Usefulness of EMA, GLUT-1, and XIAP for the Cytologic Diagnosis of Malignant Mesothelioma in Body Cavity Fluids

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Key Words: Malignant mesothelioma; Cytology; Benign effusion; Epithelial membrane antigen; EMA; Glucose transporter-1; GLUT-1; X-linked inhibitor of apoptosis; XIAP

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Abstract

We compared the effectiveness of epithelial membrane antigen (EMA) with 2 newly described markers, X-linked inhibitor of apoptosis protein (XIAP) and an isoform of glucose transporter (GLUT-1), in the distinction between malignant mesothelioma (MM) and benign effusion (BE) in body cavity fluids. Immunohistochemical studies were performed on cell block sections from 35 cases of histologically confirmed MM and 38 BEs, using antibodies to EMA, XIAP, GLUT-1 (GLUT-1m, monoclonal; GLUT-1p, polyclonal). Results were graded based on the percentage of cells staining: negative (0%), 1+ (<25%), 2+ (25%-49%), 3+ (50%-74%), and 4+ (75%-100%). The performance of each marker was compared using receiver operating characteristic curve analysis. EMA demonstrated the best accuracy, with an area under the curve of 0.91 as compared with XIAP (0.67), GLUT-1m (0.74), and GLUT-1p (0.80). Based on these findings, EMA is a better marker than XIAP or GLUT-1 for the diagnosis of MM.

In histologic samples, a reliable distinction between benign and malignant mesothelial proliferations is dependent on the presence of unequivocal invasion to adjacent tissue by tumor cells. This distinction creates a challenge in small tissue samples or cytologic specimens. Although certain cytomorphologic features, such as an abundance of large clusters (“proliferation spheres”), cellular enlargement, marked nuclear atypia, and a high mitotic rate, have been associated with malignancy and provide clues to diagnosis, a definitive characterization often cannot be made by cytologic studies alone. On the other hand, body cavity fluid is a frequent and easy-access sample source. An accurate cytologic diagnosis, therefore, is highly desirable because a benign result can prevent an unnecessary invasive surgical procedure.

Immunohistochemical analysis has been extensively applied as an aid in the distinction between malignant mesothelioma (MM) and other tumors that can involve pleura and mimic MM, particularly metastatic adenocarcinoma. Despite extensive research, however, the value of immunohistochemical features, such as an abundance of clusters (“proliferation spheres”), cellular enlargement, marked nuclear atypia, and a high mitotic rate, have been associated with malignancy and provide clues to diagnosis, a definitive characterization often cannot be made by cytologic studies alone. On the other hand, body cavity fluid is a frequent and easy-access sample source. An accurate cytologic diagnosis, therefore, is highly desirable because a benign result can prevent an unnecessary invasive surgical procedure.

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malignant effusions and a spectrum of epithelial malignancies. Although they are not specific markers for MM, recent reports on histologic specimens have demonstrated their potential as markers of mesothelial malignancy. The purpose of our study was to evaluate the diagnostic usefulness of EMA, GLUT-1, and XIAP for the distinction between MM and benign mesothelial cells in cytologic samples.

Materials and Methods

Cases

The study was performed with institutional review board approval. Case reports of pleural and peritoneal effusions between 2000 and 2006 were retrieved from the files of the Brigham and Women’s Hospital (Boston, MA), and the medical record was reviewed for each case. From this cohort, 2 groups of cases were selected and formed the basis of our study. The first group consisted of malignant effusions from patients in whom the diagnosis of MM at that site was confirmed by histologic follow-up. The second consisted of benign effusions (BEs) of any cause in patients with no history of malignancy and with a minimum of 2 years (maximum, 6 years) of clinical follow-up. Adequate cellularity of mesothelial cells was required for inclusion in the study. Body cavity washings were excluded. H&E-stained cell block material was reviewed for each case to confirm adequate cellularity for immunohistochemical analysis.

Immunohistochemical Analysis

Antibodies for EMA (clone E29, DAKO, Cambridgeshire, England), XIAP (clone 48/hILP/XIAP, BD Biosciences, San Jose, CA), GLUT-1 (GLUT-1m [monoclonal], clone SPM498, Abcam, Cambridge, MA; GLUT-1p [polyclonal], rabbit polyclonal, DAKO) were evaluated. A 4-μm-thick section from paraffin-embedded cell block material was prepared for each case. The slides were deparaffinized and treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and heat-induced epitope retrieval was used for each marker except EMA (Table 1). Then slides were incubated respectively with anti-XIAP (dilution 1:250), GLUT-1m (dilution 1:200), GLUT-1p (dilution 1:200), and EMA (dilution 1:300) in tris(hydroxymethyl)aminomethane buffer at room temperature for 1 hour, except for the GLUT-1p studies, which were incubated overnight. An overnight incubation for anti-XIAP was also performed in a subset of cases, and no difference was noted in staining results compared with those seen with a 1-hour incubation. The slides were developed using the EnVision-Plus detection system (DAKO) with 3,3′-diaminobenzidine tetrahydrochloride as the chromogen.

In a subset of cases, additional immunohistochemical analysis was also performed for mesothelial marker WT-1 (dilution 1:100; DAKO) to highlight the mesothelial cell population. For the latter study, epitope retrieval was performed using a steamer (50 minutes) and EDTA solution. Detection was performed as for other markers.

The results were blindly reviewed and scored based on the percentage of cells staining using 5 categories: negative: 0% of cells staining; 1+, fewer than 25% of cells staining; 2+, 25% to 49% of cells staining; 3+, 50% to 74% of cells staining; and 4+, 75% or more of cells staining. The sensitivity and specificity were determined at different levels of positivity (≥1+, 2+, 3+, and 4+).

Statistical Analysis

Receiver operating characteristic (ROC) curve analysis was performed for each marker by using the SPSS computer program, version 6 (SPSS, Chicago, IL).

Results

A total of 73 cases were selected for study. The MM cohort (n = 35) included 22 men and 13 women with a mean age of 64.1 years (range, 48-80 years). In this cohort, 20 cases had an equivocal initial cytologic diagnosis (ie, “atypical” or “suspicious”), and 15 had a positive cytologic diagnosis of MM. The malignancies in this group consisted of 32 pleural and 3 peritoneal MMs, which included 28 epithelioid and 7 mixed epithelioid and sarcomatoid subtypes, all determined by histologic examination. The BE group (n = 38) included

Table 1

Antibodies, Sources, and Retrieval Conditions

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Dilution</th>
<th>Epitope Retrieval</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA</td>
<td>E29</td>
<td>1:300</td>
<td>None</td>
<td>DAKO, Cambridgeshire, England</td>
</tr>
<tr>
<td>XIAP</td>
<td>48/hILP/XIAP</td>
<td>1:250</td>
<td>50 min in steamer/EDTA*</td>
<td>BD Biosciences, San Jose, CA</td>
</tr>
<tr>
<td>GLUT-1p</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>Pressure cooker/DAKO retrieval solution</td>
<td>DAKO, Carpinteria, CA</td>
</tr>
<tr>
<td>GLUT-1m</td>
<td>SPM 498</td>
<td>1:200</td>
<td>30 min in steamer/EDTA</td>
<td>Abcam, Cambridge, MA</td>
</tr>
</tbody>
</table>

EMA, epithelial membrane antigen; GLUT-1, glucose transporter-1 (m, monoclonal; p, polyclonal); XIAP, X-linked inhibitor of apoptosis.

* Invitrogen, South San Francisco, CA.
21 women and 17 men with a mean age of 61.3 years (range, 30-84 years). The samples from the BE group consisted of 32 pleural and 6 peritoneal effusions.

The results of the immunohistochemical staining reactions are summarized in Table 2. Of 35 MMs, 15 (43%) showed positive staining with all 4 markers, of which 7 cases (20%) revealed 2+ or higher (≥25% cells) staining for each marker. The sensitivity and specificity at different levels of positivity are shown in Table 3. At the level of 2+ or higher (≥25% of cells stained), EMA, XIAP, GLUT-1m, and GLUT-1p demonstrated a sensitivity of 74%, 66%, 40%, and 67% and a specificity of 97%, 58%, 95%, and 74%, respectively. At a higher threshold (≥3+, ie, ≥50% of cells stained), the sensitivity values were 71%, 46%, 34%, and 51%, and the specificity values were 100%, 79%, 95%, and 92%, respectively.

Representative staining patterns for each marker are shown in Image 1. EMA and GLUT-1 demonstrated linear membranous positivity with focal cytoplasmic accentuation. In contrast, XIAP showed a particulate or nonhomogeneous cytoplasmic positivity. Because XIAP is also expressed in histiocytes, the addition of the mesothelial cell marker WT-1 was necessary for a subset of cases for the accurate identification of the mesothelial cells when histiocytes were present in abundance. GLUT-1 is highly expressed in RBCs, and, therefore, strong background staining was observed in some cases when abundant RBCs were present. GLUT-1m and GLUT-1p showed identical staining patterns but with different intensity. In general, the polyclonal antibody demonstrated stronger immunoreactivity but with more background staining compared with the monoclonal antibody.

An ROC curve was plotted to assess the efficiency of each marker in distinguishing malignant from benign mesothelial cells Figure 1. Overall, EMA demonstrated the best efficiency with the highest sensitivity and specificity at all levels of positivity (Figure 1). The area under curve for EMA was 0.91, significantly higher than that for XIAP (0.67) and GLUT-1m (0.74) (P < .05). Although EMA also showed a higher area under curve than did GLUT-1p, this difference did not reach statistical significance (P = .09).

Discussion

Anti-EMA, an antibody to a human milk fat globule membrane that is secreted by mammary epithelial cells has been proposed as a useful marker of malignancy in mesothelial proliferations.9,17-20 Previous studies have shown that strong staining for EMA is helpful in excluding reactive

**Table 2**

Summary of Immunohistochemical Results in Benign Effusions and Effusions Caused by Malignant Mesothelioma

<table>
<thead>
<tr>
<th>Marker/Staining</th>
<th>Malignant Mesothelioma (n = 35)</th>
<th>Benign Effusion (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EMA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>5 (14)</td>
<td>33 (87)</td>
</tr>
<tr>
<td>1+</td>
<td>4 (11)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>2+</td>
<td>1 (3)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>3+</td>
<td>6 (17)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4+</td>
<td>19 (54)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>XIAP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>6 (17)</td>
<td>15 (39)</td>
</tr>
<tr>
<td>1+</td>
<td>6 (17)</td>
<td>7 (18)</td>
</tr>
<tr>
<td>2+</td>
<td>7 (20)</td>
<td>8 (21)</td>
</tr>
<tr>
<td>3+</td>
<td>5 (14)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>4+</td>
<td>11 (31)</td>
<td>4 (11)</td>
</tr>
<tr>
<td><strong>GLUT-1m</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>13 (37)</td>
<td>31 (82)</td>
</tr>
<tr>
<td>1+</td>
<td>8 (23)</td>
<td>5 (13)</td>
</tr>
<tr>
<td>2+</td>
<td>2 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3+</td>
<td>8 (23)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>4+</td>
<td>4 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>GLUT-1p</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>6 (17)</td>
<td>24 (63)</td>
</tr>
<tr>
<td>1+</td>
<td>5 (14)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>2+</td>
<td>6 (17)</td>
<td>7 (18)</td>
</tr>
<tr>
<td>3+</td>
<td>5 (14)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>4+</td>
<td>13 (37)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

EMA, epithelial membrane antigen; GLUT-1, glucose transporter-1 (m, monoclonal; p, polyclonal); XIAP, X-linked inhibitor of apoptosis.

* Data are given as number (percentage). –, 0% of cells staining; 1+, <25% of cells staining; 2+, 25%-49% of cells staining; 3+, 50%-74% of cells staining; and 4+, 75%-100% of cells staining.

**Table 3**

Sensitivity and Specificity of EMA, XIAP, GLUT-1m, and GLUT-1p for the Effusions Caused by Malignant Mesothelioma

<table>
<thead>
<tr>
<th>% of Cells Staining/ Sensitivity and Specificity</th>
<th>EMA</th>
<th>XIAP</th>
<th>GLUT-1m</th>
<th>GLUT-1p</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1 (1+) Sensitivity</td>
<td>0.86</td>
<td>0.83</td>
<td>0.63</td>
<td>0.83</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.87</td>
<td>0.40</td>
<td>0.82</td>
<td>0.63</td>
</tr>
<tr>
<td>≥2 (2+) Sensitivity</td>
<td>0.74</td>
<td>0.66</td>
<td>0.40</td>
<td>0.67</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.97</td>
<td>0.58</td>
<td>0.95</td>
<td>0.74</td>
</tr>
<tr>
<td>≥5 (3+) Sensitivity</td>
<td>0.71</td>
<td>0.46</td>
<td>0.34</td>
<td>0.51</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1.00</td>
<td>0.79</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>≥75 (4+) Sensitivity</td>
<td>0.54</td>
<td>0.31</td>
<td>0.11</td>
<td>0.37</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1.00</td>
<td>0.90</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

EMA, epithelial membrane antigen; GLUT-1, glucose transporter-1 (m, monoclonal; p, polyclonal); XIAP, X-linked inhibitor of apoptosis.

* Interpretations for 1+ through 4+ are as follows: 1+, <25% of cells staining; 2+, 25%-49% of cells staining; 3+, 50%-74% of cells staining; and 4+, 75%-100% of cells staining.
Positive staining patterns for epithelial membrane antigen (EMA), glucose transporter-1 (GLUT-1), and X-linked inhibitor of apoptosis (XIAP) in effusions with malignant mesothelioma cells. A, H&E, ×40. B, EMA, ×40. EMA shows membranous positivity with focal cytoplasmic accentuation. C, H&E, ×40. D, GLUT-1p (polyclonal), ×40. GLUT-1 shows membranous positivity with focal cytoplasmic accentuation. E, H&E, ×40. F, XIAP, ×40. XIAP shows particulate and nonhomogeneous cytoplasmic positivity.
mesothelial cells, although focal and weak positivity has been reported. The sensitivity and specificity for distinguishing malignant from benign mesothelial cells, however, have been variable in previous studies.\textsuperscript{15,17,18,34} Most found that EMA was positive in the majority of effusions from patients with MM (70%-80%) and negative in reactive mesothelial proliferations.\textsuperscript{17-20} Other reports, however, have shown that reactive mesothelium can be positive for EMA in up to 70% of cases.\textsuperscript{15,17,34} These conflicting results might be attributed to differences in patient populations, scoring systems, and antibodies and antigen retrieval methods. The histologic subtypes of MM included in each study may also affect the results. EMA is most highly expressed in epithelioid mesotheliomas and rarely in the sarcomatoid subtype. Different antibody clones may also account for the conflicting results. Saad et al\textsuperscript{17} demonstrated a significant difference in EMA positivity using different clones. Clone E29 (DAKO, Cambridgeshire, England) had a sensitivity of 75% and specificity of 100% for MM, whereas clone Mc5 (DAKO), with comparable sensitivity (70%), showed much lower specificity (40%). By using the same clone E29 (DAKO), we obtained similar results: a sensitivity of 77% and a specificity of 97% when 25% or more of cells (≥2+) stained and a sensitivity of 71% and specificity of 100% when 50% or more of cells (≥3+) stained. The scoring system for evaluating the immunohistochemical results is an important variable that can be addressed in a variety of ways. By using ROC curve analysis, we were able to incorporate the data at several different thresholds of positivity and calculated a diagnostic accuracy of 0.91 for EMA. Thus, our results confirm that EMA, specifically clone E29 (DAKO), has very good specificity and moderate sensitivity for the distinction between benign and malignant mesothelial cells in body cavity effusions.

GLUT-1, a member of the glucose transporter isoform family, functions to facilitate the entry of glucose into cells. It is largely undetected in normal epithelial tissues and benign tumors but is expressed in a variety of malignancies.\textsuperscript{25-31} This is thought to be due to the adaptation in tumor cells to their increased metabolism and glucose utilization as a result of active cellular proliferation. GLUT-1 overexpression has been revealed in a variety of carcinomas, such as those of the head and neck, bladder, thyroid, lung, and kidney.\textsuperscript{25-30} Several studies have also demonstrated that GLUT-1 is a sensitive marker of malignancy in effusions caused by a variety of carcinomas.\textsuperscript{22-24}

Recently, GLUT-1 was proposed as a discriminator between reactive and malignant mesothelium on histologic specimens, with 100% sensitivity and specificity.\textsuperscript{32} Unfortunately, we failed to reproduce these results in our cytologic samples using the polyclonal or monoclonal antibody to GLUT-1. In the present study, strong positivity for GLUT-1 was observed in some MMs but not in others. The reason for the discrepancy between our results and previously published data is unclear. The polyclonal antibody we used is similar to that used in a previous study.\textsuperscript{32} The difference in specimen types (solid tissue vs fluid suspension) may account for the discrepancy. Exfoliated cells in fluid suspension may have altered glucose metabolism compared with cells attached to extracellular matrix.

Positivity for GLUT-1 was observed in a subset of benign effusions from patients with a variety of causes for their effusions, including acute myocardial infarction, congestive heart failure, and nephrotic syndrome. Although most false-positive cases demonstrate weak and focal immunoreactivity, 3+ or stronger staining was noted in a small number of cases (2 and 3 of 38 for GLUT-1m and GLUT-1p, respectively) (Image 2). The monoclonal antibody (GLUT-1m) showed higher specificity (82%-100%) than the polyclonal antibody, but its sensitivity ranged from 11% to 63% (Table 3), which is less than acceptable for clinical practice. Polyclonal antibody (GLUT-1p) improved sensitivity (37%-83%) (Table 3) and overall accuracy to 0.80 (Figure 1), and its performance was next to that for EMA on the ROC curve (Figure 1). Owing
to the strong staining of GLUT-1p in RBCs, however, we found that the interpretation of results can be difficult. For this reason, we believe that it is not an ideal marker to use on cell block material in which abundant RBCs are often present.

XIAP, a member of the inhibitor-of-apoptosis protein family, prevents apoptosis by specifically inhibiting caspases.\textsuperscript{35-37} Its expression, like the other proteins of this family, is often up-regulated to promote cell survival over death in tumor cells. Therefore, it is not a surprise that overexpression of XIAP can be found in malignant effusions from a variety of tumors.\textsuperscript{21}

The expression of XIAP, however, is not entirely specific for malignancy because it is also detected in various normal human tissues.\textsuperscript{38} Although its expression has been shown in MM, the frequency of expression has ranged from 50\% to 80\%.\textsuperscript{33,39} This variability has been attributed in part to the nature of the lesion and the anatomic site of the tumor. Kleinberg et al\textsuperscript{39} found that XIAP is more frequently expressed in effusions than in solid lesions and in peritoneal compared with pleural mesotheliomas. In their study, XIAP was positive in 54\% of pleural mesotheliomas compared with 77\% of peritoneal mesotheliomas. They also found that XIAP positivity is linked to a poor prognosis, presumably due to an associated chemoresistance.\textsuperscript{39} Only 3 peritoneal mesotheliomas were included in our cohort, which may explain the low sensitivity of XIAP in the present study. We found, however, that XIAP also showed low specificity. Positive

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image2}
\caption{Pleural effusion from a patient with acute myocardial infarction complicated by pulmonary hemorrhage and duodenal ulcer. \textbf{A}, H&E stain of cell block section shows reactive mesothelial cells (\times40). \textbf{B}, \textbf{C}, and \textbf{D}, These reactive mesothelial cells are positively stained (3\+: 50\%-74\% of cells) for glucose transporter (GLUT)-1p (polyclonal) (\textbf{B}, \times40), GLUT-1m (monoclonal) (\textbf{C}, \times40), and X-linked inhibitor of apoptosis (\textbf{D}, \times40).}
\end{figure}
staining was identified in a substantial subset of BE cases. These findings are in disagreement with the observations made previously by Wu et al., 21 who found only 1+ to 2+ positivity in 2 (6%) of 35 benign body cavity effusions and washes. In our study, approximately 60% of BEs demonstrated different degrees of positivity, and 21% showed 3+ or stronger staining. Despite additional efforts to modify our techniques according to the previous work, such as overnight incubation of antibody, there was no major improvement in the results. 21,33 The difference between the findings of the previous study21 and our study might be due to a difference in specimen types. We included only effusions but not washings; the latter may represent a significantly different physiologic condition with a different staining pattern for XIAP. It is known that overexpression of XIAP is a result of cellular adaptation to the microenvironment for the benefit of survival when access to oxygen and nutrients is important in cell cycle regulation. Although its expression, like that of GLUT-1, can be found in malignant cells, it may also be seen in reactive conditions under cellular stress. One such example is shown in Image 2, in which positive staining for XIAP and GLUT-1 was observed. This may be a result of cellular adaptation to altered metabolism by exfoliated mesothelial cells due to limited access to oxygen and nutrients. Consistently, the reactive changes (eg, enlarged nuclei, prominent nucleoli) were also demonstrated morphologically in this case (Image 2). As described by others, 21,39 we also noted nonspecific staining of XIAP in histiocytes and inflammatory cells, which may cause a false-positive interpretation. In our study, a marker of mesothelial differentiation, such as WT-1, was necessary in a subset of cases to confirm that we were indeed evaluating the staining results of mesothelial cells.

We have confirmed previous findings that EMA is a specific marker of malignancy for mesothelioma when staining is strong and diffuse. EMA is superior to 2 newly reported markers, GLUT-1 and XIAP, for the diagnosis of MM in body cavity fluids. GLUT-1 shows strong positivity in RBCs, and XIAP demonstrates nonspecific staining in histiocytes and inflammatory cells, and, therefore, they are less suitable for use in fluid samples in which abundant RBCs and histiocytes are often present on cell block material. Although EMA only shows moderate sensitivity in distinguishing malignant from reactive mesothelium, its specificity makes it a valuable and easy marker to use in body cavity fluids. An unequivocal diagnosis, however, may not be established solely on the basis of results with this marker. Correlation with conventional cytomorphologic findings, clinical data, radiologic evidence, other immunohistochemical marker studies, and cytogenetics is often necessary for a final, conclusive interpretation. When the other information and ancillary studies are inadequate, a tissue biopsy is needed to confirm the cytologic suspicion.

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References


