Use of a Panel of Markers in the Differential Diagnosis of Adenocarcinoma and Reactive Mesothelial Cells in Fluid Cytology

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Abstract

To evaluate the use of a panel of markers to differentiate adenocarcinoma and the reactive/inflammatory process in fluid cytology, we stained 29 formalin-fixed, paraffin-embedded cell blocks of effusion fluid from patients with metastatic adenocarcinoma and 24 cell blocks from patients with benign effusion with mucicarmine and antibodies to carcinoembryonic antigen (CEA), B72.3, and calretinin. Positive staining with CEA, B72.3, and mucicarmine was seen in 22 (76%), 20 (69%), and 18 (62%) adenocarcinoma cases, respectively. All except 1 adenocarcinoma was negative for calretinin. No benign cases were positive for B72.3 and mucicarmine. In 1 benign case, scattered epithelial cells demonstrated weak positivity for CEA. The majority of combinations were 100% specific for adenocarcinoma. The highest sensitivity (86%) for adenocarcinomas was achieved with the staining combination of negative for calretinin and positive for any adenocarcinoma marker (CEA, B72.3, or mucicarmine). No benign cases were positive for B72.3 and mucicarmine. In 1 benign case, scattered epithelial cells demonstrated weak positivity for CEA. The majority of combinations were 100% specific for adenocarcinoma. The highest sensitivity (86%) for adenocarcinomas was achieved with the staining combination of negative for calretinin and positive for any adenocarcinoma marker (CEA, B72.3, or mucicarmine). The use of a panel of markers that recognize adenocarcinoma and mesothelial cells is useful in the differential diagnosis between metastatic adenocarcinoma and the reactive/inflammatory process. The profile of positive staining with at least one of the adenocarcinoma markers and negative calretinin staining is highly specific and sensitive for identifying adenocarcinoma in fluid cytology.

The cytologic differential diagnosis of mesothelial cells and adenocarcinoma in fluid specimens can be challenging. Difficulties are often due to overlap in the morphologic features of mesothelial cells and carcinomatous cells. Various ancillary studies have been used to increase the diagnostic accuracy of cytology. Immunocytochemical analysis is the most commonly used “special” technique and often involves the use of a panel of antibodies. There is a whole body of literature examining the role of immunocytochemical analysis and the value of individual and panels of markers in fluid cytology. However, there are great differences of opinion about which markers should be included in the panel for routine clinical settings.

Markers, including carcinoembryonic antigen (CEA), epithelial membrane antigen, B72.3, Ber-Ep4, and Leu-M1, that are used commonly in the panel recognize molecules expressed by adenocarcinomas but not by mesothelial cells. Markers that recognize molecules expressed by mesothelial cells are disappointingly scarce and rarely used for diagnostic purposes because of their limited specificity or sensitivity. Calretinin, a calcium-binding protein, is expressed normally in neurons of the central and peripheral nervous system. Various investigators have shown that calretinin is a reliable and specific marker for cells of mesothelial origin in both histologic and cytologic preparations. The usefulness of a panel with markers of adenocarcinomas and mesothelial cells in fluid cytology has not been studied.

The purpose of the present study was to determine the value of a panel that consisted of 3 markers that are used commonly to identify adenocarcinoma (CEA, B72.3, and mucicarmine) and a mesothelial marker (calretinin) for...
distinguishing between reactive mesothelial cells and metastatic adenocarcinoma in effusion fluids. The immunostaining results of individual markers and various combinations were analyzed to identify the immunoprofile that would be most specific and sensitive in the differential diagnosis of these two entities in fluids.

### Materials and Methods

Formalin-fixed, paraffin-embedded cell blocks from 53 cytologic specimens of pleural, pericardial, and peritoneal fluids were retrieved from the files of the Department of Pathology, University of Alabama at Birmingham. These cytologic specimens consisted of 29 metastatic adenocarcinomas from 28 patients and 24 benign specimens from 24 patients without a history of malignancy. For 1 patient with breast carcinoma, 2 specimens were available; the specimens were obtained at different times. The primary sites of origin of the metastatic adenocarcinomas were as follows: breast, 3; gastrointestinal tract, 3; lung, 11; kidney, 1; and ovary, 10. In 34 fluid specimens (20 adenocarcinomas and 14 benign cases), a corresponding pleural, pericardial, or peritoneum (omentum) biopsy specimen was available. Previous surgical pathologic materials were available for review and comparison in the remaining 9 malignant fluid specimens. The remaining 10 benign fluid specimens were from patients with no known malignant neoplasms. H&E-stained sections of each cell block were evaluated for the presence of tumor.

Five-micrometer sections were cut and deparaffinized. Immunostaining was performed using a modified avidin-biotin peroxidase technique. Heat-induced epitope retrieval was accomplished by heating in a steamer for 10 minutes in a 0.01-mol/L concentration of citrate buffer, pH 6. The antibodies used are summarized in Table 1. Immunostaining was accomplished with a semiautomated staining machine (Ventana Medical Systems, Tucson, AZ). Appropriate positive and negative controls were included.

Cytochemical staining for mucicarmine was performed using the Mayer method. Briefly, 5-µm sections were deparaffinized, rehydrated, and incubated with Weigert hematoxylin and working mucicarmine solution for 7 and 30 minutes, respectively.

The stained sections were evaluated independently by 2 observers (E.C.K., D.C.C.) who were unaware of the original cytologic diagnosis and follow-up. The results of staining with CEA, B72.3, and mucicarmine were based on cytoplasmic staining, whereas calretinin results were based on nuclear staining of the atypical cells. The distribution of staining was scored as negative or positive. Positive staining was defined as staining in more than 1% of the atypical cells regardless of the intensity of the staining.

### Results

Table 2 summarizes the results of the staining for each marker. None of the benign cases were positive for B72.3 or for mucicarmine. In 1 benign case, scattered (<10%) epithelial cells demonstrated weak positivity for CEA. The reactive mesothelial cells showed strong immunoreactivity for calretinin in all benign cases. Positive immunostaining with CEA, B72.3, and mucicarmine was seen in 22 (76%), 20 (69%), and 18 (62%) malignant cases, respectively. All except 1 malignant case demonstrated negative staining for calretinin. The adenocarcinoma that showed positive staining with calretinin was a pelvic washing specimen obtained from a patient with FIGO (International Federation of Gynecology and Obstetrics) grade III endometrioid ovarian carcinoma. The patient also had histologic evidence of omental involvement. The cytologic specimen consisted of numerous papillary groups of atypical cells that were positive for calretinin and negative for B72.3, CEA, and mucicarmine. Compared with the primary ovarian tumor, the atypical cells present in the cell block preparation had a lower nuclear grade and appeared less pleomorphic. We concluded that the atypical cells in the cytologic specimen most likely were of mesothelial origin and that this false-negative case was due to a sampling error. Four cases of metastatic adenocarcinomas, including 1 renal cell carcinoma, 1 breast carcinoma, and 2 adenocarcinomas of lung, were negative for CEA, B72.3, and mucicarmine.

The sensitivity and specificity for individual and combinations of markers for the differential diagnosis

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### Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Clone</th>
<th>Dilution</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoembryonic antigen</td>
<td>Ventana, Tucson AZ</td>
<td>TF3H8-1</td>
<td>Prediluted</td>
<td>Heat-induced</td>
</tr>
<tr>
<td>B72.3</td>
<td>Ventana</td>
<td>B72.3</td>
<td>Prediluted</td>
<td>Heat-induced</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Zymed, San Francisco, CA</td>
<td>Polyclonal</td>
<td>Prediluted</td>
<td>Heat-induced</td>
</tr>
</tbody>
</table>
between metastatic adenocarcinoma and a benign process in fluid cytology are listed in Table 2. B72.3 and mucicarmine were both specific markers (100%) for adenocarcinoma, but their sensitivity was 69% and 62%, respectively. CEA showed a specificity of 96% for adenocarcinoma and a sensitivity of 76%. Calretinin was a specific and sensitive marker for mesothelial cells with a sensitivity and specificity of 96% and 97%, respectively.

The best 2-marker combination was CEA and calretinin. The staining combination of positive for CEA and negative for calretinin was 100% specific and 76% sensitive for metastatic adenocarcinoma. The positive and negative predictive values of this combination were 100% and 77%, respectively. Many combinations of 3 or 4 markers were 100% specific for adenocarcinoma. The highest sensitivity of 86% for adenocarcinoma was achieved with the

Table 2
Results of Staining for Individual Markers and Combinations

<table>
<thead>
<tr>
<th>Marker</th>
<th>No. (%) Positive Adenocarcinoma Cases (n = 29)</th>
<th>No. (%) Positive Benign Cases (n = 24)</th>
<th>Sensitivity for ACA (%)</th>
<th>Specificity for ACA (%)</th>
<th>Likelihood Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>One marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucicarmine+</td>
<td>18 (62)</td>
<td>0 (0)</td>
<td>62</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>B72.3+</td>
<td>20 (69)</td>
<td>0 (0)</td>
<td>69</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>CEA+</td>
<td>22 (76)</td>
<td>1 (4)</td>
<td>76</td>
<td>96</td>
<td>32</td>
</tr>
<tr>
<td>Calretinin+</td>
<td>1 (3)*</td>
<td>24 (100)</td>
<td>96†</td>
<td>100†</td>
<td>55</td>
</tr>
<tr>
<td>Two markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucicarmine+/calretinin–</td>
<td>18 (62)</td>
<td>0 (0)</td>
<td>62</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>B72.3+/calretinin–</td>
<td>20 (69)</td>
<td>0 (0)</td>
<td>69</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>CEA+/calretinin–</td>
<td>22 (76)</td>
<td>0 (0)</td>
<td>76</td>
<td>100</td>
<td>39</td>
</tr>
<tr>
<td>CEA+/B72.3+</td>
<td>17 (59)</td>
<td>0 (0)</td>
<td>59</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>Other combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calretinin–/CEA+ or B72.3+</td>
<td>23 (79)</td>
<td>0 (0)</td>
<td>79</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>Positive staining in 1 or more (CEA, B72.3, mucicarmine)</td>
<td>24 (83)</td>
<td>1 (4)</td>
<td>83</td>
<td>96</td>
<td>38</td>
</tr>
<tr>
<td>Positive staining in 2 or more (CEA, B72.3, mucicarmine)</td>
<td>18 (62)</td>
<td>0 (0)</td>
<td>62</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>Calretinin– and positive staining in 1 or more (CEA, B72.3, mucicarmine)</td>
<td>25 (86)</td>
<td>0 (0)</td>
<td>86</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Calretinin– and positive staining in 2 or more (CEA, B72.3, mucicarmine)</td>
<td>18 (62)</td>
<td>0 (0)</td>
<td>62</td>
<td>100</td>
<td>29</td>
</tr>
</tbody>
</table>

ACA, adenocarcinoma; CEA, carcinoembryonic antigen; +, positive; –, negative.
* Including the false-negative case.
† For reactive mesothelial cells.

Image 11A, A small group of reactive mesothelial cells showing nuclear atypia and cytoplasmic vacuolization (H&E, ×1,000).
B, Reactive mesothelial cells showing strong positive nuclear staining and membranous accentuation, resulting in a “fried egg” appearance (calretinin, ×1,000).
immunoprofile of negative for calretinin and positive for any adenocarcinoma marker (CEA, B72.3, or mucicarmine). The positive and negative predictive values of this combination were 100% and 86%, respectively, and the likelihood ratio was 50.

**Discussion**

Based on morphologic features alone, the cytologic differentiation of reactive mesothelial cells from adenocarcinoma can be difficult. Various cytologic features are characteristic of, but not specific for, mesothelial cells. For example, intercellular spaces (windows), commonly seen in cellular aggregates of mesothelial cells, also can be identified in 13% of cases of metastatic adenocarcinoma. Therefore, ancillary studies often are performed to assist in the differential diagnosis. Electron microscopy has been used for differentiating mesothelial cells from adenocarcinoma by demonstrating long, thin, “bushy” microvilli in the mesothelial cells. Availability of adequate material, sizable costs, and long turnaround times limit the universal implementation of this technique. Furthermore, not all mesothelial cells display the aforementioned characteristic ultrastructural features. The use of cytochemical stains such as mucicarmine also has been studied. In our experience as well as in that of others,
demonstration of intracytoplasmic neutral mucin production strongly favors adenocarcinoma over mesothelial cells. However, the usefulness of cytologic stains including mucicarmine is limited because they are generally insensitive. Fewer than two thirds of the adenocarcinomas in our study demonstrated neutral mucin production. In recent years, immunocytochemical analysis has contributed greatly to the differentiation between adenocarcinoma and mesothelial cells. Most of the available markers, such as CEA, CD15 (Leu M1), BerEP4, and B72.3, recognize molecules commonly expressed by adenocarcinomas but not by mesothelial cells. A diagnosis of metastatic adenocarcinoma is favored if the neoplastic cells demonstrate positive immunoreactivity for CEA, CD15, B72.3, or BerEP4, which are expected to be absent in mesothelial cells. However, these markers are not distributed uniformly among different types of adenocarcinomas. Positive staining with at least 2 of the adenocarcinoma markers would favor a diagnosis of adenocarcinoma. Therefore, a panel of 2 or more antibodies often is used to confirm (or rule out) the possible diagnosis of metastatic adenocarcinoma but cannot support a positive identification of mesothelial cells.

CEA is the most extensively studied and widely accepted marker for differentiating adenocarcinoma and mesothelial cells. It is an oncofetal glycoprotein, originally extracted from colonic carcinomas. Most studies reported a high detection rate for adenocarcinoma with CEA. In our study, CEA was positive in 76% of adenocarcinomas. On the other hand, nearly all cases of reactive mesothelial cells reported in the literature lacked CEA staining. We noticed focal and weak CEA immunostaining in 1 benign case. Several investigators have reported that CEA stained macrophages and other inflammatory cells owing to nonspecific cross-reactivity and that it has been associated with a 5% to 15% false-positive rate.

The monoclonal antibody B72.3 is generated with a membrane-enriched fraction of human mammary carcinoma and recognizes the glycoprotein TAG-72 that is expressed in a wide variety of adenocarcinomas. In our experience, B72.3 was expressed in 69% of adenocarcinomas, and it lacked affinity for reactive mesothelial cells. These figures are similar to those reported by other investigators. It has been reported that weak and focal immunoreactivity can be seen in rare cases of malignant mesotheliomas with both CEA and B72.3. Specific markers for mesotheliomas are scarce. Furthermore, their specificity and sensitivity are limited. Among the suggested specific mesothelial markers, the ME1 monoclonal antibody, which recognizes a molecule present on the surface of mesothelial cells, reacts with up to 40% of carcinomas. The K1 monoclonal antibody, generated by immunizing mice with the OV-CAR3 ovarian cell line, reacts with epithelial and biphasic mesotheliomas as well as 70% of nonmucinous ovarian carcinomas. Thrombomodulin, a transmembrane glycoprotein normally expressed by endothelial and mesothelial cells, stained two thirds of the mesotheliomas and more than half of the adenocarcinomas in effusion cytology. HBME-1, an antigen present on the microvillus surface of the mesothelial cell, was expressed by more than half of the adenocarcinomas. N-cadherin, a member of the cadherin family, was reported to be immunoreactive with 77% of reactive mesothelial cells, 35% of malignant mesotheliomas, and 48% of adenocarcinomas.

Calretinin, a 29-kd calcium-binding protein, belongs to a large family of EF-hand proteins. In its structure, it possesses a characteristic helix-loop-helix that acts as the calcium-binding site. It is expressed in central and peripheral neural tissues and is thought to have a key role in somatosensory transduction. Several investigators have demonstrated that calretinin is a sensitive and specific marker for both benign and malignant mesothelial cells. The biologic function of calretinin in mesothelial cells is unknown. In the present study, the reactive mesothelial cells of all benign cases demonstrated strong nuclear staining with calretinin, whereas all metastatic adenocarcinomas lacked positive nuclear staining to calretinin. However, several authors have reported scattered positive staining for calretinin in 5% to 10% of adenocarcinomas, including colonic and ovarian adenocarcinomas.

Our experience, as well as that of others, has shown that a panel of antibodies is most helpful for differentiating between adenocarcinoma and mesothelial cells in fluid specimens. The advantage of using a panel of markers is to improve sensitivity and specificity. We included calretinin, a marker that recognizes mesothelial cells, in our panel so that we did not need to rely on a conclusion reached after a series of negative stainings to rule out metastatic adenocarcinoma. The majorities of the combinations of markers had high specificity for diagnosing metastatic adenocarcinoma in fluids. The 2-marker combination with the highest sensitivity (76%) for recognizing adenocarcinoma was positive staining for CEA and negative for calretinin. The best combination with the highest sensitivity (86%) and specificity (100%) for adenocarcinoma was positive staining with any one of the adenocarcinoma markers (CEA, B72.3, or mucicarmine) and negative staining with calretinin. The opposite reaction, positive for calretinin and negative for all adenocarcinoma markers, was also highly specific (97%) and sensitive (100%) for reactive mesothelial cells and had a positive predictive value of 96% in the present study.

The use of a panel, which included markers that recognized adenocarcinoma as well as cells of mesothelial origin,
aided the differentiation between metastatic adenocarcinoma and reactive mesothelial cells. The profile of positive staining with any adenocarcinoma markers and negative staining with calretinin was specific and sensitive for recognizing adenocarcinoma in fluid cytology.

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