is also expressed in the reactive stroma of chronic pleuritis and the stroma surrounding pure epithelioid MPM (Figure 17-5B and C), although it tends to diminish away from the surface in keeping with maturation of the fibroblastic stroma. Thus, this marker is of no value in distinguishing mesothelioma from reactive (atypical) mesothelial proliferations in terms of absolute expression, nor does its expression in the stroma per se support a diagnosis of biphasic MPM (Galateau-Salle et al 2016). However, it is useful in helping to identify infiltrating tumor cells into chest wall adipose tissue that may not be readily seen on routine hematoxylin and eosin (H&E) stains.

Cytokeratin stains may help in identifying sarcomatoid mesotheliomas by having an irregular staining pattern within fibrous stroma, especially at the deep aspect of the pleura, while staining of spindle cells in reactive pleuritis tends to lessen away from the surface (Figure 17-6). It is therefore important to orient samples when placing them in cassettes for sectioning.

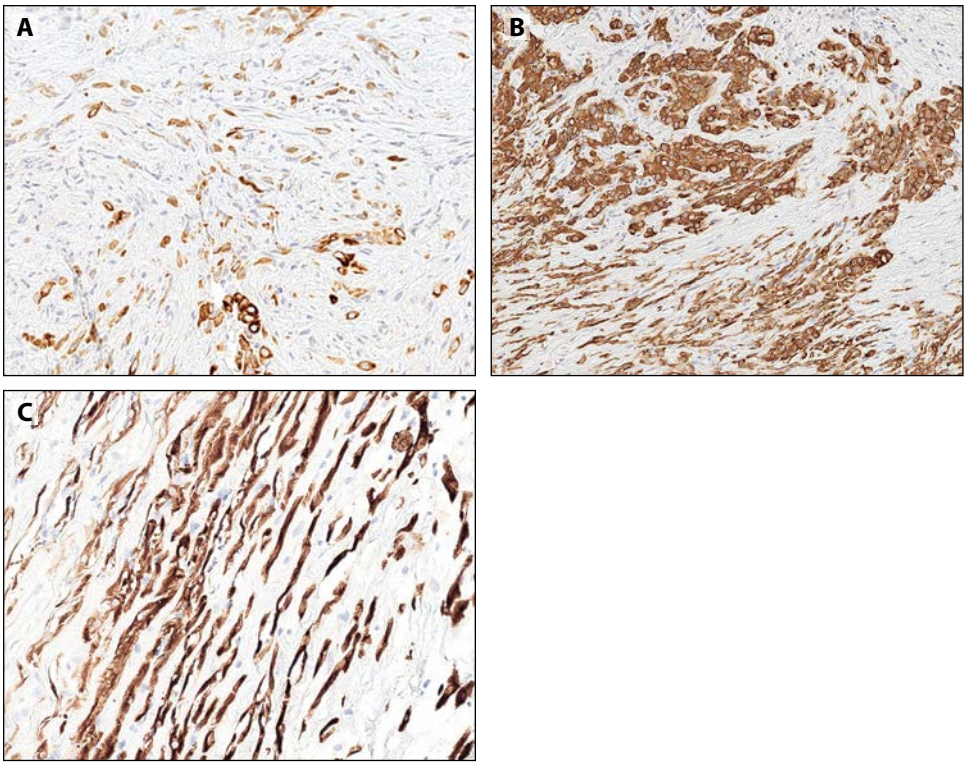


Figure 17-5. (A) High-molecular-weight cytokeratins (eg, CK5/6) are strongly expressed in epithelioid mesothelioma cell clusters and can be associated with a weak and focal staining of some nonneoplastic stromal cells. **(B and C)** Broad-spectrum cytokeratin (eg, clone AE1/AE3) as well as specific clones (eg, CK 5/6) stain both neoplastic and benign reactive mesothelial cells, and therefore this marker is of limited value in the differential diagnosis of mesothelioma from benign lesions with marked reactive atypia. The epithelioid and spindle neoplastic cells of this biphasic mesothelioma **(B)**, but also the reactive stromal cells of sclerosing organizing pleuritis **(C)** strongly express cytokeratins.

Summary Answer

Cytokeratin stains are helpful in highlighting full-thickness pleural cellularity, lack of zonation, and presence of invasion of mesothelial cells into chest wall adipose tissue.

What Immunohistochemical Markers Can Be Used to Distinguish Between Benign and Malignant Mesothelial Proliferations?

A large number of studies support the use of BRCA1-associated protein 1 (BAP1) in the differential diagnosis between benign atypical mesothelial proliferations and mesothelioma. BAP1 is a deubiquitinating protein with oncosuppressor functions and a role in cell proliferation and growth inhibition. It is expressed in the nucleus of all normal cells. Somatic alterations of the *BAP1* gene located on chromosome 3p21 cause the loss of protein expression in neoplastic cells, an event occurring in approximately 65% of epithelioid and 20% of sarcomatoid MPM (Hida et al 2017; Churg et al 2018; Galateau-Salle et al 2018). The recent recommendation is not to use this marker in isolation from other morphologic and immunophenotypic data (Nicholson et al 2020).

Except for the rare forms of BAP1 germline alterations, BAP1 immunoreactivity is preserved in all nonneoplastic cells of adipose, vascular, and connective tissues (Figure 17-7).

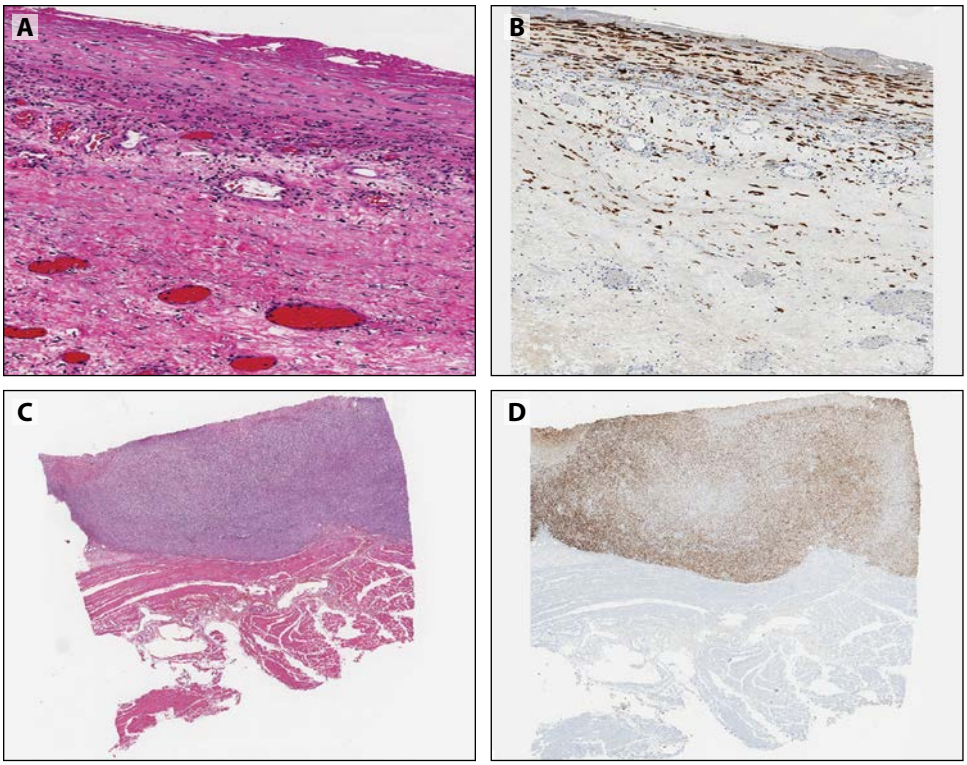


Figure 17-6. (A and B) Cytokeratin expression in reactive fibrosing pleuritis highlights zonation (hypercellularity at the surface and decreased cellularity deep toward the chest wall) and a horizontal distribution of benign mesothelial cells, while **(C and D)** malignant sarcomatoid proliferation shows haphazard growth pattern of mesothelial cells occupying the entire pleural thickness.

BAP1 IHC can be useful in distinguishing reactive atypical mesothelial proliferations from MPM. Loss of BAP1 staining is also helpful in establishing the diagnosis of epithelioid MPM in cytology fluid specimens (Figure 17-8).

BAP1 IHC can also be helpful in establishing the diagnosis of biphasic MPM. Its loss in epithelioid tumor cells, but not in stromal cells, argues against a diagnosis of biphasic MPM, even in the presence of atypical spindle cells, which rather represent a stromal reaction (Figure 17-9) (Righi et al 2016; Galateau-Salle et al 2018; Wu et al 2017). However, the interpretation should be done with caution, as occasional cases of biphasic mesothelioma may show discordant results between epithelioid and stromal components (Bueno et al 2016). In those cases, other ancillary tests such as methylthioadenosine phosphorylase (MTAP) IHC or fluorescence in situ hybridization (FISH) for *CDKN2A* homozygous deletion may be helpful.

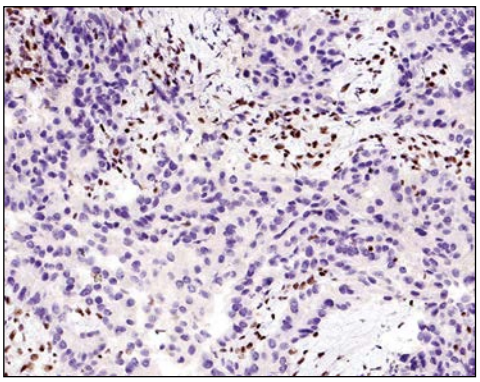


Figure 17-7. BRCA1-associated protein 1 (BAP1) is lost in this epithelioid mesothelioma as demonstrated by absence of nuclear staining in neoplastic cells and intact expression in nonneoplastic stromal cells.

MTAP IHC has been recently proposed as a surrogate marker of *CDKN2A/p16* gene alterations. Homozygous deletion of the *CDKN2A* gene located on chromosome 9p21 is a well-established

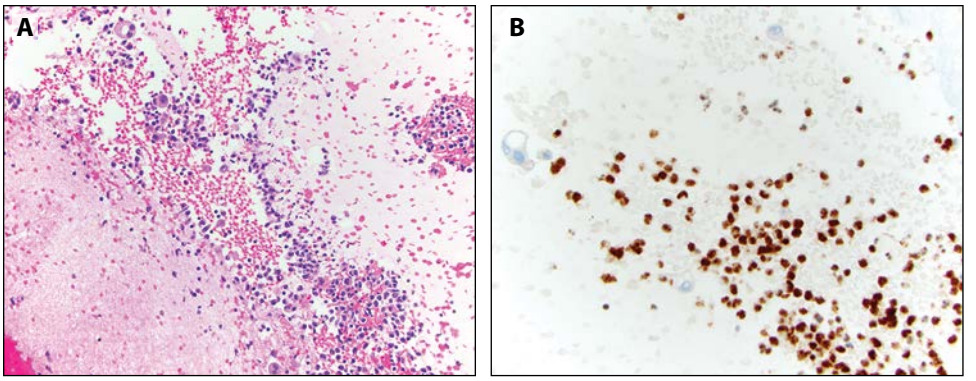


Figure 17-8. Pleural fluid specimen with scattered clusters of atypical mesothelial cells and lymphocytes. **(A)** Morphologically, this pleural fluid was interpreted as atypical. **(B)** However, loss of BRCA1-associated protein 1 (BAP1) expression in the same cluster of mesothelial cells confirms that they are indeed malignant.

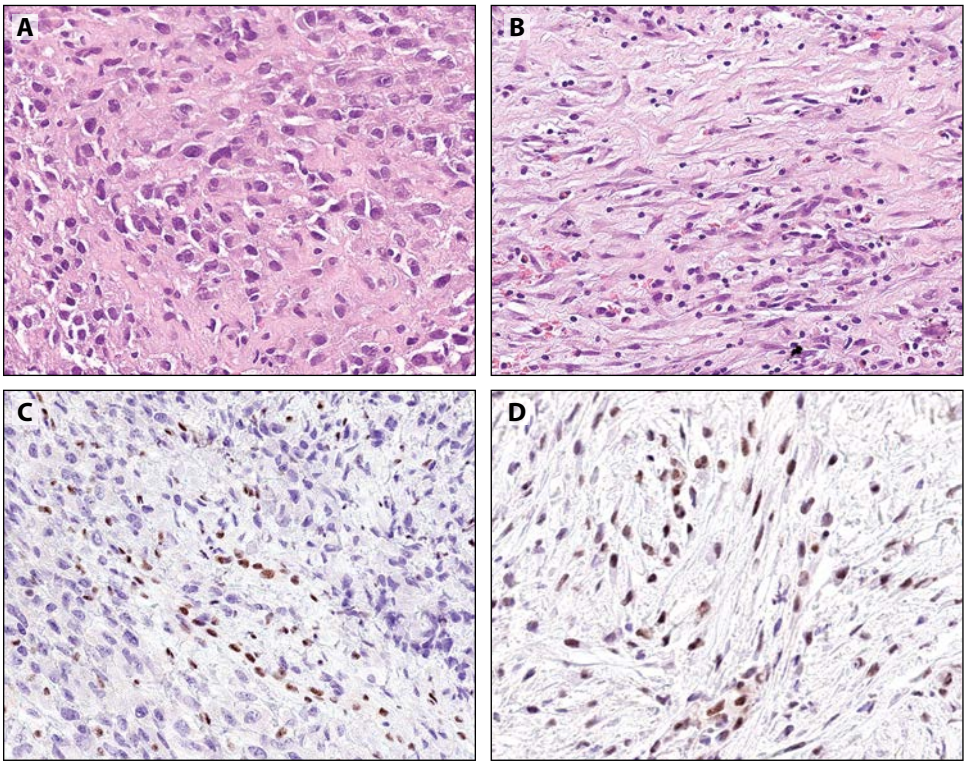


Figure 17-9. (A) Case of suspected biphasic mesothelioma with an epithelioid cell component associated to **(B)** a markedly atypical stroma. **(C)** BRCA1-associated protein 1 (BAP1) is lost only in the epithelioid component. **(D)** An intact expression of BAP1 in atypical stromal cells favors its reactive rather than neoplastic nature, thus supporting a final diagnosis of epithelioid mesothelioma (with atypical stroma reaction).

marker of malignant mesothelial proliferations that is lost in approximately 70% of epithelioid and almost 100% of sarcomatoid MPM (Illei et al 2003; Chiosea et al 2008; Husain et al 2018). MTAP is an enzyme involved in purine metabolism whose gene is located very close to *CDKN2A* at the 9p21.3 locus and has been reported to undergo deletions in tandem with *CDKN2A* in up to 100% of MPM.

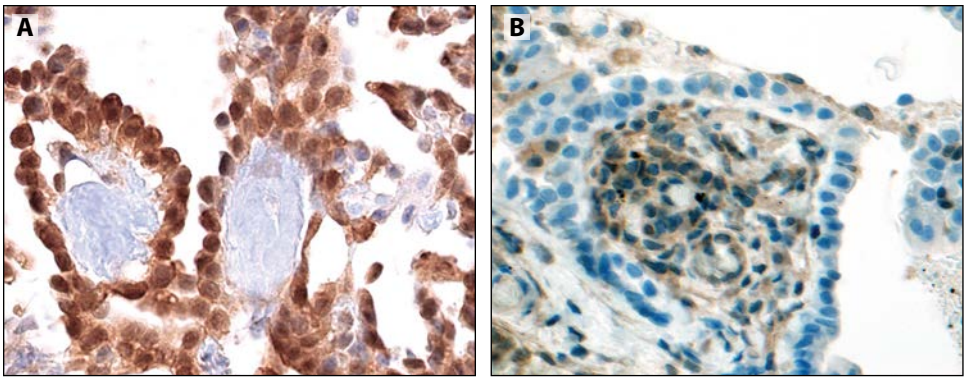


Figure 17-10. (A) A papillary mesothelioma with wild-type *CDKN2A* gene diffusely expresses methylthioadenosine phosphorylase (MTAP) in all neoplastic and also stromal cells. **(B)** An example of papillary mesothelioma with MTAP loss. Neoplastic cells lining the papillary stalk are negative while nonneoplastic stromal cells are regularly expressing the protein.

In normal human cells and in the 30% of wild-type epithelioid MPM cases, MTAP is expressed in the cytoplasm and also in the nucleus of normal and neoplastic cells (Figure 17-10A).

Gene deletions in MPM cells are associated with a loss of both nuclear and cytoplasmic immunoreactivity (Figure 17-10B), in which neoplastic mesothelial cells (negative) can be distinguished from stromal cells (that maintain their nuclear expression, if of reactive rather than neoplastic nature). When present, the MTAP loss is observed in all neoplastic cells, and only 10% of cases were found to have a partial loss in a fraction of cells (Berg et al 2018; Chapel et al 2020).

Loss of MTAP was reported also in up to 23% of reactive mesothelial proliferations (Zimling et al 2012), a finding not confirmed by other authors (Hida et al 2017) when the reactivity was interpreted as positive if of equal or higher intensity than that of inflammatory cells (internal control).

Summary Answer

BAP1 IHC and MTAP (as surrogate for *CDKN2A* homozygous deletion) can be helpful in distinguishing benign from malignant mesothelial proliferation in surgical and fluid specimens.

Conclusions

IHC is necessary to confirm the diagnosis of malignant mesothelioma, as many cases have medicolegal implications. For epithelioid mesothelioma, in a morphologically, radiologically, and clinically typical case, the recommendation is to use 2 mesothelioma markers and 2 carcinoma markers for a diagnosis. If the results are discordant, additional markers should be used. For sarcomatoid mesothelioma, cytokeratin staining should be undertaken, supplemented by empirical use of mesenchymal and mesothelial markers, with the choice of antibodies likely reflecting morphologic features. Often, as specificities and sensitivities are much lower than in epithelioid tumors, the histopathologic conclusion is a balance of probabilities, and multidisciplinary input is required. BAP1 and MTAP (as a surrogate for *CDKN2A/p16* homozygous deletion) IHC is helpful in separating benign from malignant mesothelial proliferations and for establishing the diagnosis of malignant mesothelioma in effusions and limited tissue samples.

References

Berg KB, Churg A. GATA3 immunohistochemistry for distinguishing sarcomatoid and desmoplastic mesothelioma from sarcomatoid carcinoma of the lung. *Am J Surg Pathol*. 2017;41(9):1221-1225.

Berg KB, Dacic S, Miller C, et al. Utility of methylthioadenosine phosphorylase compared with BAP1 immunohistochemistry, and CDKN2A and NF2 fluorescence in situ hybridization in separating reactive mesothelial proliferations from epithelioid malignant mesotheliomas. *Arch Pathol Lab Med*. 2018;142(12):1549-1553.

Bueno R, Stawiski EW, Goldstein LD, et al. Comprehensive genomic analysis of malignant pleural mesothelioma identifies recurrent mutations, gene fusions and splicing alterations. *Nat Genet*. 2016;48(4):407-416.

Chapel DB, Churg A, Santoni-Rugiu E, et al. Molecular pathways and diagnosis in malignant mesothelioma: a review of the 14th international conference of the international mesothelioma interest group. *Lung Cancer*. 2019;127:69-75.

Chapel DB, Schulte JJ, Berg K, et al. MTAP immunohistochemistry is an accurate and reproducible surrogate for CDKN2A fluorescence in situ hybridization in diagnosis of malignant pleural mesothelioma. *Mod Pathol*. 2020;33(2):245-254.

Chiosea S, Krasinkskas A, Cagle PT, et al. Diagnostic importance of 9p21 homozygous deletion in malignant mesotheliomas. *Mod Pathol*. 2008;21(6):742-747.

Churg A, Nabeshima K, Ail G, et al. Highlights of the 14th international mesothelioma interest group meeting: pathologic separation of benign from malignant mesothelial proliferations and histologic/molecular analysis of malignant mesothelioma subtypes. *Lung Cancer*. 2018;124:95-101.

Galateau-Salle F, Churg A, Roggli V, et al. The 2015 World Health Organization classification of tumors of the pleura: advances since the 2004 classification. *J Thorac Oncol*. 2016;11(2):142-154.

Galateau-Salle F, Le Stang N, Nicholson AG, et al. New insights on diagnostic reproducibility of biphasic mesotheliomas: a multi-institutional evaluation by the International Mesothelioma Panel from the MESOPATH Reference Center. *J Thorac Oncol*. 2018;13(8):1189-1203.

He C, Wang B, Wan C, et al. Diagnostic value of D2-40 immunostaining for malignant mesothelioma: a meta-analysis. *Oncotarget*. 2017;8(38):64407-64416.

Hida T, Hamasaki M, Matsumoto S, et al. Immunohistochemical detection of MTAP and BAP1 protein loss for mesothelioma diagnosis: comparison with 9p21 FISH and BAP1 immunohistochemistry. *Lung Cancer*. 2017;104:98-105.

Husain AN, Colby TV, Ordonez NG, et al. Guidelines for pathologic diagnosis of malignant mesothelioma 2017 update of the consensus statement from the international mesothelioma interest group. *Arch Pathol Lab Med*. 2018;142(1):89-108.

Illei PD, Rusch VS, Zakowski MF, et al. Homozygous deletion of CDKN2A and codeletion of the methylthioadenosine phosphorylase gene in the majority of pleura mesotheliomas. *Clin Cancer Res*. 2003;9(6):2108-2013.

Klebe S, Brownlee NA, Mahar A, et al. Sarcomatoid mesothelioma: a clinical-pathologic correlation of 326. *Mod Pathol*. 2010;23(3):470-479.

Le Stang N, Burke L, Blaizot G, et al. Differential diagnosis of epithelioid malignant mesothelioma with lung and breast pleural metastasis: a systematic review compared with a standardized panel of antibodies—a new proposal that may influence pathologic practice. *Arch Pathol Lab Med*. 2020;144(4):446-456.

Marchevsky AM, LeStang N, Hiroshima K, et al. The differential diagnosis between pleural sarcomatoid mesothelioma and spindle cell/pleomorphic (sarcomatoid) carcinomas of the lung: evidence-based guidelines from the International Mesothelioma Panel and the MESOPATH National Reference Center. *Hum Pathol*. 2017;67:160-168.

Nicholson AG, Sauter JL, Nowak AK, et al. EURACAN/IASLC proposals for updating the histologic classification of pleural mesothelioma: towards a more multidisciplinary approach. *J Thorac Oncol*. 2020;15(1):29-49.

Righi L, Duregon E, Vatrano S, et al. BRCA1-associated protein 1 (BAP1) immunohistochemical expression as a diagnostic tool in malignant pleural mesothelioma classification: a large retrospective study. *J Thorac Oncol*. 2016;11(11):2006-2017.

Wu D, Hiroshima K, Yusa T, et al. Usefulness of p16/CDNK2A fluorescence in situ hybridization and BAP1 immunohistochemistry for the diagnosis of biphasic mesothelioma. *Ann Diagn Pathol*. 2017;26:31-37.

Zimling ZG, Jorgensen A, Santoni-Rugiu E. The diagnostic value of immunohistochemically detected methylthioadenosine phosphorylase deficiency in malignant pleural mesotheliomas. *Histopathology*. 2012;60(6B):E96-105.

Thymic Tumors and Immunohistochemistry

18

By Andre L. Moreira and William D. Travis

Introduction

Thymic tumors are rare with an estimated prevalence of nearly 1% in the general population (Araki et al 2015; Siesling et al 2012). Management of patients with these lesions incorporates clinical, laboratory, and imaging features to determine differential diagnoses, disease extent, and, most importantly, histopathologic classification. Anterior mediastinal lesions, including those involving the thymus, are heterogeneous etiologically, which adds to the complexity of the site. This chapter provides support for the use of immunohistochemical stains in the differential diagnosis of the most commonly encountered anterior mediastinal masses and concentrates on the differential diagnosis of thymic epithelial lesions (thymoma, thymic carcinoma) and germ cell tumors.

What Are the Best Markers for the Diagnosis of Thymoma?

Despite different histologic classifications, all thymomas show a similar pattern of immunohistochemical reactivity. Thymomas are composed of thymic epithelial cells (keratin positive) and thymic immature lymphocytes (positive cells from terminal deoxynucleotidyl transferase [TdT], CD1a, or CD99) (Marx et al 2014) (Figure 18-1). This combination is diagnostic of thymoma in the appropriate morphologic setting. Stains for keratin 7 and 20 are not recommended as thymomas can be negative for both markers (Chu et al 2000; Pomplun et al 2002). Lymphomas can involve the mediastinum and enter in the differential diagnosis of thymoma World Health Organization (WHO) types B1 and B2. A pitfall is acute lymphoblastic lymphomas, which are more common in the young adult, the tumors cells are positive for TdT; however, all lymphomas lack keratin-positive cells.

Summary Answer

A combination of keratin and TdT is the best panel for the diagnosis of thymoma. CD1a or CD99 can also be used to mark thymic immature lymphocytes.

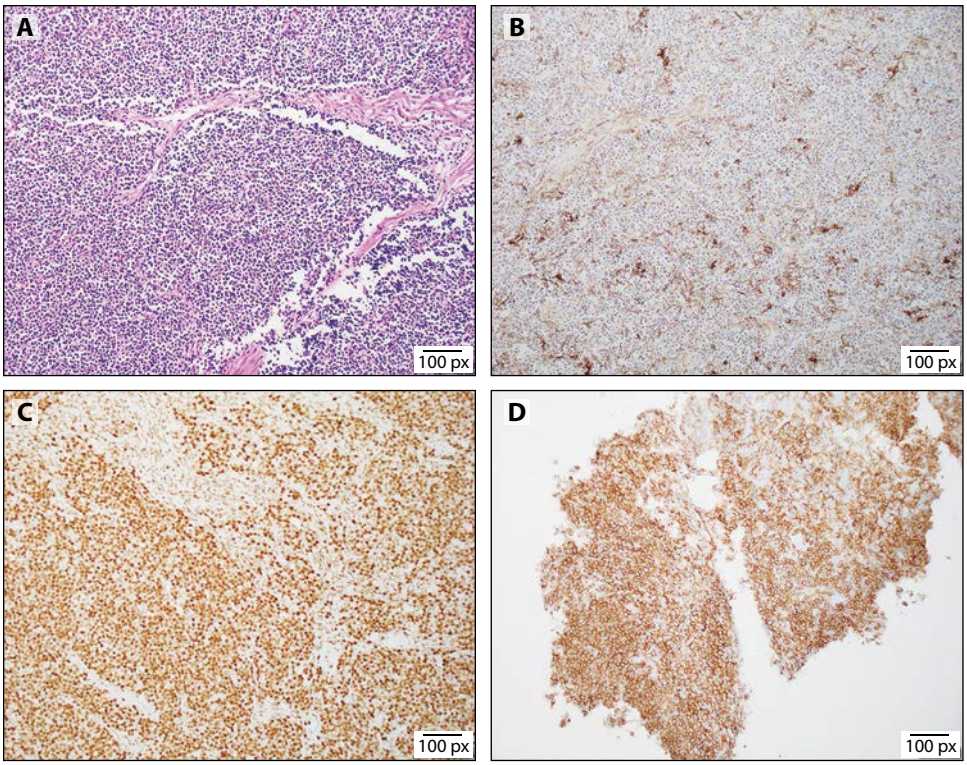


Figure 18-1. (A) H&E stained section of a World Health Organization (WHO) type B1 thymoma, showing fibrous bands, lobular growth pattern, and abundant lymphocytes. Epithelial cells are not readily apparent. **(B)** Immunohistochemical stain for keratin (AE1/AE3) highlights thymic epithelial cells in a meshlike distribution. **(C)** Immunohistochemical stain for TdT highlights thymic lymphocytes. **(D)** Immunohistochemical stain for CD1a highlights thymic lymphocytes, core biopsy of a type B2 thymoma.

What Other Markers Can Be Used to Highlight Thymic Epithelial Cells?

Thymic epithelial cells are positive for p40/p63 (Su et al 2015; Marx et al 2014) and PAX8 (Ordonez 2012; Asirvatham et al 2014), both of which stain cell nuclei (Figure 18-2). The latter can also be positive in neuroendocrine tumors of the thymus, but the reactions of thymic tumors are seen only when polyclonal PAX8 antibody is used (Toriyama et al 2014).

Summary Answer

Polyclonal PAX8 and p40/p63 can be added to the panel of keratin and TdT for the characterization of thymomas.

How Can Immunohistochemistry Help in the Classification of Thymomas?

Immunohistochemical stains can improve the reproducibility of WHO type B thymoma classification (Figure 18-3). B1 thymomas have a meshlike distribution of keratin-positive cells (Figure 18-1B), whereas B2 thymomas show clusters of keratin-positive cells.

Summary Answer

A keratin stain can reveal the pattern of epithelial cells, which helps in the classification between B1 and B2 thymoma.

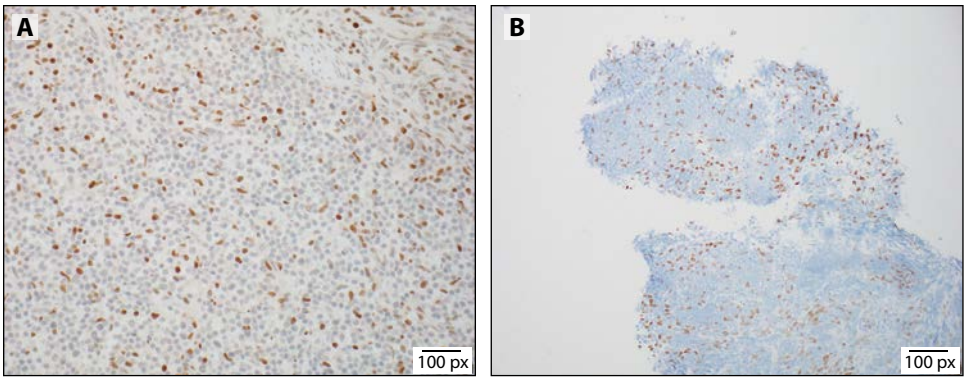


Figure 18-2. (A) Immunohistochemical stain for PAX8 highlights thymic epithelial cells. **(B)** Immunohistochemical stain for p40 highlights thymic epithelial cells in a core biopsy of a type B2 thymoma.

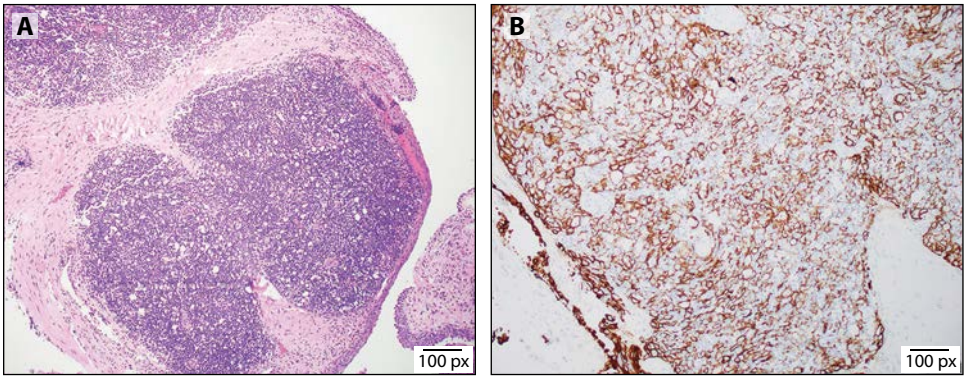


Figure 18-3. (A) H&E stained section of a World Health Organization (WHO) type B thymoma. **(B)** Immunohistochemical stain for keratin (AE1/AE3) shows clusters of epithelial cells, thus fulfilling a criterion for the classification of type B2 thymoma. Compare with Figure 18-1B that shows a meshlike network of keratin-positive cells in a type B1 thymoma.

How Can Thymoma Be Differentiated from Thymic Carcinoma?

The differential diagnosis of a B3 thymoma and thymic carcinoma can be challenging, especially in a small biopsy. The diagnosis relies on morphologic features of tumor cells, but immunohistochemistry (IHC) can help in the diagnosis (Figure 18-4). WHO type B3 thymoma is associated with TdT-positive lymphocytes, whereas thymic carcinoma is not associated with thymic lymphocytes, so a TdT or CD1a immunohistochemical stain is negative in these carcinomas (Weissferdt et al 2016; Marx et al 2014). Most thymic carcinomas are squamous cell carcinomas; thus, these tumors are positive for p40/p63 and PAX8. Although these markers are not specific, the positive reaction is suggestive of thymic origin. Moreover, 60% to 70% of thymic carcinomas express positivity for KIT (CD117) and CD5 in epithelial cells in contrast to lack of KIT and CD5–dual-positive reaction in lung cancer. These 4 markers can increase the diagnostic yield in terms of differential diagnoses of thymic carcinoma from B3 thymoma and lung squamous cell carcinoma (Asirvatham et al 2014; Su et al 2015; Kriegsmann et al 2015).

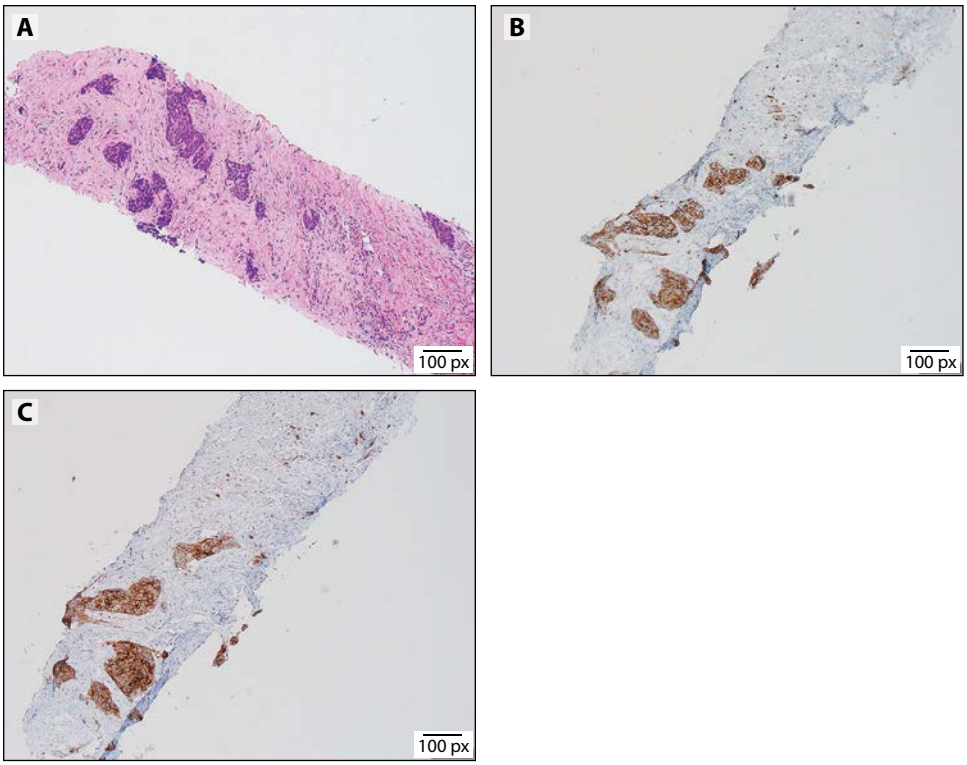


Figure 18-4. (A) H&E stained section of thymic carcinoma, core biopsy, showing clusters of epithelial cells in a fibrous stroma. Immunohistochemical stain for **(B)** CD5 and **(C)** CD117, showing **(B)** a positive membranous and cytoplasmic reaction in epithelial cells. The epithelial tumor cells were also positive for p40, and there were no TdT-positive lymphocytes associated with the tumor (*not shown*).

Summary Answer

The differential diagnosis of WHO type B3 thymoma and thymic carcinoma can be challenging in small biopsy specimens. The presence of TdT-positive lymphocytes is in favor of the diagnosis of thymoma. Positive CD5 and/or CD117 expression helps in the diagnosis of thymic carcinoma as well as the differential diagnosis from lung squamous cell carcinoma.

Which Stains Are Useful in Diagnosing Germ Cell Tumors?

The diagnosis of mediastinal germ cell tumors relies heavily on clinical, radiographic, and histologic information. Germ cell tumors are prevalent in young adult men (Calaminus and Joffe 2016) and have an association with increased serum markers such as α-fetoprotein (AFP), and β-human chorionic gonadotropin (β-HCG) for yolk sac tumor and choriocarcinoma, respectively (Salem and Gilligan 2011), despite no serum markers for seminoma, teratoma, or embryonal carcinoma. The components of a germ cell tumor can be determined by histology and IHC because each tumor has specific immunoreactivity patterns, except teratoma, as no specific markers have been described for this tumor. To differentiate from other tumors, sal-like protein 4 (SALL4) is most widely expressed across the germ cell tumors (Figure 18-5), including teratoma. Although some acute lymphoblastic B-cell leukemia and acute myeloid leukemia are also positive, SALL4 expression suggests a diagnosis of mediastinal germ cell tumor. Clinically, distinction between seminoma and non-seminomatous germ cell tumors is important; thus, for this distinction, OCT3/4, KIT (CD117), CD30, and glypican 3 are the

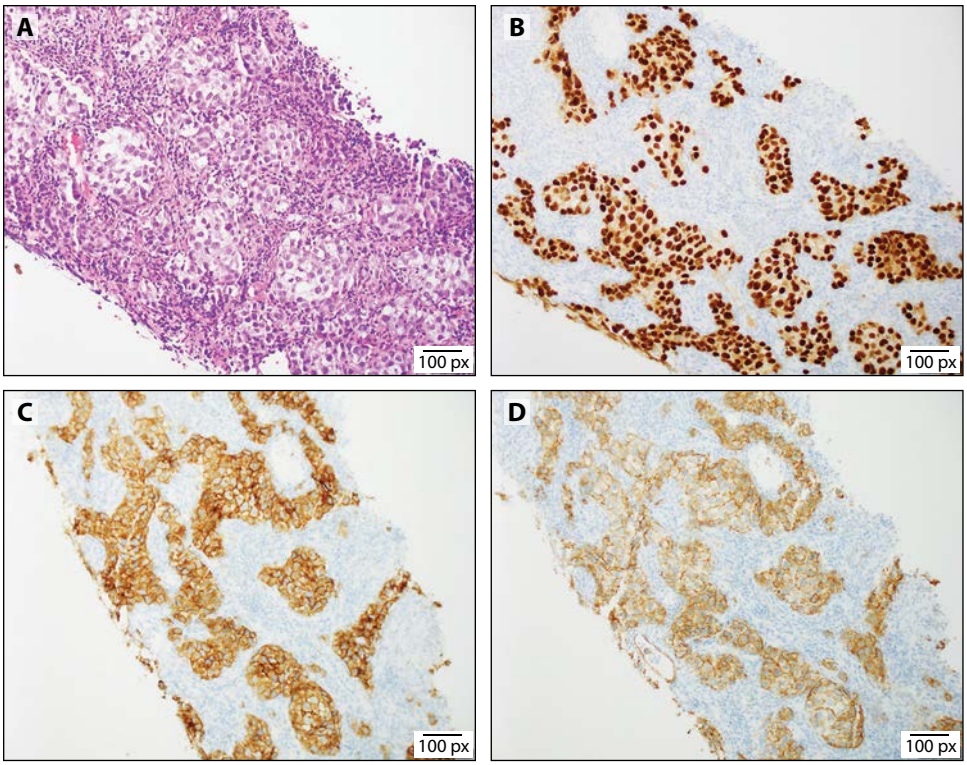


Figure 18-5. (A) H&E stained slide of a mediastinal seminoma, core biopsy. Nests of large tumor cells surrounded by lymphocytes. **(B)** Seminoma is positive for SALL4 (nuclear stain), a pan-germ cell tumor marker, OCT4 (*not shown*), **(C)** CD117, and **(D)** D2-40.

recommended markers (Figure 18-5) (Ulbright et al 2014), whereas most tumors are mixed germ cell tumors and contain any combination of tumors. OCT3/4 is positive in seminoma and embryonal carcinoma but negative in yolk sac tumors (Figure 18-6) and choriocarcinoma. CD117 is expressed in almost all seminomas but is negative in embryonal carcinomas and shows variable expression in yolk sac tumors. CD30 is positive in most embryonal carcinomas (Figure 18-7) but is negative in other germ cell tumors, while glypican 3 is seen in yolk sac tumors and most choriocarcinomas but is usually negative in embryonal carcinomas and seminomas. Most yolk sac tumors are AFP positive, but this may be focal. Choriocarcinomas also express HCG (Figure 18-8) (Ulbright et al 2014). In contrast to testis, mediastinal germ cell tumors can be positive for cytokeratin (Suster et al 1998; Weissferdt et al 2015), so positive keratin does not immediately indicate an epithelial tumor (Figure 18-9).

Summary Answer

SALL4 is a pan-germ cell tumor marker that should be included in a panel to work up these tumors (Camparo and Comperat 2013). Once positive SALL4 suggests germ cell tumor, OCT3/4, KIT (CD117), CD30, and glypican 3 can be used as classifiers for seminoma and non-seminomatous germ cell tumors (Cheng et al 2007; Weissferdt et al 2019). Other markers should be included after histologic examination and added accordingly (Ulbright 2005; Weissferdt et al 2015).

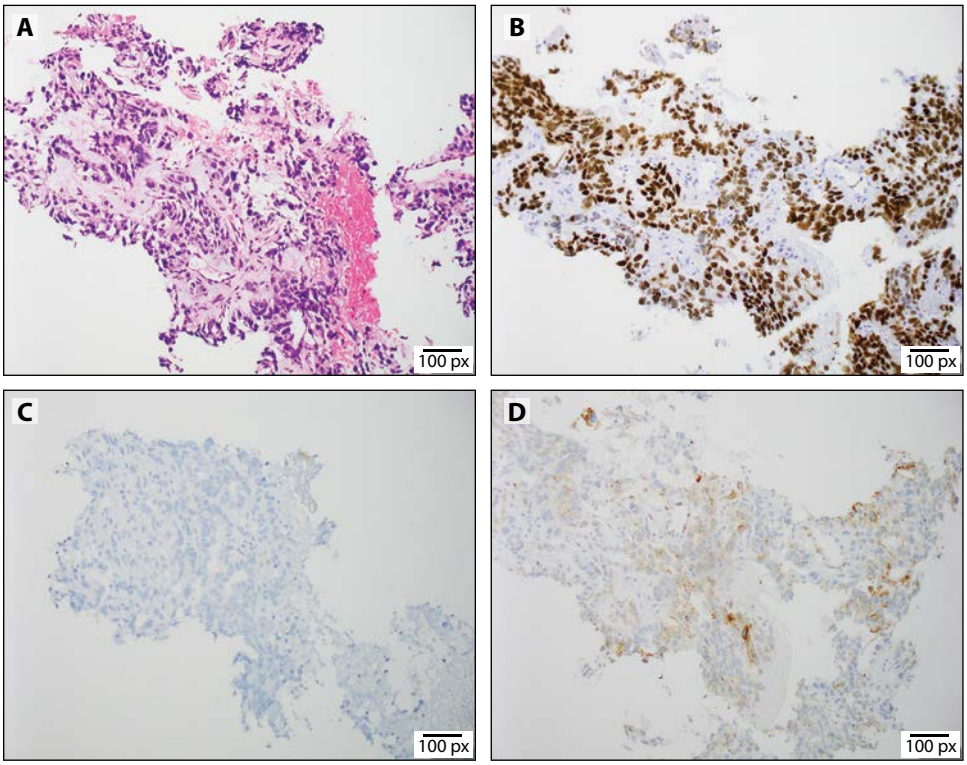


Figure 18-6. (A) H&E stained slide of a mediastinal yolk sac tumor forming histologic patterns that resemble embryonic structures. Yolk sac tumors are **(B)** positive for SALL4 and **(C)** negative for OCT4. Yolk sac tumors are also **(D)** positive for glypican 3, often in a patchy distribution. The tumor is also positive for α-fetoprotein (AFP) (*not shown*).

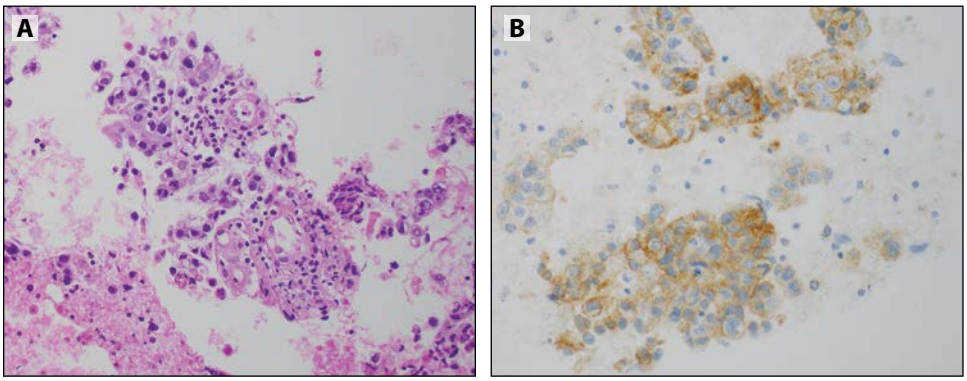


Figure 18-7. H&E stained slides of embryonal carcinoma, cell block. **(A)** Tumor cells are larger and more pleomorphic than yolk sac tumor. **(B)** Embryonal carcinomas are positive for CD30. Tumor cells are also positive for SALL4 and OCT4 (*not shown*).

Conclusions

Thymic tumors are rare and include a variety of histologic subtypes. IHC provides a substantial aid for differential diagnosis of thymic tumors, particularly in small biopsy specimens. In addition to thymic epithelial tumors and germ cell tumors that have been discussed in this chapter, lymphomas, neuroendocrine tumors, and some other rare tumors can also develop in the thymus. The differential diagnoses from these tumors is described elsewhere (Marx et al 2015).

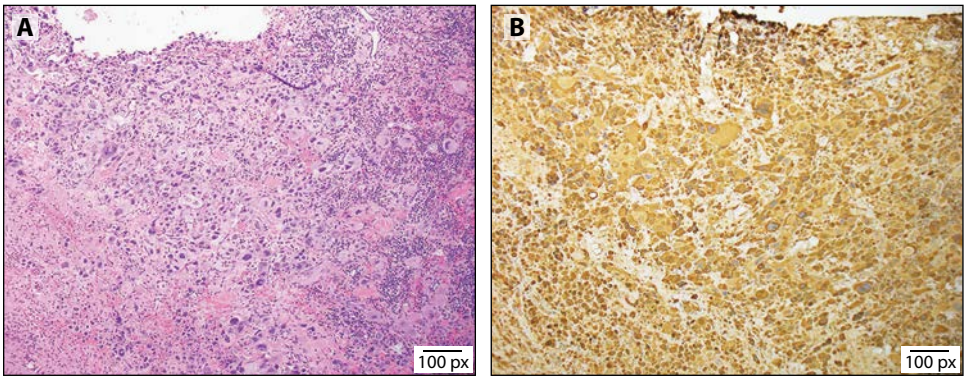


Figure 18-8. H&E stained slide of a choriocarcinoma. **(A)** Large pleomorphic, multinucleated cells often associated with hemorrhage. **(B)** Choriocarcinoma are positive for β-human chorionic gonadotropin (β-HCG), and often negative for all other germ cell tumor markers.

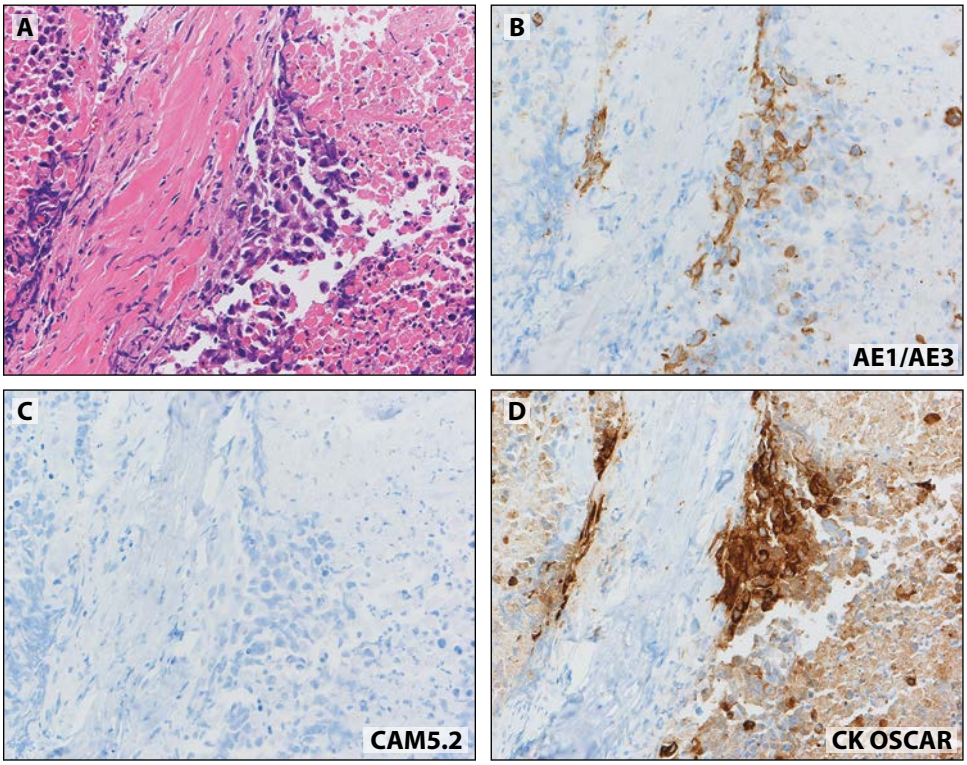


Figure 18-9. Cytokeratin staining in seminoma. Because seminoma can be positive for cytokeratin, the positivity does not directly indicate an epithelial tumor.

References

Araki T, Nishino M, Gao W, et al. Anterior mediastinal masses in the Framingham Heart Study: prevalence and CT image characteristics. *Eur J Radiol Open*. 2015;2:26-31.

Asirvatham JR, Esposito MJ, Bhuiya TA. Role of PAX-8, CD5, and CD117 in distinguishing thymic carcinoma from poorly differentiated lung carcinoma. *Appl Immunohistochem Mol Morphol*. 2014;22:372-376.

Calaminus G, Joffe J. Germ cell tumors in adolescents and young adults. *Prog Tumor Res*. 2016;43:115-127.

Camparo P, Comperat EM. SALL4 is a useful marker in the diagnostic work-up of germ cell tumors in extra-testicular locations. *Virchows Arch*. 2013;462:337-341.

Cheng L, Sung MT, Cossu-Rocca P, et al. OCT4: biological functions and clinical applications as a marker of germ cell neoplasia. *J Pathol*. 2007;211:1-9.

Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol*. 2000;13:962-972.

Kriegsmann M, Muley T, Harms A, et al. Differential diagnostic value of CD5 and CD117 expression in thoracic tumors: a large scale study of 1465 non-small cell lung cancer cases. *Diagn Pathol*. 2015;10:210.

Marx A, Chan JK, Coindre JM, et al. The 2015 World Health Organization classification of tumors of the thymus: continuity and changes. *J Thorac Oncol*. 2015;10(10):1383-1395.

Marx A, Strobel P, Badve SS, et al. ITMIG consensus statement on the use of the WHO histological classification of thymoma and thymic carcinoma: refined definitions, histological criteria, and reporting. *J Thorac Oncol*. 2014;9(5):596-611.

Ordonez NG. Value of PAX 8 immunostaining in tumor diagnosis: a review and update. *Adv Anat Pathol*. 2012;19:140-151.

Pomplun S, Wotherspoon AC, Shah G, et al. Immunohistochemical markers in the differentiation of thymic and pulmonary neoplasms. *Histopathology*. 2002;40:152-158.

Salem M, Gilligan T. Serum tumor markers and their utilization in the management of germ-cell tumors in adult males. *Expert Rev Anticancer Ther*. 2011;11:1-4.

Siesling S, van der Zwan JM, Izarzugaza, I, et al. Rare thoracic cancers, including peritoneum mesothelioma. *Eur J Cancer*. 2012;48(7):949-960.

Su XY, Wang WY, Li JN, et al. Immunohistochemical differentiation between type B3 thymomas and thymic squamous cell carcinomas. *Int J Clin Exp Pathol*. 2015;8:5354-5362.

Suster S, Moran CA, Dominguez-Malagon H et al. Germ cell tumors of the mediastinum and testis: a comparative immunohistochemical study of 120 cases. *Hum Pathol*. 1998;29(7):737-742.

Toriyama A, Mori T, Sekine S, et al. Utility of PAX8 mouse monoclonal antibody in the diagnosis of thyroid, thymic, pleural and lung tumours: a comparison with polyclonal PAX8 antibody. *Histopathology*. 2014;65(4):465-472.

Ulbright TM. Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Mod Pathol*. 2005;18(suppl 2):S61-79.

Ulbright TM, Tickoo SK, Berney DM, et al. Best practices recommendations in the application of immunohistochemistry in testicular tumors: a report from the International Society of Urological Pathology consensus conference. *Am J Surg Pathol*. 2014;38(8):e50-59.

Weissferdt A, Kalhor N, Moran CA. Thymomas with extensive clear cell component: a clinicopathologic and immunohistochemical study of nine cases. *Am J Clin Pathol*. 2016;146:132-136.

Weissferdt A, Kalhor N, Rodriguez Canales J, et al. Primary mediastinal yolk sac tumors: an immunohistochemical analysis of 14 cases. *Appl Immunohistochem Mol Morphol*. 2019;27:125-133.

Weissferdt A, Rodriguez-Canales J, Liu H, et al. Primary mediastinal seminomas: a comprehensive immunohistochemical study with a focus on novel markers. *Hum Pathol*. 2015;46(3):376-383.

Use of Immunohistochemistry in Predictive Biomarker Testing

19

By Keith M. Kerr, Ming Sound Tsao, Fred R. Hirsch, and Ignacio I. Wistuba

Introduction

As has been extensively discussed elsewhere in this book, immunohistochemistry (IHC) is largely concerned with the detection of proteins in tissue sections. Conversely, with a few exceptions, the cellular oncogenic alterations that make tumors susceptible to molecularly targeted drugs usually concern gene alterations at a DNA and/or RNA level. Nonetheless, it is the altered protein that exerts the oncogenic activity, and it is this protein, and not the altered nucleic acid sequence, that is the target of the drug.

The introduction of human epidermal growth factor receptor 2 (HER2) IHC in breast cancer testing was a difficult process in the early 1990s and made many oncologists wary of IHC in general. Some IHC-based biomarkers in non-small cell carcinoma (NSCC) have come and gone (thymidylate synthase, epidermal growth factor receptor [EGFR], and MET), but some of these may make a comeback (see the following sections). More recently, however, programmed death ligand-1 (PD-L1) IHC has established this technique firmly at the core of NSCC biomarker testing.

Does IHC Have a Role in Detecting EGFR Alterations?

IHC assays against EGFR wild-type (WT) protein were investigated during the search for biomarkers to select patients for EGFR tyrosine kinase inhibitor (TKI) therapy, until it was discovered that it was a more specific group of patients with a range of activating and sensitizing mutations in the TK domain of *EGFR* who benefited from these drugs (Clark et al 2006; Eberhard et al 2008). In the FLEX trial, EGFR WT IHC was effective in selecting patients who were more likely to benefit from the anti-EGFR monoclonal antibody cetuximab (Douillard et al 2014), but this agent failed to obtain regulatory approval, so EGFR WT IHC testing never reached routine clinical practice. This biomarker was also used in trials of necitumumab, another anti-EGFR monoclonal antibody therapeutic, but failed to demonstrate effective patient selection (Diaz-Serrano et al 2019).

Antibodies are available for use in IHC assays to detect mutant EGFR proteins for the *EGFR* L858R exon 21 mutation and for some of the exon 19 deletion mutant proteins. Although the sensitivity and specificity of the anti-L858R protein is acceptable, the range of exon 19 deletion mutations found in clinical practice renders the antibody unable to identify all mutant proteins as the antibody only detects 15 base pair deletions and no other sensitizing deletions in the exon 19 (Cooper et al 2013; Chen et al 2014). Consequently, the sensitivity for all exon 19 deletion mutations is low and falls below a level routinely acceptable in clinical practice. These IHC tests are, however, used in clinical practice where mutation sequence testing is not possible, or where longer turnaround times for mutation testing leads to requests for this IHC test with a 1- to 2-day turnaround time (Figure 19-1). This approach to *EGFR* mutation testing, using IHC, is not recommended for routine practice in the latest version of the molecular testing guideline given by the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC), and Association for Molecular Pathology (AMP) (Lindeman et al 2018).

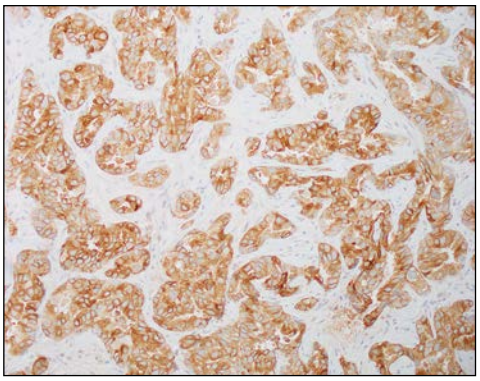


Figure 19-1. Metastatic adenocarcinoma of the lung in a breast core biopsy in an 86-year-old woman. The tumor is positive for *EGFR* exon 19 deletion mutation specific antibody (E746_A750del- clone 6B6, Roche Tissue Diagnostics).

Summary Answer

EGFR WT IHC has no current routinely recommended clinical use. IHC to detect a limited range of mutant EGFR proteins is occasionally used in particular circumstances.

What Is the Role of IHC in Detecting Tumors Bearing *ALK* Gene Rearrangements?

Anaplastic lymphoma kinase (ALK) IHC plays a pivotal role in the detection of patients with an *ALK* gene rearrangement. It is sometimes used as a screening tool, but it has also been accepted by the U.S. Food and Drug Administration (FDA) when the VENTANA ALK (D5F3) CDx Assay is used as a therapy-determining test without actual direct proof of an *ALK* gene rearrangement (Lindeman et al 2018).

There is a modest elevation in ALK protein in the cytoplasm of NSCC tumor cells bearing an *ALK* gene rearrangement. Except for some neural tissues, ALK protein is not found in normal adult tissues (Hallberg and Palmer 2013). As a result, ALK IHC screening was adopted as a fast and cheap method to identify NSCC patients whose samples should be submitted for confirmatory fluorescence in situ hybridization (FISH) or other molecular testing. This saves laboratories from performing a large number of expensive FISH tests to identify a molecular abnormality present in only approximately 4% of the test population. Studies using the ALK1 clone, which was in common use for the diagnosis of anaplastic lymphoma where ALK protein levels are high, proved inadequately sensitive to reliably detect the lower levels of ALK protein found in *ALK*-rearranged NSCC (Mino-Kenudson et al 2010). Assays using the 5A4 and D5F3 anti-ALK clones were developed, and several studies have shown

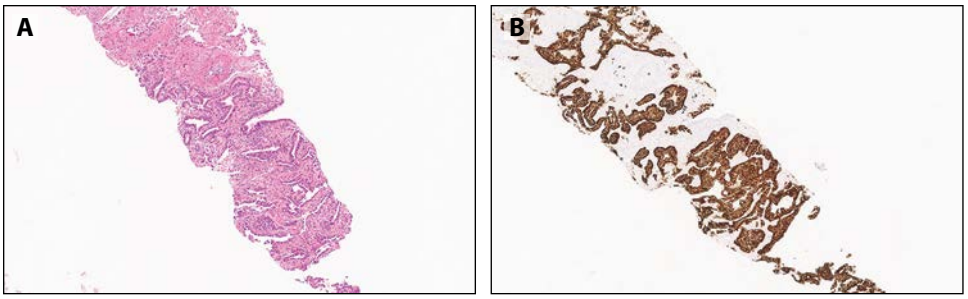


Figure 19-2. (A) Core biopsy of the lung showing acinar pattern adenocarcinoma. **(B)** This tumor has anaplastic lymphoma kinase (*ALK*) gene rearrangement and was strongly positive on ALK immunohistochemistry (IHC) using a 5A4 clone-based laboratory-developed assay.

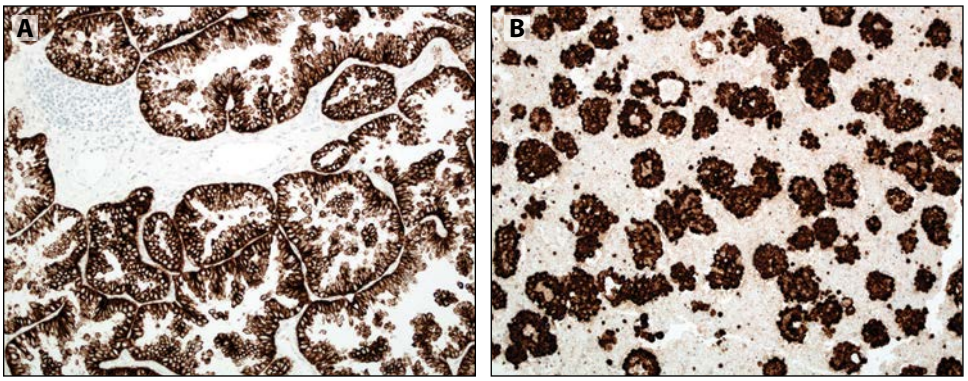


Figure 19-3. Adenocarcinoma of lung showing strong and diffuse staining for anaplastic lymphoma kinase (ALK) using the VENTANA ALK (D5F3) CDx Assay in **(A)** a surgical biopsy and **(B)** a pleural effusion cell block. Both cases showed *ALK* gene rearrangement on fluorescence in situ hybridization (FISH) testing.

that these assays can be used to screen populations of NSCC samples and identify cases with *ALK* gene rearrangement (Figures 19-2 and 19-3) (Tsao et al 2016). Although the sensitivity is generally adequate, depending on the detection chemistry of any particular laboratory-developed test (LDT), specificity is lower, especially when IHC positivity is at lower levels (Tsao et al 2013; Blackhall et al 2014; von Laffert et al 2014; Minca et al 2013; Selinger et al 2013; Nitta et al 2013). For this reason, such assays are best used as screening tools to select patients for confirmatory FISH or other molecular testing (Lindeman et al 2018). Cancer care teams may, however, validate their own ALK IHC LDT assay, and if it has an acceptably high specificity, and relevant regulatory authorities allow, they may choose to use it directly for therapy decisions. It should be noted, however, that the performance of ALK LDTs testing in some external quality assurance schemes falls short of acceptable performance (Ibrahim et al 2016). The VENTANA ALK (D5F3) CDx Assay is a highly sensitive and specific assay that is approved for the treatment selection of patients for ALK TKIs in advanced-stage NSCC. This assay utilizes a tyramide amplification step in the detection system that eliminates the dynamic range of staining in *ALK*-rearranged NSCC samples, creating an effectively binary readout: positive or negative. The VENTANA ALK (D5F3) CDx Assay demonstrates high correlation with the presence of an *ALK* gene rearrangement in NSCC (Nitta et al 2013).

In an interesting twist in the story of *ALK* rearrangement testing in NSCC, there is now evidence from both academic studies and the ALEX trial, that patients whose tumors have

an *ALK* gene rearrangement and whose tumors are IHC positive show higher response rates than patients who are rearrangement positive but are IHC negative (van der Wekken et al 2017; Mok et al 2017). This makes sense because the protein is the oncogenic moiety and is the target of the drug. It also signals a potential role for ALK IHC in patients whose *ALK* gene rearrangement is detected by next-generation sequencing (NGS) (see "Conclusions").

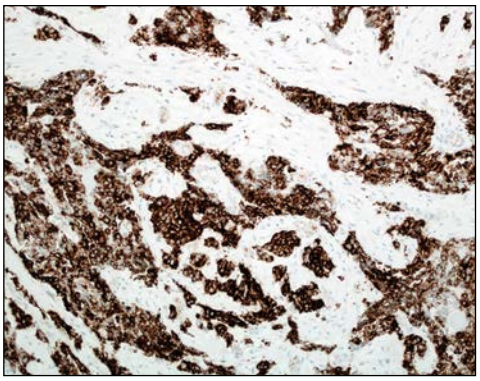


Figure 19-4. Endobronchial biopsy sample showing small cell carcinoma positive using the VENTANA ALK (D5F3) CDx Assay. There was no rearrangement on fluorescence in situ hybridization (FISH) testing.

Finally, a word of caution when using ALK IHC without FISH or molecular confirmation as just described. Up to approximately 20% of high-grade lung neuroendocrine carcinomas express sometimes strong and diffuse ALK IHC positivity in the absence of a gene rearrangement (Figure 19-4). Occasionally, such tumors may be misdiagnosed as adenocarcinoma on the basis of thyroid transcription factor-1 (TTF1) IHC positivity, especially in small, morphologically challenged samples (Kondoh et al 2019).

Summary Answer

ALK IHC has a pivotal role in ALK predictive biomarker testing in patients with advanced-stage NSCC.

What Is the Role of IHC in Detecting Tumors Bearing *ROS1* Gene Rearrangements?

In comparison to ALK testing, c-ros oncogene 1 (*ROS1*) gene rearrangement testing is less mature. There is no companion diagnostic ROS1 IHC test available, and although the use of ROS1 IHC is well established and recommended, this methodology should only be used as a screening tool to enrich a test population for FISH or molecular testing for *ROS1* gene rearrangements (Lindeman et al 2018; Tsao et al 2016). All ROS1 IHC tests currently in use, therefore, are by definition, LDTs. The D4D6 clone is frequently used in clinical practice. Published data suggest it is possible to develop an adequately sensitive assay (Figure 19-5), but the specificity is generally lower than the best ALK IHC assays (Bubendorf et al 2016; Selinger et al 2017). ROS1 protein levels may be variable in different parts of a tumor bearing a rearrangement, opening the possibility for biopsy sampling error, and expression can be very low in the presence of some rearrangements, leaving potential for some fusion genes to be missed by IHC screening. On the contrary, some adenocarcinomas may show focal moderate to strong positivity in the absence

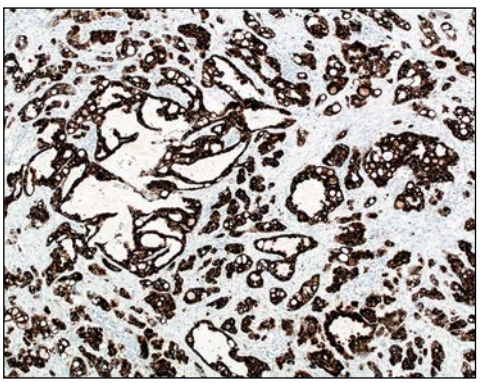


Figure 19-5. Diffuse staining in lung adenocarcinoma for c-ros oncogene 1 (*ROS1*) using a D4D6-based assay. This case showed *ROS1* gene rearrangement on fluorescence in situ hybridization (FISH) testing.

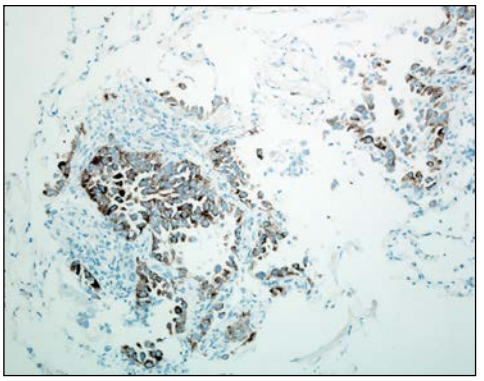


Figure 19-6. This lung core biopsy shows adenocarcinoma with c-ros oncogene 1 (*ROS1*) positive staining using a D4D6-based assay. There was no gene rearrangement of fluorescence in situ hybridization (FISH) testing.

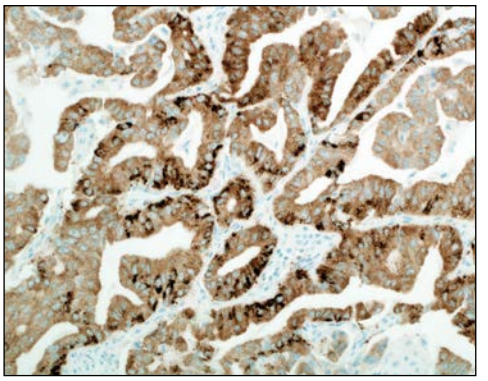


Figure 19-7. Lung adenocarcinoma with c-ros oncogene 1 (*ROS1*) gene rearrangement and positive staining for *ROS1* using the SP384 clone. This case nicely demonstrates variability in intensity and granular character of staining often seen in such cases.

of a *ROS1* fusion (Figure 19-6). Data have shown that *ROS1* gene rearrangements may be found in as few as 20% of ROS1 IHC positive cases (Tsao et al 2016). Recently the anti-ROS1 SP384 clone has become available (Roche Tissue Diagnostics) and is also an effective screening tool (Figure 19-7) (Huang et al 2019; Conde et al 2019; Hofman et al 2019).

There are as yet no data showing a relationship between therapy response and IHC positivity in this context, but it is certainly something worthy of study.

Summary Answer

ROS1 IHC is an established technique for the enrichment of a patient population with advanced-stage NSCC to have confirmation of *ROS1* gene rearrangement by an alternative molecular method.

Does IHC Have a Role in the Identification of Tumors with *NTRK1-3* Gene Rearrangements?

When compared with the preceding alterations, there is even less experience in NSCC, of testing for neurotrophic tyrosine receptor kinase (*NTRK*) fusion genes, but once again, the IHC positivity screening paradigm has emerged. This is especially so since *NTRK* rearrangements are extremely rare in NSCC (0.1%-1%) (Farago et al 2018), and for many laboratories, it would not be feasible to screen all relevant cases using a stand-alone FISH test for example. Data are limited, but pan-TRK IHC using clone EPR17341 (Figure 19-8) (Abcam) has shown a correlation with the presence of *NTRK1-3* gene rearrangements found by other means and represents a feasible screening strategy (Hechtman et al 2017). Approaches to this difficult area of NSCC biomarker testing are still being developed, but some recommendations have now been published (Penault-Llorca et al 2019; Marchio et al 2019). NTRK IHC positivity may also occur in neuroendocrine tumors where there is no gene rearrangement (Figure 19-9) (K.M. Kerr, personal observation).

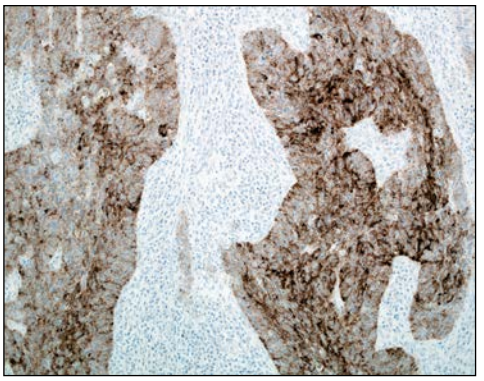


Figure 19-8. Lung adenocarcinoma showing positive staining using pan-TRK immunohistochemistry.

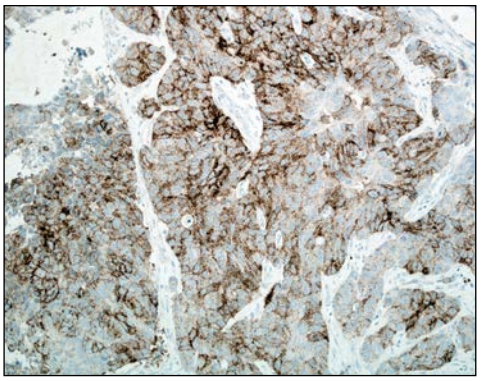


Figure 19-9. Large cell neuroendocrine carcinoma of the lung showing pan-TRK immunohistochemistry (IHC) positivity but no evidence of neurotrophic tyrosine receptor kinase (*NTRK*) gene rearrangement.

Summary Answer

IHC testing to assist the detection of *NTRK1-3* gene rearrangements is an emerging technique, but without an established role.

Does IHC Testing Have Any Role in Predictive Biomarker Testing for Any Other Targetable Genomic Alterations?

B-raf proto-oncogene protein (BRAF) and MEK kinase inhibitors are approved in many countries for use in patients with BRAF V600E mutations (Planchard et al 2017; Planchard and Johnson 2018; Leonetti et al 2018). The approved methodology for identifying patients for such therapy is some form of sequencing. Antibodies for use in IHC assays are available to identify the V600E mutant protein. Although these are sometimes used in patients with malignant melanoma, their use in patients with NSCC is not recommended (Lindeman et al 2018).

IHC assays for MET were introduced as a putative selective biomarker for use with the anti-MET monoclonal antibody therapeutic onartuzumab (Spigel et al 2017). Although high levels of MET expression, which were shown using an assay based on the SP44 clone did appear to enrich for improved patient outcomes, this therapy failed to reach regulatory approval. More recently, MET alterations in NSCC are once again drug targets of interest in 2 particular settings: de novo MET gene amplification, or more importantly perhaps, in the context of a resistance mechanism of relapse after EGFR TKI therapy as a biomarker for MET-targeted therapy (Salgia 2017; Wang et al 2019). Such treatments are also being investigated in patients whose tumors bear a wide range of mutations in and around exon 14 of the MET gene, alterations, which cause skipping of exon 14 during transcription and oncogenic drive because of impaired degradation of MET protein (Salgia 2017; Paik et al 2015). In both scenarios, but more so in the case of MET exon 14 skipping, it has been reasoned that elevated levels of protein might be an IHC marker that can be used to screen tumors for MET-targeted molecular testing. To date, literature appears to suggest that although positive IHC for MET is associated with the presence of the genomic changes, IHC approaches so far investigated are not sensitive and specific enough to be used as screening tools and many

cases would be missed (Baldacci et al 2020; Guo et al 2019). The future for MET IHC in NSCC in this context is uncertain.

Although HER2 IHC is well established as a predictive diagnostic test in breast cancer, it has no currently approved role in lung cancer testing. HER2 mutations are rare in lung cancer, but protein over-expression is more common. Recent data have raised the possibility of an effective targeted therapy for these patient groups (Smit et al 2020). While HER2 IHC is not part of the current testing scenario, this may change in the future.

Gene rearrangements of the ret proto-oncogene (*RET*) are a promising if rare target in lung cancer with several new agents effective against NSCC with this alteration. Following the paradigm already described for ALK, ROS1, and NTRK testing, RET IHC is of interest and possible value, but there are currently no data available.

NRG1 gene rearrangements are found in cases of NSCC, especially in invasive mucinous adenocarcinomas, and promising efficacy from EGFR TKI therapy has been shown. Positive IHC for NRG1 protein has been described in these cases, but the role of such an assay is yet to be established (Nagasaka and Ou 2019; Trombetta et al 2018).

In small cell carcinoma, IHC testing for DLL3 was recently pursued as an enrichment strategy for the antibody-cytotoxic drug conjugate rovalpituzumab tesirine (Morgensztern et al 2019). This drug is not currently being taken forward to regulatory approval because of disappointing results in trials and considerable toxicity (Morgensztern et al 2019; Mullard 2019).

Summary Answer

There are no established roles for IHC testing for the identification of patients bearing other targetable alterations just discussed.

What Is the Role of PD-L1 IHC in Selecting Patients with NSCC for Immunotherapy?

IHC for PD-L1 is now well established in the routine biomarker testing algorithm for stages III and IV NSCC. This topic has been extensively reviewed elsewhere, including in the *IASLC Atlas of PD-L1 Immunohistochemistry Testing in Lung Cancer* (Tsao et al 2017). Here, only a brief review of some key points is presented.

PD-L1 IHC expression has consistently shown the ability to enrich populations of NSCC patients for benefit from anti-programmed cell death protein-1 (PD1) or PD-L1 drugs. The greater the proportion of tumor cells that express PD-L1 on cell membranes at any level of intensity, the higher the probability of patient benefit (Figure 19-10). The PD-L1 IHC biomarker for most drugs investigated in patients with NSCC concerns expression only in tumor cells, but atezolizumab has been developed using a PD-L1 assay based on the SP142 clone and expression of PD-L1 in both tumor and immune cells (Figure 19-11) (Fehrenbacher et al 2016).

Although the dose–response relationship between PD-L1 expression levels and clinical outcomes is preserved in clinical trials of second-line or greater immunotherapy, only a prescription of pembrolizumab in this setting requires a PD-L1 tumor proportion score (TPS) greater than or equal to 1% (Herbst et al 2016). Other approved inhibitors may be given regardless of PD-L1 expression; testing in this situation is considered complementary (Brahmer et al 2015; Borghaei et al 2015; Fehrenbacher et al 2016). A PD-L1 IHC TPS of 50% or more is required for the use of first-line pembrolizumab monotherapy, and both the 22C3

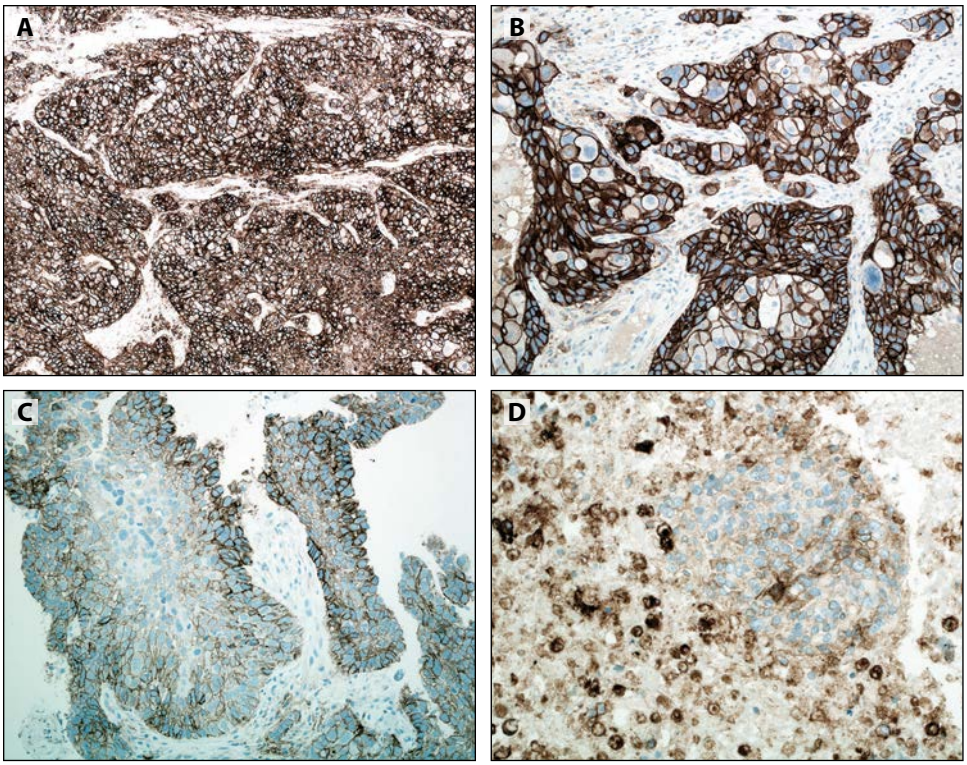


Figure 19-10. (A and B) Poorly differentiated adenocarcinoma showing strong and widespread membrane positivity for programmed death ligand-1 (PD-L1). **(C)** Squamous cell carcinoma showing a characteristic pattern of variable tumor cell membrane staining. Metastatic non-small cell carcinoma (NSCC) in an endobronchial ultrasound (EBUS) cytology cell block showing very focal tumor cell staining, but also **(D)** staining in macrophages and cellular debris. Staining using the Dako 22C3 pharmDx assay.

and SP263 assays (see the following paragraphs) are approved as companion diagnostics in this setting (Reck et al 2016; VENTANA PD-L1 [SP263] assay). Combinations of first-line pembrolizumab and platinum doublet chemotherapy have regulatory approval regardless of PD-L1 expression, despite substantial differences in outcome depending on PD-L1 IHC TPS; and the diagnostic is generally regarded as a useful complementary diagnostic for nuanced therapy choices for individual patients (Mok et al 2019; Peters et al 2019b). In stage III NSCC, durvalumab, a PD-L1 inhibitor, is approved for use following chemoradiotherapy. In the United States, this approval is agnostic of PD-L1 expression, although greater probability of benefit is correlated with higher PD-L1 TPS (Antonia et al 2017, 2018). In Europe, a controversial post hoc analysis of the Pacific trial data showed no overall survival benefit in the less than 1% TPS cohort, so PD-L1 IHC testing and a TPS of 1% or more was mandated by the European Medicines Agency for use of the Pacific regimen (Peters et al 2019a).

The 5 anti-PD1 or PD-L1 drugs that are at the most advanced stages of development, including approved indications mentioned earlier, are pembrolizumab, nivolumab, durvalumab, atezolizumab, and avelumab. Each of these has been developed through clinical trials using PD-L1 IHC assays using anti-PD-L1 IHC clones 22C3, 28-8, SP263, SP142, and 73-10 respectively. The multiplicity of different PD-L1 assays has caused some confusion among oncologists and consternation in pathology. The main dilemma for pathology has been the question of which tests to perform? Most laboratories cannot reasonably offer

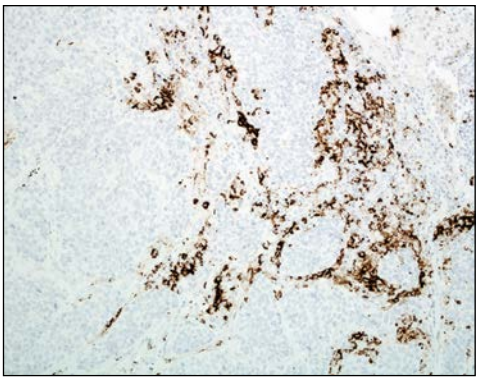


Figure 19-11. Distinct and marked staining of programmed death ligand-1 (PD-L1) in stromal immune cells surrounding solid pattern adenocarcinoma of the lung, which shows no tumor cell positivity. Staining using the VENTANA PD-L1 (SP142) assay.

all these assays, so can a single assay be performed and the results used for all the preceding clinical indications? How similar are these trial-validated assays? A number of assay comparison studies, including the IASLC BluePrint study, have demonstrated acceptable concordance between the 22C3, 28-8, and VENTANA PD-L1 (SP263) assays for assessing NSCC TPS (Hirsch et al 2017; Scheel et al 2016; Ratcliffe et al 2017; Hendry et al 2018; Adam et al 2018; Tsao et al 2018; Rimm et al 2017; Torlakovic et al 2020). The VENTANA PD-L1 (SP142) assay has consistently shown lower TPS scores, whereas the 73-10 assay is significantly more sensitive; in avelumab trials, a TPS of 80% has been considered equivalent to a score of 50% using the 22C3 assay (Barlesi et al 2018). There are also some emerging data suggesting that the VENTANA PD-L1 (SP263) assay may actually be more sensitive than the 22C3 assay, although the degree and significance of this is still unclear (Hendry et al 2018; Munari et al 2018).

Some laboratories will use their own PD-L1 LDT assay, rather than a commercial trial-validated assay. Although these LDTs can match the technical performance of a trial-validated assay (Adam et al 2018; Neuman et al 2016; Roge et al 2017), there is no guarantee that they will, and rigorous in-house and external quality assurance validation is required (Cheung et al 2019).

Cytology-type samples were not used in any of the above-mentioned clinical trials or during commercial assay development. There has, therefore, been reluctance to use cytology-type material for clinical PD-L1 testing. There is now, however, a substantial literature that demonstrates acceptable equivalent performance of PD-L1 assessment for TPS using trial-validated assays, comparing cytology versus tissue biopsy samples from the same tumor (Heymann et al 2017; Buttner et al 2017; Skov and Skov 2017; Gosney et al 2020; Yatabe et al 2019). Such is the variability in the way in which cytology-type samples are fixed, processed, and prepared for staining, that careful attention to these pre-analytic steps is required, and standardization will help in translating these study observations into routine clinical practice. It is recommended that laboratories using cytology-type samples for clinical PD-L1 testing undertake internal validation of their in-house procedures to ensure equivalent performance.

Summary Answer

Although alternative biomarkers for use with immunotherapy in NSCC are actively being sought, it seems highly likely that PD-L1 IHC will stay as part of the required assessment of NSCC clinical samples in relation to anti-PD1 and PD-L1 immunotherapy. Questions, however, remain about different assays, sample types, expression in tumor versus immune cells, and how the IHC data should be used for clinical decision-making.

Can IHC Be Used to Assess the Tumor Microenvironment to Select Patients for Immunotherapy?

IHC can potentially have a role in the assessment of the degree of tumor inflammation at a cellular level, or for the assessment of other regulatory molecules that may imply susceptibility or resistance to current immunotherapies in NSCC. There are, however, relatively few data, and all are still investigational as none of these factors are accepted for approved therapies. CD8 IHC has been used to assess tumor inflammation (Conde et al 2018), and multiplex IHC is being explored as a way of assessing multiple cellular factors simultaneously in the same tumor sample (Lu et al 2019).

Summary Answer

How tumor microenvironmental factors might, in the future, be assessed and utilized remains to be determined. This must be based on sound evidence and clinical trials.

Conclusions

IHC is a relatively simple, inexpensive yet powerful tool for directly investigating the status of proteins, often the oncogenic moieties and drug targets, in clinical NSCC samples. The molecular revolution in personalized therapy for NSCC has largely concentrated on genomic alterations, and the plethora of mutational and other genomic data are scientifically seductive and persuasive. The preceding discussion, however, clearly shows that proteomic assessments using IHC still have a pivotal role in NSCC diagnostics to ensure that patients receive the most effective therapy. As the number of clinically relevant biomarkers in the tumors of our patients with NSCC increases, so does the attraction of using multiplex, parallel NGS as a "one-stop shop" for genomic biomarker information, mutations, fusion genes, and even gene copy number. As NGS becomes the standard molecular testing platform, might the use of IHC as just described diminish or disappear? Contemporary practice in some centers would certainly suggest so, but what about the data on the importance of protein expression associated with *ALK* gene rearrangement? Could there emerge a paradigm whereby a fusion gene detected by NGS would require demonstration of the protein in order to better predict drug efficacy? This is a pertinent and logical question. It remains to be seen whether either pharma-sponsored trials or the scientific community will provide the data to give us an answer.

References

Adam J, Le Stang N, Rouquette I, et al. Multicenter harmonization study for PD-L1 IHC testing in non-small-cell lung cancer. *Ann Oncol*. 2018;29(4):953-958.

Antonia SJ, Villegas A, Daniel D, et al. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. *N Engl J Med*. 2017;377(20):1919-1929.

Antonia SJ, Villegas A, Daniel D, et al. Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. *N Engl J Med*. 2018;379(24):2342-2350.

Baldacci S, Figeac M, Antoine M, et al. High MET overexpression does not predict the presence of MET exon 14 splice mutations in MSCLC: results from the IFCT PREDICT.amm study. *J Thorac Oncol*. 2020;15(1):120-124.

Barlesi F, Vansteenkiste J, Spigel D, et al. Avelumab versus docetaxel in patients with platinum-treated advanced non-small-cell lung cancer (JAVELIN Lung 200): an open-label, randomised, phase 3 study. *Lancet Oncol*. 2018;19(11):1468-1479.

Blackhall FH, Peters S, Bubendorf L, et al. Prevalence and clinical outcomes from patients with ALK-positive resected stage I to III adenocarcinoma: results from the European Thoracic Oncology Platform Lungscape Project. *J Clin Oncol*. 2014;32(25):2780-2878.

Borghaei H, Ares-Paz L, Horn L, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med*. 2015;373(17):1627-1639.

Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med*. 2015;373(2):123-135.

Bubendorf L, Buttner R, Al-Dayel F, et al. Testing for ROS1 in non-small lung cancer: a review with recommendations. *Virchows Arch*. 2016;469(5):489-503.

Buttner R, Gosney JR, Skov BG, et al. Programmed death ligand-1 immunohistochemistry testing: a review of analytical assays and clinical implementation in non-small-cell lung cancer. *J Clin Oncol*. 2017;35(34):3867-3876.

Chen Z, Liu HB, Yu CH, et al. Diagnostic value of mutation-specific antibodies for immunohistochemical detection of epidermal growth factor receptor mutations in non-small cell lung cancer: a meta-analysis. *PLoS One*. 2014;9(9):e105940.

Cheung CC, Lim HJ, Garatt J, et al. Diagnostic accuracy in fit-for-purpose PD-L1 testing. *Appl Immunohistochem Mol Morphol*. 2019;27(4):251-257.

Clark GM, Zborowski DM, Culbertson JL et al. Clinical utility of epidermal growth factor receptor expression for selecting patients with advanced non-small cell lung cancer for treatment with erlotinib. *J Thorac Oncol*. 2006;1(8):837-846.

Conde E, Caminoa A, Dominguez C, et al. Aligning digital CD8+ scoring and targeted next-generation sequencing with programmed death ligand 1 expression: a pragmatic approach in early-stage squamous cell lung carcinoma. *Histopathology*. 2018;72(2):270-284.

Conde E, Hernandez S, Martinez R, et al. Assessment of a new ROS1 immunohistochemistry clone (SP384) for the identification of ROS1 rearrangements in patients with non-small cell lung carcinoma: the ROSING Study. *J Thorac Oncol*. 2019;14(12):2120-2132.

Cooper WA, Yu B, Yip PY, et al. EGFR mutant-specific immunohistochemistry has high specificity and sensitivity for detecting targeted activating EGFR mutations in lung adenocarcinoma. *J Clin Pathol*. 2013;66(9):744-748.

Diaz-Serrano A, Sanchez-Torre A, Paz-Ares L. Necitumumab for the treatment of advanced non-small-cell lung cancer. *Future Oncol*. 2019;15(7):705-716.

Douillard JY, Pirker R, O'Byrne KJ, et al. Relationship between EGFR expression, EGFR mutation status, and the efficacy of chemotherapy plus cetuximab in FLEX study patients with advanced non-small-cell lung cancer. *J Thorac Oncol*. 2014;9(5):717-724.

Eberhard DA, Giaccone G, Johnson BE, et al. Biomarkers of response to epidermal growth factor receptor inhibitors in Non-Small-Cell Lung Cancer Working Group: standardization in the clinical trial setting. *J Clin Oncol*. 2008;26(6):983-994.

Farago AF, Taylor MS, Doebele RC, et al. Clinicopathologic features of non-small-cell lung cancer harbouring an NTRK gene fusion. *JCO Precis Oncol*. 2018. doi:10.1200/PO.18.00037

Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomized controlled trial. *Lancet*. 2016;387(10030):1837-1846.

Gosney JR, Boothman AM, Ratcliffe M, et al. Cytology for PD-L1 testing: a systematic review. *Lung Cancer*. 2020;141:101-106.

Guo R, Berry LD, Aisner DL, et al. MET IHC is a poor screen for MET amplification of MET exon 14 mutations in lung adenocarcinomas: data from tri-institutional cohort of the Lung Cancer Mutation Consortium. *J Thorac Oncol*. 2019;14(9):1666-1671.

Hallberg B, Palmer RH. Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. *Nat Rev Cancer*. 2013;13(10):685-700.

Hechtman JF, Benayed R, Hyman DM, et al. Pan-TRK immunohistochemistry is an efficient and reliable screen for the detection of NTRK fusions. *Am J Surg Pathol*. 2017;41(11):1547-1551.

Hendry S, Byrne DJ, Wright GM, et al. Comparison of four PD-L1 immunohistochemical assays in lung cancer. *J Thorac Oncol*. 2018;13(3):367-376.

Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet*. 2016;387(10027):1540-1550.

Heymann JJ, Bulman WA, Swinarski D, et al. PD-L1 expression in non-small cell lung carcinoma: comparison among cytology, small biopsy, and surgical resection in specimens. *Cancer Cytopathol*. 2017;125(12):896-907.

Hirsch FR, McElhinny A, Stanforth D, et al. PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the BluePrint PD-L1 IHC assay comparison project. *J Thorac Oncol*. 2017;12(2):208-222.

Hofman V, Rouquette I, Long-Mira E, et al. Multicenter evaluation of a novel ROS1 immunohistochemistry assay (SP384) for detection of ROS1 rearrangements in a large cohort of lung adenocarcinoma patients. *J Thorac Oncol*. 2019;14(7):1204-1212.

Huang RSP, Smith D, Le CH, et al. Correlation of ROS1 immunohistochemistry with *ROS1* fusion status determined by fluorescence in situ hybridization. *Arch Pathol Lab Med*. Published online September 11, 2019. doi:10.5858/arpa.2019-0085-OA

Ibrahim M, Parry S, Wilkinson D, et al. ALK immunohistochemistry in NSCLC: discordant staining can

impact patient treatment regimen. *J Thorac Oncol*. 2016;11(12):2241-2247.

Kondoh C, Horio Y, Hayashi Y, et al. Anaplastic lymphoma kinase expression in small-cell lung cancer. *Histopathology*. 2019;75(1):20-28.

Leonetti A, Facchinetti F, Rossi G, et al. BRAF in non-small cell lung cancer (NSCLC): pickaxing another brick in the wall. *Cancer Treat Rev*. 2018;66:82-94.

Lindeman NI, Cagle PT, Aisner DL, 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. *J Thorac Oncol*. 2018;13(3):323-358.

Lu S, Stein JE, Rimm DL, et al. Comparison of biomarker modalities for predicting response to PD-1/PD-L1 checkpoint blockade: a systematic review and meta-analysis. *JAMA Oncol*. 2019;5(8):1195-1204.

Marchio C, Scaltriti M, Ladanyi M, et al. ESMO recommendations on the standard methods to detect NTRK fusions in daily practice and clinical research. *Ann Oncol*. 2019;30(9):1417-1427.

Minca EC, Portier BP, Wang Z, et al. ALK status testing in non-small cell lung carcinoma: a correlation between ultrasensitive IHC and FISH. *J Mol Diagn*. 2013;15(3):341-346.

Mino-Kenudson M, Chirieac LR, Law K, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res*. 2010;16(5):1561-1571.

Mok T, Peters, S, Camidge DR, et al. Patients with ALK IHC-positive/FISH-negative NSCLC benefit from ALK TKI treatment: response data from the global ALEX trial. *J Thorac Oncol*. 2017;12(11):S1739-S1749.

Mok TSK, Wu YL, Kudaba I, et al. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-024): a randomised, open-label, controlled, phase 3 trial. *Lancet*. 2019;393(10183):1819-1830.

Morgensztern D, Besse B, Greillier L, et al. Efficacy and safety of rovalpituzumab tesirine in third-line and beyond patients with DLL3-expressing, relapsed/refractory small-cell lung cancer: results from the phase II TRINITY study. *Clin Cancer Res*. 2019;25(23):6958-6966.

Mullard A. Cancer stem cell candidate Rova-T discontinued. *Nat Rev Drug Discov*. 2019;18(11):814.

Munari E, Rossi G, Zamboni G, et al. PD-L1 assays 22C3 and SP263 are not interchangeable in non-small cell lung cancer when considering clinically relevant cutoffs: an interclone evaluation by differently trained pathologists. *Am J Surg Pathol*. 2018;42(10):1384-1389.

Nagasaka M, Ou SI. Neuregulin 1 fusion-positive NSCLC. *J Thorac Oncol*. 2019;14(8):1354-1359.

Neuman T, London M, Kania-Almog J, et al. A harmonization study for the use of 22C3 PD-L1 immunohistochemical staining on Ventana's platform. *J Thorac Oncol*. 2016;11(11):1863-1868.

Nitta H, Tsuta K, Yoshida A, et al. New methods for ALK status diagnosis in non-small-cell lung cancer: an improved ALK immunohistochemical assay and a new, Brightfield, dual ALK IHC-in situ hybridization assay. *J Thorac Oncol*. 2013;8(8):1019-1031.

Paik PK, Drilon A, Fan PD, et al. Response to MET inhibitors in patients with stage IV lung adenocarcinomas harbouring MET mutations causing exon 14 skipping. *Cancer Discov*. 2015;5(8):842-849.

Penault-Llorca F, Rudzinski ER, Sepulveda AR. Testing algorithm for identification of patients with TRK fusion cancer. *J Clin Pathol*. 2019;72(7):460-467.

Peters S, Dafni U, Boyer M, et al. Position of a panel of international lung cancer experts on the approval decision for use of durvalumab in stage III non-small-cell lung cancer (NSCLC) by the Committee for Medicinal Products for Human Use (CHMP). *Ann Oncol*. 2019;30(2):161-165. (a)

Peters S, Reck M, Smit EF, et al. How to make the best use of immunotherapy as first-line treatment of advanced/metastatic non-small-cell lung cancer. *Ann Oncol*. 2019;30(6):884-896. (b)

Planchard D, Johnson BE. BRAF adds an additional piece of the puzzle to precision oncology-based treatment strategies in lung cancer. *Arch Pathol Lab Med*. 2018;142(7):796-797.

Planchard D, Smit EF, Groen HJM, et al. Dabrafenib plus trametinib in patients with previously untreated $BRAF^{V600E}$-mutant metastatic non-small-cell lung cancer: an open-label, phase 2 trial. *Lancet Oncol*. 2017;18(10):1307-1316.

Ratcliffe MJ, Sharpe A, Midha A, et al. Agreement between programmed cell death ligand-1 diagnostic assays across multiple protein expression cutoffs in non-small cell lung cancer. *Clin Cancer Res*. 2017;23(14):3585-3591.

Reck M, Rodriguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med*. 2016;375(19):1823-1833.

Rimm DL, Han G, Taube JM, et al. A prospective, multi-institutional, pathologist-based assessment of 4 immunohistochemistry assays for PD-L1 expression in non-small cell lung cancer. *JAMA Oncol*. 2017;3(8):1051-1058.

Roge R, Vyberg M, Nielsen S. Accurate PD-L1 protocols for non-small cell lung cancer can be developed for automated staining platforms with clone 22C3. *Appl Immunohistochem Mol Morphol*. 2017;25(6):381-385.

Salgia R. MET in lung cancer: biomarker selection based on scientific rationale. *Mol Cancer Ther*. 2017;16(4):555-565.

Scheel AH, Dietele M, Heukamp LC, et al. Harmonized PD-L1 immunohistochemistry for pulmonary squamous-cell and adenocarcinomas. *Mod Pathol*. 2016;29(10):1165-1172.

Selinger CI, Li BT, Pavlakis N, et al. Screening for ROS1 gene rearrangements in non-small-cell lung cancers using immunohistochemistry with FISH confirmation is an effective method to identify this rare target. *Histopathology*. 2017;70(3):402-411.

Selinger CI, Rogers TM, Russell PA, et al. Testing for ALK rearrangement in lung adenocarcinoma: a multicentre comparison of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol*. 2013;26(12):1545-1553.

Skov BG, Skov T. Paired comparison of PD-L1 expression on cytologic and histologic specimens from malignancies in the lung assessed with PD-L1 IHC 28-8pharmDx and PD-L1 IHC 22C3pharmDx. *Appl Immunohistochem Mol Morphol*. 2017;25(7):453-459.

Smit EF, Nakagawa K, Nagasaka M, et al. Trastuzumab deruxtecan (T-DXd; DS-8201) in patients with HER2-mutated metastatic non-small cell lung cancer (NSCLC): interim results of DESTINY-Lung 01. *J Clin Oncol*. 2020;38 (suppl; abstr 9504).

Spigel DR, Edelman MJ, O'Byrne K, et al. Results from the phase III randomized trial of onartuzumab plus erlotinib versus erlotinib in previously treated stage IIIB or IV non-small-cell lung cancer: METLung. *J Clin Oncol*. 2017;35(4):412-420.

Torlakovic E, Lim HJ, Adam J, et al. "Interchangeability" of PD-L1 immunohistochemistry assays: a meta-analysis of diagnostic accuracy. *Mod Pathol*. 2020;33(1):4-17.

Trombetta D, Graziano P, Scarpa A. Frequent NRG1 fusions in Caucasian pulmonary mucinous adenocarcinoma predicted by phosphor-ErbB3 expression. *Oncotarget*. 2018;9(11):9661-9671.

Tsao MS, Hirsch FR, Yatabe Y, eds. *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer*. 2nd ed. IASLC; 2016.

Tsao MS, Hirsch FR, Yatabe Y, eds. *IASLC Atlas of ALK Testing in Lung Cancer*. IASLC; 2013.

Tsao MS, Kerr KM, Dacic S, et al., eds. *IASLC Atlas of PD-L1 Immunohistochemistry Testing in Lung Cancer*. IASLC; 2017.

Tsao MS, Kerr KM, Kockx M, et al. PD-L1 immunohistochemistry comparability study in real-life clinical samples: results of BluePrint phase 2 project. *J Thorac Oncol*. 2018;13(9):1302-1311.

van der Wekken AJ, Pelgrim R, 't Hart N, et al. Dichotomous ALK-IHC is a better predictor for ALK inhibition outcome than traditional ALK-FISH in advanced non-small cell lung cancer. *Clin Cancer Res*. 2017;23(15):4251-4258.

von Laffert M, Warth A, Penzel R, et al. Multicenter immunohistochemical ALK-testing of non-small-cell lung cancer shows high concordance after harmonization of techniques and interpretation criteria. *J Thorac Oncol*. 2014;9(11):1685-1692.

Wang Q, Yang S, Wang K, et al. MET inhibitors for targeted therapy of EGFR TKI-resistant lung cancer. *J Hematol Oncol*. 2019;12(1):63.

Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. *J Thorac Oncol*. 2019;14(3):377-407.

Concluding Perspective

By Keith M. Kerr, Alain C. Borczuk, Wendy A. Cooper, Sanja Dacic, Andre L. Moreira, Yasushi Yatabe, and Ming Sound Tsao

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This atlas provides an extensive illustration of the crucial place that immunohistochemistry (IHC) has in the diagnosis of thoracic malignancies. This technique has roles to play at every point in the diagnostic journey that our patients' tissue samples take.

We have described the basic principles and biochemical basis of IHC, and the ways in which IHC techniques contribute to the diagnosis in numerous different scenarios in thoracic malignancies, reviewing the essence of the World Health Organization (WHO) classification, and how diagnoses can be reached in both surgically resected cases and in small biopsy and cytology samples. This latter section in the WHO classification and in this atlas is of crucial importance because, in the lung cancer world, at least two-thirds of patients have advanced-stage disease at presentation and therefore, pathologists have only small biopsy and/or cytology samples available for diagnosis and predictive biomarker testing.

This atlas has also provided specific discussion on the individual markers that we use most frequently and the various clones that are available. The variations that can occur between clones marketed to identify the same entity are of considerable importance and represent an issue that is sometimes underestimated by pathologists. Differences in clones can result in misinterpretation and misdiagnosis if the characteristics and specificities of those clones are not understood. Furthermore, most of our diagnostic IHC assays are essentially laboratory-developed tests, meaning that there can be considerable variation between laboratories in how many of these assays perform. Therefore, robust internal laboratory validation of the assay protocol for each marker should be performed prior to its clinical use. Additional issues may be identified and rectified through participation in IHC external quality assurance (EQA) schemes.

Contemporary pathologic diagnostic practice is heavily dependent on IHC. Pathologists training in an era where a plethora of IHC markers are available to identify innumerable macromolecules are tempted to use the technology "because they can" on occasions when it is perhaps not really required, but the greater diagnostic certainty that IHC can provide is always welcome. Conversely, as eloquently stated by Juan Rosai in the foreword to the third

edition of David J. Dabb's *Diagnostic Immunohistochemistry* (Dabbs 2010), the overuse, or over-reliance, on IHC in routine diagnosis can lead the pathologist astray. Very few of our IHC markers are specific for any particular diagnosis, and the vast majority of these diagnostic markers are physiologically expressed. It is, therefore, of the utmost importance that the IHC findings are always considered in the context of the tumor morphology.

And so it is with lung cancer diagnosis, particularly in the context of diagnosis on small samples. The importance of not overusing IHC is emphasized in the WHO classification and elsewhere (Lindeman et al 2018), as too much IHC carried out for initial diagnosis may leave insufficient material for subsequent predictive biomarker testing, especially molecular testing (except for fluorescence in situ hybridization), which usually requires more materials than is needed for IHC. This is a major problem for many oncologists and patients (Cane et al 2015).

How might IHC change in the future? Undoubtedly, more clones and markers will become available. Much of our assessment of IHC is qualitative rather than quantitative, and with the rise of digital pathology, one can envisage an easier route to quantification of IHC marker expression. In lung cancer diagnosis, assessment of Ki-67 might be an example of where such technology can help, but evidence is needed to show that the availability of more accurate and granular expression data for any of the markers we use can actually be of diagnostic value. If this were to happen, then it would become even more important for our IHC techniques to become more standardized and controlled.

More markers, and more permutations of complex expression profiles, but only small amounts of tissue available poses challenges. Rapid technologic advances have now made multiplex IHC techniques, both bright-field and immunofluorescence based, feasible for clinical adoption and routine diagnosis. This could provide solutions to squeeze more information from limited tissue. Allied with digital technology, these techniques can provide spatial data on co-expression that are hitherto very difficult to generate. As the complexity of data increase, as well the number of permutations of findings as more data points (markers) are added, artificial intelligence platforms may increasingly find a role. Once again, however, for this approach to move from research into routine practice, clinical value remains to be demonstrated. There is often a risk that our technology and ability to interrogate a lung cancer specimen is well ahead of developments in oncology that can take advantage of these extra data. This is why the interface between research and clinical practice is so important. It is perhaps more likely that such developments on multiplex IHC might find more value in predictive biomarker testing, both in the complex field of immunotherapy and in the interaction of aberrant signaling pathways and regulatory proteins, in cells driven by addictive oncogenes, especially when tumors develop resistance to targeted therapies, rather than at the initial stage of diagnosis and classification of lung cancer.

It is likely, however, that genomics, and therefore proteomics, will play an increasing role in the diagnosis and classification of lung cancer. The 2015 WHO classification saw a pivotal change in incorporating IHC marker expression in the basic definitions of squamous cell and adenocarcinoma. We can envisage more such changes as important clinical subgroups of lung cancer are identified and defined at a molecular level. However, because IHC provides single-cell resolution on specific marker expression using minimal tissue material, and because proteins are the ultimate oncogenic effectors and therapeutic targets, this technique will continue to play a key role in the diagnostic and biomarker testing world (Tsao and

Yatabe 2019). An understanding of how IHC works and what may confound assay performance will therefore become even more important.

IHC is an extremely valuable and powerful technique, which when correctly and appropriately applied, can greatly increase the diagnostic accuracy in cases of lung cancer and other thoracic tumors. This, by helping determine treatment options, has clearly been demonstrated as a benefit to our patients suffering from this biologically diverse yet frequently deadly group of diseases. This atlas, we hope, provides a resource not only for pathologists, but also for anyone else interested in lung cancer and its management. This volume on diagnostic IHC in lung cancer adds to the series of diagnostic atlases provided by the International Association for the Study of Lung Cancer (IASLC) and will surely be followed by other new titles.

References

Cane P, Linklater KM, Nicholson AG, et al. Morphological and genetic classification of lung cancer: variation in practice and implications for tailored treatment. *Histopathology*. 2015;67(2):216-224.

Dabbs DJ, ed. *Diagnostic Immunohistochemistry: Theranostic and Genomic Applications*. 3rd ed. Saunders Elsevier; 2010.

Lindeman NI, Cagle PT, Aisner DL 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. *J Thorac Oncol*. 2018;13(3):323-358.

Tsao MS, Yatabe Y. Old soldiers never die: is there still a role for immunohistochemistry in the era of next-generation sequencing panel testing? *J Thorac Oncol*. 2019;14(12):2035-2038.

Appendix A: Antibody List

Antibody name	Function	Species	Clone	Atlas reference page
Actin, smooth muscle	SMA (alpha smooth muscle actin)	Mouse	1A4	113, 120-121, 134-135, 139
		Mouse	ASM-1	
AE1/AE3	Pancytokeratin	Mouse	AE1/3	49, 67-68, 71-73, 79, 115, 118, 122, 125, 138, 159-160, 162, 168-169
		Mouse	AE1/AE3/PCK26	
ALK	Gene product of anaplastic lymphoma kinase, p80	Mouse	ALK1	6-8, 17, 25, 28, 39-40, 62, 95, 98-99, 133-134, 176-178, 181, 184
		Mouse	5A4	
		Rabbit	D5F3	
B72.3	TAG-72 (tumor-associated glycoprotein 72)	Mouse	B72.3	157, 159
BAP1	BRCA1-associated protein 1, of which product binds to the breast cancer type 1 susceptibility protein (BRCA1) via the RING finger domain of the latter and acts as a tumor suppressor	Mouse	C4	4, 162-166
Ber-EP4	Ep-CAM	Mouse	Ber-EP4	157, 159
34βE12	Cytokeratin, mostly reacted to cytokeratins 1, 5, 10, and 14 (high-molecular-weight keratin)	Mouse	34βE12	68-69, 71, 73
		Mouse	Cocktail (34βE12+p63)	
BG8, Lewis y	Lewis y blood antigen	Mouse	F3	157
CAM5.2	CK8, CK7 (lesser extent)	Mouse	CAM5.2	68, 72, 79-80, 119, 159, 173
		Mouse	5D3	
Calretinin	Calcium-binding protein of 29 kD that is a member of the family of so-called EF-hand proteins, which also includes S-100 proteins	Mouse	CAL6	4, 69, 136, 138, 157-161
		Mouse	DAK-Calret 1	
		Rabbit	DC8	
		Rabbit	SP65	
Catenin	β-catenin	Mouse	β-catenin-1	48, 109-112
		Mouse	14	
		Mouse	17C2	
	p120 catenin	Mouse	98	
		Rabbit	EP66	

Antibody name	Function	Species	Clone	Atlas reference page
CD5	Type I transmembrane glycoprotein found on the surface of thymocytes, T lymphocytes, and a subset of B lymphocytes, which may act as a receptor to regulate T-cell proliferation	Mouse	4C7	69, 169-170
		Rabbit	SP19	
CD31 (PECAM-1)	Platelet and endothelial cell adhesion molecule 1 (PECAM1)	Mouse	JC70A	128-129, 159
		Mouse	1A10	
CD34	Plays a role in the attachment of stem cells to the bone marrow extracellular matrix or to stromal cells	Mouse	QBEnd 10	113, 115, 123, 126, 128-130, 132, 159
		Mouse	MY10	
CD45	Leukocyte common antigen	Mouse	2B11+PD7/26	37, 96
		Mouse	RP2/18	
CD56 (NCAM)	Neural cell adhesion molecule 1	Mouse	123C3	37, 75-78, 80-82, 92, 130, 136-137, 139
		Rabbit	MRQ-42	
		Mouse	CD564	
CD99 (MIC2)	MIC2 gene products, Ewing sarcoma marker	Mouse	12E7	115, 136-137, 167
		Mouse	O13	
		Mouse	PCB1	
CD117 (KIT)	KIT proto-oncogene, receptor tyrosine kinase	Mouse	EP10	120-121, 169-171
		Rabbit	Polyclonal	
		Rabbit	9.7	
CDK4	Cyclin-dependent kinase 4	Mouse	DCS-31	134-135
CDX2	Caudal-type homeobox 2	Mouse	DAK-CDX2	104-105, 107-110, 114, 144-146, 154-157
		Rabbit	EPR2764Y	
		Rabbit	EP25	
CEA	Carcinoembryonic antigen	Mouse	CEA31	157, 159
		Mouse	COL-1	
		Mouse	II-7	
		Mouse	TF3H8-1	
Chromogranin A	Member of the chromogranin/secretogranin family of neuroendocrine secretory proteins, found in secretory vesicles of neurons and endocrine cells	Mouse	DAK-A3	36, 76, 111
		Mouse	LK2H10	
		Mouse	5H7	
		Rabbit	EP1030Y	
CK5/6	Cytokeratin 5/6	Mouse	D5/16B4	36, 41-42, 45, 51, 64-65, 69, 71-73, 114-115, 119, 156-157, 161-162
CK5/6/8/18	Cytokeratin, multi (5/6/8/18), NCL-CK5/6/8/18, NCL-L-CK5/6/8/18, and RTU-CK5/6/8/18	Mouse	5D3/LP34	67-68, 79
CK7	Cytokeratin 7	Mouse	OV-TL12/30	45-47, 51, 67-73, 79, 103-105, 107-110, 114, 119, 144-146, 149-152, 154
		Mouse	RN7	
		Rabbit	SP52	
CK20	Cytokeratin 20	Mouse	Ks20.8	71-73, 79, 104-105, 107-110, 114, 144-146, 149-151
		Mouse	PW31	
		Rabbit	SP33	

Antibody name	Function	Species	Clone	Atlas reference page
CK OSCAR	Wide-spectrum cytokeratin	Mouse	OSCAR	67-68, 114, 117, 159, 173
Claudin 4	Integral membrane proteins that are components of the epithelial cell tight junctions	Mouse	3E2C1	126, 157, 159
Desmin	Intermediate filament	Mouse	D33	127, 134-135, 137-139, 159
		Mouse	DE-R-11	
D2-40	Podoplanin (D2-40)	Mouse	D2-40	157, 159-160, 166, 171
EMA	Epithelial membrane antigen	Mouse	E29	113-116, 120, 130
		Mouse	GP1.4	
ER	Estrogen receptor	Mouse	6F11	13, 28, 31, 108, 110-111, 146-147, 186
		Rabbit	SP1	
	Estrogen receptor α chain	Mouse	1D5	
		Rabbit	EP1	
	Estrogen receptor β1	Mouse	PPG5/10	
GATA3	GATA binding protein 3	Mouse	HG3-31	19, 108, 146-147, 149, 155-156, 160, 166
		Mouse	L50-823	
GCDFP15	Gross cystic disease fluid protein-15	Mouse	23A3	155-156
		Rabbit	EP1582Y	
Glypican-3	Member of cell-surface heparan sulfate proteoglycans	Mouse	GC33, 1G12	110, 152, 170-172
		Mouse	IG12	
H3K27me3	Histone H3 lysine 27 trimethylation	Rabbit	C36B11	135-136, 142
HMB45	Melanoma-associated antigen	Mouse	HMB45	139-140, 159
		Mouse	Triple cocktail (HMB45+A103 +T311)	
HNF4α	Hepatocyte nuclear factor 4 alpha	Mouse	H1415	106, 145, 156
		Rabbit	C11F12	
ISMN1	Insulinoma-associated protein 1	Mouse	A-8	37-38, 75-77, 81, 136
Ki-67	Nuclear protein associated with cellular proliferation	Mouse	MIB-1	5-6, 28, 37, 81, 85-89, 93, 114, 116, 190
		Mouse	MM1	
		Mouse	K2	
		Rabbit	30-9	
KL1	Pancytokeratin	Mouse	KL1	67-68, 159
Lu5	Pancytokeratin	Mouse	Lu5	68
MDM2	Nuclear-localized E3 ubiquitin ligase, which promotes tumor formation by targeting tumor suppressor proteins, such as p53	Mouse	IF2	134-136
MNF116	CK5/6/8/17	Mouse	MNF116	67-68, 72
MOC31	Ep-CAM/epithelial specific antigen	Mouse	MOC31	157, 159
MTAP	Methylthioadenosine phosphorylase, used as a surrogate marker for homozygous loss of p16	Mouse	2G4	163-166

Antibody name	Function	Species	Clone	Atlas reference page
Napsin A	Member of the peptidase A1 family of aspartic proteases	Mouse	MRQ-60	35-36, 40-41, 44-45, 50-51, 69, 92, 103, 105, 108, 110, 114, 118-119, 124, 144-147, 149-150, 153-154, 156-157
		Mouse	IP64	
		Rabbit	Polyclonal	
NKX3.1	NK3 homeobox 1, which encodes a homeobox-containing transcription factor functioning as a negative regulator of epithelial cell growth in prostate tissue	Rabbit	EP356	151, 154-155, 157
NUT	Nuclear protein in testis	Rabbit	C52B1	5, 7, 14, 46-47, 51, 63, 75, 82, 113-118, 122-124, 127
OCT3/4	POU class 5 homeobox 1, which encodes a transcription factor containing a POU homeodomain that plays a key role in embryonic development and stem cell pluripotency	Mouse	N1NK	170-171
p16	Cyclin-dependent kinase inhibitor 2A, CDKN2A	Mouse	E6H4	143-144, 163, 165-166
		Mouse	G175-405	
		Mouse	JC8	
p40	Isoform of TP63 product, also known as ΔNP63	Mouse	BC28	2, 28, 35-38, 41-48, 50-51, 56, 60-65, 69-70, 74, 78, 80-81, 92, 105-106, 111, 114-117, 119-121, 130, 149, 152, 157, 159, 168-170
p63	Product of TP63	Mouse	4A4	2, 28, 41-42, 45, 50-52, 60-65, 74, 80-81, 100, 114-115, 119-122, 149, 156-157, 168-169
		Mouse	Cocktail (34βE12+p63)	
		Mouse	DAK-p63	
		Mouse	7JUL	
PAX8	Paired box 8, a transcription factor to regulate development of the thyroid	Mouse	MRQ-50	108, 110-111, 148-150, 153-157, 168-169, 174
		Mouse	PAXR1	
S100	Calcium-binding protein	Rabbit	Polyclonal	113, 120-122, 127, 135, 139, 159
SALL4	Spalt-like transcription factor 4, which encodes a zinc finger transcription factor thought to play a role in the development of abducens motor neurons	Mouse	6E3	110-111, 126, 170-173
SMARCA4/BRG1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4, which encodes a member of the SWI/SNF family of proteins	Mouse	G-7	5, 7, 12, 51, 125-128
		Rabbit	EPNCIR111A	
SMARCB1 (BAF47, hSNF5, INI1)	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1, which is a part of a complex that relieves repressive chromatin structures	Mouse	25/BAF47	126, 130-131
SOX10	SRY-box transcription factor 10, encodes a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development and in the determination of the cell fate	Rabbit	SP267	135, 146-147
		Mouse	Polyclonal	

Antibody name	Function	Species	Clone	Atlas reference page
STAT6	Signal transducer and activator of transcription 6, which encodes a member of the STAT family of transcription factors	Rabbit	Polyclonal	130-133, 159
		Rabbit	Polyclonal (SC-20)	
		Rabbit	YE361	
Synaptophysin	Integral membrane protein of small synaptic vesicles in brain and endocrine cells	Mouse	DAK-SYNAP	37, 40, 75-81, 93, 109-110, 130-131, 136-137, 139
		Mouse	27G12	
		Rabbit	MRQ-40	
		Rabbit	SP11	
TTF1	Thyroid transcription factor-1, also known as NKX2-1; a transcription factor to regulate development of thyroid and lung	Mouse	8G7G3/1	2, 28, 34-36, 38-40, 43-48, 50-51, 53-59, 62, 65, 69-70, 72, 92-93, 96, 103-111, 113-121, 127, 139, 144-148, 150-153, 158-159, 178
		Mouse	SPT24	
		Rabbit	SP141	
WT1	Wilms tumor protein	Mouse	6F-H2	4, 69, 136-138, 157-160
		Mouse	WT49	

Appendix B: Manufacturers

The following manufacturers and their products are noted in this Atlas. The locations given for each manufacturer is not the only location; most manufacturers have offices worldwide.

Abcam
Cambridge, United Kingdom

Abnova
Taipei, Taiwan

Active Motif
Carlsbad, California, United States

Agilent Dakot
Santa Clara, California, United States

BD Biosciences
San Jose, California, United States

BioLegend
San Diego, California, United States

Cell Signaling Technology, Inc.
Danvers, Massachusetts, United States

Invitrogen - Thermo Fisher Scientific
Carlsbad, California, United States

Leica Biosystems
Buffalo Grove, Illinois, United States

Nichirei Bioscience
Tokyo, Japan

Roche Tissue Diagnostics
Tucson, Arizona, United States

Santa Cruz Biotechnology, Inc.
Dallas, Texas, United States

Sigma-Aldrich
St. Louis, Missouri, United States

Signet Laboratories, Inc.
Dedham, Massachusetts, United States