PAX8 Reliably Distinguishes Ovarian Serous Tumors From Malignant Mesothelioma

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Abstract: Ovarian serous neoplasms can have morphologic overlap with malignant mesothelioma. The distinction is clinically important, yet most studies have failed to identify immunostains that reliably distinguish these 2 tumor types. Recently, transcription factor PAX8 was shown to be a sensitive and relatively specific marker for Müllerian tumors. In addition, some studies suggest that h-caldesmon is sensitive and specific for mesothelioma when compared with serous ovarian tumors. The goal of this study was to evaluate whether PAX8 and h-caldesmon expression can successfully distinguish mesothelioma from serous ovarian tumors. Immunohistochemistry was carried out using PAX8 and h-caldesmon antibodies on archival tissue from 254 ovarian serous tumors and 50 mesothelial tumors. Nuclear and cytoplasmic immunoreactivity were considered positive for PAX8 and h-caldesmon, respectively. PAX8 staining was present in 99% of high-grade serous ovarian carcinomas and all (100%) low-grade ovarian carcinomas and serous borderline tumors; however, only 74% of these cases (188/254) were diffusely positive in more than 50% of tumors cells, and intensity ranged from strong to weak. None of the pleural malignant mesotheliomas were reactive with PAX8. However, 2/23 (9%) peritoneal malignant mesotheliomas showed focal and/or weak staining for PAX8; the remaining cases were negative. Two well-differentiated papillary mesotheliomas and 1 multicystic mesothelioma each showed some staining for PAX8. h-caldesmon was negative in all serous neoplasms and all mesothelial neoplasms, except 1 pleural malignant mesothelioma which showed patchy immunoreactivity. Strong PAX8 staining is highly specific ($P < 0.00001$) for ovarian serous tumors when compared with malignant mesotheliomas of the peritoneum and pleura. The presence of weak staining for PAX8 in the 3 ''noninvasive'' mesotheliomas questions the use for PAX8 in this differential diagnosis. On the basis of this study, h-caldesmon is not a useful marker for mesothelioma.

Key Words: PAX8, h-caldesmon, serous, borderline, mesothelioma, peritoneal

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The clinical and morphologic distinction of serous ovarian neoplasms and malignant mesothelioma can be difficult. This is especially true when the former is of low morphologic grade and is associated with diffuse invasive peritoneal implants, or when an ovarian tumor (most frequently a high-grade serous carcinoma) has metastasized to the pleura. For this reason, a number of studies have attempted to use a variety of immunohistochemical stains to distinguish these 2 tumor types. Unfortunately, very few immunostains have proven to be particularly specific or sensitive. For example, expression of calretinin, WT-1, D2-40, and mesothelin are expressed in the majority of mesotheliomas (high sensitivity); however, these markers can also be expressed in a significant subset of serous ovarian neoplasms (low specificity).[2,8,11,18,23,39] Other markers, including BER-EP4, HE4, CD15, and B72.3 have been shown to be expressed more frequently in ovarian serous tumors than mesotheliomas, but decreased sensitivity and/or specificity of these markers has also limited their use as reliable discriminators.[2,11,13,19,23,30,39]

Earlier studies by Comin et al[11] suggest that serous ovarian tumors and peritoneal mesotheliomas can be distinguished with high specificity and sensitivity using antibodies to estrogen receptor (positive in 95% of the serous tumors and none of the mesotheliomas) and h-caldesmon, a specific smooth muscle marker (positive in 5% of the serous tumors and all of the mesotheliomas), respectively. However, it was not clear from this study whether low-grade and/or high-grade serous ovarian carcinomas were evaluated, and other studies have shown that the ER receptor is positive in fewer (approximately 55%) high-grade serous ovarian carcinomas.[27] The high specificity and sensitivity of h-caldesmon reported in this study is encouraging, but only a small number of cases were tested, and other studies have shown that h-caldesmon is negative in epithelial tissues and tumors.[3,35,43,52]

PAX8 is a member of the paired box (PAX) family of transcription factors, and is important in organogenesis of

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the thyroid gland, kidney, and Müllerian system.[7,28,42] By immunohistochemistry, PAX8 is a nuclear marker and interpretation is straightforward. In the Müllerian system, PAX8 is expressed in a variety of ovarian tumors, especially serous carcinoma, but also in endometrioid and clear cell carcinomas of the ovary.[5,23,36] Secretory cells of the normal fallopian tube are positive for PAX8, and these cells are believed to be the origin for many of the serous ovarian cancers.[5,22,25,26]

The diagnostic use of PAX8 as a clinical marker was recently shown by its ability to reliably discriminate between ovarian and breast carcinomas.[36] It has also been shown to stain thyroid epithelial neoplasms,[37] renal epithelial neoplasms,[51] and clear cell adenocarcinoma of the bladder.[50] The aim of this study was to determine whether PAX8 can distinguish serous ovarian neoplasms from malignant epithelioid mesotheliomas of the peritoneum and pleura. In addition, cases were stained with h-caldesmon in an attempt to verify its use as an adjunctive immunostain for this differential diagnosis.

MATERIALS AND METHODS

After approval from the Institutional Review Board at Brigham and Women's Hospital, 304 cases between 1998 and 2009 were obtained from the archival files in the Department of Pathology at Brigham and Women's Hospital, Boston, MA. These cases include: (i) 50 mesotheliomas (23 peritoneal malignant epithelioid mesotheliomas, 2 perintoneal well-differentiated papillary mesotheliomas (WDPM), 1 case of perintoneal multiloculated inclusion cyst (also known as "multicystic mesothelioma"), and 24 pleural malignant epithelioid mesotheliomas), (ii) 102 low-grade ovarian serous tumors (92 serous borderline tumors (SBTs), and 10 low-grade serous carcinomas), and (iii) 152 high-grade serous ovarian carcinomas. Hematoxylin and eosin-stained slides generated from formalin fixed, paraffin-embedded tissue were reviewed to confirm the diagnoses before inclusion in the study. A panel of immunostains, including calretinin, WT-1, and D2-40, were used during clinical work-up to support all mesothelioma diagnoses; these immunostains were not repeated for this study. In addition, cytogenetic and/or fluorescence in situ hybridization (FISH) results typical of malignant mesothelioma[15,33] supported the diagnosis of malignant peritoneal mesotheliomas in 11 of the 23 (48%) cases.

Immunohistochemistry was carried out using the Envision Plus/Horseradish Peroxidase system (Dako, Carpinteria, CA), a polyclonal antibody to PAX8 (Proteintech Group Inc, Chicago, IL, 1:800 dilution), and a monoclonal antibody to h-caldesmon (Dako, clone h-CD, all cases stained at a dilution of 1:300; at a dilution of 1:300; a subset of these cases were additionally stained using a dilution of 1:100). In brief, paraffin-embedded sections were incubated in hydrogen peroxidase and absolute alcohol for 30 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out using pressure cooker pretreatment in citrate buffer (pH = 6.0). Tissue sections were subsequently incubated with the primary antibody for 40 minutes at 25°C. After TBS rinses, the tissue was incubated using the Envision Plus secondary antibody for 30 minutes followed by diaminobenzidine for 5 minutes. Appropriate positive (tonsil lymphocytes for PAX8 and a segment of colon for h-caldesmon) and negative (incubation with secondary antibody only) controls were stained in parallel for each round of immunohistochemistry.

PAX8 was evaluated for nuclear staining and h-caldesmon was evaluated for a cytoplasmic reaction. Immunoreactivity was graded based on intensity (weak, moderate, strong) and extent (semiquantitatively; 0, no staining; 1+, < 5% cells staining; 2+, 5% to 10% cells staining; 3+ 11% to 50% cells staining; 4+, > 50% cells staining). Internal positive controls (ie, B lymphocytes for PAX8 and vascular smooth muscle for h-caldesmon) were noted and used as an intensity reference when present. Statistical comparisons of categoric data were carried out using χ^2 or Fisher exact test, depending upon the sample size. A P value of < 0.05 was considered statistically significant.

RESULTS

Morphology

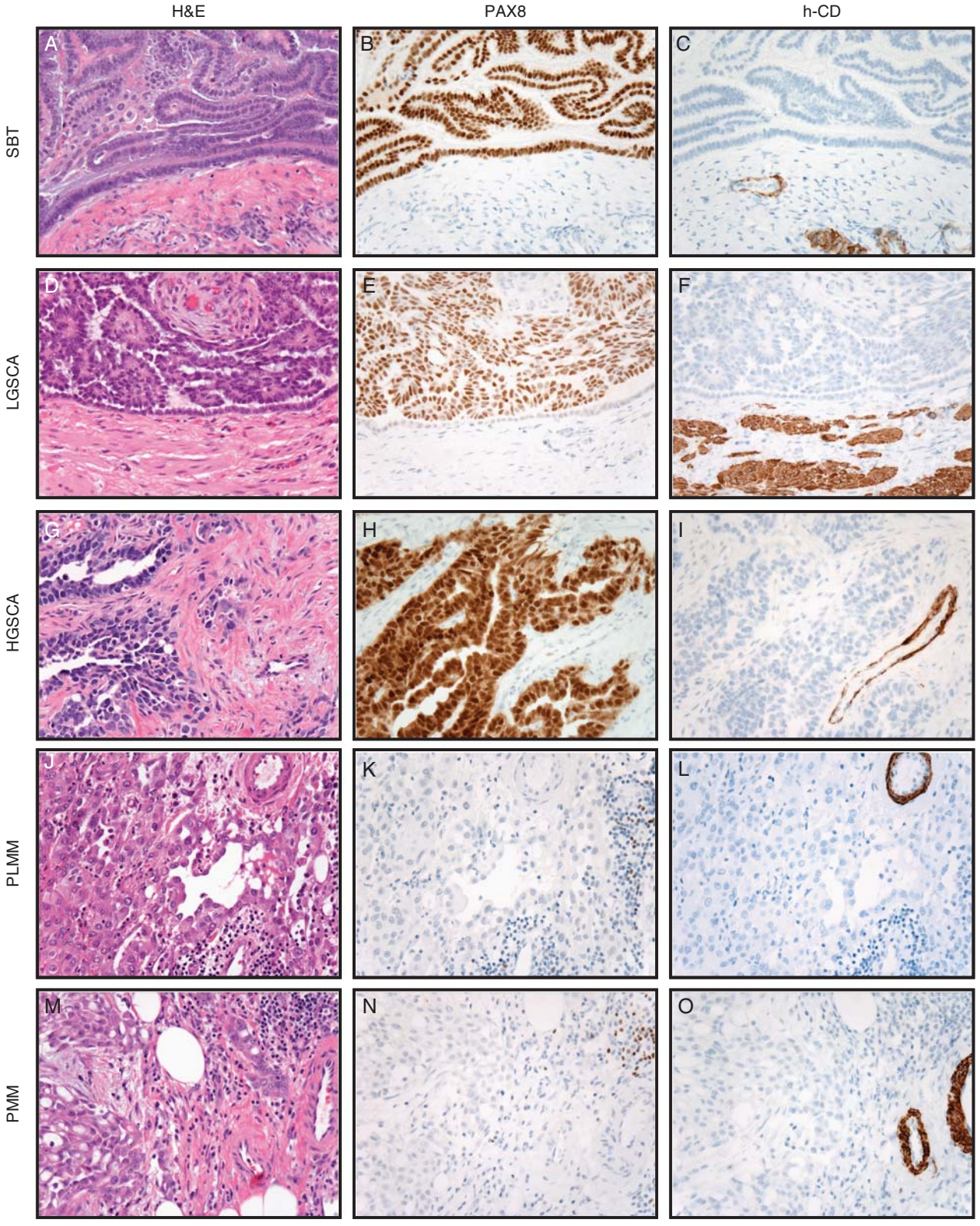
Serous Tumors of the Ovary

The 92 SBTs showed characteristic papillary epithelial proliferation with hierarchical branching and minimal nuclear atypia (Figs. 1A–C). The 10 low-grade serous carcinomas were distinguished from SBTs by more significant nuclear atypia, nucleoli, and pleomorphism of the malignant epithelial cells lining the papillae (Figs. 1D–F), and by foci of stromal invasion. The 152 high-grade serous carcinomas contained hyperchromatic and pleomorphic cells, with high nuclear/cytoplasmic ratios, necrosis, and areas of solid and/or papillary growth patterns (Figs. 1G–I).

FIGURE 1. PAX8 and h-caldesmon staining in serous ovarian tumors and malignant mesotheliomas. A to C, Serous borderline tumors (SBT) of the ovary were PAX8 positive and h-caldesmon negative. D to F, Low-grade ovarian serous carcinomas (LGSCA) were PAX8 positive and h-caldesmon negative. G to I, All but 1 high-grade serous ovarian carcinoma (HGSCA) was PAX8 positive; all high-grade serous ovarian carcinomas were h-caldesmon negative. J to L, Pleural malignant mesotheliomas (PLMM) were all negative for PAX8; 23 of 24 pleural malignant mesotheliomas were negative for h-caldesmon. M to O, 21 of 23 peritoneal malignant mesotheliomas (PMM) were negative for PAX8; all peritoneal malignant mesotheliomas were negative for h-caldesmon. Note immunoreactivity of internal control tissues: PAX8 with B lymphocytes (K and N) and h-caldesmon with smooth muscle in vessel walls (C, I, L, O) or adnexal tissue (F). h-CD indicates h-caldesmon.

Mesothelial Neoplasms

The mesothelial neoplasms were composed of bland to mildly atypical, monotonous cuboidal cells with well-demarcated cell borders and variably prominent nucleoli. A majority (23/24) of the pleural malignant mesotheliomas (Figs. 1J–L) were of the epithelioid (epithelial) type;



the 1 remaining case was predominantly epithelioid, but also had a minor sarcomatoid component. Of the primary peritoneal mesotheliomas (Figs. 1M–O), 21 were purely epithelioid and 2 were predominantly epithelioid with a minor spindle cell component. Two additional cases from the peritoneum, consistent with WDPM, were composed of wide fibrovascular papillary cores lined by a single layer of bland, monotonous cuboidal cells; no stromal invasion was identified (Figs. 2C, D). One additional case, consistent with so-called multilocular mesothelioma (peritoneal inclusion cysts) was composed of variably sized, multiloculated, thin walled cysts lined by bland, flattened mesothelial cells (Figs. 2E, F). Of note, 17 of the 23 (74%) malignant peritoneal mesotheliomas occurred in men; the WDPMs and the case of peritoneal inclusion cysts affected women.

Immunohistochemistry

Immunohistochemical results are summarized and presented in Table 1.

Serous Tumors of the Ovary

PAX8 was immunoreactive in 151/152 (99%) high-grade serous carcinomas (Fig. 1H) and all 102 (100%) low-grade serous neoplasms of the ovary, including both the SBTs (Fig. 1B) and the well-differentiated serous carcinomas (Fig. 1E) of the ovary. The majority of the borderline tumors (79/92, 86%) showed a diffuse (4+, > 50% of tumor cells) nuclear staining pattern, and the intensity was consistently moderate to strong in 69 (75%) of these cases. The remaining 13 SBT cases (14%) showed focal (10% to 50%) staining; of these 6 (7%) had moderate or strong staining, and 7 (8%) showed weak immunoreactivity. Nine of the 10 (90%) low-grade serous carcinomas were diffusely and strongly positive for PAX8 in tumor cells; the remaining case was focally (3+), but still strongly immunoreactive. Of the 151 PAX8 positive high-grade serous carcinomas, 100 cases (66%) were diffusely positive (4+), but approximately half of these cases showed moderate to weak staining patterns. Fifty (33%) of the remaining high-grade serous carcinomas were strongly positive for PAX8, but only in a subset of tumor cells (3+); the last case (1%) was focally and weakly positive. The sole PAX8 negative serous carcinoma (by TMA) was morphologically consistent with a high-grade ovarian serous carcinoma (resection specimen and metastases reviewed), and it is possible that the absence of staining may be secondary to a sampling error (false negative).

h-caldesmon (diluted at 1:300) was negative in all 152 ovarian serous tumors (strong immunoreactivity with the smooth muscle in vessels walls and/or adnexal structures was present at this dilution as a positive internal control on all slides) (Figs. 1C, F, I).

Mesothelial Neoplasms

All 24 (100%) malignant mesotheliomas of the pleura were completely negative for PAX8 (Fig. 1K). Two of the 23 (9%) malignant mesotheliomas of the peritoneum, both with classic cytogenetic or fluorescence in situ hybridization findings that supported the morphologic diagnosis, showed focal and/or weak staining for PAX8 (Figs. 2A, B); the remaining 21 cases (91%) were entirely negative for PAX8 (Fig. 1N). All 3 of the well-differentiated peritoneal mesotheliomas showed immunoreactivity with PAX8; however, the 2 WDPMs were only weakly positive (Fig. 2D), whereas the 1 case of multilocular mesothelioma (peritoneal inclusion cysts) was moderately and diffusely positive for PAX8 (Fig. 2F).

h-caldesmon (diluted 1:300) was negative in all 26 mesothelial tumors of the peritoneum (Fig. 1O). Only 1 of the 24 (4%) pleural malignant mesotheliomas showed patchy immunoreactivity with h-caldesmon (Figs. 3A, B); tumor in the remaining 23 pleural cases was completely negative for h-caldesmon (Fig. 1L). Strong immunoreactivity with the smooth muscle in vessels walls was present at this dilution as a positive internal control on the slides of every mesothelial neoplasm (Figs. 1L, O). As prior studies have claimed that h-caldesmon reacts with mesotheliomas at a dilution of 1:100, a subset of peritoneal cases (N = 17) was also stained using this concentration of antibody. In our laboratory, all 17 peritoneal mesothelial neoplasms were negative for h-caldesmon when using a dilution of 1:100 (positive internal controls noted in all cases) (data not shown).

DISCUSSION

Malignant mesothelioma, occurring in either the pleura or peritoneum, is a rare neoplasm (approximately 2000 new cases each year in the United States of America) with an aggressive clinical course. Even with maximal medical and surgical therapy, malignant mesotheliomas of the peritoneum (also known as "primary peritoneal mesothelioma" or "diffuse malignant peritoneal mesothelioma") have median and 5-year survival rates of 50 months and 35% to 45%, respectively; slightly worse, pleura malignant mesotheliomas have median and 5-year survival rates of approximately 11 months and 10% to 15%, respectively.[9,16,32,45,54] Epithelioid malignant mesotheliomas occurring in either the pleura or peritoneum are characterized by a monotonous, deceptively bland proliferation of cuboidal epithelioid cells that invade adjacent tissue (Figs. 1J, 1M, 2A, 3A). The cells often have prominent nucleoli, well-demarcated cell borders, and abundant eosinophilic cytoplasm. Occasionally, increased cytologic atypia is encountered. Architectural findings range, even within individual tumors, from large sheets of polygonal cells to prominent tubulopapillary structures. Mitoses are rare, and psammoma bodies may be seen, particularly within the tubulopapillary pattern of mesothelioma.

In contrast, WDPM and multicystic mesothelioma (also known as multiloculated peritoneal inclusion cysts) are generally considered benign entities.[9] WDPM is composed of broad papillary to tubulopapillary structures, lined by a single layer of bland epithelioid cells, with or without psammoma bodies (Fig. 2C). The

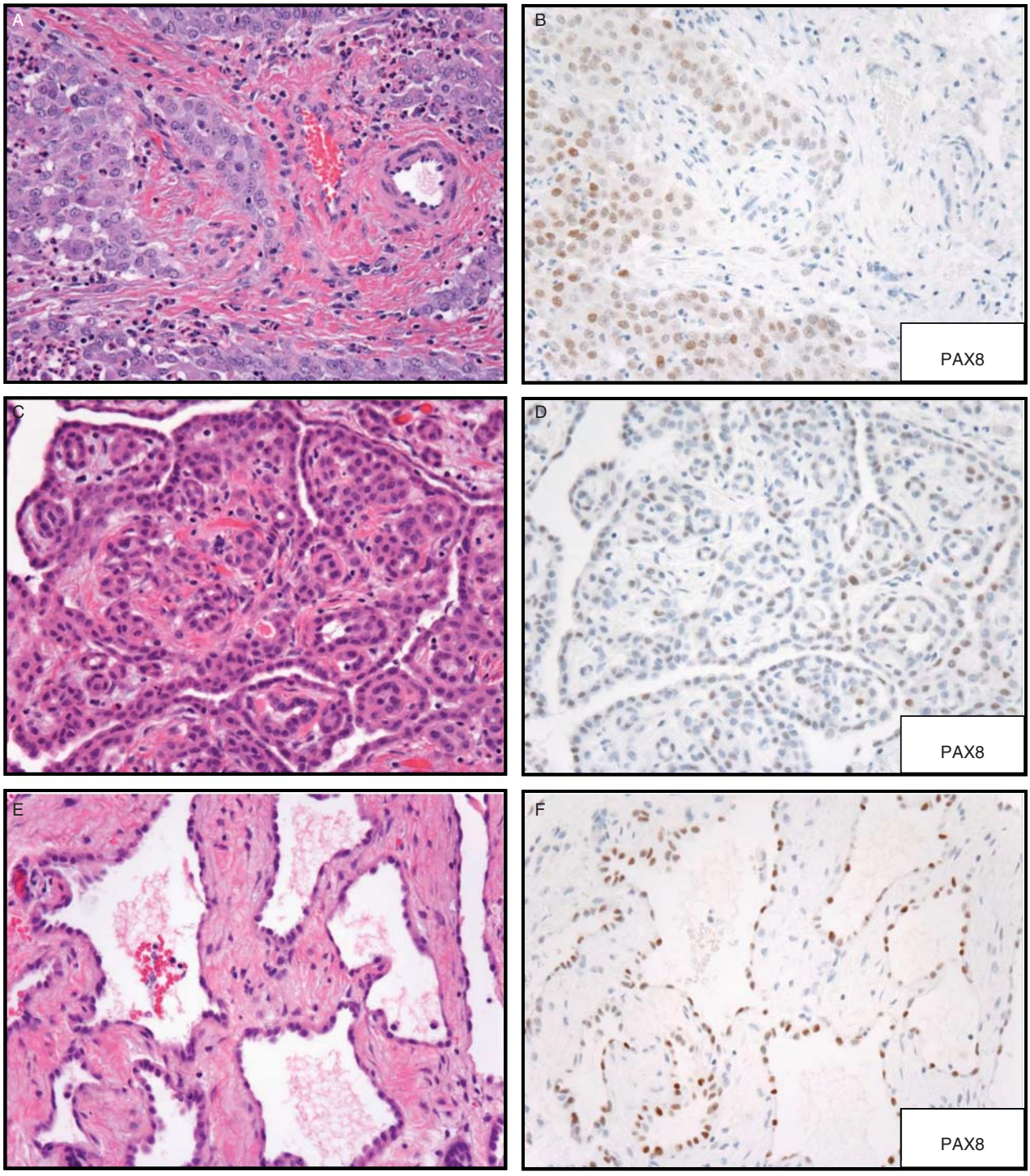


FIGURE 2. PAX8 immunoreactivity in mesothelial neoplasms. A and B, Only 2 of 23 peritoneal malignant mesotheliomas showed multifocal weak staining for PAX8. C and D, Both well-differentiated papillary mesotheliomas showed weak multifocal staining for PAX8. E and F, The multiloculated cystic mesothelioma was PAX8 positive.

epithelioid cells are flat to cuboidal, lack prominent nucleoli, and have only occasional mild cytologic atypia and rare mitoses. WDPM may be associated with extensive fibrosis; however, invasion of adjacent tissue is not seen. Multicystic mesothelioma/multiloculated peritoneal inclusion cyst is characterized by multiple, variably sized, cystic structures lined by a single layer of bland, flattened mesothelial cells (Fig. 2E). These cells can

TABLE 1. PAX8 and h-Caldesmon Staining in Ovarian Serous Tumors and Mesothelial Neoplasms

Diagnosis	PAX8 # POS/TOTAL (%)	4+	3+	2+	1+	h-Caldesmon # POS/TOTAL (%)
SBT	92/92 (100)	79	10	2	1	0/92 (0)
LGSCA	10/10 (100)	9	1	0	0	0/10 (0)
HGSCA	151/152 (99)	100	50	0	1	0/153 (0)
PLMM	0/24 (0)	0	0	0	0	1/24 (4)
PMM	2/23 (9)	0	1	0	1	0/23 (0)
WDPM	2/2 (100)	0	2	0	0	0/2 (0)
MLCM	1/1 (100)	1	0	0	0	0/2 (0)

0, no staining; 1+, < 5% cells staining; 2+, 5-10% cells staining; 3+, 11-50% cells staining; 4+, > 50% cells staining.
HGSCA, high-grade serous carcinoma of the ovary; LGSCA, low-grade serous carcinoma of the ovary; MLCM, multiloculated cystic mesothelioma; PLMM, pleural malignant mesothelioma; PMM, peritoneal malignant mesothelioma; SBT, serous borderline tumor of the ovary; WDPM, well-differentiated papillary mesothelioma.

exhibit occasional mild atypia, but invasion of adjacent tissue is absent.

Serous carcinoma of the ovary is the eighth most common cancer in woman with approximately 21,000 new cases diagnosed per year in the United States of America. Although this incidence is approximately 10 times more common than malignant mesothelioma, it has a similarly dismal prognosis with an overall 5-year survival rate of < 40%.[6,21,40,46] However, as evidenced by biomarker expression and genetic studies, serous tumors of the ovary are a heterogenous group of neoplasms both morphologically (Figs. 1A, D, G) and biologically with significant differences in survival (ie, higher grade and/or higher stage tumors have a worse prognosis).[4,6,23,46–49] These differences are addressed in the 2-pathway model proposed by Shih and Kurman.[22,48] In this model, Type II tumors include the high-grade serous carcinomas, which are associated with *TP53* mutations and a rapidly progressive clinical course. The Type I tumors, associated with *KRAS* and *BRAF* mutations, include SBTs (also known as "serous tumors of low malignant potential" or "atypical proliferative serous tumors") and low-grade serous carcinomas, which are more frequently indolent with a reported 5-year survival of 80% in patients with noninvasive implants. It is the low grade, Type I tumors (especially when associated with invasive implants) that are morphologically and immunohistochemically most difficult to distinguish from malignant mesothelioma in the peritoneum. Additionally metastases of low or high-grade ovarian serous carcinomas to the pleura can be difficult to distinguish from primary pleural tumors.

Lineage specific markers are a useful tool for distinguishing among poorly differentiated neoplasms; however, few highly specific proteins have been identified to date. Some examples include TTF1,[44] RCC antigen,[1] and Mammaglobin.[20,53] Nevertheless, many poorly differentiated tumors are still difficult to distinguish as sensitivity of these lineage specific markers is less than perfect. In some cases a panel of immunostains, plus morphology, clinical history, and molecular studies can aid in making an accurate diagnosis.

PAX8 is a 48 kD protein, encoded at locus 2q12-14, that was first described in mouse thyroid organogenesis,

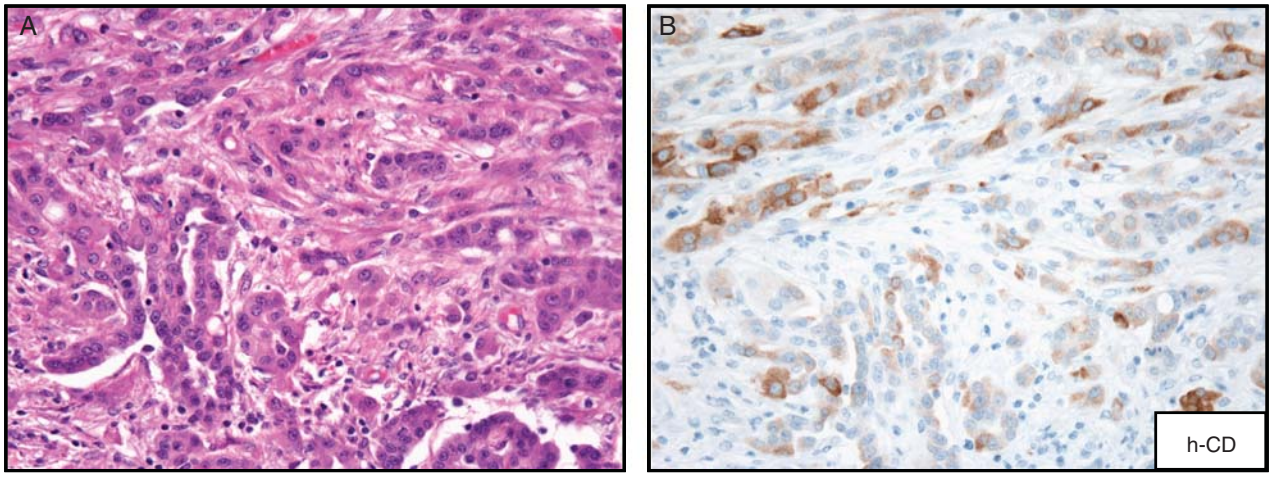


FIGURE 3. h-caldesmon immunoreactivity in a pleural malignant mesothelioma. All neoplasms in this study were negative for h-caldesmon except 1 pleural malignant mesothelioma (shown, A, B). h-CD indicates h-caldesmon.

and was later implicated in follicular thyroid carcinoma.[41,42] PAX8-PPAR gamma rearrangement occurs in 30% of conventional follicular thyroid carcinomas but not in other subtypes of thyroid carcinoma.[14,17,24,34] PAX8 has also been shown to be a lineage-specific protein in the kidney. In embryonic development of the murine kidney, PAX8, together with PAX2, regulates branching morphogenesis and nephron differentiation.[31] PAX8 is expressed in all RCC subtypes, but not in bladder urothelial carcinomas, which originate from a different embryologic origin.[51] Interestingly, a small subset of renal pelvis urothelial carcinomas, clear cell carcinomas of the bladder and nephrogenic adenomas have been shown to be immunoreactive for PAX8.[50,51]

The third major organ system to be regulated developmentally by PAX8 is the embryonic Müllerian system, which gives rise to the female genital tract. Female mice lacking PAX8 are infertile, owing to defects in genital tract formation.[29] PAX8 has also been shown to be expressed in the human Müllerian tract—including the secretory cells of fallopian tubes.[5,26] Consistent with the role of PAX8 as a lineage-specific marker, it is also expressed in tumors derived from the Müllerian tract epithelium. In fact, recent reports suggest that malignant transformation of secretory cells of the fallopian tube is likely the source for many ovarian/pelvic serous carcinomas.[12,25] Clinically, PAX8 has been shown to be of use in distinguishing breast and ovarian carcinomas, which can have significant morphologic and immuophenotypic overlap.[36]

The differentiation between ovarian carcinoma and mesothelioma is a particular challenge owing to similarities in both histologic appearances and immunophenotypic profiles. Calretinin, CK5/6, D2-40, and WT1 are positive in more than 90% of mesotheliomas; however, they have also been shown to be expressed in approximately 10%, 25%, 65%, and 90% of serous ovarian carcinomas, respectively.[23] This study confirms the high sensitivity of PAX8 for ovarian serous neoplasms, as 253/254 (99.6%) cases were positive for this marker (Table 1 and Figs. 1B, E, H). In contrast, PAX8 was expressed in very few (5 of 50; 10%) mesothelial neoplasms: all pleural malignant mesotheliomas were negative for PAX8 (Fig. 1K), whereas 2/23 (9%) peritoneal malignant mesotheliomas (1 from a man and 1 from a woman, both diagnoses supported by cytogenetic/FISH studies) (Fig. 2B), and the 2 WDPMs showed focal and/or weak PAX8 staining (Fig. 2D); the 1 multicystic mesothelioma showed a relatively diffuse and moderately strong staining pattern (Fig. 2F). Overall, these results yield a sensitivity of 99.6% and a specificity of 95.7% when comparing serous ovarian neoplasms with malignant mesothelial tumors ($P < 0.00001$), and suggest that PAX8 is an excellent immunostain that will help distinguish between these 2 challenging malignancies with similar morphologies.

Of note, 2 other markers were recently described to differentiate ovarian carcinoma and mesothelioma: Tenascin-X[55] was reported as positive in 73% of malignant peritoneal mesotheliomas and absent in ovarian carcinomas, and h-caldesmon[11] was reportedly positive in 100% of malignant peritoneal mesotheliomas, and 97% of pleural mesotheliomas,[10] and 5% of ovarian carcinomas. However, h-caldesmon was evaluated in this study and found to stain only 1 of the mesothelial tumors (Fig. 2B) and none of the serous tumors (Figs. 1C, F, I). The reason for this major discrepancy is uncertain as the only difference in staining protocol between the 2 studies was the dilution of the antibody, which was purchased from the same company. In this study the antibody was diluted 1:300 (the standard dilution used by our laboratory) for all cases, whereas in the study by Comin et al the antibody was more concentrated (1:100). To address the discrepancy, we chose to stain a subset of 17 peritoneal mesotheliomas with the h-caldesmon antibody at a dilution of 1:100; under these conditions all cases remained negative for this marker. Positive internal control tissue (smooth muscle of blood vessel walls and/or adnexal smooth muscle) was strongly immunoreactive in every case at both dilutions in this study showing that the dilution of antibody and the immunohistochemical technique used were sufficient and appropriate. Moreover, h-caldesmon was shown in earlier studies to be a relatively specific smooth muscle marker, with very little, if any, staining in epithelial cells/neoplasms.[3,35,38,43,52] These overall findings raise question as to the use of h-caldesmon in distinguishing ovarian serous tumors from mesothelioma, and furthermore support the use of PAX8 as the most sensitive and specific antibody for this differential diagnosis thus far.

In summary, this study shows that PAX8 is highly sensitive and specific for ovarian serous tumors when compared with malignant/invasive mesothelial tumors. In particular, PAX8 may prove to be most useful and helpful when evaluating small pleural or peritoneal biopsies.

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