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Original Article

Pathology Section

Diagnostic Utility of Cell Block and Immunocytochemistry in Differentiating Malignant from Non-malignant Effusions: A Cross-sectional Study

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ABSTRACT

Introduction: Cytological evaluation of body cavity fluids has been widely employed in diagnosing the underlying aetiology. Differentiating reactive mesothelial cell proliferations from metastatic malignant cells based on cytomorphology alone is challenging. Therefore, the use of cell blocks in conjunction with immunocytochemistry can significantly improve diagnostic accuracy.

Aim: To investigate the role of cell blocks and immunocytochemistry in distinguishing malignant from non-malignant effusion fluids.

Materials and Methods: A prospective cross-sectional study was conducted on 70 serous effusion fluid samples (ascitic and pleural fluids) received in the Cytology section of the Pathology Department from October 2021 to November 2022. Relevant clinical details, including demographic information, age, gender, presenting complaints, and radiological and laboratory investigations, were documented from the patients' medical records. All fluids underwent conventional cytology, and the remaining fluid was used for cell block preparations. Immunocytochemistry was performed using Ber-EP4 and Calretinin immuno-markers to differentiate between reactive mesothelial cells and malignant cells. The association between cytosmear/cell block and immunocytochemistry was calculated using the chi-square test.

Results: Out of the 70 cases, 44 were pleural fluids, and 26 were ascitic fluids. Conventional cytology identified 11 positive cases, 20 suspicious cases, and 39 negative cases for malignancy. However, after cell block examination, the total number of positive cases reduced to 9, suspicious cases decreased to 12, and negative cases increased to 49, resulting in a 14.3% increase in diagnostic accuracy. Immunocytochemistry using Ber-EP4 showed strong positivity in 12 cases, indicating epithelial malignancy (adenocarcinoma), while 9 cases were Ber-EP4 negative. Calretinin positivity was observed in the mesothelial cells of all 21 cases where immunohistochemistry was performed, resulting in a 17.5% increase in diagnostic accuracy.

Conclusion: The combination of cell block technique with conventional cytology improves the diagnostic yield and accuracy by providing better interpretation of architectural patterns and cytomorphology. Additionally, the application of immunocytochemistry using Ber-EP4 and Calretinin aids in distinguishing malignant from non-malignant serous effusions.

Keywords: Ber-EP4, Calretinin, Conventional smears, Mesothelial cell

INTRODUCTION

Cytological examination of serous effusion fluids is of paramount importance as it not only helps in making the diagnosis but also aids in explaining the underlying aetiology. The presence of malignant cells indicates an advanced stage of the disease. However, cytological assessment of exfoliated cells in effusion samples is one of the most challenging areas in clinical cytopathology. Moreover, detecting tumour cells in effusion fluids is crucial in managing many cancers where surgical intervention is contraindicated. Conventional smears often pose diagnostic challenges when differentiating between reactive mesothelial cells and malignant cells, especially when the differences are marginal. In such cases, the cell block technique offers the advantage of better preservation of cytomorphologic details and histological patterns. This material can be further utilised for ancillary studies, including immunohistochemistry and molecular testing, to confirm the diagnosis [1].

Reactive mesothelial cells have variable cytological appearances and can phenotypically mimic neoplastic cells [2]. Several immune markers have been studied in the past to define mesothelial and epithelial cells; however, none have produced optimal results. It has been suggested that a minimum of two markers should be selected, as the expression of antigens in metastatic malignancies is usually heterogeneous [3]. A combination of epithelial and mesothelial markers can be applied to interpret the cell of origin.

To the best of our knowledge, the present study is the first of its kind to be conducted in the northeastern part of India. For all screening purposes, the use of Ber-EP4 and Calretinin has tremendously reduced the strenuous task of distinguishing malignant effusions from benign ones in resource-restricted areas. In this study, the authors aim to investigate the diagnostic utility of cell block and immunocytochemistry in differentiating epithelial malignancies from mesothelial proliferations in serous effusions.

MATERIALS AND METHODS

A prospective cross-sectional study was conducted at a tertiary care centre over a 14-month period from October 2021 to November 2022, following approval from the Institutional Ethics Committee (IEC No. HIMS/IRB/2021-22/S178). A total of 70 samples, including 44 pleural fluids and 26 ascitic fluids, were collected and studied in the cytology section of the pathology department. Relevant clinical details, including demographic information, age, gender, presenting complaints, and radiological and laboratory investigations, were documented from the patients' medical records.

Inclusion criteria: All consecutive 70 effusion fluids (pleural and ascitic) received in the Department of Pathology during the study period from October 2021 to November 2022.

Exclusion criteria: Fluids other than pleural and ascitic, samples less than 10 mL, markedly degenerated fluids, and clotted samples were excluded from the study.

Methodology

All serous effusion fluid samples underwent physical examination followed by cytological examination. A 10-milliliter portion of fresh pleural and ascitic fluid samples was divided equally into two parts of five milliliters each. One part was used for conventional smear cytology, and the other part was used for cell block preparation. The sediment obtained from centrifuging 5 ml of the sample at 2000 rpm for 15 minutes was used to prepare cytosmears, which were then stained with haematoxylin and eosin, Papanicolaou (PAP), and May-Grünwald-Giemsa (MGG) stains.

The remaining 5 ml of fluid was used to prepare cell blocks using the plasma thromboplastin method and processed along with other histopathological specimens. Paraffin-embedded cell button sections of 4-6 µm thickness were prepared and stained with haematoxylin and eosin. Immunocytochemical stains, including Ber-EP4 (mouse monoclonal antibody-Cell Marque, dilution 1:200) and Calretinin (mouse monoclonal antibody-Dako, dilution 1:100), were applied as needed. Ovarian adenocarcinoma and Purkinje cells of the normal cerebellum were used as positive controls for Ber-EP4 and Calretinin, respectively. Negative controls were obtained by omitting the primary antibody in the immunocytochemistry procedure and using TRIS buffer solution instead. Ber-EP4 positivity was assessed based on brown cytoplasmic and/or membranous staining of malignant epithelial cells. The intensity of staining (score 0: no staining; 1: weak; 2: moderate; 3: strong) and the percentage of stained cells (score 0: no staining; 1: <10%; 2: 10-50%; 3: >50%) were scored on a four-tiered scale. The scores were added to obtain the staining index, and a cutoff value of score 2 was used to determine positivity. Calretinin positivity was defined as strong nuclear with or without cytoplasmic positivity in at least 10% of the mesothelial cells [4].

Cytological evaluation was performed, considering all available clinical data and various investigation reports. The samples were categorised as negative for malignancy, suspicious of malignancy, or positive for malignancy. Cellularity was qualitatively graded in each case (No cellularity: no cells; Mild: <10% of cells; Moderate: 10-50%; and High: >50%) [5]. All cytological diagnoses were confirmed either by histopathology or the clinico-radiological profile of the patients.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) Statistics version 28.0. Discrete variables were presented as frequencies and percentages, while continuous variables were expressed as means. The statistical association between cytosmear/cell block and immunocytochemistry was calculated using the chi-square test, and a p-value <0.05 was considered statistically significant.

RESULTS

Out of the 70 effusion fluids received, pleural fluids were more common (n=44; 62.9%) compared to ascitic fluids (n=26; 37.1%). The most common age group was 41 to 60 years (n=29; 41.4%), followed by 61 to 80 years (n=23; 32.9%), 21 to 40 years (n=17; 24.3%), and 0 to 20 years (n=1; 1.4%). The mean age of the patients was 51.8±10.2 years. The study showed a slight male preponderance (n=36; 51.4%) with a male-to-female ratio of 1.06:1. Haemorrhagic fluids accounted for 30% (n=21) of all the fluids received during the study period. Haemorrhagic fluids were defined as those with an RBC count of more than 1 million/µL on microscopy.

The most common presenting complaint was breathlessness (n=39; 55.7%), followed by chest pain (n=27; 38.6%), abdominal distension (n=20; 28.6%), and pain abdomen (n=19; 27.1%). Other complaints included cough (n=17; 24.3%), fever (n=15; 21.4%), body ache (n=8; 11.4%), loss of appetite (n=8; 10%), abdominal discomfort (n=5; 7.1%), vomiting (n=4; 5.7%), generalised swelling (n=1; 1.4%), and loss of weight (n=1; 1.4%).

In the present study, conventional cytology showed no cellularity in 3 cases, mild cellularity in 15 cases, moderate cellularity in 36 cases, and high cellularity in 16 cases. In cell block preparations, 2 samples showed no cellularity, 5 samples showed mild cellularity, 19 samples showed moderate cellularity, and 44 samples showed high cellularity. Cell blocks increased the diagnostic yield by 40%.

On conventional cytology, 39 cases were negative for malignancy, 20 cases were suspicious, and 11 cases were positive for malignancy. After cell block preparations, 49 cases were negative, 12 cases were suspicious, and 9 cases were positive. Comparative analysis showed a reduction of 8 suspicious cases and 2 positive cases. The diagnostic accuracy was increased by 14.3% [Table/Fig-1].

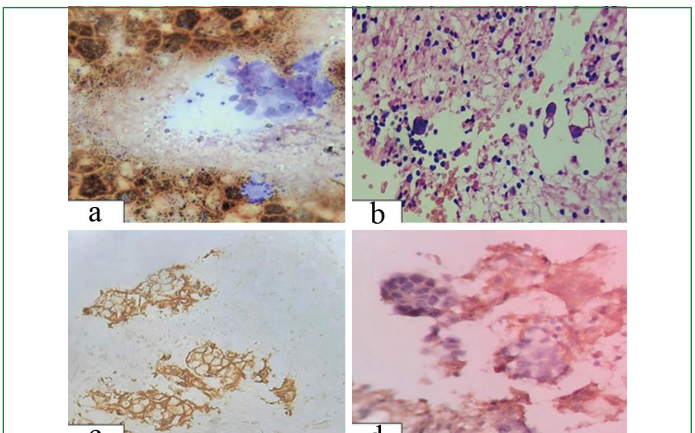
	Cytosmear		Cell block		ICC	
Diagnosis	Number	Percentage	Number	Percentage	Number	Percentage
Negative for malignancy	39	55.7%	49	70%	58	82.9%
Suspicious of malignancy	20	28.6%	12	17.1%	0	0
Positive for malignancy	11	15.7%	09	12.9%	12	17.1%
Total	70	100%	70	100%	70	100%
Diagnostic accuracy increased by			14.3%		17.5%	

[Table/Fig-1]: Comparative analysis of cytosmears, cell block and immunocytochemistry.

Immunocytochemistry was performed on cell blocks of all suspicious (n=12) and positive (n=9) cases [Table/Fig-2]. Out of these 21 cases, Ber-EP4 positivity was seen in 12 cases, with diffuse and strong positivity observed in all cell block positive cases. Three additional cases showed strong Ber-EP4 positivity in atypical cells [Table/Fig-3]. All these cases were negative for Calretinin. One case

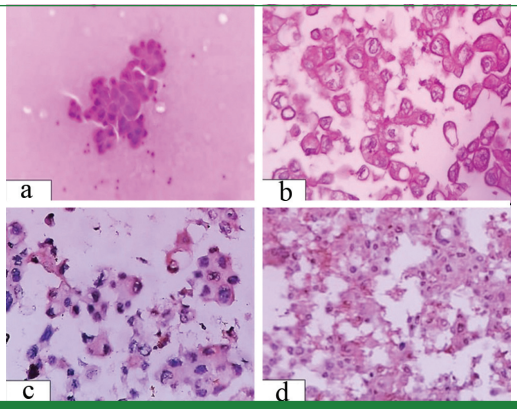
Cell block diagnosis	No. of cases	Immunocytochemistry	
		Ber-EP4	Calretinin
Suspicious of malignancy	12 (57.1%)	3 (14.3%)	9 (42.9%)
Positive for malignancy	9 (42.9%)	9 (42.9%)	0
Total	21	12	9

[Table/Fig-2]: Expression of immunomarkers on all suspicious and positive effusions on cell block.



[Table/Fig-3]: a) Cytosmears showing suspicious looking cluster of cells (100X, H&E). b) Cell block showing singly lying and occasional clusters of atypical cells (100X, H&E). c) Tumour cells showing membranous and cytoplasmic Ber-EP4 positivity (100X, IHC). d) Tumour cells are negative for Calretinin (400X, IHC).

that was suspicious on both conventional smears and cell block did not show positivity with either Ber-EP4 or Calretinin. Due to an insignificant clinical profile, this case was diagnosed as Negative for Malignancy [Table/Fig-4]. Immunocytochemistry increased the diagnostic accuracy by 17.5%.



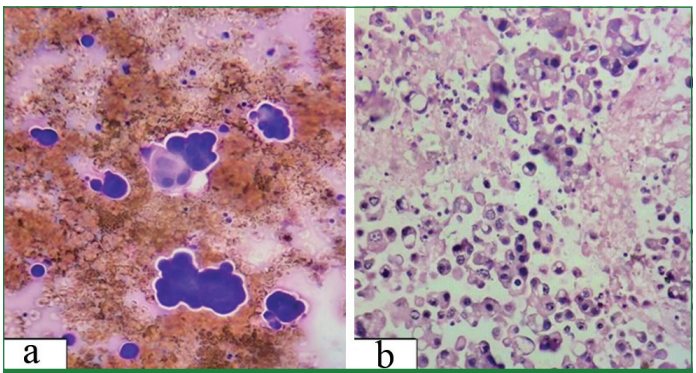
[Table/Fig-4]: a) Cytosmears showing occasional cluster of suspicious looking cells (100X, H&E) b) Cell block showing many clusters of atypical cells (400X, H&E); c) Atypical looking cells are negative for Ber-Ep4 (400X, IHC) d) Atypical cells showing Calretinin negativity (100X, IHC).

Statistical analysis showed a significant association between all three methods (Conventional cytology/Cell block/Immunocytochemistry) (p-value <0.01) [Table/Fig-5].

Sample	Cell block	ICC	Chi-square test
Negative for malignancy	70%	82.9%	**0.0014**
Suspicious of malignancy	17.1%	0%	
Positive for malignancy	12.9%	17.1%	
Total	100%	100%	

[Table/Fig-5]: Statistical association between cell block and immunocytochemistry. Bold p-value is significant

Cell blocks in the present study preserved cytomorphologic details and allowed for better appreciation of histological patterns, resulting in increased diagnostic accuracy [Table/Fig-6]. Cell blocks also reduced obscuring elements such as haemorrhage, debris, and necrosis. Homogenous expression of immunomarkers on cell blocks facilitated quick interpretation of results compared to the heterogeneous and aberrant expressions observed on conventional cytological smears [Table/Fig-7].



[Table/Fig-6]: a) Cytosmears showing clusters of atypical cells on a haemorrhagic background (400X, H&E). b) Cell blocks showing better preservation of cytomorphologic details of tumour cells (400X, H&E).

DISCUSSION

Mesothelial proliferations in body cavity fluids can often have deceptive cytomorphologic features, making it necessary to distinguish them from malignant cells. The detection of tumour cells in fluid cytology indicates advanced stage cancer and poor survival outcomes. Immunohistochemistry can help differentiate between the two populations of cells, but no single marker is absolutely specific. Therefore, it is recommended to study a panel of immunomarkers in effusion fluids to address this challenge [6].

Morphological features	Cytosmear	Cell block
Cellularity	**Number of cases**	**Number of cases**
1. High	16	44
2. Moderate	36	19
3. Low	15	05
4. Acellular	03	02
Architecture		
1. Diffuse sheets/Singly lying	54 (B*=39, M† and S‡=15)	47 (B*=46, M†=01)
2. 3D ball clusters	09	10
3. Acini/glandular pattern	03	10
4. Papillary pattern	02	03
5. Signet ring cells	02	00
Obscuring elements		
1. Haemorrhage	20	02
2. Debris	30	11
3. Necrosis	06	01
Immunocytochemistry		
Expression of immunomarkers	Mostly aberrant	Usually adequate

[Table/Fig-7]: Comparison of morphological features of Cell Block over Conventional Cytosmear.
Where B*=Benign; M†=Malignant; S‡=Suspicious

In the present study, pleural fluids accounted for 62.9% of the fluids received, followed by ascitic fluids at 37.1%. Of the fluids received, 30% were haemorrhagic. Another study by Saha R et al., also reported pleural fluids as the most common, followed by peritoneal fluids [7]. However, Nautiyal N et al., found ascitic fluid to be the most common, likely due to a high incidence of pelvic inflammatory diseases in women of reproductive age [8].

Most of the patients in the present study were in the age group of 41 to 60 years, with a slight male preponderance. This is consistent with findings from other studies, where the most common age range was 51 to 60 years [4,9]. However, Dey S et al., found that the most common age group was 61 to 70 years, with females outnumbering males. These variations may be attributed to differences in the study populations [10].

In the present study, 30% of the samples were haemorrhagic, with 29% of these being positive for malignancy. Shukla P et al., described 28% of fluids as haemorrhagic, with 54% of them being malignant. This suggests that the presence of a haemorrhagic effusion strongly favours malignancy [11]. Comparative assessment of cellularity on conventional smears and cell blocks in the present study revealed that cell blocks contributed significantly to a higher diagnostic yield. Arora et al., and Shukla P et al., also noted increased cellularity in cell block preparations [6,11]. The study of Kushwaha R et.al. also depicted cells in the pleural fluid and their significance in differential diagnosis [12].

Cell block preparations in the present study allowed for better appreciation of architectural patterns, such as 3-dimensional cell ball clusters, glandular patterns, acini, and papillary patterns. Assawasaksakul T et al., also observed an increased sensitivity in diagnosing malignant effusions on cell blocks due to better demonstration of architectural patterns [13]. Therefore, cell block technique offers the additional benefit of recognising histological patterns that cannot be identified on conventional smear preparations [14,15]. In the present study, the diagnostic accuracy of cell block method was increased by 14.3% compared to conventional cytology. Shivakumarswamy U et al., also found that cell block examination increased the diagnostic accuracy by 15% [1].

Among the 11 cases that were initially reported as positive for malignancy on conventional cytology in the present study, two cases turned out to be negative for malignancy on cell blocks. This

false positivity could be attributed to florid hyperplasia of mesothelial cells that appeared highly suspicious on conventional smears. However, better cytomorphologic details and the application of immunohistochemistry helped clarify this confusion on cell blocks. Shivkumarswamy U et al., discussed various discrepancies that can arise while examining conventional smears and advised the use of cell block examination as an adjunct to conventional cytology [1].

In the present study, immunocytochemistry with BerEp4 and Calretinin on cell blocks helped in confirming the diagnosis of malignancy in 12 cases that were either suspicious or positive on cell blocks. BerEp4 showed strong positivity in all nine positive cases, while Calretinin positivity was seen only in the mesothelial cells. Khurram N et al., also observed in their study that immunostaining with BerEp4 and Calretinin on cell block preparations helped confirm the diagnosis in the majority of suspicious cases [2]. This suggests that BerEp4 is a specific and sensitive marker for adenocarcinoma cells, while Calretinin is a marker of reactive mesothelial cells [16].

Although most authors recommend using at least two mesothelial and two epithelial markers for a correct diagnosis, in resource-restricted settings where effusion fluids are routinely screened for tumour cells, a judicious utilisation of two primary immunomarkers can help in rapid and accurate diagnosis in most challenging cases [17-19]. Overall, the present study highlights the importance of cell block preparations and immunocytochemistry in improving the diagnostic accuracy of effusion fluid cytology. These techniques allow for better appreciation of architectural patterns and aid in distinguishing between mesothelial proliferations and malignant cells.

Limitation(s)

A limited number of samples were evaluated in the present study.

CONCLUSION(S)

The cell block method was found to increase the diagnostic yield compared to conventional cytosmears. Additionally, cytological architecture and morphological features were better appreciated on cell blocks than on conventional cytosmears. Furthermore, the combination of conventional cytology with the cell block method and immunocytochemistry was shown to increase the diagnostic yield. This study utilised a limited panel of BerEp4 and calretinin, which proved to be a cost-effective and time-saving technique. Therefore, the authors conclude that the combination of BerEp4 and Calretinin can be independently used to distinguish malignant from non-malignant serous effusion fluids.

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