Original Article

Using EMA and Calretinin on Cell Blocks for the Differentiation of Reactive Mesothelial Cells from Adenocarcinoma Cells in Pleural Effusions

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ABSTRACT

Background: Effusion in body cavities can be considered as the presentation of malignancy or inflammatory conditions. Pleural fluid cytology is a popular diagnostic tool for the differentiation of adenocarcinoma cells (AC) from reactive mesothelial cells (RMC). However, there are many sources of controversies and errors in this technique that should be addressed.

Methods: This case-control study aimed to evaluate the use of immunohistochemistry markers, namely epithelial membrane antigen (EMA) and calretinin, on cell blocks to differentiate between RMC and AC in pleural effusions. Suspected malignant effusions were selected according to the clinical data and their equivocal cytological smears. A total of 80 samples corresponding to the fresh specimens sent from the Department of Internal Medicine to the Cytology Laboratory of Faghihi Hospital during Jan 2017-Feb 2018 comprised the case group. In addition, the control group entailed 80 non-malignant pleural samples with RMC.

Results: We observed that 74 (out of 80) effusion samples were strongly positive for EMA (92.5%). The sensitivity, specificity, and efficiency of the EMA marker were 92.5%, 95%, and 93.7%, respectively. The results of the calretinin assessment indicated 78 (out of 80) positive cases in the control group (97.5%). The sensitivity, specificity, and efficiency of calretinin staining were 97.5%, 98.7%, and 98.1%, respectively.

Conclusion: According to the results of the current study, EMA and calretinin are two reliable markers with acceptable accuracy in differentiating between RMC and AC.

Keywords: Adenocarcinoma, Calretinin, EMA, Pleural effusions

Introduction

Different malignant tumors, including carcinomas, sarcomas, and lymphomas, commonly cause a metastatic involvement of the pleura. Moreover, direct invasion of the pleura by primary lung tumors is frequent. Overall, 75% of malignant tumors originating from pleura are carcinomas (1). Malignant pleural effusion with adenocarcinoma cells (AC) in the pleural fluid is a challenging subject, and in some cases, it is challenging to differentiate them from reactive mesothelial cells (RMC) (2). Although pleural fluid cytology is a popular diagnostic tool to differentiate AC from RMC, there are many sources of controversies and errors that should be taken into consideration (3). Therefore, cytopathologist needs an accurate ancillary test to address such problems. Although several Immunocytochemistry (ICC) panels of markers have been suggested, there is no consensus on any panel as a standard (4).

No single marker has been able to differentiate RMC from AC. Most studies recommend a panel of antibodies to make a definitive diagnosis. Some investigations concluded that ICC markers MOC-31 and calretinin, as a limited panel, can be useful for differentiating RMC from AC in difficult cytologic smears. The results of these studies have revealed a high sensitivity for these markers (4, 5). Others have proposed epithelial membrane antigen (EMA) and calretinin as useful markers for distinguishing AC from RMC (3). The results of some studies have showed that claudin-4 is less frequently expressed in RMC and consequently may help differentiate AC

from RMC in pleural and peritoneal fluid cytology smears (6).

Immunohistochemistry (IHC) is applicable to both cytological smears and cell blocks. It seems that Hematoxylin and Eosin (H&E) staining of cell blocks could be useful in differentiating AC from RMC, particularly in combination with cytomorphology. Furthermore, the application of IHC on cell block sections is more accurate compared to smears (5, 6). Several studies recommend using a combined antibody panel that includes mesothelial and epithelial markers to distinguish mesothelial cells from adenocarcinoma cells in serous effusions (7). The goal of this study was to assess the usefulness of immunohistochemical markers for identifying malignant glandular epithelial cells and benign reactive mesothelial cells on sections of cell blocks prepared from pleural effusions. We, also, aimed to compare cytodiagnosis on cell blocks with cytodiagnosis on traditional smears.

Materials and Methods

This study evaluated the fresh specimens of pleura sent from the Department of Internal Medicine to the Cytology Laboratory of Faghihi Hospital affiliated to Shiraz University of Medical Sciences during Jan 2017-Feb 2018. All suspected malignant pleural effusions were selected according to the relevant clinical data and equivocal cytological smears. A total of 80 consecutive cases were investigated following excluding the specimens which did not meet the inclusion criteria. The cytology smears and histopathological H&E slides (Figure 1) of all patients were evaluated.
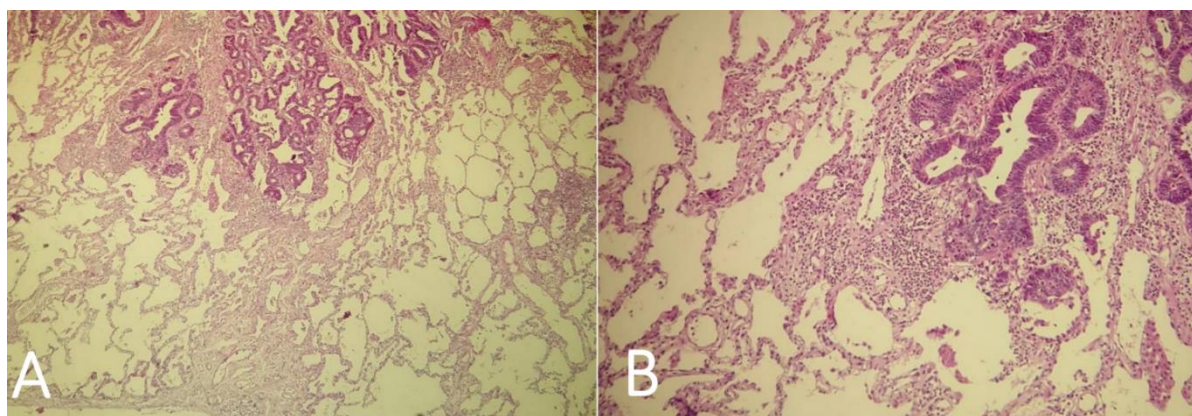

Figure 1. Hematoxyline and Eosine staining of lung adenocarcinoma. A: Lung adenocarcinoma lipidic pattern (×100). B: The neoplastic cells grow along the alveolar walls (×400).

Our investigation was conducted by two different cytopathologists with experience in diagnostic cytopathology. The fluid from half of the fresh samples was submitted for standard processing, and the other half was submitted for cell blocking using the plasma thrombin procedure. Following the initial diagnosis based on cytological data, the samples were subjected to cell block examination and immunohistochemistry analysis.

The histopathology report of adenocarcinoma was considered the gold standard test. The surgical biopsies of selected cases had definite histological evidence of primary or metastatic adenocarcinoma. All the 80 cases diagnosed as positive (56 cases, 70%) or suspicious (24 cases, 30%) for malignancy in cytology were confirmed by histological examination. They were reported as adenocarcinoma (51 cases, 64%) and metastatic adenocarcinoma (29 cases, 36%) of breast and stomach origin (Table 1). On the other hand, 80 cellular samples from the pleura with RMC that lacked cytomorphological, histological, and clinical evidence of adenocarcinoma or malignant mesothelioma were chosen as the control group. None of these samples had a pathological report of malignancy, and both of our cytopathologists reported all cases as negative.

Table 1. Distribution of the cases of pleural effusion cytology according to the histological diagnosis

Parameters	Diagnosis	No. of cases	Percentage
Benign	Congestive heart failure	37	46%
	Cirrhosis	21	26%
	Pneumonia	13	16%
	Collagen vascular disease	9	12%
	Total	80	100%
Malignant	Lung adenocarcinoma	51	64%
	Metastasis	29	36%
	Total	80	100%

We followed up the clinical diagnosis of these cases, and the results indicated congestive heart failure (37 cases, 46%), cirrhosis (21 cases, 26%), pneumonia (13 cases, 16%), and collagen vascular disease (9 cases, 11%). The results have been shown in Table 1. The exclusion criteria were (a) positive cases with any scanty malignant cells in their smears, (b) being positive for cytomorphology without confirmatory pathology report, and (c) containing a high number of mesothelial cells in positive effusion. Cell blocks were prepared for all case and control samples using the thrombin method. After the evaluation of cell blocks and H&E slides, 5 µm unstained samples were prepared for Immunohistochemical study.

Several studies recommended using at least two markers for carcinoma and two markers for mesothelial cells to reduce the number of false positive and false negative results. In addition, there are recommendations for using a panel of two antibodies, one for epithelial cells and the other for mesothelial cells, which is more cost-effective (1, 2). This study applied a panel of two antibodies, namely EMA (E29, BioGenex, USA) and anti-calretinin (DAK-Calret 1, Dako, Denmark), to differentiate RMC from AC. We used a mesothelioma sample as a positive control for calretinin staining. For IHC, sections of 2-4 microns in thickness from the paraffin embedded cell blocks were made and taken on Poly-L lysine coated slides. They were subjected to immunostaining using EMA and CAL by an indirect method employing rabbit polyclonal antibodies against CAL (Cell Marque dilution 1:500,) and monoclonal mouse anti-EMA antibodies (Cell Marque, dilution 1:300). A brown colored precipitate indicated a positive stain in the following manner:

1. Cells labeled with calretinin displayed nuclear and cytoplasmic staining.

2. Cells labeled with EMA showed cytoplasmic staining (with membranous accentuation).

In this case-control study, the student t-test was applied to compare the means of the two groups, and P-value < 0.05 was considered statistically significant. All cell blocks, as well as IHC results, were studied by a pathologist in a double-blind design. Finally, the sensitivity,

specificity, and accuracy of each marker were determined.

Results

The current study was conducted on 80 cell blocks of malignant effusions with adenocarcinoma and 80 negative cases as the control group. The specimens were taken from people aged 25-81 years with a median age of 60 years and a male to female ratio of 1:0.85. The results of the EMA assessment that showed strong cytoplasmic staining were defined as a positive result (Figure 2). We observed that 74 (out of 80) positive effusion specimens showed strong positivity for EMA (92.5%). In the control group, 4 (out of 80) cases showed positive results (Table 2). The expression of EMA was observed in isolated mesothelial cells in these cases. The sensitivity, specificity, and accuracy for the EMA marker were 92.5%, 95%, and 93.7%, respectively.
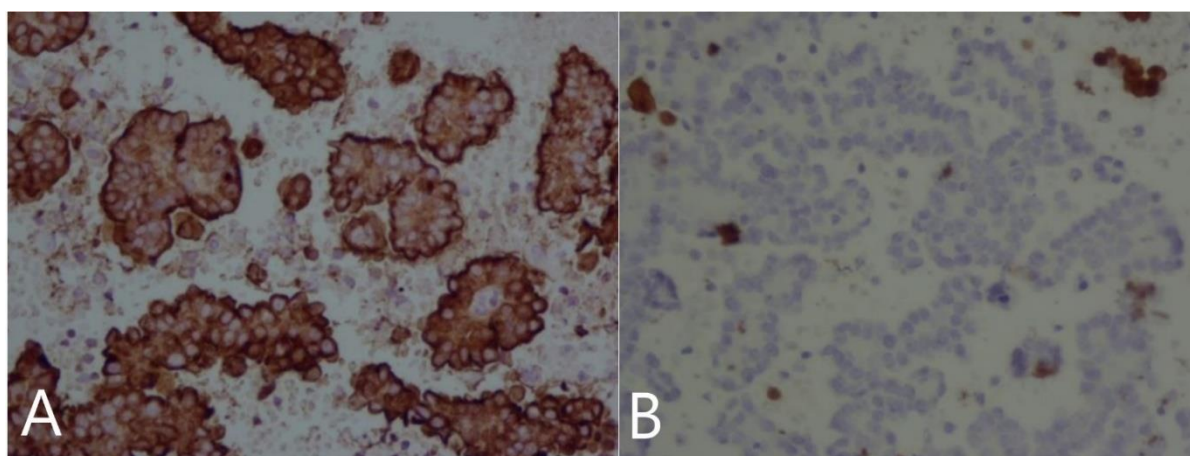

Figure 2. EMA and calretinin immunostaining of the same malignant cell block. A: EMA immunostaining. The clusters of malignant adenocarcinoma cells are strongly positive (×400). B: Calretinin immunostaining. The clusters of malignant adenocarcinoma cells are negative, whereas there are few strongly positive reactive mesothelial cells in the background (×400).

Table 2. Result of EMA and calretinin for pleural cell blocks

Histopathological diagnosis	EMA			Calretinin		
	Positive	Negative	Total	Positive	Negative	Total
Reactive mesothelial cells	4	76	80	78	2	80
Malignant cells	74	6	80	1	79	80

The results of calretinin evaluation demonstrated that in the control group, 78 (out of 80, 97.5%) specimens were positive for calretinin (Figure 3). Positive immunostaining for calretinin was noted only in one of the 80 malignant effusion cases. This case was diagnosed as poorly differentiated adenocarcinoma of the lung using an IHC panel. The sensitivity, specificity, and accuracy of calretinin staining were 97.5%, 98.7%, and 98.1%, respectively. In order to confirm the usefulness of any biomarker, sensitivity and specificity should be accompanied by a confidence interval. We calculated the confidence interval based on specificity, sensitivity, and sample size for each biomarker (Table 3). The study found that all cases between the two cytopathologists were in agreement. On standard cytological inspection of 80 confirmed adenocarcinoma on H&E slides, 56 instances (70%) of pleural fluid effusion were positive for malignancy, and 14 cases (30%) were identified as suspicions, which increased to 80 cases when cell block was used (100%).
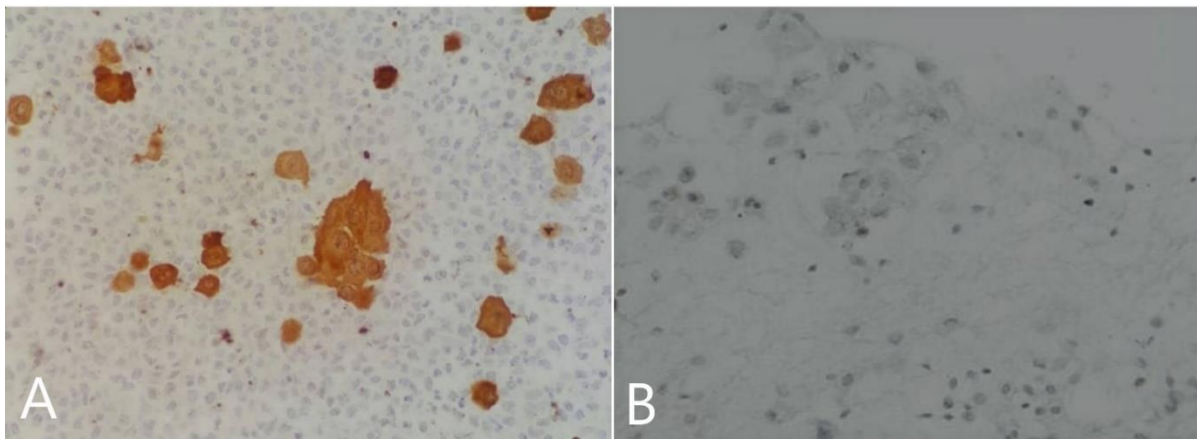
Figure 3. EMA and calretinin immunostaining of the same malignant cell block. A: Calretinin immunostaining of reactive mesothelial cells (×400). B: The clusters of reactive mesothelial cells are negative for EMA (×400).

Table 3. Sensitivity, specificity, accuracy, and confidence interval of EMA and calretinin tests on pleural cell blocks

	Sensitivity (%)	Specificity (%)	Accuracy (%)	Confidence interval
EMA	92.5	95	93.7	0.96-1.01
Calretinin	97.5	98.7	98.1	0.94-1.01

Discussion

Cytological diagnosis of malignant effusions is a routine practice all around the world. Evaluation of these specimens is sometimes challenging for cytopathologists due to the presence of RMC, which may be indistinguishable from cells shed from AC. The RMCs represent the three-dimensional clusters of cohesive cells with foamy cytoplasm, fine chromatin, and prominent nucleoli in morphology (1, 2).

The cell block is a simple and useful technique that helps to make a definite diagnosis in cytology smears. In cell block sections, the morphology of cells is similar to paraffin block sections. Cell block sections can be used for special stains and immunohistochemistry (4). As a result, sections from cell blocks and ICC can supplement cytological smears for definitive diagnosis in equivocal cases. We analyzed cell block preparations for immunostaining and used them to differentiate malignant effusions from reactive ones (2, 3).

Several studies utilized ICC analysis to differentiate between adenocarcinoma and mesothelial cells. However, there is no consensus on a unique panel of antibodies that can be used generally. Among various available markers, carcinoembryonic antigen, Leu M1, BerEP4, and B72.3 are expressed by adenocarcinoma but not by mesothelial cells. However, the expression of these markers was not diagnostic to determine the primary site of these malignancies and was not uniform among different types of adenocarcinoma (5, 7).

According to the general guidelines, when adequate tissue is available, it is recommended to use a panel of four antibodies, comprising of two positive and two negative ones, which yield reliable results. In cytology cases, the same approach is not applicable due to the scattered and limited neoplastic cells in cell blocks (8, 9). No single marker has been capable of differentiating RMC from AC. Many authors recommended using a panel of two antibodies, one positive for epithelial cells and the other positive for mesothelial cells, which is more cost-effective and results in an accurate diagnosis in most cases (10, 11). In this study, we used EMA and calretinin to differentiate between AC and RMC.

In the course of the current study, which lasted about one year, we did not come across any cases of malignant mesothelioma. However, the differentiation between RMC and mesothelioma could be made based on the cytological features. In the current study, staining with EMA and calretinin was performed on 80 cell blocks with adenocarcinoma (malignant effusions) and 80 negative cases in the control group. The sensitivity, specificity, and accuracy for the EMA marker were 92.5%, 95%, and 93.7%, respectively. The six malignant cases negative for EMA were later diagnosed as poorly differentiated adenocarcinoma of the lung using an IHC panel.For calretinin staining,

sensitivity, specificity, and accuracy were found to be 97.5%, 98.7%, and 98.1%, respectively. Mesothelial cells were observed in all benign effusions, while neutrophils with very occasional mesothelial cells were found in two cases, which calretinin was negative in both cases. The combination of these markers results in excellent accuracy for the differentiation of adenocarcinoma from reactive effusions.

Murugan *et al.* reported that EMA showed 100% sensitivity and 97% specificity for adenocarcinoma (12, 13). Yahya *et al.* found the sensitivity of calretinin for mesothelial cells to be 90%. These authors concluded 100% specificity and 96% accuracy, which are very similar to our findings (13). Likewise, other authors have reported a sensitivity of 90%-100% and specificity of 92%-98% for calretinin (3). Masanori Matsuda *et al.* reported the expression of calretinin in 307 consecutive cases of lung cancer evaluated immunohistochemically. Calretinin expression was identified in 15% of adenocarcinomas. They concluded that calretinin-positive lung tumors share common characteristics with those of advanced stages and smoker cases. These tumors can be differentiated from mesothelioma using other mesothelial and epithelial markers (14).

Consistent with the findings of some other investigations (15-21), the results of the current study showed that no single marker is capable of predicting the final diagnosis with 100% accuracy. However, a combination of calretinin and EMA presented excellent sensitivity and specificity. Therefore, a combination of at least two markers, one positive and one negative for epithelial cells, is suggested as the best approach for cytological cell blocks. The application of a large panel of antibodies may result in false reactions because of the overlapping immunoreactivity of several neoplasms (22-24).

In our investigation, cell block examination was helpful in confirming malignancy, and an increase in the sensitivity of diagnosis by cell block method was observed in cases that were reported as suspicious for malignancy by conventional smears. Bhanvadia VM *et al.* and Thapar M *et al.* found that using the cell block approach in addition to routine cytological examination increased diagnostic yield by 14 percent and 10%, respectively (6,10). In a similar study, Udasimath *et al.* looked at cell block sections of pleural fluid and were able to diagnose six more patients, increasing the diagnostic yield for malignancy by 14% (11,13).

The cell block method is a simple, safe, and inexpensive method that is useful in evaluating fluid cytology (7). In comparison to traditional smears, cell block smears showed enhanced cellularity and improved morphological details, such as preservation of architectural patterns such as three-dimensional clusters, better nuclear and cytoplasmic preservation, and an intact cell membrane (9). The cell block technique also has the advantage of reliably identifying histological patterns of disease that are difficult to detect in smear preparation (9,15).

A limitation of using immunochemistry in differentiating between reactive and malignant effusions is the false-positive and false-negative results of these antibodies, particularly in the case of poorly differentiated carcinomas. The other limitations entail technical errors and the subjective interpretation of the results.

Conclusion

According to the findings of the present study, the cytological examination of pleural fluid is a reliable, cost-effective diagnostic method. Supplementary techniques, such as cell block and ICC can be used to cross-check the final diagnosis. The ICC method with a panel of two markers is useful in confirming RMC or AC. Our research revealed that EMA and calretinin are two reliable markers with ideal specificity and sensitivity in differentiating between RMC and AC.

Disclosure Statement

The authors declare no financial or non-financial conflict of interests.

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Ethical and Legal Considerations

The research was performed following the World Medical Association Declaration of Helsinki. Written informed consent was taken from all patients and the healthy participants of the current study. The present investigation was approved by the Ethics Committee of Shiraz University of Medical Sciences, Iran (code: 1390.3010).

Acknowledgments

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