Practical Applications in Immunohistochemistry

Carcinomas of Unknown Primary Site

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• ***Context.***—**Identification of the site of origin of carcinoma of unknown primary using immunohistochemistry is a frequent requirement of anatomic pathologists. Diagnostic accuracy is crucial, particularly in the current era of targeted therapies and smaller sample sizes.**

Objectives.—**To provide practical guidance and suggestions for classifying carcinoma of unknown primary using both proven and new antibodies, as well as targeting panels based on integration of morphologic and clinical features.**

Data Sources.—**Literature review, the authors' practice experience, and authors' research.**

Conclusions.—**With well-performed and interpreted immunohistochemistry panels, anatomic pathologists can successfully identify the site of origin of carcinoma of unknown primary. It is crucial to understand not only the diagnostic uses of the many available antibodies but also the potential limits and pitfalls.**

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It is estimated that approximately 4% of all patients with cancer present with carcinomas of unknown primary (CUPs), representing a higher incidence than known malignancies such as non-Hodgkin lymphoma or ovarian cancer.[1] The identification of a primary site in such a setting has taken on dramatically increased clinical relevance, given the differences in prognosis and treatment, particularly targeted therapies of carcinomas of various primary sites. By integrating morphology with well-performed and well-interpreted immunohistochemistry (IHC), the pathologist can frequently provide definitive diagnostic information in most cases regarding the most likely primary site or sites of the carcinoma presenting as metastases. With the ongoing additions of lineage-specific transcription factors, pathologists have available an increasing number of relatively inexpensive IHC "tools," which more accurately identify CUP. In this era of health care cost containment, and the need to provide clinicians with a relatively quick diagnosis, IHC remains the gold standard at diagnosing CUP. There have been a number of recent publications advocating for the use of gene expression-based tests in the setting of CUP.[2–4] Both methodologies offer a similar range of accuracy in tumor classification (ranging from around 75% and greater); however, in our practice, gene expression-based tests are rarely used or required. Although the proposed algorithm of using gene expression profiling when the initial round of IHC panel is inconclusive may be a useful complement to IHC in some laboratories, in our practice, we often include an additional round of carefully selected and targeted IHC stains in such a scenario, which frequently leads to a diagnosis.

In general, there are 2 classes of antibody markers that can be of assistance in the workup of CUP: (A) antibodies to keratins, and (B) antibodies to organ-restricted markers.

KERATINS

Low–Molecular-Weight Keratins Versus High–Molecular-Weight Keratins

Keratins, previously referred to as *cytokeratins*, have recently undergone a change in nomenclature to accommodate the sequencing of the human genome and discovery of several novel keratin genes.[5] The somewhat arbitrary division of the keratin universe into "high– versus low–" molecular-weight keratins corresponds to certain aspects of the tissue distribution of keratins. Thus, low–molecular-weight keratins (eg, keratin [K] 8, K18) are expressed by "simple" epithelium, such as glandular epithelium of the gastrointestinal (GI) tract, hepatocytes, among others, and high–molecular-weight keratins (eg, K5, K14, K17) are expressed by "complex" epithelium, such as stratified (squamous, transitional) epithelium, as well as ductal and

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Table 1. Distribution of "Modal" Keratins 7 and 20 Immunophenotypes in Different Types of Carcinomas			
Carcinoma Types	Keratin 7	Keratin 20	Immunophenotype,[a] %
Colorectal adenocarcinoma	−	+	75–95
Lung adenocarcinoma	+	−	90
Breast ductal carcinoma	+	−	80–95
Ovarian serous papillary carcinoma	+	−	>90
Endometrial adenocarcinoma	+	−	80–100
Hepatocellular carcinoma	−	−	71–89
Lung neuroendocrine carcinoma	−	−	60–80
Renal cell carcinoma	−	−	70–90
Prostatic adenocarcinoma	−	−	60–100
Lung squamous cell carcinoma	−	−	50–90
Transitional cell carcinoma	+	+	25–90

[a] Data derived from Wang et al[7] and Chu et al.[8]

basal cells.[6] The subclassification of carcinomas by high– and low–molecular-weight keratins, however, has largely been superseded by subclassification using antibodies to K7 and K20, which is a far more powerful discriminator.

K7 and K20

These 2 individual keratin proteins have a partially overlapping but unique distribution among normal epithelium and its corresponding carcinomas. Although K7 is found in some simple epithelia (eg, lung pneumocytes and breast acinar epithelium but not hepatocytes), it is not found in all epithelia, whereas K20 is generally expressed in only a restricted subset of epithelia, such as the epithelium of the GI tract, especially colorectum, the urothelial umbrella cells, and Merkel cells of the epidermis. This relatively limited K20 tissue distribution has, therefore, been useful in the identification of the primary site of carcinomas. The 2 most comprehensive and authoritative studies of coordinate K7 and K20 expression in carcinomas at various sites are those of Wang et al[7] and Chu et al.[8] (Please see Table 1 for the modal distribution of K7 and K20.)

Carcinomas of certain primary sites (eg, stomach) are notable for their lack of a modal or dominant K7/K20 immunophenotype. As a general rule, gastric adenocarcinomas can manifest almost any K7/K20 immunophenotype, pancreatic carcinomas generally show a $K7^+/K20^+$ immunophenotype, with a large subset showing a colorectal immunophenotype ($K7^-/C20^+$), and cholangiocarcinomas generally show a close immunophenotypic overlap with pancreatic carcinomas.[9–11] In reality, the utility of antibodies to K7 and K20 in determining primary site of origin is limited and can help point toward diagnoses that must be confirmed by IHC studies employing organ-restricted markers.

Other Keratins

Expression of a few other keratins has been demonstrated to manifest organ restriction of potential use in the diagnosis of CUP. The most important of these is keratin 5 (and its "pair," keratin 14), which can be employed as markers of squamous, transitional cell, myoepithelial, and mesothelial differentiation. The second is keratin 17, which, when expressed at high levels, appears to be a good marker for distinguishing carcinomas of pancreatobiliary tract origin from gastric carcinomas.[12–14]

ORGAN-SPECIFIC MARKERS OF CARCINOMAS

There are 2 classes of tumor-specific antibodies: cytoplasmic (and/or membranous) markers of differentiation, and nuclear transcription factors. The former include cytoplasmic markers, such as the breast-restricted marker, gross cystic disease fluid protein 15 (GCDFP-15), and membranous markers, such as the GI tract-restricted marker, villin. The level of expression, and, in general, the fraction of tumor cells found to be positive with these cytoplasmic markers, are generally a function of the state of differentiation of the tumor, so that one finds fewer marker-positive cells in poorly differentiated, compared with well-differentiated, tumors. In contrast, nuclear transcription factors, when positive, are generally expressed in the entire tumor cell population, and expression is generally independent of the state of differentiation of the tumor. Table 2 contains a summary of the organ-specific markers.

Breast Cancer Markers

Estrogen Receptor.—Estrogen receptor (ER) has a limited role in the identification of the primary site of carcinomas presenting at a metastatic site, given that ER is expressed in only two-thirds to three-quarters of primary breast cancers and a lower fraction of breast cancers in a metastatic site.[15,16]

Furthermore, ER is expressed in a wide subset of carcinomas, including those primary to the endometrium and ovary, but also in "unexpected" sites, such as papillary carcinomas of the thyroid[17,18] and adnexal tumors of the skin.[19,20] Most important from a diagnostic standpoint, a significant number of primary lung adenocarcinomas (approximately 10%–20%) can also show positive immunostaining, although in general these tumors show only focal ER expression. In contrast, ER expression is exceedingly rare in adenocarcinomas of the GI tract, especially colorectal adenocarcinomas.[21–24]

GCDFP-15 and Mammaglobin A.—The GCDFP-15, as described by Mazoujian and colleagues,[25] exhibited an overall sensitivity of approximately 55% in breast carcinomas,[26] and in more-recent studies the reported sensitivity (using different monoclonal and polyclonal antibodies) has been between 23% and 73%.[27–29] In our experience, using the 23A3 monoclonal antibody, the sensitivity is close to 80%.[30] This sensitivity of GCDFP-15 as a breast marker is a function of histologic subtype and is generally greatest in lobular carcinoma (particularly those with signet ring cells) as well as tumors showing apocrine features. Additionally, the level of expression of GCDFP-15 may be focal in breast cancer. In contrast, only a very small fraction of basallike carcinomas exhibit positive staining. The strong immunophenotypic overlap among breast cancers, salivary gland carcinomas, and sweat gland carcinomas of the skin with GCDFP-15 expression has been well documented.[31] The expression of GCDFP-15 is seen in 5% to 10% of primary

Table 2. Summary of Carcinoma (Tumor)-Specific Antibody Reagents

Carcinoma Subtype	Antibodies to:	Localization of Signal	Sensitivity	Specificity	Also Identifies
Breast	Estrogen receptors	Nuclear	Moderate	Moderate	Endometrioid adenocarcinoma, ovarian serous CA
Breast	GCDFP-15	Cytoplasmic	Low	Moderate	Salivary gland, sweat gland tumors
Breast	Mammaglobin	Cytoplasmic	Low	Moderate	Salivary gland, sweat gland tumors
Breast	GATA3	Nuclear	High	Moderate	Salivary gland, transitional cell CAs, skin adnexal tumors
Colorectal and GI	Villin	Membranous brush border	High	Moderate	Subset of lung carcinomas, ovarian and endometrial CAs
Colorectal	CDX2	Nuclear	High	High	Subset of pancreatic, gastric CAs
Hepatocellular	HepPar1	Cytoplasmic	Moderate	High	Hepatoid adenocarcinomas
Hepatocellular	Arginase	Nuclear and cytoplasmic	High	High	Hepatocellular CAs
Lung adenocarcinoma and thyroid, including NE	TTF-1	Nuclear	High	High	Neuroendocrine CAs of other sites
Lung adenocarcinoma	Napsin A	Cytoplasmic	High	High	GYN clear cell CAs, subset of renal cell and thyroid CAs
GYN	PAX8	Nuclear	Very high	Moderate	Thyroid CA, renal cell CA
Ovarian serous	WT1	Nuclear	Very high	High	Mesothelioma
Prostate	Prostate-specific antigen	Cytoplasmic	Very high	Very high	. . .
Prostate	NKX3.1	Nuclear	Very high	Very high	. . .
Renal cell	PAX8	Nuclear	Moderate	Moderate	GYN and thyroid CAs
Squamous, transitional cell	p63	Nuclear	Very high	Very high	Thymoma, salivary gland tumors, some neuroendocrine CAs, trophoblastic tumors
Squamous, transitional cell	P40	Nuclear	Very high	Very high	Thymoma, salivary gland tumors, trophoblastic tumors
Thyroid	Thyroglobulin	Cytoplasmic	High	Very high	. . .
Thyroid	PAX8	Nuclear	Very high	Moderate	GYN and renal CAs
Transitional cell	Uroplakin	Cell membranous	Low	High	. . .
Transitional cell	GATA3	Nuclear	High	Moderate	Breast cancers, salivary gland CAs, skin adnexal tumors

Abbreviations: CA, carcinoma; GI, gastrointestinal; GYN, gynecologic; NE, neuroendocrine.

ovarian (as well as endometrial) carcinomas. A few lung adenocarcinomas (5%–6%) are also GCDFP-15^{+} [32,33]; most adenocarcinomas of other sites, including the GI tract and genitourinary tract, are usually negative.

Mammaglobin A is a 10-kDa protein initially identified through the gene discovery process.[34] The sensitivity of mammaglobin as a marker of breast carcinoma is somewhat less than that of GCDFP-15, according to our experience and published reports (again, using different antibodies) and is between 50% and 70%.[23,24,35,25] Our experience dictates, furthermore, that approximately 7% of breast cancers are mammaglobin^{+} but GCDFP-15^{-}, yielding a combined sensitivity of 86%. Mammaglobin can also be identified in approximately 10% of endometrial/ovarian carcinomas and shows, similar to GCDFP-15, expression in salivary gland and adnexal neoplasms.

GATA Binding Protein 3.—GATA binding protein 3 (GATA3) is 1 of 6 members of a zinc finger transcription factor family and is crucial to differentiation of many tissues, including breast glandular epithelial cells, hair follicles, T lymphocytes, adipose tissue, kidney, and nervous system. Recent studies have shown GATA3 to be a very sensitive marker for breast carcinomas (and urothelial carcinomas).[36,37] The level of sensitivity of expression of GATA3 in breast carcinomas is reported at 91% and 100% for ductal and lobular types, respectively, with most of those tumors showing diffuse and strong nuclear staining.[24] Additionally, unlike mammaglobin and GCDFP-15, GATA3 expression is seen in 43% of triple-negative and 54% of metaplastic breast carcinomas.[33] Expression of GATA3 is also maintained in metastatic breast carcinomas (>90%), and other than high-level of expression in urothelial carcinomas, GATA3 is also identified in a subset of adenocarcinomas, with only a significant minority of endometrial, pancreatic, and salivary gland carcinomas showing expression. Of note is the high level of expression of GATA3 in skin adnexal tumors (similar to GCDFP-15 and mammaglobin). In addition, GATA3 is reported in most mesotheliomas, chromophobe renal cell

carcinomas, trophoblastic germ cell neoplasms, and paragangliomas.[32,38–40]

Suggestion.—To achieve maximal sensitivity, it is suggested that all 3 breast-restricted markers, GATA3, GCDFP-15, and mammaglobin, are used.

Lung Cancer Markers

Thyroid Transcription Factor 1.—*Thyroid Transcription Factor 1*.—Thyroid transcription factor 1 (TTF-1) is a 38-kDa member of the NKX2 family of DNA-binding transcription factors; TTF-1 is selectively expressed during embryogenesis in the thyroid, the diencephalon, and in respiratory epithelium.[41,42] Although TTF-1 is expressed by both neuroendocrine and nonneuroendocrine carcinomas of the lung, its frequency of expression varies markedly among the different histologic subtypes.

Nonneuroendocrine Carcinomas.—The sensitivity of TTF-1 is greatest among adenocarcinomas and nonmucinous bronchioloalveolar carcinomas (adenocarcinoma with lepidic pattern, Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification),[43] in which it exceeds 90%, and is lowest in mucinous adenocarcinomas and squamous cell carcinomas, in which expression frequency is exceedingly low.[44–48] Among conventional lung adenocarcinomas, the sensitivity is in the range of 90%,[49] despite earlier reported sensitivities ranging from 65% to 75%,[46,50] and higher if mucinous carcinomas were eliminated from the analysis.[43] In the context of lung carcinomas presenting at metastatic sites, TTF-1 appears to retain similar sensitivity.[51–55]

The sensitivity of TTF-1 is also a function of the antibody clone employed, and the SPT24 clone manifests significantly greater sensitivity than does the older 8G7G1/1 clone, on which almost all of the published TTF-1 data are based.

In addition, TTF-1 expression has been demonstrated in a small subset of ovarian, endometrial,[56–59] and colorectal[60,61] carcinomas, although the extent of positivity is usually focal, often in isolated clusters of cells. A very small fraction of breast carcinomas can also express TTF-1.[62]

Neuroendocrine Carcinomas.—Striking differences in sensitivity have also been found among the spectrum of neuroendocrine carcinomas of the lung, varying from nearly 90% in small cell carcinomas to approximately 50% in large cell neuroendocrine carcinomas to less than 50% in carcinoid tumors.[42,44,63,64]

However, there are caveats for the application of this antibody to the study of metastatic neuroendocrine carcinomas. Despite its very high sensitivity in primary, high-grade, particularly small cell, lung neuroendocrine carcinomas, TTF-1 expression cannot be considered specific for high-grade neuroendocrine carcinomas of lung origin. Studies have demonstrated TTF-1 expression in a variable subset of small cell (neuroendocrine) carcinomas of the genitourinary and gynecologic (GYN) tract.[65–68] However, more-limited differential diagnoses may be addressable with TTF-1 expression because TTF-1 expression in Merkel cell tumors of the skin is exceedingly rare.[69,70]

Another limitation of this antigen is its relatively poor preservation in alcohol-fixed materials, eg, aspirate smears, in which the sensitivity is quite low.[71] In our experience, cell blocks of pleural fluids, which contain material that has been either fixed in alcohol or is nonfixed before creation of a formalin-fixed cell pellet, can manifest a profound loss of TTF-1 antigenicity.

Napsin A.—Napsin A is an aspartic protease that is crucial to the maturation of surfactant B and present in the cytoplasm of type 2 pneumocytes and alveolar macrophages.[72,73] It is a very sensitive marker for detecting pulmonary adenocarcinomas with a level of sensitivity reported at 79% to more than 90% of tumors, with some studies reporting a small subset of napsin A^+/TTF-1^- primary lung adenocarcinomas.[70,74] The specificity of coexpression of TTF-1 and napsin A is extremely high for pulmonary adenocarcinomas; however, napsin A can also be identified in a subset of renal cell carcinomas (most frequently papillary in which up to 80% show napsin expression) as well as in a minority of endometrial adenocarcinomas and papillary thyroid carcinomas.[69,70] Additionally, as described below under GYN tract carcinomas, studies in our laboratory have shown high-level expression of napsin A in virtually all cases of clear cell carcinomas of the ovary.[75]

GI Tract Cancer Markers

CDX2.—CDX2 is a nuclear transcription factor that has a key role in controlling the proliferation and differentiation of intestinal epithelial cells.[76,77] As demonstrated in our study of nearly 500 carcinomas,[78] CDX2 is expressed in virtually 100% of colorectal adenocarcinomas. (However, the subset of colorectal adenocarcinomas displaying the microsatellite unstable genotype generally displayed reduced or even absent CDX2 expression).[79] The pattern of positivity with antibodies to CDX2 can also be of diagnostic significance because most adenocarcinomas of the stomach, pancreas, and biliary tract that are $CDX2^+$ show a much more variegated or even focal pattern of CDX2 expression, when compared with the uniform expression characteristic of colorectal adenocarcinomas.[76] CDX2 is expressed in approximately one-half of gastric adenocarcinomas, and at an even higher frequency in the intestinal-type adenocarcinoma subset, and in approximately one-third of pancreatobiliary tract carcinomas.[76] Adenocarcinomas of other sites, which manifest colorectal and noncolorectal GI-like histologic appearances, such as ovarian mucinous carcinomas, bladder adenocarcinomas, and sinonasal intestinal-type adenocarcinomas, all express CDX2 at high frequencies,[80–84] minate adenocarcinomas arising at those sites from true GI tract adenocarcinomas. CDX2 expression has also been described in a limited subset of mucinous and nonmucinous pulmonary adenocarcinomas (enteric subtype)[85–87]; 40% to 50% of the nonmucinous pulmonary adenocarcinomas, however, also express the lung-restricted nuclear transcription factor TTF-1.[83]

CDX2 expression is also seen in GI neuroendocrine tumors, including those primary to the intestine (eg, carcinoid tumors) and, to a variable degree, the pancreas (islet cell tumors),[88,89] although the intensity of CDX2 expression is generally much weaker and more focal than it is in adenocarcinomas of these sites.[86,87,90,91] High-grade GI tract neuroendocrine carcinomas also express CDX2 at a high frequency, but, within the context of high-grade neuroendocrine carcinomas, CDX2 can be expressed in non-GI tract high-grade neuroendocrine carcinomas, such as those of the bladder and lung.[87]

Among nonneuroendocrine carcinomas, CDX2 displays a high specificity, with virtually no expression in nonneuroendocrine carcinomas of the breast, kidney, and salivary gland.[92–94] A subset of endocervical and endometrial adenocarcinomas can express CDX2, usually in the variegated pattern seen in noncolorectal GI tumors, and often in

areas showing mucinous differentiation.[95,96] Furthermore, a curious pattern of CDX2 expression has been documented in the "squamous" morules of endometrioid hyperplasia and carcinoma.[97,98] Adenocarcinomas arising within germ cell tumors often show intestinal differentiation, as evidenced by CDX2 expression.[83,84]

Villin.—Villin is an actin-binding protein, found preferentially in microvilli, expression of which is largely (but not entirely) restricted to glandular epithelium and corresponding adenocarcinomas of the GI tract.[99] As with CDX2, expression is greatest and most reliably found in colorectal adenocarcinomas, but lower levels of expression are found in adenocarcinomas primary to the pancreatobiliary tract and stomach.[82,100,101] Our experience dictates that scoring of the membranous or "brush border" signal is most significant; cytoplasmic immunostaining can be seen in other types of tumors, particularly neuroendocrine carcinomas.[102] The overall sensitivity of antibodies to villin, in our study,[101] was approximately 75% for colonic adenocarcinomas and approximately 40% for adenocarcinomas of the pancreas and stomach.[82]

As with CDX2, villin expression can also be seen in adenocarcinomas of other sites that display a GI-type histology and immunophenotype, including adenocarcinomas of the lung, nasopharynx, ovary, and bladder[82,79]; villin expression may also be seen in a subset endometrioid adenocarcinomas.[79]

Suggestion.—To achieve maximal sensitivity and aid in the identification of the primary site, both GI tract–restricted markers, CDX2 and villin, should be employed, particularly when the differential diagnosis includes carcinoma arising in the upper GI tract, such as the pancreatobiliary tract and the stomach. Although the individual sensitivities of CDX2 and villin as markers of the latter tumors are each approximately 50%, their combined sensitivity is in excess of 75%.

Markers of Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) certainly often enters into the differential diagnosis of tumors in the liver, which is one of the most common single sites of metastatic presentation in CUPs sites. The differential diagnosis, if any, lies with primary biliary tract or metastatic adenocarcinoma or neuroendocrine carcinoma. Although α-fetoprotein has, in the past, been considered the gold standard marker for hepatocellular differentiation,[103,104] that marker can no longer be recommended because several other markers have greater sensitivity and specificity, including the hepatocyte paraffin 1 (Hep-Par 1) antibody and arginase-1.[105,106]

Hep-Par 1 Antibody (CPS1).—The monoclonal antibody Hep-Par 1 detects a liver (hepatocyte)-specific marker, subsequently found to represent the enzyme carbamoyl phosphate synthase.[107–109]

In one of the first immunohistochemical surveys, Hep-Par 1 manifested a sensitivity of 82% and a specificity of 90% for the detection of hepatocellular neoplasms.[110] In more recent studies,[111–114] the sensitivity of Hep-Par 1 expression in HCCs has been found to range between 70% and 100%, although in our experience, the sensitivity is in the middle of that range. The Hep-Par 1 antibody is most helpful in the analysis of tumors in the liver, helping to distinguish metastatic carcinomas from primary HCCs in the appropriate clinical context. However, most large studies have demonstrated expression, even at high levels, in a small but significant (1%–10%) subset of adenocarcinomas primary to the lung, pancreas, stomach, ovaries, and adrenal cortex,[110,112] making it important to use the Hep-Par 1 antibody as part of a panel of antibodies in determining the most likely primary site; these Hep-Par 1^+ tumors are frequently, albeit not exclusively, tumors with hepatoid morphology.[115,116] Because expression of CPS1 is observed in nonneoplastic liver and benign hepatocellular lesions, the use of Hep-Par 1 antibody cannot be used to distinguish benign from malignant liver lesions.

Arginase-1.—As first described by Yan et al[117] arginase-1, an enzyme involved in the urea cycle, appears to represent the most-sensitive (and, perhaps, most-specific) marker of HCC to date. The immunostaining is generally in a cytoplasmic, granular pattern. Yan and colleagues[117] found a sensitivity of 96% and a specificity of essentially 100%, exceeding that of all other hepatocellular markers. Importantly, arginase-1 maintained a high level of sensitivity even in the context of high-grade HCC, where its sensitivity was 86%, compared with 46% for the Hep Par-1 antigen. The other major advantage of arginase-1 is that it is not expressed in "hepatoid" and other non-HCCs (particularly carcinomas of the lung, stomach, and kidney), which can be seen with antibodies to the Hep Par-1 antigen.[118] Although there are few published studies on arginase-1, our experience suggests that this is the marker of choice for identifying HCC.

Glypican-3.—Glypican-3 is an oncofetal protein that has proven useful in distinguishing HCC from nonneoplastic hepatic lesions and hepatic adenomas[119]; however, its use in the IHC workup of CUP presenting in the liver is limited in our practice. Although there are a small number of studies showing some utility in identifying HCC as opposed to metastatic carcinoma to the liver, we have found the high level of sensitivity and specificity of arginase-1 to surpass the use of glypcian-3 in this setting.[117,120,121]

GYN Cancer Markers

Wilms Tumor Antibody.—Wilms tumor antibody (WT1) encodes a nuclear transcription factor implicated in tumorigenesis and in specifying normal urogenital development.[122] In adult healthy tissues, however, WT1 is expressed by a very restricted subset of cells and tissues, that is, mesothelial cells, ovarian surface epithelium, mesangial cells in the kidney, a subset of smooth muscle cells, and granulocytic cells and precursors. Several studies have documented the specificity of WT1 as a marker of ovarian carcinomas in the context of adenocarcinomas. In addition, WT1 has important applications as a marker of mesothelioma, distinguishing it from nonovarian adenocarcinomas, and as a marker of desmoplastic small, round cell tumors. The major application of antibodies to WT1 in the context of CUPs is their identification of ovarian serous carcinomas, primary peritoneal adenocarcinomas, and fallopian tube serous carcinomas, with very high sensitivity and specificity, both in excess of 90%.[123–126] In a poorly differentiated ovarian carcinoma, nuclear WT1 reactivity favors a serous neoplasm because endometrioid, clear cell, and mucinous carcinomas are negative.[123] Most uterine serous carcinomas are negative or focally reactive, although the literature is somewhat contradictory.[127] In the breast, WT1 is expressed in around 6% of the cases, usually at low levels in pure mucinous (65%) and mixed mucinous (33%) subtypes.[123,128]

Estrogen Receptor.—Many healthy tissues and tumors, including a subset of carcinomas arising within the female

genital tract, exhibit nuclear expression for ER. In endometrial carcinomas of endometrioid type (type 1), ER antibodies are reactive, whereas in uterine serous and clear cell carcinomas (type 2), they usually are not.[129] Assessment of ER by IHC can be part of a panel (which also includes monoclonal carcinoembryonic antigen, vimentin, and p16) to differentiate endometrial adenocarcinoma of the endometrium from endocervical adenocarcinoma[130] because endometrioid carcinomas are generally diffusely ER$^+$, whereas endocervical adenocarcinomas are ER$^-$ or, at most, focally reactive.[131] Depending on the clinical setting, antibodies to ER can be most helpful in corroborating the diagnosis of an ovarian carcinoma primary, particularly because 85% to 90% of ovarian serous carcinomas are ER$^+$,[132] and ER expression, despite some reports to the contrary,[21–24] is exceedingly rare in carcinomas of the GI tract, especially the colorectum. Although a large subset of endometrioid ovarian adenocarcinomas also express ER,[133] despite reports to the contrary,[134] the mucinous and clear cell variants of ovarian carcinoma do not express ER.[77] Estrogen receptor is of no value in the distinction between a primary ovarian adenocarcinoma (mainly including endometrioid and serous carcinoma) and a metastasis from the breast or from elsewhere within the female genital tract. Again, additional markers (such as GCDFP-15 and mammaglobin A) should be used in these clinical settings.

PAX2.—The use of antibodies to PAX2 has been largely supplanted by the use of antibodies to PAX8, which is a more-sensitive and robust reagent for the identification of GYN carcinomas.

PAX8.—PAX8 is a transcription factor that is critical to embryogenesis of the thyroid gland, kidney, and müllerian system. PAX8 is expressed in nonciliated, mucosal cells of the fallopian tubes, endocervix, endometrium, and simple ovarian inclusion cysts but not on the surface of the epithelial cells of the ovary.[135,136] PAX8 shows a high level of expression in nonmucinous ovarian carcinomas and has been seen by some studies in up to 90% to 100% of serous, endometrioid, clear cell, and transitional cell carcinomas (TCCs). In contrast, mucinous carcinomas of the ovary show a much lower level of expression and, when positive, are typically focal, with studies reporting 0% to 50% of tumors showing expression. PAX8 is highly expressed in endometrioid adenocarcinomas (98%) and also in uterine serous carcinomas and endometrial clear cell carcinomas (although few tumors have been evaluated in these studies). Expression of PAX8 in the setting of invasive cervical adenocarcinomas is less well studied, with only a few reported as positive.[137,138] Studies have also shown that PAX8 is not expressed in mammary carcinomas, including ductal and lobular types. Because the ovary is a common site of involvement for metastasis by breast carcinoma, PAX8 can be a useful marker in the differential diagnosis of ovarian and breast carcinomas.

Napsin A.—Studies in this laboratory have shown that the lung adenocarcinoma–associated marker napsin A is highly expressed in clear cell carcinomas of the ovary, with 100% of tumors showing high-level expression. In contrast, only 10% of endometrioid carcinomas and none of the papillary serous carcinomas or serous borderline tumors were napsin$^+$.[73] Napsin A expression has also been reported in clear cell carcinomas of the endometrium (82%), a few endometrial serous carcinomas (8%), and no endometrial endometrioid carcinomas.[139] Table 3 contains a summary of WT1, PAX8, and napsin in ovarian, renal, and breast carcinoma.

Table 3. Comparison of WT1, Napsin A, and PAX8 in Ovarian, Breast, and Renal Carcinomas

Carcinoma	WT1, %	Napsin A, %	PAX8, %
Ovarian serous	>80	<10	>80
Ovarian endometrioid	<10	<10	>80
Ovarian clear cell	<10	>80	>80
Ovarian mucinous	<10	<10	50–80
Breast	<10	<10	<10
Renal	<10	50–80	>80

Prostate Markers

Prostatic adenocarcinoma causes 2% of CUPs, with the main metastatic site being bone and inguinal lymph nodes.[140] Prostatic cancer has perhaps the most-specific and sensitive site-predictive markers of all: prostatic-specific antigen (PSA) and the more recently described NKX3.1.

Prostatic-Specific Antigen.—Antibodies to PSA were first described as an immunohistochemical marker by Nadji et al[141] in the early 1980s, which demonstrated near perfect sensitivity and specificity in the initial published study. Subsequent studies have confirmed the very high sensitivity of this marker, apparently independent of Gleason score,[142] with an overall sensitivity in the range of 95% and specificity approaching 100%.[143] However, PSA is also expressed by a subset of breast cancers[144] (which should only very rarely pose a diagnostic problem) and is also expressed focally in salivary gland and pancreatic carcinomas.[145]

NKX3.1.—The antibody to the prostatic tumor suppressor gene *NKX3.1* has been recently reported to be an extremely sensitive marker for identifying metastatic prostatic adenocarcinoma (positive in 99%), slightly surpassing the sensitivity of PSA; however, similar to the specificity of PSA, the specificity of NKX3.1 approaches 100% (identified in only 1 of 349 nonprostatic carcinomas, a lobular carcinoma of the breast).[146] Furthermore, the level of sensitivity of NKX3.1 is maintained in high-grade prostatic carcinomas (Gleason score, 8–10), seen in up to 95% of cases.[147]

Prostatic Acid Phosphatase.—Prostatic acid phosphatase is a protein, and expression is largely restricted to the prostatic glands and neoplasms derived from them. Subsequent studies have tempered the initial enthusiasm for this marker and shown it to lack the specificity of PSA. Indeed, given the availability of more-robust prostatic markers, such as PSA and NKX3.1, use of prostatic-specific acid phosphatase antibodies in this clinical setting cannot be recommended.

Suggestions.—Given the high sensitivity and specificity of antibodies to PSA for prostatic adenocarcinoma, it is probably not necessary to supplement this in a screening antibody panel, although we have found that, in some clinical settings with antigenically compromised tissues, the addition of NXK3.1 has proven helpful.

TCC Markers

p63 and p40.—p63 and p40, markers for both squamous and transitional cell differentiation, are discussed below.

GATA3.—The nuclear transcription factor GATA3, as indicated earlier, is highly expressed in breast carcinomas and TCCs. More than 90% of urothelial carcinomas are positive for GATA3, with most showing diffuse and strong nuclear staining.[32,34] GATA3 expression is a useful marker in

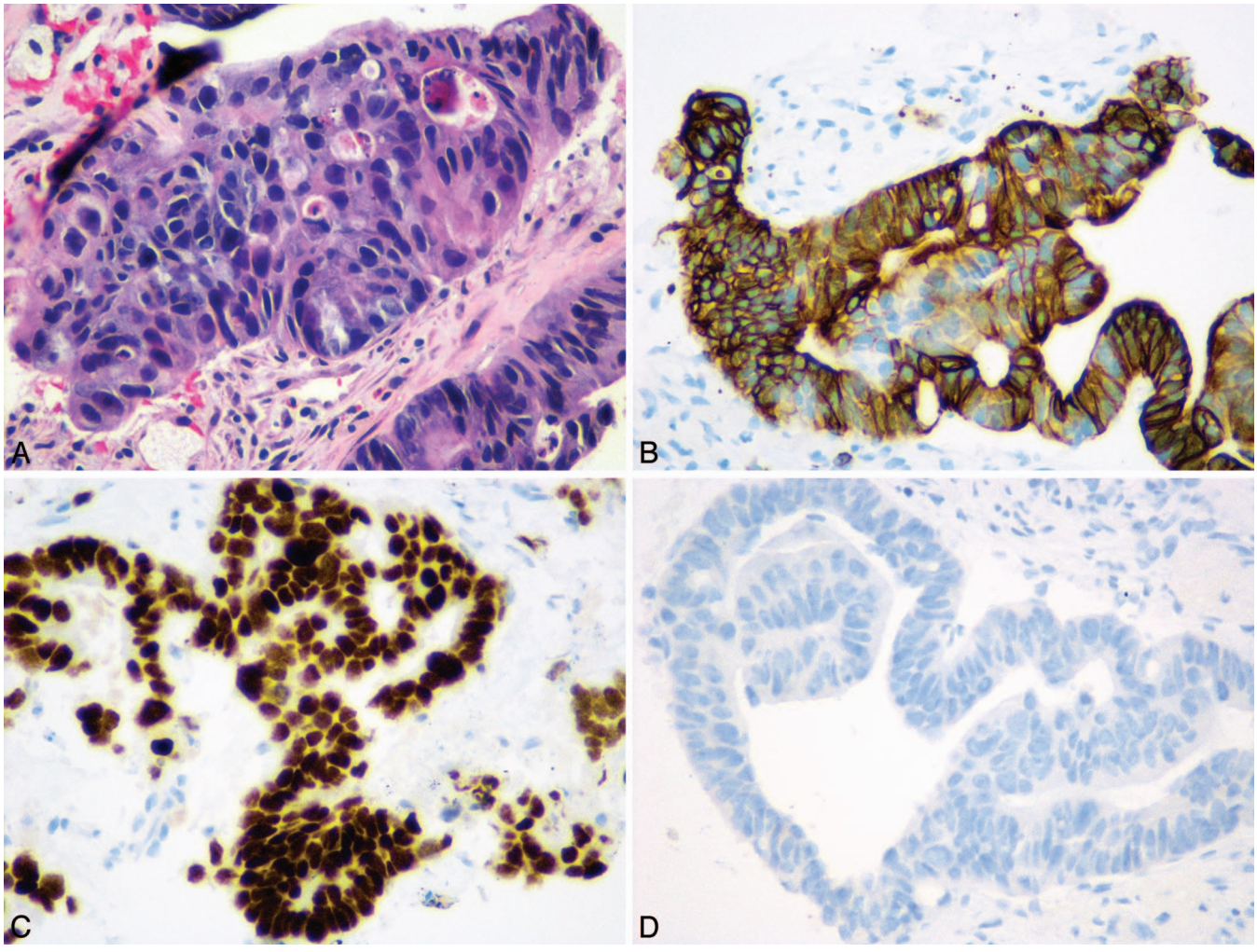

Figure 1. *Metastatic adenocarcinoma from rectosigmoid colon (A), Keratin 20^{+++} (B), CDX2^{+++} (C), and TTF-1^{-} (D). Not shown: negative keratin 7 and napsin A. Note: $^{+++}$ equals >75% cells positive (hematoxylin-eosin, original magnification ×40 [A]; original magnification ×40 [B through D]).*

distinguishing TCC from other non–small cell carcinomas potentially in the differential diagnosis, such as, prostatic adenocarcinoma. GATA3 is also expressed, however, in carcinomas primary to the breast and salivary gland[36] and in a smaller subset of genitourinary tract and lung squamous cell carcinomas.[148] Furthermore, GATA3 is useful in distinguishing TCC from high-grade prostatic adenocarcinomas, which are typically negative for this marker.[149]

Uroplakin.—Uroplakin is a glycoprotein of the asymmetrical unit membrane, which forms plaques on the apical surfaces of urothelial umbrella cells and was the first, specific, urothelial-restricted marker described, initially exhibiting a relatively high rate of sensitivity in the setting of noninvasive TCCs approaching 90%, but exhibiting a lower rate of sensitivity in the setting of invasive and metastatic TCC (approximately 50%–60%).[150–155] Uroplakin does exhibit an extremely high rate of specificity for identifying TCC and is generally not identified in non-urothelial neoplasms. Nevertheless, uroplakin's low rate of sensitivity in invasive and metastatic TCC limits the use of this antibody in the setting of metastatic CUPs and has recently been supplanted by GATA3, the more-sensitive marker of TCC.

Germ Cell Markers

Although germ cell tumors, in the appropriate clinical pathologic setting, may enter into the differential diagnosis of metastatic CUPs, a thorough discussion of the currently available germ cell markers is beyond the scope of this review. Instead, we refer our readers to the recent review by Ulbright et al.[156]

Renal Cell Carcinoma Markers

PAX2.—The nuclear transcription factor PAX2 is expressed overall in approximately 70% to 80% of renal cell carcinomas. However, use of PAX2 as a marker to detect metastatic renal cell carcinoma has largely been supplanted by the more-sensitive and robust marker, PAX8.

PAX8.—The transcription factor PAX8, critical to the embryogenesis of the kidney, is identified in renal tubular epithelium and vas deferens, but not glomeruli.[140] PAX8 identifies most of the renal epithelial neoplasms, with many cases of conventional (clear cell) renal cell carcinoma exhibiting a sensitivity of 88% to 98% and a similarly high level of sensitivity seen in papillary renal cell carcinomas, varying from 71% to 100%. Although fewer chromophobe renal cell carcinomas have been studied, rates of expression of PAX8 have been reported to vary from 57% to 95% and is

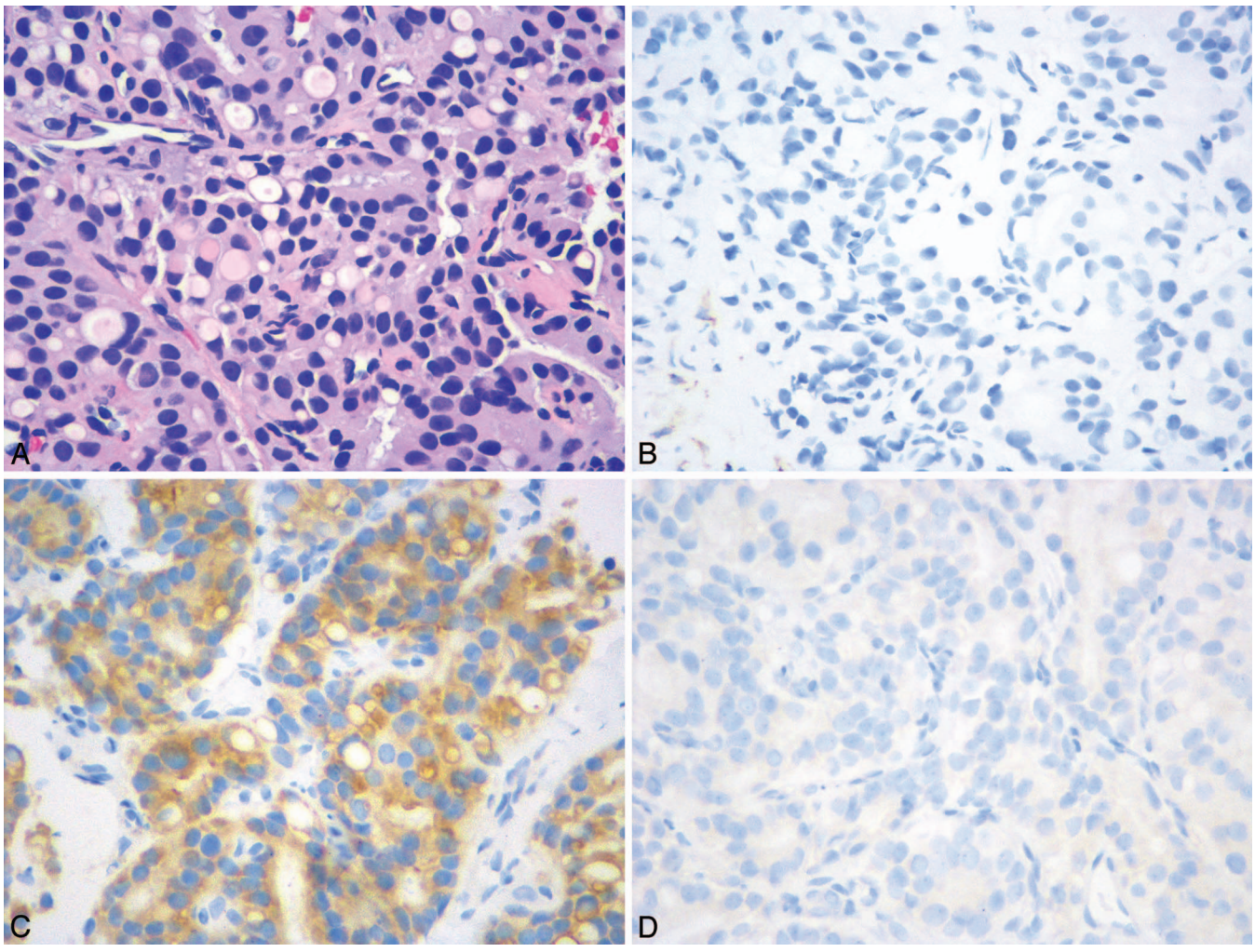

Figure 2. *Metastatic prostatic adenocarcinoma (A), Keratin 7^{-} (B), PSA^{+++} (C), and Villin^{-} (D). Not shown: negative keratin 20, TTF-1, napsin A, and CDX2. Note: $^{+++}$ equals >75% cells positive (hematoxylin-eosin, original magnification ×40 [A]; original magnification ×40 [B through D]).*

similar in sarcomatoid and Xp11 translocation renal cell carcinomas; although few tumors have been evaluated, rates of sensitivity vary from 44% to 100% and 50% to 80%, respectively.[139,140,157–159] Although PAX8 is not expressed in bladder TCCs, PAX8 expression has been described in a subset of renal pelvic urothelial carcinomas.[139,140] The finding of PAX8 expression in a number of non-GYN or genitourinary carcinomas has been called into question; those studies employed a polyclonal anti-PAX8 antibody that has subsequently been demonstrated to cross-react with PAX6 and PAX5.[160]

Thyroid Markers

Thyroglobulin.—Antibodies to thyroglobulin have long been considered specific and sensitive markers of both primary and metastatic carcinomas of the thyroid,[161,162] although there can be considerable technical difficulties using antithyroglobulin antibodies in tumors in and around the thyroid tissue, with the real potential for misinterpretation of false-positive immunostaining. Thyroglobulin is an excellent marker of papillary and follicular carcinomas but is a poor marker of anaplastic thyroid carcinomas and, as might be expected, is not a marker of medullary (neuroendocrine) carcinomas of the thyroid.[163]

TTF-1.—Importantly, TTF-1 is an even more-sensitive marker of thyroid carcinomas than thyroglobulin is and is expressed in more than 90% of thyroid carcinomas, with the solitary exception of the anaplastic variant, in which the sensitivity is close to zero. In addition, TTF-1 can be employed as a marker of neuroendocrine carcinomas of the thyroid, such as medullary carcinomas.[164,165]

PAX8.—PAX8 is critical to the organogenesis of the thyroid gland and is highly expressed in the thyroid follicular epithelium.[159] In papillary and follicular carcinomas of the thyroid, PAX8 is expressed in 100% cases (as reported in multiple studies). In contrast to TTF-1, PAX8 expression has been identified in the setting of anaplastic thyroid carcinoma, with a level of sensitivity reported at approximately 80%. In the setting of metastatic CUP, PAX8 is useful in discriminating between a TTF-1^{+} lung adenocarcinoma and a TTF-1^{+} thyroid carcinoma because PAX8 expression has not been identified in primary lung adenocarcinomas.*

(Please also see renal and GYN tract sections for descriptions of PAX8 expressions in these tumors.)

* *References 139, 140, 158, 159, 166, 167.*

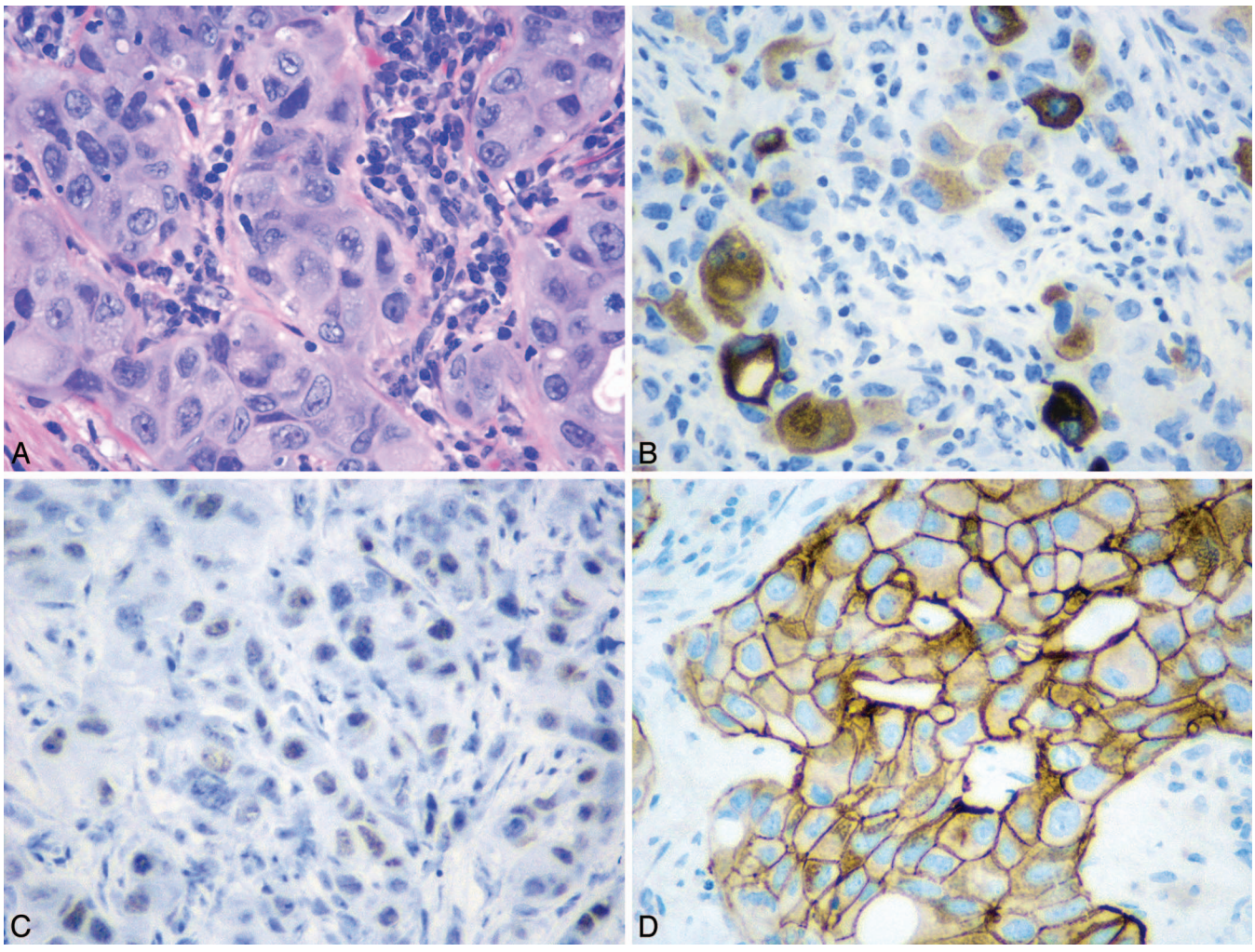

Figure 3. *Metastatic ductal adenocarcinoma from breast (A), GCDFP-15^{++} (B), GATA3^{++} (C), and HER2^{+++} (positive for overexpression) (D). Not shown: mammaglobin^{+}, keratin 5/6^{++}; negative ER, TTF-1, and p63. Note: $^{+++}$ equals >75% cells positive; $^{++}$ equals 26%–75% cells positive; $^{+}$ equals 1%–25% cells positive (hematoxylin-eosin, original magnification ×40 [A]; original magnification ×40 [B through D]).*

Adrenal Markers

Inhibin-α.—Inhibin is a protein expressed in a restricted subset of healthy cells, including ovarian granulosa cells, testicular Leydig cells, and adrenal cortical epithelium.

Antibodies to the α-chain of inhibin can serve as an excellent marker for the identification of primary adrenal cortical tumors and their distinction from metastatic carcinomas to the adrenal gland.[168–172] The overall sensitivity of antibodies to the α-subchain of inhibin is in the range of 80% to 90%.[173] Inhibin-α expression also characterizes ovarian and testicular stromal tumors.

MART1 Antigen.—An alternative or supplementary marker of adrenal cortical differentiation is the MART1 (Melan-A) antigen, first defined as a melanocytic/melanoma marker but fortuitously found to also be expressed in adrenal cortical cells and tumors.[174] The sensitivity of MART1/Melan-A as a marker of adrenal cortical tumors is comparable to, or even greater than, inhibin-α.[174,175]

Steroidogenic Factor 1.—Recent studies have shown the transcription factor steroidogenic factor 1 is an extremely sensitive marker at identifying adrenal cortical neoplasms (ranging from 85% to 100%) and, furthermore, exhibits 100% specificity at discriminating these neoplasms from other tumors with clear cell morphology, such as renal cell carcinoma, ovarian clear cell carcinoma, and chordomas[176,177] This nuclear marker is identified at high levels in sex cord-stromal tumors of the ovary and at lower levels in testicular sex cord-stromal tumors.[178]

Anomalous Findings in Adrenal Cortical Tumors.—Adrenal cortical tumors are unique among epithelial tumors in the very low level of keratins that these tumors can sometimes express.[179]

In addition, adrenal cortical tumors are unique among nonneuroendocrine tumors in their expression of synaptophysin.[180]

Identification of Neuroendocrine Tumors

Antibodies to neuroendocrine markers are often included in a panel of markers used to identify the primary site of carcinomas, particularly when the histologic setting raises the possibility of the presence of neuroendocrine differentiation. Although other markers, such as CD56,[181] have been used by some researchers, the most-sensitive and specific neuroendocrine markers, which have extensive track re-

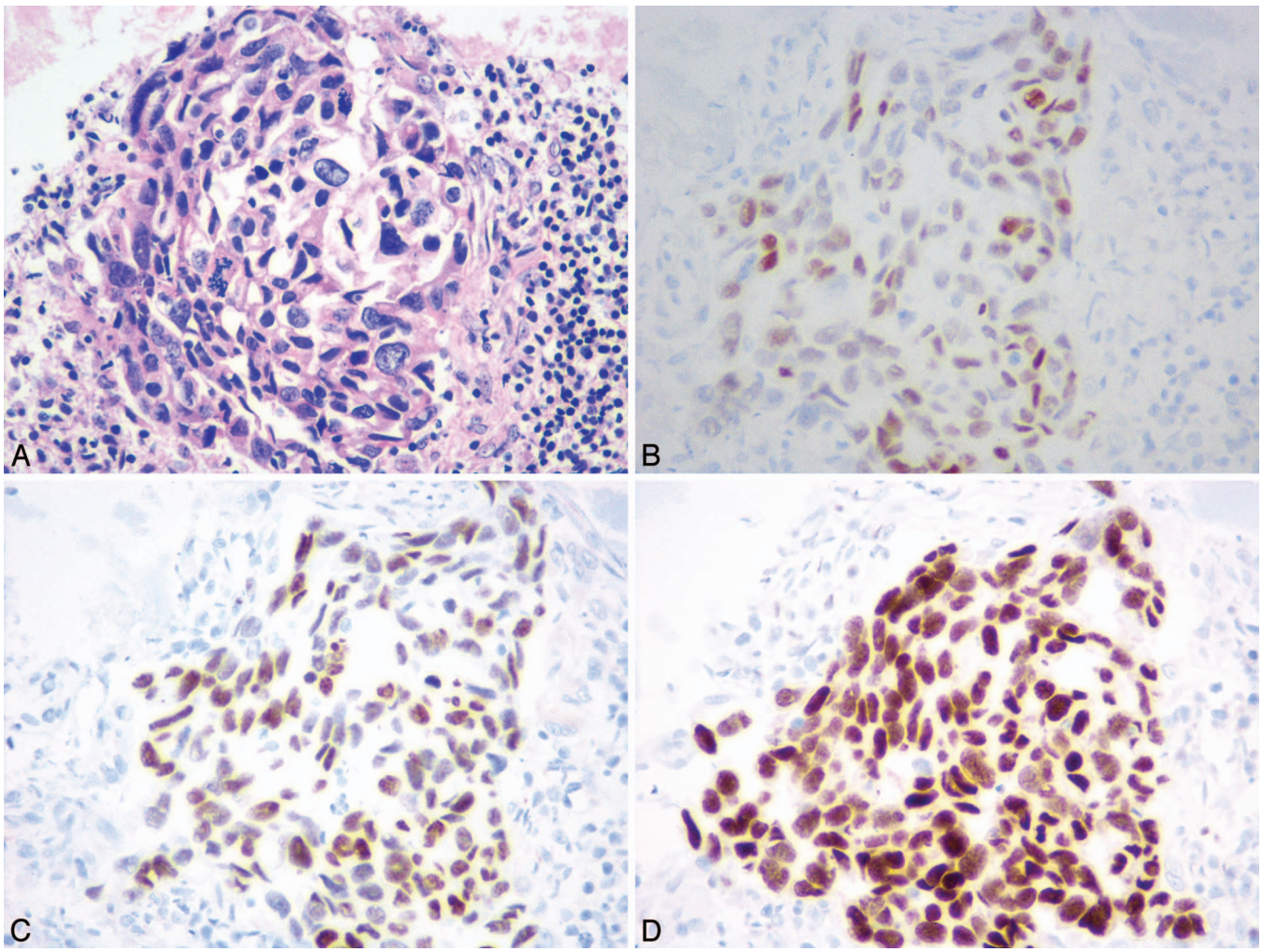

Figure 4. *Metastatic high-grade serous carcinoma of ovary (A), ER^{++} (B), WT1^{++} (C), and PAX8^{+++} (D). Not shown: keratin 7^{+++}; negative keratin 20, GATA3, p63, and CDX2. Note: $^{+++}$ equals >75% cells positive; $^{++}$ equals 26%–75% cells positive (hematoxylin-eosin, original magnification ×40 [A]; original magnification ×40 [B through D]).*

cords, are chromogranin A[182] and synaptophysin.[183] Neuron-specific enolase should not be used as a neuroendocrine marker because it lacks specificity.[184,185] There is a direct correlation between the degree of differentiation of the tumor (well-differentiated types, eg, carcinoid and pancreatic endocrine tumor, to poorly differentiated types, eg, small cell carcinoma) and the level of chromogranin A expression, as evidenced by the intensity of immunostaining and the fraction of positive tumor cells. Antibodies to chromogranin A, synaptophysin, or both, alone or in combination, will identify greater than 90% of neuroendocrine carcinomas, including small cell carcinomas, which correspond to "poorly differentiated" neuroendocrine carcinomas, particularly in the setting of carcinomas of the lung, where this distinction is most critical.[186] Focal neuroendocrine differentiation can be found in nonneuroendocrine tumors, exemplified by scattered chromogranin A^{+}, synaptophysin^{+}, or both positive cells through the tumor (a pattern not uncommon in adenocarcinomas of the GI tract) or in breast and prostate carcinomas.

Squamous/Transitional Cell Markers

p63 and p40.—Although generally referred to as a single molecule, the transcription factor p63 actually consists of at least 2 isoforms, referred to as TAp63 and ΔNp63. Antibodies to p63 have been used for many years to identify myoepithelium in the breast, the outer cell layer in prostatic glands, and squamous (and transitional) cell differentiation. The 4A4 anti-p63 clone in use for many years is actually a "pan-p63" antibody, identifying both the ΔNp63 and the TAp63 isoforms; antibodies to p40 can be employed as a more squamous-specific marker. Another advantage of p40, rather than p63, antibodies is the absence of low level immunostaining in selected adenocarcinomas as well as neuroendocrine carcinomas, for example, of the lung.[187]

Squamous Differentiation.—Pure squamous cell carcinomas, such as those arising in the lung or cervix, are uniformly and strongly p63^{+} and p40^{+}, but there are other tumors, such as thymomas,[188,189] that also manifest a squamous immunophenotype and are also universally positive for expression of p63 and p40. However, in the context of identifying squamous cell carcinomas, p63 and p40 are not organ-specific markers.

Transitional Differentiation.—Transitional cell carcinomas generally manifest uniform expression of p63 and p40, even in the setting of poorly differentiated tumors, such as spindle cell bladder TCC.[190,191]

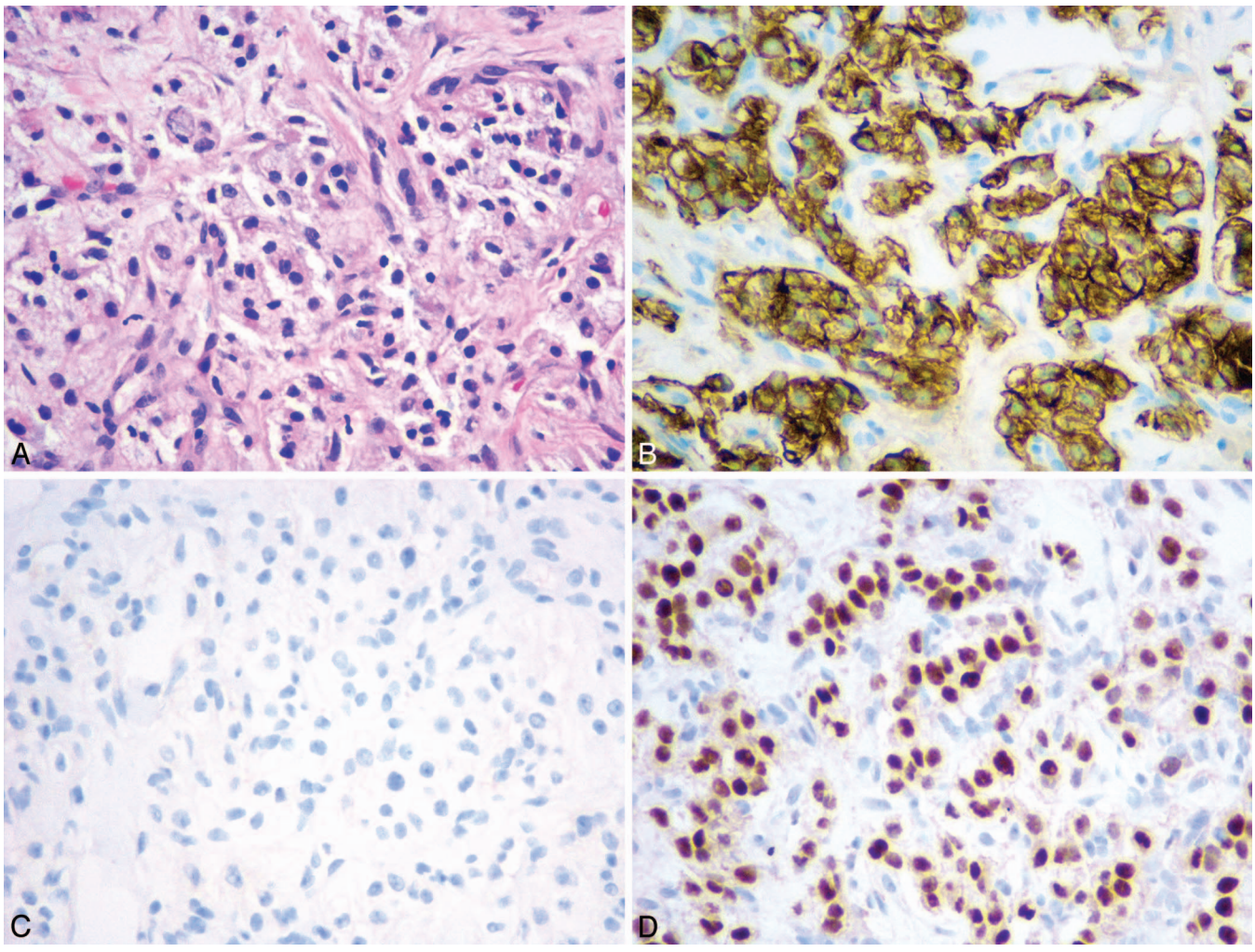

Figure 5. *Metastatic renal cell carcinoma (A), broad spectrum keratins (OSCAR^{+++}) (B), PSA^{-} (C), and PAX8^{+++} (D). Not shown: negative keratin 7 and TTF-1. Note: $^{+++}$ equals >75% cells positive (hematoxylin-eosin, original magnification ×40 [A]; original magnification ×40 [B through D]).*

Nonsquamous, Nontransitional Carcinomas.—p63 and p40 are also expressed in carcinomas demonstrating myoepithelial differentiation (eg, adenoid cystic and other salivary gland carcinomas) and in carcinomas demonstrating trophoblastic differentiation.[192]

Keratin 5.—Antibodies against K5 are useful in corroborating the presence of squamous differentiation in poorly differentiated carcinomas, when used in conjunction with antibodies to p63, p40, or both. Kaufmann and colleagues[193] reported an 84% sensitivity and 79% specificity for K5 in squamous cell carcinoma. Keratin 5 can also be positive in a subset of breast, urothelial, ovarian, pancreatic, and endometrioid carcinomas (50%).[194] However, the pattern of K5 expression in these latter tumors is almost always variable, in contrast to the uniform pattern of expression in squamous carcinomas.

CONCLUSIONS

In conclusion, with well-performed and well-interpreted IHC panels, anatomic pathologists can successfully identify the site of origin of CUPs; however, it is crucial to understand not only the diagnostic uses of the many available antibodies but also their potential limits and pitfalls. To illustrate this, 5 representative case studies (from P.K.) from patient files are provided next, including pertinent clinical histories and stains.

Case 1

Figure 1, A through D, shows a needle biopsy of single left lower lobe lung nodule from a 73-year-old man, who was a long-term smoker, with a history of a biopsy-proven rectosigmoid colon adenocarcinoma 1 month before the lung biopsy.

Diagnosis.—The case was diagnosed as metastatic adenocarcinoma from rectosigmoid colon (based on K7^{-}/K20^{+} profile, high-level expression of the GI marker CDX2, and absence of lung marker TTF-1/napsin expression); the enteric subset of lung adenocarcinomas can exhibit a similar immunophenotype but, typically, those tumors show expression of K7.

Case 2

Figure 2, A through D, shows a needle biopsy of a cervical lymph node from a 63-year-old man with no known primary carcinoma, who presented with cervical adenopathy that was clinically suspicious for lymphoma.

Diagnosis.—The case was diagnosed as metastatic prostatic adenocarcinoma (based on expression of the

prostatic restricted marker PSA, typical K7$^-$ profile, and absence of expression of GI markers villin/CDX2 and lung markers TTF-1/napsin).

Case 3

Figure 3, A through D, shows a right axillary lymph node biopsy from a 55-year-old woman with ill-defined density seen on mammogram of the right breast and positron emission tomography–positive up-take in right parotid gland.

Diagnosis.—The case was diagnosed as metastatic ductal adenocarcinoma from the breast versus the salivary gland (based on coexpression of GCDFP-15, mammaglobin, and GATA3 and overexpression of HER2; patient was subsequently shown to have biopsy-proven benign lesion of the parotid gland and infiltrating ductal carcinoma of the breast).

Case 4

Figure 4, A through D, shows a needle biopsy of a retroperitoneal lymph node containing scant material that included a single focus of carcinoma from a 73-year-old woman with history of a hysterectomy for unknown reasons, who presented with retroperitoneal adenopathy, possible splenic metastases, and left pelvic sidewall mass on computed tomography scan interpreted as probable residual ovary.

Diagnosis.—The case was diagnosed as metastatic, high-grade serous carcinoma from the ovary (given the specific immunophenotypic coexpression of PAX8, WT1, and ER and the absence of expression from markers indicating squamous [p63], urothelial [p63/GATA3], or GI tract [CDX2] origin).

Case 5

Figure 5, A through D, shows a needle biopsy of a mediastinal lymph node from a 58-year-old man with a recent diagnosis of prostate adenocarcinoma (Gleason pattern 4, "hypernephroid"), a nephrectomy 5 years prior for "sarcomatoid" renal cell carcinoma, and a remote history of melanoma who presented with mediastinal and lung masses.

Diagnosis.—The case was diagnosed as metastatic renal cell carcinoma, conventional type (given expression of PAX8, typical K7$^-$ profile, and absence of prostatic marker PSA; PAX8 expression in a male is essentially limited to renal and thyroid carcinomas, and in this case, the negative TTF-1 ruled against a tumor of thyroid or lung origin).

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