ORIGINAL ARTICLE

Diagnostic Accuracy of a Limited Immuno-panel of Calretinin and Ber-EP4 for Diagnosis of Malignant Effusions

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ABSTRACT

Objective: To differentiate between adenocarcinoma cells and reactive mesothelial cells (RMC) in serous effusions using a limited immuno-panel of Ber-EP4 and Calretinin.

Study Design: Descriptive study.

Place and Duration of Study: Department of Pathology, Allama Iqbal Medical College, Lahore, in collaboration with the Departments of Surgery, Pulmonology and Oncology, Jinnah Hospital, Lahore, from March 2015 to March 2016.

Methodology: Ninety-seven clinically and radiologically proven cases of peritoneal and pleural effusion and peritoneal wash of patients with suspicion of malignancy were included in the present study. Diagnostic accuracy of a limited immuno-panel of Calretinin and Ber-EP4 for diagnosis of malignant effusions was calculated using histopathology as gold standard.

Results: The sensitivity of Ber-EP4 for malignant cases was 98.6%, specificity 100%, positive predictive value (PPV) 100%, negative predictive value (NPV) 96%, and diagnostic accuracy 98.9%. Sensitivity of Calretinin as positive staining for RMC was 79.2%, specificity and positive predictive value (PPV) 100%, negative predictive value (NPV) 93.6%, and diagnostic accuracy 94.8%.

Conclusion: Limited immuno-panel of Calretinin and Ber-EP4 had a high positive and negative predictive value and is cost-effective in resource limited set-up for identification of adenocarcinoma cells and reactive mesothelial cells in challenging cases of serous effusions.

Key Words: *Reactive mesothelial cells, Adenocarcinoma cells, Serous effusion.*

INTRODUCTION

Primary malignant tumors of the mesothelium are uncommon as compared to involvement of serous membranes by secondary metastatic tumor deposits.[1] Among all malignant tumors, adenocarcinomas[2] are the commonest which involve serous membranes with resultant malignant or reactive effusions. Cytological examination of aspirated body cavity fluids for diagnosis of malignant cells is a mandatory diagnostic procedure for correct tumor staging.[3]

Reactive mesothelial cells (RMC) are invariably present in effusions.[4] These cells can have variable cytological appearance and may resemble neoplastic cells phenotypically.[5] In such cases, immunohistochemical markers are helpful in differentiating the reactive mesothelial *versus* malignant cells.[6] Most studies suggest an extensive antibody panel comprised of a combination of mesothelial and epithelial markers.[7,8] However, its application is not cost-effective. It remains non-feasible, therefore, for routine use in a resource limited setup.[9]

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In a developing country like Pakistan, there is a need to develop cost-effective diagnostic techniques. The present study was, therefore, designed to address this common problem of differentiating reactive and malignant exfoliated cells in clinically suspected malignant effusions by using a panel of two immuno-markers only.

The objective of this study was to evaluate the diagnostic accuracy of limited immuno-panel of two antibodies for discrimination of reactive mesothelial cells and malignant epithelial cells in effusions, Calretinin and Ber-EP4, respectively.

METHODOLOGY

Ninety-seven pleural and peritoneal effusion samples with provisional clinical diagnosis of benign effusion or suspected malignant effusion were collected from the outpatient and indoor departments of Surgery, Pulmonology and Oncology, Jinnah Hospital, Lahore.

The samples of pleural and peritoneal fluids and washings were received fresh in the Pathology Laboratory. The samples were examined for gross apperance, and findings were noted. Each sample was divided into two equal parts and transferred into two separate test tubes.

Test tube #1 was processed for cytological examination. It was centrifuged at 2000 revolutions per minute for 5 minutes. The supernatant was discarded. Smears

were prepared on glass slides from the deposit obtained after centrifugation. Minimum of two slides were prepared from each sample. One of these slides were air dried for Giemsa stain,[10] and other was fixed in ethanol for Hematoxylin and Eosin staining.[11]

Test tube #2 was processed for cell block preparation. For hemorrhagic effusions 1 to 2 drops of 1% glacial acetic acid was added for lysis of RBC. The sample was centrifuged for 5 minutes at 1500 rpm. Supernatant was discarded. The deposit was then fixed in 1:1 solution of 10% formalin and centrifuged for 10 minutes again at 2500 rpm. The sediment was left in test tube for overnight. Then further sample processing and hematoxylin and eosin (H&E) was done.[12]

The cell block slides were examined using the Olympus binocular microscope, CX-21. The scanner lens was used to examine the cellularity, architecture and pattern of the cells. Then low and high power objective lenses were used to examine the cytologic details to categorise the cell block as reactive, suspicious for positive malignant cells or positive for malignant cells. After that unstained slides were prepared from cell blocks and were subjected to immunohistochemistry (IHC) for confirmation of cell block diagnosis. For this purpose, two antibodies, Calretinin and Ber-EP4 were used. Calretinin is positive immunohistochemical marker for RMC and negative for adenocarcinoma cells while Ber-EP4 is positive marker for adenocarcinoma cells and is negative for RMC.[13]

Biopsy samples or surgically excised specimens of the suspected malignant cases were also received and were processed for histological examination. The cytological diagnosis of malignant cells was verified with the histopathological diagnosis on biopsy tissue and the immunohistochemistry results on cell blocks. For cases with provisional benign diagnosis clinic-radiological correlation and follow-up was used for verification. Study variables and information collected were entered into SPSS version 20.0 and analysed through its statistical programme. Immunohistochemical results were listed as positive or negative for presence or absence of adenocarcinoma cells and RMC. Descriptive statistics were presented as frequencies and percentages. Cross-tabulation was done for IHC Ber-EP4 and IHC Calretinin with histopathology. Diagnostic accuracy was calculated using histopathology as gold standard.

RESULTS

Ninety-seven smears, prepared from centrifuged deposits of the aspirates, were examined for cytological features. Fifty-five (56.7%) cases were reported positive for malignant cells; whereas, 21 (21.6%) cases were reported as negative. Out of these negative 21 cases, three (14.3%) cases showed acellular smears. In another 21 (21.6%) cases, definitive diagnosis could not be rendered. These were reported as suspicious for malignant cells and clinical and radiological correlation was advised.

Cell blocks were prepared from the centrifuged deposit of all the 97 cases. Three unstained slides were made from cell block of each fluid, one slide was stained with H&E and rest of the 2 slides were used for application of Ber-EP4 and Calretinin each. Control of Ber-EP4 used was appendicular mucosa and control for Calretinin was malignant mesothelioma.

Out of 97 cases, Ber-EP4 showed positive staining (membranous staining) in 72 (74.2%) cases and negative staining in 25 (25.8%) cases. Clinical and radiological correlation was carried out. Histopathological diagnosis on biopsy tissue which was taken as gold standard revealed 73 (75.3%) cases were actually to be positive for malignant cells; whereas, 24 (24.7%) cases were benign. These results indicate that Ber-EP4 showed positivity in all the malignant effusion cases except one case. All the benign cases showed negative results.

Out of 97 cases, Calretinin showed positive (nuclear and cytoplasmic staining) staining in 19 (19.6%) cases and negative staining in 78 (80.4%) cases. All cases were clinically and radiologically correlated. This revealed 24 (24.7%) cases were actually benign, and 73 (75.3%) cases were malignant. Out of those 24 benign cases, only 19 (79.2%) cases showed positive staining; whereas, all malignant effusions were negative for Calretinin staining. Results of Calretinin as positive staining in reactive mesothelial cells is also cross-tabulated. Histological, clinical, and radiological correlation of all study cases were done.

Results of Ber-EP4 and Calretinin positivity and negativity were cross-tabulated with histopathological and clinical diagnosis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were calculated.

Sensitivity of Ber-EP4 for malignant cases is 98.6%, specificity is 100%, PPV is 100% and NPV is 96% with diagnostic accuracy of 98.9%.

Sensitivity of Calretinin as positive staining for RMC is 79.2%, specificity is 100%, PPV is 100%, and NPV is 93.6% with diagnostic accuracy of 94.8% (Table I).

Table I: Diagnostic accuracy of IHC Ber-EP4 and IHC Calretinin.

Diagnostic accuracy	IHC Ber-EP4	IHC Calretinin
Sensitivity	98.6%	79.2%
Specificity	100.0%	100.0%
PPV	100.0%	100.0%
NPV	96.0%	93.6%
Diagnostic accuracy	98.9%	94.8%

DISCUSSION

Distinction of reactive mesothelial cells from adeno-carcinoma cells is critical in cytological diagnosis of body

cavity effusions. The overlapping phenotypic features between these two types of cells pose a major diagnostic challenge in routine cytology practice.[9]

The work-up of body effusions includes examination of the cytological smears as a mandatory step; however, the diagnostic accuracy of serous effusion cytology using routine smear is low.[14]

Oyafuso *et al*. reviewed the cytological diagnosis of 4,297 serous fluid samples. According to their results, 1,982 false negative, 21 false positive, 1,588 true positive and 468 true negative were obtained; whereas, 161 were still suspicious for malignant cells and 77 were inconclusive. The authors inferred that cytological diagnosis alone cannot be 100% accurate for malignant effusions.[15]

The results of present study concur with these findings when out of 97 cases, 50 true positive, 5 false positive, 16 true negative, and 2 false negative results were obtained. This led to the diagnosis of 68% of the malignant cases and 66% of benign only on the basis of cytology. These results were confirmed with histopathological diagnosis and/or clinical and radiological diagnosis.

To complement the fluid cytology, technique of cell block enhances the sensitivity and specificity of cytological diagnosis. Nair, and Manjula, in their study on 148 effusion samples, compared the results of regular smears and cell blocks taking biopsy examination as the gold standard. Their results indicated that sensitivity of cell block was almost double than that of routine cytology. The results of present study tally with these authors, when 80% of malignant cases and 70% of benign/reactive cases were correctly diagnosed using the cell block technique.[16]

The cell block technique improves diagnosis by revealing better architectural pattern.[17] Compact arrangement of cells in cell block along with least amount of background staining helps easy interpretation as compared to traditional smear. In addition, various sections can be obtained from single sample. Despite these advantages of cell block, in several cases due to variation in size and shape of reactive mesothelial cells and overlying phenotypic features with adenocarcinoma cells, ancillary techniques need to be used. Immunohistochemistry can greatly help in resolving challenging cases.[18]

A variety of mesothelial cell and epithelial cell antibodies have been used to assist in this differentiation. Studies have been performed to explore the diagnostic efficacy of different combinations of immunohistochemical markers for diagnosis of malignant serous effusions.

A study was done by Su *et al*., they also evaluated six immunohistochemical markers, three for metastatic adenocarcinoma cells (CEA, MOC31and Ber-EP4) and three for RMC (Calretinin, HBME1 and thrombomodulin). Sensitivity and specificity of each maker was calculated. Results showed 86.7%, 80%, and 76.4% sensitivity for CEA, MOC31 and Ber-EP4, respectively and specificity calculated was 98.1%, 92.5% and 86.8%, respectively. The sensitivity of Calretinin, HBME-1, and thrombomodulin for RMC was calculated as 83%, 79.2%, and 47.2%, respectively. The specificity was 88.3%, 21.7%, and 70%, respectively.[19]

Grefte and his co-authors studied six immunohistochemical markers. Among those were three mesothelial markers (Calretinin, EMA and HMGF1), and three epithelial markers (Ber-EP4, B72.3, and CEA). All six antibodies were applied on each cell block prepared from serous effusion samples. Their results showed that Calretinin is very sensitive marker for mesothelial cells. They also suggest that at least one antibody for epithelial cells along with Calretinin should be used for accurate diagnosis of malignant effusions. Finally, they concluded that Ber-EP4 is more sensitive as compared to rest of the two markers, by revealing 100% positive immunostaining in malignant cells and all reactive cases were negatively stained.[20] The present results also tally with the conclusion of these authors.

Politi *et al*. used HBME1, Calretinin, Moc 31, Ber-EP4 and BG 8 for differentiation of adenocarcinomas cells *versus* reactive mesothelial cells in 134 serous effusions. According to their findings, the sensitivity of HBME1 and Calretinin for mesothelial cells was 98% and 100%, respectively. The sensitivity of the stains for adenocarcinoma cells was 86.25% for Moc31, 77.5% for Ber-EP4 and 67.5% for BG8; whereas, combined calculated sensitivity was 100%. The results proved that Calretinin is an ideal marker for mesothelial cells.[21]

Fetsch and Abati calculated the percentage of immunoreactivity of frequently used antibodies for segregation of adenocarcinoma cells and reactive mesothelial cells in various studies. The findings showed 96% staining of adenocarcinoma cells with Ber-EP4 and 80 to 100% staining of reactive mesothelial cells with Calretinin.[22]

These studies indicate that compared to other makers, Calretinin and Ber-EP4 has more sensitivity for mesothelial cells and adenocarcinoma cells, respectively. Ber-EP4 is considered to be one of the best available antibody for the panel used for differentiation of ACA and RMC in effusion cytology. However, its use in combination with a mesothelial marker is recommended for better diagnosis.[23] As far as mesothelial markers are concerned, results indicated that Calretinin is specific and sensitive marker for reactive and neoplastic mesothelial cells.[24,25]

The number of antibodies that can be used in immunocytochemistry has increased dramatically over the past few years, and the future of diagnostic cytopathology will

continue to expand as more and more immuno-histochemical markers are validated and experimentally proved to be used. Yet for resource-limited set-ups, priority remains the cost-effectiveness; hence the hunt for a short but accurate immuno-panel.

Differentiation between RMC and MM is not possible with this limited immuno-panel of antibodies. Effusions containing cells of both malignant mesothelioma and reactive mesothelium, show positive immunoreactivity with Calretinin and negative results with Ber-EP4. Primary cause of malignant effusion cannot be diagnosed with this limited immuno-panel. Effusions due to malignant melanoma, lymphoma and sarcoma, although rare, cannot be diagnosed. Exclusion is possible, however.

CONCLUSION

Application of limited panel of Ber-EP4 and Calretinin on cell block preparations of serous fluid is cost-effective and time-saving technique, which can be used as regular diagnostic procedure along with cytological smear preparations. It is especially useful at resource-limited centres with heavy workload as in our public-sector health institutions. The use of this combination of antibodies at the primary diagnostic level can aid in rapid and accurate diagnosis in morphologically difficult cases ensuring cost-effectiveness.

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