Immunohistochemistry in Peritoneal Mesothelioma

A Single-Center Experience of 244 Cases

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Malignant mesothelioma is a primary tumor arising from the mesothelium of the pleura, peritoneum, pericardium, and tunica vaginalis. Its occurrence is correlated with asbestos exposure and is estimated between 2 to 30 per million by population. Malignant mesothelioma incidence continues to rise worldwide with peak incidence expected by 2030.

Mesothelioma currently presents challenges in both diagnosis and management. Mesothelioma characteristically exhibits a wide variety of histologic and cytomorphologic patterns and features. Morphologic appearance ranges from epithelioid, sarcomatoid, and biphasic subtypes, but variants with pleomorphic, decidual, small, vacuolated, and clear cells have also been described. Overlapping features among malignancies, such as adenocarcinomas and mesotheliomas with papillary or solid components, sarcomatoid carcinomas or sarcomas with pleomorphic or spindle cell components and sarcomatoid mesotheliomas, and non–small cell carcinomas or serous carcinomas and epithelioid mesothelioma, are typical, and thus, the diagnosis of mesothelioma on the basis of morphology alone is frequently not possible. Immunohistochemistry is recognized as the best, widely used, ancillary technique in the differential diagnosis of mesothelioma; however, a single marker for mesothelioma has yet to be identified, and information on markers can be contradictory and limited in this rare tumor. In addition to immunostaining inadequacies, up to 44% of mesothelioma diagnoses are incorrect because of specimen insufficiency and physician inexperience with this rare malignancy.

Accordingly, diagnostic panels are under constant scrutiny, and novel markers are sought to improve diagnostic accuracy.

Abdominal malignant mesothelioma represents a less common subset of these tumors. Because of demographic differences when compared with pleural disease, for example, a greater proportion of female patients and its abdominal location, the differential diagnosis includes carcinomas of gastrointestinal, gynecologic, and renal origin. This creates a greater challenge in diagnosis requiring a wider array of immunohistochemistry studies.

In this study, we report and discuss the results of 17 immunohistochemistry markers used in the differential
diagnosis of mesothelioma—anti-TAG72 antibody (B72.3), breast cancer 1–associated protein (BAP1), anti-epithelial cell adhesion molecule antibody (Ber-EP4), calretinin, syndecan-1 (CD138), carinoembryonic antigen (CEA), cytokeratin 20 (CK20), cytokeratin 5/6 (CK5/6), cytokeratin 7 (CK7), anti-oncofetal protein M2A antibody (D2-40), estrogen receptor (ER), Lewis X antibody (CD15), mesothelin, tumor protein 63 (p63), paired box gene 8 (PAX8), progesterone receptor (PR), and Wilms tumor protein (WT1)—in a large series of peritoneal mesothelioma cases.

### MATERIALS AND METHODS

This was a retrospective analysis performed with approval of the Columbia University Medical Center Review Board. The pathology records from 1999 to 2014 at Columbia University Medical Center (New York, New York) were reviewed for the diagnosis of malignant mesothelioma and were further analyzed for tumors whose primary presentation was in the abdomen. Patients with disease originating in the pleura were, therefore, excluded, as were malignant mesothelioma of the tunica vaginalis and multicystic mesothelioma for a total of 244 peritoneal tumors.

The diagnosis was rendered with a combination of morphologic and immunohistochemistry studies. The diagnosis of biphasic disease required the identification of a sarcomatous component of 5% or greater. Pure sarcomatoid malignant mesotheliomas were not identified in this series.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue. Most cases were tested with clones and antibody dilutions, as noted in Table 1. In some cases, immunohistochemistry was submitted as part of consultation cases, and the specific immunohistochemistry method was not available. All antibodies were scored as positive or negative as part of diagnostic evaluation and were not assessed semiquantitatively.

### RESULTS

Immunohistochemistry results are shown in Table 2 and described below.

### Table 1. Antibodies Used for Immunohistochemistry in This Study

| Marker | Source                          | Clone | Dilution |
|--------|---------------------------------|-------|----------|
| B72.3  | Signet Laboratories (Dedham, Massachusetts) | B72.3 | 1:40     |
| BAP1   | Santa Cruz Biotecnology (Santa Cruz, California) | C-4 mAb | 1:50     |
| Ber-EP4| DAKO (Carpinteria, California)     | Ber-EP4 mAb | 1:200   |
| Calretinin | DAKO                            | DAK-calret 1 mAb | 1:50     |
| CD138  | DAKO                            | M15 mAb | 1:60     |
| CD15   | DAKO                            | CD1    | 1:100    |
| CEA    | BioGenex Laboratories (Fremont, California) | B01-94-11M-P mAb | 1:800    |
| CK5/6  | DAKO                            | D5/16 B4 mAb | 1:40     |
| CK7    | DAKO                            | OV-TL 12/30 mAb | 1:200    |
| CK20   | DAKO                            | K_20.8 mAb | 1:200    |
| D2-40  | DAKO                            | D2-40 mAb | 1:40     |
| ER     | DAKO                            | 1D5 mAb | 1:160    |
| Mesothelin | BioGenex Laboratories          | 5B2 mAb | 1:20     |
| p63    | DAKO                            | 4A4 mAb | 1:100    |
| PAX8   | Proteintech Group (Rosemont, Illinois) | pAb   | 1:200    |
| PR     | DAKO                            | PgR 636 | 1:100    |
| WT1    | Cell Marque (Rocklin, California) | 6f-H2 mAb | 1:40     |

Abbreviations: BAP1, breast cancer 1–associated protein; CEA, carinoembryonic antigen; ER, estrogen receptor; mAb, monoclonal antibody; pAb, polyclonal antibody; PAX8, paired box gene 8; PR, progesterone receptor; WT1, Wilms tumor protein.

### Table 2. Immunohistochemistry Results in Peritoneal Mesothelioma, N = 244

| Marker | All Types | Epithelioid | Biphasic | WDPM |
|--------|-----------|-------------|----------|------|
|        | Total Cases | Positive Cases, No. (%) | Total Cases | Positive Cases, No. (%) | Total Cases | Positive Cases, No. (%) | Total Cases | Positive Cases, No. (%) |
| B72.3  | 196       | 6 (3)       | 147      | 4 (3)      | 41          | 2 (5)       | 8          | 0 (0)     |
| BAP1   | 181       | 82 (45)     | 137      | 58 (42)    | 40          | 20 (50)     | 4          | 4 (100)   |
| Ber-EP4| 209       | 17 (7)      | 153      | 11 (8)     | 44          | 11 (27)     | 12         | 3 (81)    |
| Calretinin | 244 | 244 (100) | 177      | 177 (100)  | 50          | 50 (100)    | 17         | 17 (100)  |
| CD138  | 91        | 9 (10)      | 68       | 8 (12)     | 18          | 1 (6)       | 5          | 0 (0)     |
| CD15   | 192       | 7 (4)       | 141      | 4 (3)      | 43          | 3 (7)       | 8          | 0 (0)     |
| CEA    | 199       | 9 (5)       | 147      | 6 (4)      | 43          | 3 (7)       | 9          | 0 (0)     |
| CK5/6  | 194       | 137 (70)    | 148      | 135 (91)   | 38          | 32 (84)     | 8          | 6 (75)    |
| CK7    | 132       | 123 (93)    | 92       | 84 (91)    | 35          | 34 (97)     | 5          | 5 (100)   |
| CK20   | 116       | 5 (4)       | 82       | 4 (5)      | 31          | 1 (3)       | 3          | 0 (0)     |
| D2-40  | 97        | 78 (80)     | 74       | 63 (85)    | 20          | 12 (60)     | 3          | 3 (100)   |
| ER     | 84        | 2 (2)       | 60       | 1 (2)      | 20          | 1 (5)       | 4          | 0 (0)     |
| Mesothelin | 150 | 130 (87)    | 112      | 105 (94)   | 34          | 24 (71)     | 4          | 3 (75)    |
| p63    | 63        | 3 (5)       | 43       | 2 (5)      | 16          | 1 (6)       | 3          | 0 (0)     |
| PAX8   | 191       | 12 (6)      | 142      | 8 (6)      | 43          | 3 (7)       | 6          | 1 (17)    |
| PR     | 71        | 5 (7)       | 50       | 3 (6)      | 18          | 1 (6)       | 3          | 1 (3)     |
| WT1    | 218       | 205 (94)    | 159      | 153 (96)   | 45          | 38 (84)     | 14         | 14 (100)  |

Abbreviations: BAP1, breast cancer 1–associated protein; CEA, carinoembryonic antigen; ER, estrogen receptor; p63, paired box gene 8; PAX8, paired box gene 8; PR, progesterone receptor; WDPM, well-differentiated papillary mesothelioma; WT1, Wilms tumor protein.
Immunohistochemistry Markers With Positive Findings in Mesothelioma

Calretinin, WT1, CK5/6, D2-40, and mesothelin are generally immunoreactive in peritoneal mesothelioma but can also be positive in gynecologic and nongynecologic adenocarcinoma. All 244 (100%) of the peritoneal mesotheliomas in this cohort were immunoreactive for calretinin. Of the 218 peritoneal mesotheliomas tested, 205 (94%) displayed WT1 expression, which included 153 of 159 (96%) of the epithelioid subtype tumors, and 38 of 45 (84%) biphasic mesotheliomas. Of the 13 WT1− cases, calretinin immunoreactivity (n = 13) and lack of reactivity for B72.3 (n = 13), CEA (n = 11), Ber-EP4 (n = 12), and CD15 (n = 12) were used in support of the diagnosis. Of note, 7 of 10 of those cases (70%) were immunoreactive for mesothelin, 8 of 9 cases (88%) were CK5/6 reactive, and 10 of 11 cases (91%) were PAX8−. Therefore, an approach of 2 mesothelial markers and up to 4 negative “carcinoma” markers was diagnostically helpful in those WT1− cases.

The CK5/6 marker was immunoreactive in 173 of 194 cases (89%); 135 of 148 epithelioid tumors (91%) were positive, and 32 of 38 biphasic tumors (84%) were positive. Of the 21 tumors not immunoreactive for CK5/6, all 21 (100%) stained positive for calretinin, and all but one of 19 (95%) tested were WT1−.

Eighty percent (78 of 97) of the tumors stained positive for D2-40, with 63 of 74 epithelioid tumors (85%) and 12 of 20 (60%) biphasic tumors. Of the 19 tumors not reactive to the D2-40 antibody, 19 of 19 (100%) were immunoreactive to calretinin, 16 of 19 (84%) expressed WT1, and 12 of 16 (75%) were CK5/6−; for 2 cases, calretinin was the only positive mesothelioma marker. Mesothelin may have utility in the monitoring and treatment of mesothelioma, and 132 of 150 tumors (88%) showed mesothelin immunoreactivity (105 of 112 [94%] epithelioid tumors and 24 of 34 [71%] biphasic tumors; Figure 1, A and B).

Markers Generally Positive in Carcinoma

The markers B72.3, Ber-EP4, CD138, CEA, and CD15 are used in the diagnosis of carcinoma but can also be immunoreactive in mesothelioma. A low, but potentially confounding, rate of immunoreactivity was seen for those markers in peritoneal mesothelioma, with B72.3 positive in 6 of 196 cases (3%), Ber-EP4 positive in 17 of 209 cases (8%), CD138 positive in 9 of 91 cases (10%), CEA positive in 9 of 199 cases (5%), and CD15 positive in 7 of 192 cases (4%). Surprisingly, those rates were similar or greater in biphasic tumors as compared with epithelioid histology.

Markers Used in the Differential Diagnosis of Carcinoma

Tumor protein 63 was seen in 3 of 62 peritoneal mesotheliomas (5%); 2 of 43 epithelioid tumors (5%) stained positive, and 1 of 16 biphasic tumors (6%) were positive. Estrogen receptor was found in 2 of 84 mesotheliomas (2%); 1 of 60 epithelioid tumors (2%), and 1 of 20 biphasic tumors (5%) were immunoreactive, with both patients being female. Progesterone receptor was seen in 5 of 71 tumors (7%) tested, which included 3 of 50 epithelioid tumors (6%) and 1 of 18 biphasic tumors (6%), with 3 female (2 epithelioid, 1 biphasic) patients and 1 male (biphasic) patient. In that cohort, 12 of 191 (6%) of the tumors showed PAX8 immunoreactivity, with 8 of 142 (6%) in epithelioid tumors (Figure 2, A), 3 of 43 (7%) in biphasic tumors (Figure 2, B), and 1 of 6 (17%) in well-differentiated papillary mesotheliomas (Figure 2, C).

CK7 and CK20 are frequently used in the diagnosis of carcinoma of unknown primary. However, in peritoneal mesothelioma, CK7 was frequently positive (93%; 123 of 132 patients) and was, therefore, not helpful in distinguishing mesothelioma from carcinomas in that differential diagnosis because CK7 was typically positive in both. On the other hand, CK20 was infrequently positive in mesothelioma, as confirmed in our cohort (5 of 116; 4%).

BAP1

There is increasing use of BAP1 in mesothelioma diagnosis, with loss of expression supporting a malignant diagnosis (Figure 3, A). In peritoneal mesothelioma, 82 of 181 (45%) of the total cohort were BAP1−, which included 58 of 137 epithelioid tumors (42%), 20 of 40 biphasic tumors (50%; Figure 3, B), and all 4 well-
differentiated papillary mesotheliomas (100%) tested (Figure 3, C).

DISCUSSION
Diagnosing peritoneal mesothelioma is contingent on the results of immunohistochemical panels composed of markers positive for mesothelioma versus markers positive in carcinoma. In addition, there is increasing use of markers that are relatively tissue-specific transcription factors.

Of the conventional markers, calretinin was the most sensitive (100%), followed by WT1 (94%), CK5/6 (89%), and D2-40 (80%). Markers generally positive in carcinoma, in decreasing order of frequency in mesothelioma were CD138 (10%), Ber-EP4 (8%), CEA (5%), CD15 (4%), and B72.3 (3%). Markers used in the differential diagnosis of carcinoma and mesothelioma showed frequency in peritoneal mesothelioma as follows: PR (7%), PAX8 (6%), p63 (5%), CK20 (4%), and ER (2%). As a result, a panel approach can be useful to differentiate between mesothelioma and carcinoma.

The PAX8 staining is well-documented as a renal cell carcinoma–associated marker11,21,22 and is also commonly expressed in carcinomas of the thyroid and parathyroid, ovary, endometrium, and thymus.5 PAX8, a transcription factor having an important role in embryogenesis of the thyroid, urinary system, areas of the nervous system, and organs derived from the Wolffian and Müllerian ducts, is sometimes positive in mesothelioma. Although it has not been described in pleural mesothelioma,11,23 immunoreactivity in prior studies of peritoneal mesothelioma has ranged from 0 to 9%. In one study, 2 of 23 peritoneal epithelioid mesotheliomas (9%) displayed focal and/or weak staining for PAX8,23 and in another, 1 of 64 diffuse peritoneal mesotheliomas (2%) showed diffuse nuclear positivity.24 In one recent study, 0 of 40 cases (0%) of peritoneal mesothelioma stained positive for PAX8.5 These findings have indicated PAX8 as a key immunohistochemical marker in the differential diagnosis between carcinomas and mesothelioma. In the present study, we found 12 of 191 peritoneal mesotheliomas (6%) to stain positive for PAX8, which included 8 of 142 epithelioid tumors (6%), 3 of 43 (7%) of the biphasic subtype, and 1 of 6 well-differentiated papillary mesotheliomas (17%). Although useful, PAX8 cannot, therefore, be considered a definitive negative

Figure 2. Immunohistochemistry for PAX8 in peritoneal mesothelioma. A, An epithelioid malignant mesothelioma with strong PAX8 staining. B, PAX8 immunoreactivity in a biphasic peritoneal mesothelioma. C, Strong nuclear PAX8 immunoreactivity is shown in a well-differentiated papillary mesothelioma (3,3′-diaminobenzidine, original magnification ×100 [A through C]).
marker of mesothelioma in immunohistochemical panels for the differential diagnosis of carcinoma and mesothelioma.

BAP1, a tumor-suppressor gene, binds to the ring finger domain of BRCA1, catalyzes the removal of ubiquitin chains from ubiquitinated proteins, and enhances BRCA1-mediated cell growth suppression. BAP1 loss in mesothelioma is used to establish malignancy because benign proliferations rarely exhibit BAP1 loss. Germline BAP1 mutations are associated with a high incidence of many malignancies, particularly mesothelioma and uveal melanoma. We found that BAP1 was expressed in 82 of 181 (45%) of the cases, that is, it was lost in 99 of 181 (55%) of the cases. By subtype, BAP1 positivity was observed in our study in 58 of 137 epithelioid tumors (42%), 20 of 40 biphasic tumors (50%), and 4 of 4 well-differentiated papillary tumors (100%). Although the overall rate of BAP1 loss in peritoneal mesothelioma was consistent with past data (Table 3), our results do not coincide with findings on nonepithelioid morphologic patterns in which more than 90% of nonepithelioid mesotheliomas were BAP1+.

Mesothelin, a cell-surface glycoprotein and tumor-differentiation antigen, was highly expressed in epithelioid mesothelioma. Sarcomatoid and biphasic mesotheliomas have shown limited positivity. Mesothelin is associated with a poorer prognosis in several cancers. Its exact biological role remains unknown. Mesothelin staining in mesothelioma appears strong and occurs along the cell membrane, whereas it is often focal and cytoplasmic in other cancers, such as lung adenocarcinoma and squamous carcinoma. Limited expression in healthy human

| Source, y            | Total Peritoneal Mesothelioma Cases, No. | BAP1 Loss in Peritoneal Mesothelioma, No. (%) |
|----------------------|------------------------------------------|---------------------------------------------|
| This study           | 181                                      | 99 (55)                                     |
| Andrici et al,11 2015| 9                                       | 6 (67)                                      |
| Singhi et al,12 2015 | 86                                      | 49 (57)                                     |
| Kato et al,37 2016   | 11                                      | 3 (27)                                      |
tissues and high expression in cancers renders mesothelin an attractive candidate for immunotherapy, that is, in therapies that target cell-surface mesothelin or elicit an immune response against it. In our cohort, 133 of 152 (88%) of the mesotheliomas were positive for mesothelin, 106 of 113 epithelioid mesotheliomas (94%) stained positive, 24 of 35 (69%) of the biphasic mesotheliomas stained positive, and 3 of 4 well-differentiated papillary mesotheliomas (75%) expressed mesothelin.

SS1P in an antimesothelin immunotoxin. Two of 2 patients (100%) with epithelioid peritoneal mesothelioma who were treated with SS1P exhibited tumor shrinkage (44% and 70%) after treatment.7 In vitro studies of amatuximab (MORAb-009, Morphotek, Exton, Pennsylvania), an antimesothelin antibody, showed antibody-dependent cell cytotoxicity in mesothelin–positive cells. In phase I trials, a number of patients with pleural and peritoneal mesothelioma had stable disease.14 A phase II trial of a combination regimen of pemtrexed and cisplatin plus amatuximab in patients with epithelial or biphasic pleural mesothelioma with low sarcomatous content were suggestive of enhanced antitumor effects.15 Our findings highlight the frequency of mesothelin immunoreactivity in various peritoneal mesothelioma subtypes. In our study, mesothelin, although less likely to be positive in biphasic disease, was still seen in this mesothelioma subtype.

Several studies have been published on the expression of estrogen receptor in mesothelioma. The anti–ER 1D5 used in this study did not recognize ER–β, previously found nuclearily expressed in 33 of 42 (79%) malignant peritoneal mesotheliomas, with cytoplasmic expression in 9 (21%) cases.16 ER–α is rarely expressed in mesothelioma, with most studies showing expression to be absent in both pleural and peritoneal disease.5,31,32 Studies show ER–α at a maximum rate of 10% (4 of 42)16,33,34 in peritoneal mesothelioma, in female patients only, with one exception of a male patient.16 Here, we present 2 of 84 patients (2%) who tested positive for ER (ER–α). Both patients were women. One patient had the epithelioid subtype and the other had the biphasic subtype. Similarly, PR is generally reported as negative in peritoneal mesothelioma.5,31,32,34 Here, we present 5 of 71 patients (7%) with positive immunoreactivity to PR.

Few studies have reported on p63 in peritoneal mesothelioma. p63, a tumor protein 53–related gene product, is generally positive in adenocarcinoma and squamous cell carcinoma. A 2006 study18 published data on 2 of 30 pleural malignant mesotheliomas (7%) displaying p63 positivity. In our study, tests from 3 of 62 patients (5%) with peritoneal mesothelioma were p63+.

CONCLUSIONS

The results show that, among mesothelial markers, calretinin and WT1 are the most sensitive. PAX8 may be useful in distinguishing mesotheliomas from gynecologic and renal malignancy but it stains positive at a low rate in peritoneal mesothelioma. A similar observation can be made for ER and p63, which are also useful as part of a panel, but do show low rates of immunoreactivity. These stains cannot exclude peritoneal mesothelioma as single markers. Immunoreactivity for mesothelin may have a role as a predictive marker in immune targeting. BAP1 loss is described in a large cohort of malignant peritoneal mesothelioma and confirms a 55% rate of loss, which underscores that retention of BAP1 in 45% of peritoneal mesothelioma limits the significance of this marker to BAP1 cases.

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