Evaluation of 12 antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma: identification of a three-antibody immunohistochemical panel with maximal sensitivity and specificity

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We evaluated the sensitivity and specificity of 10 monoclonal and two polyclonal antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma (AdCA) using immunohistochemistry (IHC). The antibodies were directed against the mesothelial-associated antigens mesothelin, calretinin, cytokeratin 5, thrombomodulin, Wilms' tumor-1 (WT-1) gene product and HBME-1, and the nonmesothelial antigens Lewis-Y blood group (antibody BG8), MOC-31, BerEp4, CD15, and carcinoembryonic antigen (CEA) family. The 133 tumors evaluated included 65 malignant epithelioid mesotheliomas, 22 lung AdCAs, 27 ovarian serous carcinomas, 24 breast carcinomas, and five gastric carcinomas. Diagnoses were based on clinical, histologic, ultrastructural, and/or IHC findings. Calretinin had the best sensitivity for mesothelioma (95%), followed by HBME-1 (84%), WT-1 (78%), cytokeratin 5 (76%), mesothelin (75%), and vimentin and thrombomodulin (68%). Thrombomodulin had the best specificity for mesothelioma (92%), followed by cytokeratin 5 (89%), calretinin (87%) vimentin (84%), and HBME-1 (45%). When ovarian carcinomas were excluded from the analysis, the specificity of mesothelin and WT-1 for the diagnosis of mesothelioma increased to 90 and 81%, respectively. The sensitivity of the nonmesothelial antigens for AdCA was organ dependent, with BG8 performing best in the breast cancer group (96%), and BerEp4, BG8, MOC-31 performing best in the lung cancer group (100%). The specificity of the nonmesothelial antigens for AdCA was 98% for BG8 and CEA, 97% for CD15, 95% for BerEp4, and 87% for MOC-31. A novel statistical analysis technique employing logic regression analysis identified a three-antibody immunohistochemical panel including calretinin, BG8, and MOC-31, which provided over 96% sensitivity and specificity for distinguishing epithelioid mesothelioma from AdCA.
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The histologic distinction between malignant epithelioid mesothelioma (MEM) and adenocarcinoma (AdCA) is often difficult and requires ancillary studies. For many years, electron microscopy was the preferred ancillary method in this differential diagnosis. Typical ultrastructural features of MEM include long slender microvilli devoid of gylocalyx over much of the cell membrane, and aberrant microvilli projecting through deficiencies in the basal lamina.[1,2] However, these features are often absent in the less-differentiated MEM.

The development of antibodies to molecular markers of tissue differentiation has enabled immunohistochemistry (IHC) to supplant electron microscopy in distinguishing MEM from AdCA. Initially, antibodies to antigens commonly expressed by AdCA, such as carcinoembryonic antigen (CEA), LeuM1 (CD15), BerEp4, and B72.3 were used to distinguish MEM from AdCA. Thus, the diagnosis of MEM was based on the absence of expression of

these proteins, an unsatisfying approach. More recently, several antigens expressed on mesothelial cells have been described. These proteins include calretinin,[3,4] Wilms' tumor gene product (WT-1),[5,6] mesothelin,[7,8] cytokeratin 5,[9,10] HBME-1,[11,12] and thrombomodulin.[3,13]

Calretinin, a calcium-binding protein widely expressed throughout the central and peripheral nervous systems,[14] is also expressed by mesothelial cells and their tumors. Like the structurally related S-100 protein, calretinin is expressed in both the nucleus and cytoplasm. The earliest publications on calretinin reported low expression in MEM, but these studies employed a relatively insensitive antibody raised against guinea pig calretinin.[11] Although Doglioni et al[3] suggested that calretinin was mesothelium specific, subsequent studies have demonstrated its expression in a small but significant fraction of AdCAs.[4,11]

WT-1 is a DNA-binding protein predominantly located in the nucleus that plays a critical role in the development of the genitourinary tract. The Wilms' tumor gene is thought to represent a tumor suppressor gene; unlike more familiar tumor suppressor genes like p53, however, it appears to be operative only in selected cell types. In adult tissues, it is expressed by mesangial cells of the kidney, Sertoli cells of the testis, ovarian stromal cells and surface epithelium, mesothelial cells, and some smooth muscle and other stromal cells in the female genital tract (eg, myometrium, endometrial stromal cells). WT-1 is also expressed by MEM and, to a lesser extent, sarcomatoid mesothelioma,[5,15] as well as tumors derived from the ovarian surface epithelium, which may be considered a modified mesothelium. Recent studies have confirmed the specificity of WT-1 for mesothelioma,[16] but some studies have reported a relatively low sensitivity.[17,18]

Cytokeratin 5 is expressed by normal mesothelium, squamous and transitional epithelia, and myoepithelial cells.[9,10,19–22] It is often expressed by MEM and squamous cell carcinoma,[23] and can be an excellent marker for either one of these neoplasms.[9,10] However, cytokeratin 5 is also expressed by a significant proportion of breast carcinomas.[24]

Mesothelin was originally known as the CAK1 antigen identified by the antibody K1.[7,8,25,26] It is a 40 kD cell surface protein that may be involved in cell–cell adhesion, and can now be identified by 'second generation' antimesothelin antibodies such as the 5B2 clone. This protein is highly specific for mesothelial cells and surface ovarian epithelial cells, and possibly pancreatic ductal carcinomas,[27] and demonstrates a thick, linear, cell surface immunostaining pattern.

The following antigens are commonly expressed by AdCA, but not MEM: CEA family,[28,29] CD15 (LeuM1)[29–31] epithelial glycoprotein (B72.3)[32,33] tumor glycoprotein (BerEp4),[34,35] epithelial glycoprotein (Bg8),[11] E-cadherin,[18] and tumor glycoprotein (MOC-31).[17,36]

The availability of antibodies to the above-mentioned antigens has led to numerous publications with a wide spectrum of reported antibody performances. Given the large number of potential antigens one can evaluate to distinguish MEM from AdCA, there is clearly a need for critical evaluation of these antigens and their cognate antibodies, to establish a hierarchy of antigen/antibody pairs in terms of sensitivity and specificity in the diagnosis of MEM and AdCA. Our study was designed to ascertain the sensitivity and specificity of each of these mesothelial markers and, using receiver operating characteristic (ROC) curve analysis, to identify an immunodiagnostic panel ideally suited for differentiating between MEM and AdCA.

Materials and methods

IHC Methodology

A total of 133 tumors were evaluated for the study, including 65 malignant epithelial mesotheliomas, 22 lung AdCAs, 27 ovarian serous carcinomas, 24 breast carcinomas, and five gastric carcinomas. Diagnoses had been made on clinical, histologic, ultrastructural, and/or IHC findings by one of the pathologists involved in this study. For each tumor, 4–6 μm sections were cut, and one hematoxylin- and eosin-stained section was prepared. Deparaffinized sections were immunostained with the 13 antibodies listed in Table 1. Of these, 12 antibodies were of interest for the study, while the 13th antibody (antipancytokeratin monoclonal antibody cocktail AE1/AE3) was used to confirm the antigenicity of the tissue. Note that these immunostains were performed in addition to any that had been performed to aid in the initial diagnosis of the case. The specific heat-induced epitope retrieval and detection methods (eg, avidin–biotin–peroxidase complex vs Envision™ plus polymer) were selected based on prior optimization steps for each antibody. For details of the antibody clones, dilutions, and pretreatment see Table 1.

The immunostained slides were simultaneously reviewed and scored by at least three of the pathologist participants and independently reviewed by all participants. Discrepancy in scoring was very infrequent ($<2\%$ of slides), and was resolved by a consensus review of all sections. A semiquantitative scoring system was used, reflecting the percentage of positive tumor cells to the nearest 10%. For the sake of statistical analysis, the percentage cutoff for positive results was adjusted using several options including binary results (see below). Results for each antibody were tabulated from each of the 133 cases examined.

Table 1 Antibodies used in this study

Antibody	Clone	Source	Dilution	Pretreatment
Calretinin	5A5	Novocastra	1:50	8 min in pressure cooker, citrate buffer pH 6.0
Mesothelin	5B2	Novocastra	1:250	8 min in pressure cooker, citrate buffer pH 6.0
WT-1	Polyclonal	Santa Cruz	1:1000	8 min in pressure cooker, citrate buffer pH 6.0
Anti-human mesothelial cell	HBME-1	DAKO	1:250	8 min in pressure cooker, citrate buffer pH 6.0
Thrombomodulin	1009	Novocastra	1:250	Steam × 20 min in citrate+10 min in pronase
Cytokeratin 5/6	D5/16B4	Chemicon	1:2000	Steam × 20 min in citrate+10 min in pronase
Vimentin	V9	DAKO	1:1000	Steam × 20 min in EDTA pH 8.0
Epithelial glycoprotein	MOC-31	DAKO	1:50	Steam × 20 min in citrate pH 6.0
Epithelial glycoprotein	BER-EP4	DAKO	1:250	Protease XXIV × 5 min
Epithelial glycoprotein (BG8)	F3	Signet	1:250	Protease XXIV × 5 min
CEA	Polyclonal	DAKO	1:4000	Steam × 20 min in citrate pH 6.0
CD15	MMA	Becton Dickinson	1:50	Steam × 20 min in citrate+10 min in pronase
Pan-cytokeratin (to confirm antigenicity)	AE1/AE3	Chemicon	1:000	8 min in pressure cooker, citrate buffer pH 6.0

Statistical Analysis

Initially, univariate analysis (Wilcoxon rank-sum test) was used to determine whether the individual antibodies could distinguish MEM from AdCA. ROC curves were then generated for each antibody. ROC curves are a graphical representation of the relationship between sensitivity and specificity over all possible diagnostic cutoff values. The area under the curve (AUC) in ROC analysis was used to compare the ability of an antibody to discriminate between mesothelioma and AdCA, allowing for visual identification of the antibodies with the greatest discriminatory power.

Multivariate statistical analysis was subsequently performed to identify the value of biomarker combinations.[37] An adaptive binary regression method, logic regression (LR),[38,39] was chosen over classical logistic regression because LR is more appropriate for combining binary predictor[38–42] LR methodology, which has been previously used for combining biomarkers[41–44] produces 'and' and 'or' (Boolean) classification rules to identify combinations of variables best able to predict a certain outcome, and are easily interpreted by clinicians. In this study, we sought the combination of antibodies best suited to distinguish MEM from AdCA. Specifically, LR analysis was performed on a randomly selected series representing 50% of all tumors (the training data set). LR was then employed on the data from the remaining 50% of tumors (the validation data set), in an effort to confirm the results from the training set.

Results

As illustrated in Figure 1, the mesothelium-associated antigens demonstrated the following cellular localization patterns: nuclear (WT-1, Figure 1a); cytoplasmic (cytokeratin 5/6, Figure 1b and vimentin (not shown); combined nuclear and cytoplasmic (calretinin, Figure 1c); and membranous (meso-

thelin, Figure 1d, thrombomodulin Figure 1e, and HBME-1, Figure 1f). The AdCA-associated antigens demonstrated the following cellular localization patterns (data not shown): membranous (BerEP4, CD15, MOC-31) and cytoplasmic (Bg8, polyclonal CEA).

Univariate statistical analysis using the Wilcoxon rank-sum significance test, a nonparametric statistical test, was performed to test the ability of each of the antibodies to distinguish MEM from AdCA (see Table 2, column 2). With the exception of the pan-cytokeratin cocktail AE1/AE3, which was only used to confirm that the tissues were antigenically intact, all 12 antibodies demonstrated a highly significant ability ($P < 0.001$) to distinguish MEM from AdCA, as indicated in the second column of Table 2. Further analysis of specific subtypes of AdCA was not undertaken because of the relatively small numbers of carcinomas of each organ type.

The relative ability of each antibody to distinguish MEM from AdCA was then formally determined by performing ROC curve analysis (see Figure 2). ROC curves demonstrate the coordinate variation in sensitivity (shown on the Y-axis) and specificity (1-specificity is shown on the X-axis) of a test as the threshold for defining test positivity varies over the entire range of possible test outcomes. Elevating the threshold will improve specificity but will sacrifice sensitivity, and vice versa. In this study, since antigen expression was determined to the nearest 10% of tumor cells positive, thresholds for defining positive antigen expression could vary from 0 to 100% in increments of 10%. If one considers the mesothelin ROC curve as an example, this curve is flat along the horizontal axis until a false-positive rate of about 15% (1-specificity = 0.15, at left part of curve), indicating that when the threshold of mesothelin positivity used to define MEM is set high, to allow very high specificity, this marker suffers from very low sensitivity. In contrast, when the threshold of mesothelin positivity used to define MEM is
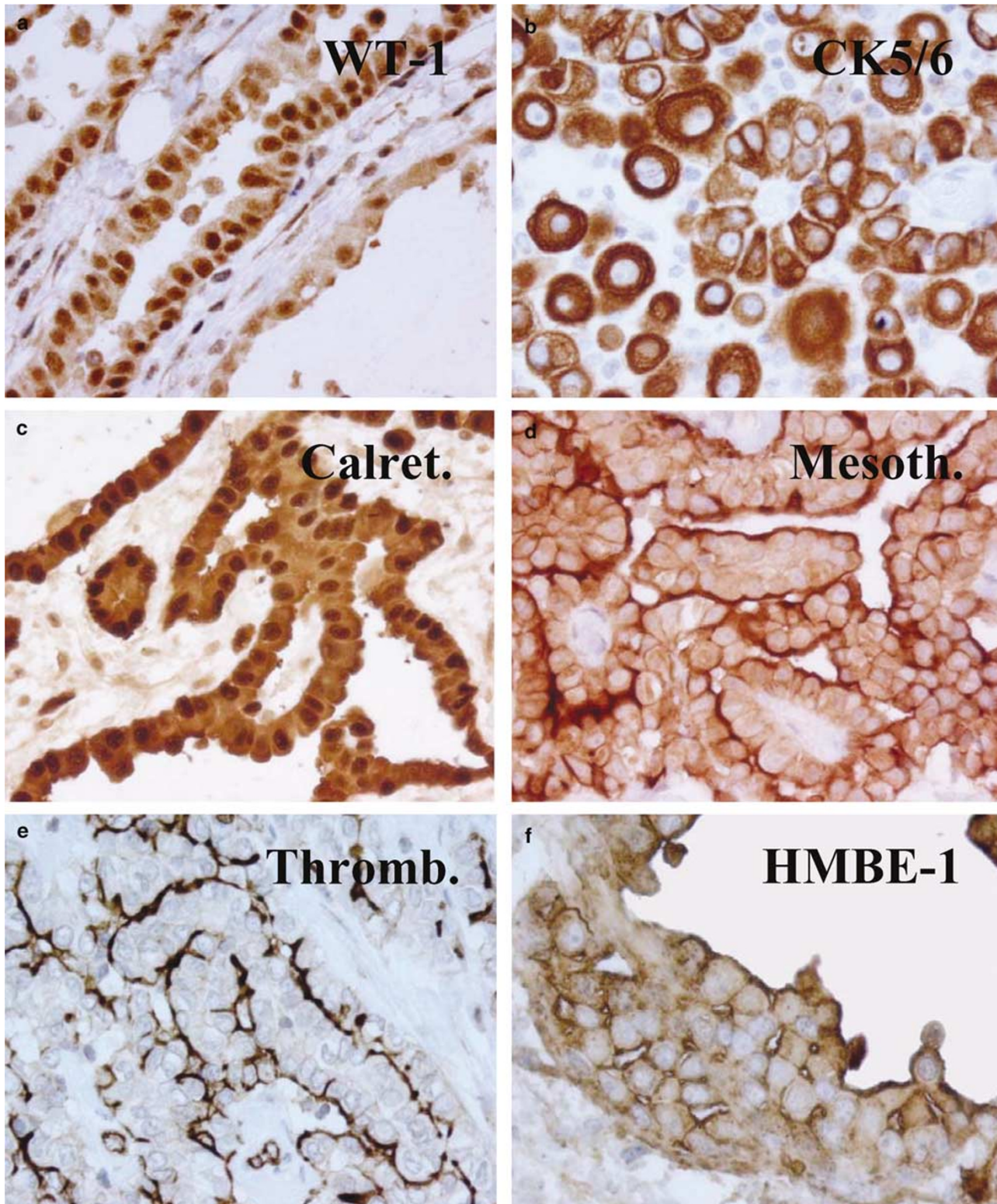
Figure 1 Summary of immunoreactivity patterns on MEMs. Immunohistochemical evaluation of a representative malignant epithelioid mesothelioma, demonstrating expression of the following antigens according to cellular localization pattern: nuclear (WT-1, **a**); cytoplasmic (cytokeratin 5/6, **b**); combined nuclear and cytoplasmic (calretinin, **c**); and membranous (mesothelin, **d**; thrombomodulin, **e**; and HBME-1, **f**).

lowered somewhat, the sensitivity first increases steeply with only a slight loss in specificity (steeply rising portion of curve), until sensitivity reaches a maximum. Further lowering the threshold for mesothelin fails to improve sensitivity, with a progressive decrease in specificity.

The area under the ROC curve (or AUC) represents an optimal summary statistic for comparing the sensitivities and specificities of the 12 antibodies (see Table 2, column 3). The most intuitive way to interpret the AUC is to think of it as an average sensitivity of the marker (averaged over the possible specificities). Therefore, tests with a higher AUC are typically considered superior to tests with a lower AUC. A perfect test/marker has AUC of 1.0, and a noninformative test/marker has an AUC of 0.5. The AUC values are listed in the third column of Table 2, while the actual ROC curves appear in Figure 2. Note that calretinin had the greatest AUC, by far, among the MEM-associated antibodies, while Bg8 and MOC-31 clearly had the greatest AUCs among the AdCA-associated antibodies.

In Table 3a, the sensitivities and specificities for each of the MEM-associated antibodies was determined for the diagnosis of MEM, while in Table 3b the sensitivities and specificities for each of the AdCA-associated antibodies was determined for the diagnosis of AdCA. In both cases, using a fixed positive cutoff of 10% antigen-positive tumor cells

Table 2 Summary of discriminatory abilities of each marker in univariate analysis

Marker	Wilcoxon rank-sum test (P-value)	ROC curve analysis (area under curve)
Mesothelin	<0.001	0.613
Calretinin	<0.001	0.906
Ck5/6	<0.001	0.722
WT-1	<0.001	0.637
Thrombomodulin	<0.001	0.662
HBME-1	<0.001	0.679
Vimentin	<0.001	0.622
Bg8	<0.001	0.956
BerEP4	<0.001	0.745
CD15	<0.001	0.531
Polyclonal CEA	<0.001	0.631
MOC-31	<0.001	0.899

Table 3 Sensitivity of specificity of each marker, using a fixed 10% positive cutoff

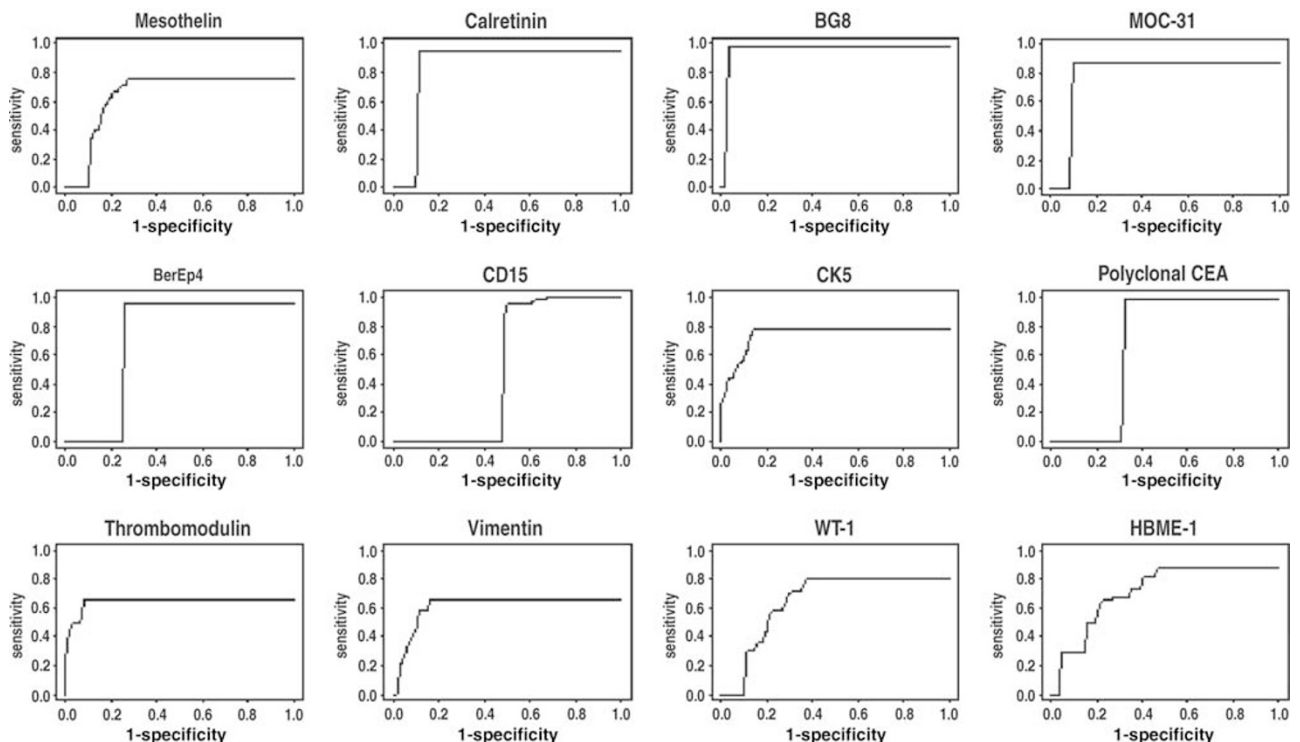| For Dx. of mesothelioma	Sensitivity	Specificity	Specificity excluding ovarian tumors
Mesothelin	0.75	0.71	0.9
WT-1	0.78	0.62	0.81
Calretinin	0.95	0.87	
Cytokeratin 5/6	0.76	0.89	
Thrombomodulin	0.68	0.92	
HBME-1	0.84	0.48	
Vimentin	0.69	0.84	
For Dx. of adenocarcinoma			
BG8	0.95	0.98	
BerEP4	0.74	0.95	
CD15	0.51	0.97	
CEA	0.63	0.98	
MOC-31	0.92	0.87	

Figure 2 ROC curve analysis for each of the 12 antibodies evaluated.

Mesothelioma Diagnosis is confirmed whenever neoplastic cells are:

BG8-negative   AND   either

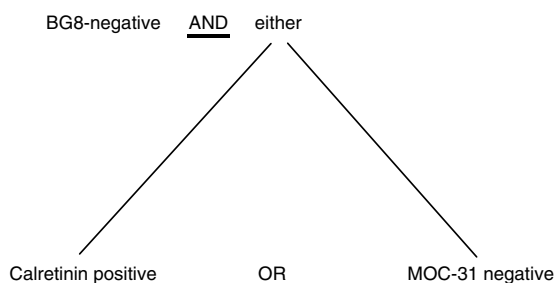Calretinin positive   OR   MOC-31 negative

Figure 3 Logic tree for discriminating MEM from AdCA. This approach correctly identified 63 of 65 MEMs, with no false-positive MEMs identified.

was used. The 10% positive cutoff was chosen because only a minimal number of the specimens in this study showed immunoreactivity on 0–10% of the tumor cells. Strikingly, the antibody to calretinin was over 90% sensitive for the identification of MEM *vs* AdCA, while the antibody to thrombomodulin was over 90% specific for the diagnosis of MEM *vs* AdCA (see Table 3). Not surprisingly, the specificity of the mesothelin and WT-1 antibodies for mesothelioma could be increased about 30% if ovarian serous carcinomas, which coexpress mesothelin and WT-1, were excluded from the analysis. In contrast to the MEM data, the antibodies to BG8 and MOC-31 were over 90% sensitive for the identification of AdCA *vs* MEM, while the antibodies to BG8, BerEP4, CD15, and CEA were all over 90% specific for the diagnosis of AdCA *vs* MEM.

Logic (not logistic) regression (LR), which uses Boolean 'and' and 'or' rules to develop a model for predicting an outcome of interest, was applied to determine the optimal combination of antibodies to distinguish carcinomas and mesotheliomas with few errors. In this analysis, 'or' rules improve sensitivity and 'and' rules improve specificity. Immunoreactivity for each antibody was treated as a dichotomous variable (positive *vs* negative depending on whether or not 10% of the tumor cells expressed the marker of interest). It has been shown that in such settings the general principle of using binary regression methods to combine markers is optimal,[37] and logistic regression is a binary approach suitable for dichotomous variables.[40,42]

LR analysis was first applied to the training data set of 50% of the cases. This analysis demonstrated that the number of antibodies required for optimal discrimination of MEM from AdCA could be reduced to three—BG8, calretinin, and MOC31—without decreasing the sensitivity or specificity to less than 96% (only one mesothelioma case was incorrectly classified as AdCA). Specifically, the combination of BG8 negativity with either calretinin positivity or MOC31 negativity demonstrated a sensitivity of 100% and specificity of 96% in confirming the diagnosis of mesothelioma (see Figure 3 for a schematic depiction of an algorithm for distinguishing MEM from AdCA based on this data). Both the training data set and the validation data set each failed to identify only one tumor as mesothelioma.

Discussion

Various tumors with epithelioid histology can affect the peripheral lung, pleura, and chest wall. These include epithelioid mesotheliomas, AdCAs, epithelioid nerve sheath tumors, melanomas, and pseudomesotheliomatous angiosarcomas.[45] By far, the most common tumors in this category are AdCA and mesothelioma. Therefore, our study focused on the most common differential diagnostic issue in this setting, namely, the distinction between epithelioid mesothelioma and AdCA involving serous surfaces.

There have been some excellent reviews of the immunohistochemical approaches to this problem.[1,11,12,46–52] The importance of using a panel of markers rather than a single one was well recognized in the early years of IHC.[53–55] Recent studies identified some antibodies with acceptable rates of specificity and sensitivity in distinguishing MEM from AdCA[3,4,10,16,22,35,56–59] although other antibodies have yielded suboptimal results.[60–63]

Two major strengths of our study were the utilization of a broad panel of antibodies to the most common markers of mesotheliomas and AdCAs, and the large number of cases evaluated. Selection of the specific antibodies was based on both evaluation of the literature[17,48,64–68] and on clinical experience in our laboratories. A few potentially useful markers, such as E-cadherin and N-cadherin, were omitted because of conflicting reports about their performance in distinguishing mesothelioma from AdCA.[57,69–72]

A third strength of our study was the detailed statistical analysis, which distinguishes this study from most previous studies in this area. Not only were all antibodies evaluated by univariate analysis to determine their abilities to distinguish MEM from all AdCA (Table 2, column 2), but the relative utility of each antibody was quantified by ROC curve analysis (Table 2, column 3), the relevant sensitivities and specificities were calculated for each MEM- and AdCA-associated antibody (Table 3), and LR was applied to determine the optimal combination of antibodies for distinguishing MEM from AdCA.

The quality of a marker is determined by the extent to which sensitivity and specificity both remain high at the threshold set to define marker positivity. As shown in Figure 2, the AUC is a convenient measurement for comparing the

overall quality of markers. Calretinin is clearly the best marker for positively identifying MEM, in that it has the highest sensitivity, with relatively high specificity; HBME-1 achieves over 80% sensitivity in identifying MEM, but with a specificity of about 50%. While BerEP4, BG8, CD15, polyclonal CEA, and MOC-31 all are capable of identifying AdCA with high sensitivity, concurrent high specificity clearly makes BG8 and MOC-31 the optimal markers for positively identifying AdCA.

Importantly, the LR model identified three antibodies (BG8, calretinin, and MOC-31) capable of distinguishing MEM from AdCA with 100% specificity and 96% sensitivity when this model was applied to the validation data set. Since LR analysis was so effective at identifying optimal markers for distinguishing MEM from AdCA, this is likely to represent a general method for optimizing antibody panels to distinguish between other histologically similar entities.

Despite the power of LR as an analytical technique, it is critical to remember that the quality of any immunohistochemical panel to distinguish MEM from AdCA will be influenced by the choice of antigens evaluated, the choice of antibody clones, the type of tissue, and the gender of the patient. While the influence of the type of tissue (eg, surgical biopsy material vs cell block of cytological material vs autopsy material) on the quality of immuno-histochemical data is obvious, the importance of antigen and antibody choice is exemplified by a recent study[63] in which an anticalretinin antibody with relatively poor specificity was employed, and antibodies to sensitive and specific mesothelial-restricted proteins (eg, mesothelin, cytokeratin 5, WT-1) were not employed. Patient gender directly impacts the specificity of both mesothelin and WT-1 to distinguish MEM from AdCA, since both antigens are expressed by ovarian serous carcinomas. Therefore, we advocate the use of 'gender-neutral' panel, which includes calretinin instead of WT-1 and mesothelin. The combination of calretinin, BG8, and MOC-31 would seem to be applicable across most clinicopathologic scenarios.

There are several caveats regarding these findings. First, although tumor cells in fluids typically maintain their immunophenotypes, we did not confirm our classification algorithm in malignant pleural fluid effusions. Second, we did not confirm our classification algorithm among peritoneal mesotheliomas, although such mesotheliomas appear to have slightly different characteristics than pleural mesotheliomas.[73] Third, we did not study metastatic carcinomas to the pleura, but focused on primary tumors of the respective organs (lung, ovary, breast, stomach). While immunophenotypic fidelity of metastatic tumors compared to their origins has been documented,[74] it is possible that a particular antigen expressed at high level by a primary tumor could be lost or significantly reduced in the metastatic setting. Fourth, we did not specifically address the differential diagnosis of MEM vs other types of AdCA, including prostate, pancreatic, and renal cell carcinomas, although the latter issue was recently addressed.[75] Fifth, we did not evaluate monoclonal antibodies to D2-40 and podoplanin, which also appear to be highly sensitive for identifying MEM.[76–78] Note that D2-40, like WT-1 and mesothelin, also recognized ovarian serous carcinomas in one of these two studies,[76] and therefore does not appear to be highly specific for MEM. The two papers describing podoplanin in MEM and AdCA[77,78] suggest that it is highly specific for MEM. We are uncertain whether adding either D2-40 or podoplanin to the combination of BG8, calretinin, and MOC-31 will improve the ability to discriminate MEM from AdCA.

In conclusion, our study goes beyond most previous publications in this area, in the following ways: (a) we evaluated a particularly large number of markers, for which the immunohistochemical techniques were previously screened and optimized; (b) we evaluated a very large set of MEM and AdCA from numerous geographic locations; and (c) we used a variety of robust statistical methods[38–42] to design a cost-effective panel of only three monoclonal antibodies, without compromising sensitivity or specificity.

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