Epithelial Membrane Antigen, Vimentin, Desmin, Calretinin, E-Cadherin on Cell Block Preparations to Distinguish Well Differentiated Adenocarcinoma from Benign, Reactive, Atypical Mesothelial Cells

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ABSTRACT

BACKGROUND
Inconclusive cytomorphology often results due to failure to distinguish between adenocarcinoma cells from benign, reactive, atypical mesothelial cells in effusion specimens. To resolve such dilemmas, auxiliary techniques like immunohistochemistry were utilised to reach a definitive diagnosis for better treatment and management of patients. We wanted to compare cytodiagnosis achieved on cell block preparations with the cytodiagnosis on conventional smear and perform immunohistochemistry for epithelial membrane antigen (EMA), calretinin, desmin, vimentin and E-cadherin on cell block preparation of the fluids in cases of indistinguishable cytomorphology of adenocarcinoma and reactive, atypical, and benign mesothelial hyperplasia.

METHODS
The immunohistochemical markers namely EMA, calretinin, vimentin, desmin and E-cadherin were applied on cell blocks employing streptavidin-biotin method. Immunohistochemistry was interpreted by giving scores to the percentage of stained cells.

RESULTS
EMA and E-cadherin had 100 % sensitivity in diagnosing adenocarcinoma whereas calretinin, vimentin and desmin were 100 % sensitive in diagnosing reactive, atypical mesothelial carcinoma on the cell block preparations.

CONCLUSIONS
Immunocytochemistry of fluid should be carried out on the cell block preparation where cytological diagnosis on conventional smear and cell block fails to detect malignant cells in the preparation.

KEY WORDS
Cell Block, Adenocarcinoma, Mesothelial Cells, Immunohistochemistry, EMA, Calretinin, Vimentin, Desmin, E-Cadherin

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Exfoliative cytology remained a mainstay diagnostic tool in evaluation of fluids in the potential spaces located at pleura, peritoneum and pericardium. The biochemical analysis of these fluids provides the information but not to the extent to arrive at a certain diagnosis which could only be achieved until the fluid is submitted for microscopy. The conventional cytological method is used to examine the fluid for its sediment smear preparations. Conventional cytology preparations have drawbacks of non-sampling of cells, multiple preparations, cellular artefacts or preservations and most importantly the situations of indistinguishable cytomorphology. The sensitivity and specificity of conventional cytology as literature was quoted to have a long range of 80 % - 94 % and 85 % - 95 % respectively. Therefore, attempts were made to, concentrate on the cell for its yield and preservation of morphology by upgraded techniques such as liquid based cytology. However, the life-like arrangement of the cells, avoidance of artefact and problems of indistinguishable cytomorphology still remain.

Cell block preparations of the fluid which runs distinct for its advantages of diagnostic evaluation remains as one of the alternative for its better figures of diagnostic accuracy reaching of up to 97 % and typing of various neoplastic and non-neoplastic lesions in the submitted sample of fluid originating at various sites. However the cell blocks on occasions found to be unrewarding at a few diagnostic dilemma that exist with conventional cytology, liquid based cytology and cell block studies to distinguish between the overlap cytomorphology of reactive atypical mesothelial cells and cells of low-grade adenocarcinoma infiltrate in pleural, peritoneal, pericardial and other fluids.

The cell block with its several advantages offers a distinct advantage that its section can be submitted for immunohistochemistry. The immunohistochemistry on the cell block often helps to resolve the dilemma of interpretation of overlap cytomorphology. Cell block have therefore been popular diagnostic tool as it limits the microscopic field, high cell yield, maintains doseness of cellular structure much similar to histomorphology and are suitable for molecular studies. It is difficult and incomplete to separate benign atypical mesothelial cells from cells of low-grade adenocarcinoma on light microscopy in sections of cell block. Immunohistochemistry for EMA, calretinin, vimentin, desmin, E-cadherin on sections of cell block identifies epithelial cells while immunohistochemistry of calretinin, vimentin and desmin identified reactive mesothelial cells. The practicing pathologist often face this dilemma of interpretation of misdiagnosing mesothelial cells as adenocarcinoma cells and vice-versa which has affected the tumour evaluation for its staging and treatment. Detecting malignant cells by immunohistochemical means has increased the diagnostic sensitivity and specificity of cell block studies to a greater extent.

Epithelial membrane antigen and E-cadherin are molecular markers that are known to exist in the cells of epithelium while vimentin, desmin and calretinin are known to exist in the mesothelial cells irrespective of the morphological type. This molecular expression of EMA and E-cadherin for epithelial cells and of vimentin, desmin and calretinin for mesothelial cells therefore provides definite diagnostic support when it is demonstrated by immunohistochemical and immunocytochemical primary monoclonal antibodies. The present study has been planned to explore the immunohistochemistry characterization of epithelial cells, reactive atypical mesothelial cells and distinction between the two if their morphologies are not concluded distinctly on conventional smear and cell block preparation.

Objectives

- To evaluate immunohistochemical identification of malignant glandular epithelial cells and benign reactive atypical mesothelial cells on sections of cell block prepared from effusions originating at various places.
- To compare cytodiagnosis achieved on cell block preparation with the cytodiagnosis on conventional smear.
- To perform immunohistochemistry for EMA, calretinin, desmin, vimentin and E-cadherin on cell block preparation of the fluids in cases of indistinguishable cytomorphology of adenocarcinoma and reactive, atypical, benign mesothelial hyperplasia.

METHODS

The present study was carried out in the Department of Pathology in the division of Cytopathology, Jawaharlal Nehru Medical College, Sawangi, Wardha for a period of 2 years (1st of August 2018 - 31st of July 2020) with the following material and methods. A total of seventy-five cases of effusions underwent conventional smear examination, cell block study and immunohistochemistry by a panel of five antibodies consisting of EMA, vimentin, desmin, calretinin and E-cadherin.

Sample size determined by using Krejcie and Morgan Methodology evaluating the justified sample size. Following equation was utilized.

\[
S = \frac{x^2 \cdot N \cdot P (1 - P)}{d^2 (N - 1) + \left[2^2 \cdot P (1 - P) \right]}
\]

Where -

- S – Justified sample size to be evaluate
- Statistical analysis: \(X^2\) – Chi square value for 1 degree of freedom at some desired probability, this is 3.84 at 5 % level of significance.
- N – Average number of patients diagnosed with adenocarcinoma and benign atypical mesothelial reaction on fluids.
- P - 50 % (0.5) proportion, ‘q’ = 1 - P = 50 % (where P is Known event and q is taken as unknown event)
- d - Degree of accuracy (5 %), expressed as a proportion (.05), it is margin of error.

Study subjects were divided into 3 groups of twenty-five patients each.

- Group A - Twenty-five samples of cyto-diagnosed as Adenocarcinoma on conventional cytology and confirmed on cell block from various body fluids.

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• Group B - Twenty-five samples of cyto-diagnosed as Reactive mesothelial cell hyperplasia and confirmed on cell block from various body fluids. Group A and B were utilized as controls for the comparisons of the results of Immunohistochemistry with study Group C.
• Group C - Twenty-five cases of indistinguishable cytomorphology or overlap cytomorphology that failed to make definite diagnosis either of adenocarcinoma or of atypical reactive mesothelial cell hyperplasia on preparation of conventional cytopathology.

Conventional Smear Preparation\(^5,4,3,5,6\)
Fresh fluid samples of effusion were taken in 50 ml test tubes and centrifuged for 15 minutes at 1500 rpm. The supernatant was discarded and the sediment obtained was used to prepare smear. A total of six smears were made from each sample. Three of the smears were wet fixed with 95% ethyl alcohol and were stained by Papanicolaou stain. Dry smears were stained by standard steps of May-Grunwald Giemsa.

Cell Block Technique\(^16,37,38,21\)
Fluid samples were centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded. Then, 2 - 3 drops of pooled plasma were mixed with the sediment followed by addition of 4 drops of thromboplastin and was mixed again. Then, the tube was kept undisturbed for five minutes for formation of cell button. For fixation the clot was transferred to a filter paper pre-moistened with formalin fixative. The filter paper with the sediment was wrapped and placed in tissue cassettes. The cassettes were processed by conventional histopathological technique. Cell blocks obtained were sectioned at 5 micrometre thickness and was stained by haematoxylin and eosin stain.

Immunohistochemistry Technique\(^39,48,30,40\)
Immunohistochemistry was performed on two sections of 4 - 5 micron thick from each block. Heat induced antigen retrieval was performed with the help of a pressure cooker. 3 % hydrogen peroxide was applied for 10 minutes for peroxidase blocking. The primary antibody was applied to the slides and was allowed to react for 30 minutes at room temperature followed by a wash with phosphate buffer saline. Then, the secondary antibody (streptavidin-biotin) was applied and allowed to react for 30 minutes at room temperature and then was washed with Peripheral blood smear (PBS). The slides were treated with 3, 3-diaminobenzidine for 15 minutes for colour development. Sections were washed, counterstained with haematoxylin and dehydrated. Xylene was used for clearing the slides. The slides were mounted with DPX.

Statistical Analysis
X2 – Chi square value for 1 degree of freedom at some desired probability, this is 3.84 at 5 % level of significance.
N – Average number of patients diagnosed with adenocarcinoma and benign atypical mesothelial reaction on fluids.
P - 50 % (0.5) proportion, ‘q’ = 1-P = 50 % (where P is Known event and q is taken as unknown event)
d – Degree of accuracy (5 %), expressed as a proportion (.05), it is margin of error.

RESULTS
All these cases of group A, B and C were studied by cell block. The comparison between conventional cytology with that of cell block diagnosis is shown in Table 4.

The cell block study could additionally bring out the cytdiagnosis of adenocarcinoma in a single case. Conventional cytology of this case was indistinguishable but stratified as favour atypical, reactive mesothelial cells hyperplasia. 16 diagnosis which were inconclusive in Group C of indistinguishable cytomorphology remained still inconclusive on cell block study. The correlation for accuracy of group A is 100 %, group B is 100 % & for group C is 98.66 %.

Immunocytochemistry
The control group A showed a well-demarcated pattern of immunoreactivity for the cells of adenocarcinoma. In group A of adenocarcinoma EMA and E-cadherin were positive with grade 2 and grade 3 scores while the other marker of calretinin, vimentin and desmin showed mostly the negative results for adenocarcinoma cells. Group of atypical, reactive mesothelial cells showed positivity for calretinin, vimentin and desmin with 2 + and 3 + score in all 25 cases and showed negative results for EMA and E-cadherin. The group C of indistinguishable cytomorphology upon the immunohistochemical evaluation of EMA, calretinin, vimentin, desmin, E-cadherin on cell block sections have shown the re-assignment of diagnosis in 5 cases.
DISCUSSION

The present study has observed 100% correlation in group A and B between the cytodiagnostics on conventional smear cytology and cell block studies that is group A of adenocarcinoma and group B of reactive mesothelial cells respectively. Such a confirmation on comparison for accuracy of conventional cytology and cell block studies have been reported in the studies of Murugan et al., Bista P et al., Nathan et al., Amiri et al. However, in the studies of Shivkumarswamy et al., Shukla et al., Bansode et al., Katti et al., Sharma et al., Aggarwal et al., and Nautiyal et al. showed that cell block performed more efficiently than conventional cytology. The present study for Group C that is of indistinguishable cytomorphology when carried out and compared between the conventional cytology and cell block studies has observed that a case has changed its category to adenocarcinoma Group yielding the percent of correlation of 98.66%.

The present study has appreciated the advantage of cell block in its utility for immunohistochemistry as it offers not only histology like sections, but it also limits the area of microscopy similar for this advantage of cell block with many other studies.

Epithelial Membrane Antigen

The sensitivity of EMA at detecting adenocarcinoma cells as reviewed in the present work is 100% which is similar to the sensitivity quoted in the studies of Singh et al. (100%), Murugan et al. (100%), Hasteh et al. (100%) and Aggarwal et al. (100%).

The present study has reported specificity of 73.68% for EMA at diagnosis of adenocarcinoma on the cell block study which is not in agreement with the studies of Murugan et al.

E-Cadherin

The present study has observed sensitivity of 100% for E-cadherin at detection of adenocarcinoma cells which is similar with the sensitivity quoted in the studies of Hirome et al. (97%), Murugan et al. (97 %) and Moghaddam et al. (88 %). The specificity of 73.68% was performed for E-cadherin in the detection of adenocarcinoma which is lesser than the specificity quoted by Xue ying su et al. (100%) and Moghaddam et al. (92%) but higher than the specificity quoted by Murugan et al. (68.42%). The present study observed PPV of 54.55% and NPV of 100% which does not correspond to the PPV values but corresponds to the NPV values in the results of Murugan et al. (PPV 97.5%, NPV 100%) and Aggarwal et al. (PPV 90% NPV 100%). The present study has likelihood ratio of 3.80 and P-value of 0.001 which is significant and is similar to that of results of Hasteh et al. (0.001).

Calretinin

The present study has observed the sensitivity of 100% which is similar to the sensitivity quoted in the studies of Murugan et al. (97.37%), Aggarwal et al. (93.75%), Nautiyal et al. (100%), Subbarayan et al. (92.86%) for high rate of specificity approaching to 100%. The present study has observed PPV of 54.55% and NPV of 100% which does not correspond to the PPV values but corresponds to the NPV values in the results of Murugan et al. (PPV 97.5%, NPV 100%) and Aggarwal et al. (PPV 90% NPV 100%).
The present study reported a specificity of 54.55 % for calretinin in diagnosis of reactive mesothelial cells which is not in agreement with the studies of Nautiyal et al.51 (100 %), Subbarayan et al.52 (97.62 %), Heon kim et al.29 (98 %), Aggarwal et al. (94.4 %), Murugan et al. (92.31 %).

The present study has observed the PPV of 73.68 % which does not correspond to the values recorded in the studies of Murugan et al (92.68 %), and Subbarayan et al. (97.67 %) while the NPV of 100 % recorded in the present study is in concordance with the studies of Aggarwal et al, Murugan et al and Subbarayan et al.

The likelihood ratio for calretinin in diagnosis of reactive mesothelial cells reported in the present study was 2.20 and P-value of 0.001 which is significant and similar to the results of Murugan et al. (LR-13.000, P value < 0.0001).

**Vimentin**

The present study observed sensitivity of 100 % which is similar to the study of Keith et al.31 (100 %) but dissimilar to the sensitivity quoted in the study of Murugan et al. (74 %). The present study observed specificity of 54.55 % which is lesser than the one reported by Murugan et al. (74.9 %). The PPV of vimentin for recognition of reactive mesothelial cells recorded in the present study was 73.68 % which corresponds to the values recorded in the study of Murugan et al. (77.78 %) and NPV of 100 % which did not correspond to the values recorded in the study of Murugan et al. (NPV 75.61 %). The present study has likelihood ratio of 2.20 and P value of 0.001 which is significant and is similar to that of the results of Murugan et al. (LR 3.592, P-value < 0.0001).

**Desmin**

Murugan et al. in their evaluation of desmin as a marker for reactive mesothelial cells has observed the sensitivity of 55 %, specificity of 95.12 %, positive predictive value of 91.3 %, negative predictive value of 69.64 %, likelihood ratio of 11.329 and P-value of 0.0001. The present study observed sensitivity of 100 %, specificity of 54.55 %, positive predictive value of 73.68 %, and negative predictive value of 100 % and P-value of 0.001.

The sensitivity reported in the present study is 100 % which is higher than what is reported by Murugan et al.30 (55 %) and Hasteh et al44 (84 %) and is similar to Gill et al.25 (100 %). The study of Gill et al.25 and Hasteh et al. also reported the P value for its diagnostic capacity and showed it to be significant, <0.0001 and 0.001 respectively. The present study also observed high P-value of 0.001 for the detection of reactive mesothelial cells which is similar to that of the results of Hasteh et al.44

If the five molecular markers were used in combination for the 25 cases of study group (C) have shown the sensitivity of 54.55 %, specificity of 100 %, positive predictive value of 100 %, negative predictive value of 73.68 % and diagnostic accuracy of 80 % as depicted in Table 7.

The individual marker and their sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio is shown in Table 8.

EMA and E-cadherin was found to have the highest sensitivity of 100 % for adenocarcinoma cells and specificity of 73.68 % with the similar positive predictive value (54.55 %), negative predictive value (100 %) and likelihood ratio of 3.8.

Vimentin, desmin, calretinin for their capacity to identify the mesothelial cells was found to have sensitivity of 100 % and specificity of 54.55 % individually. The positive predictive value and negative predictive value of all these three markers were 73.68 % and 100 % for mesothelial cells, respectively.

The likelihood ratio for vimentin, desmin, calretinin individually was 2.20.

**CONCLUSIONS**

Cell block studies offer the advantages of limited areas of microscopy and lifelike cytoarchitectural features similar to histology and are suitable for immunohistochemical examination. Immunocytochemistry on the cell block preparation of the fluids of pleural, peritoneal, and pericardial type is of immense diagnostic value if epithelial (EMA and E-cadherin) and mesothelial markers (calretinin, vimentin and desmin) are combined in a panel. Such application resolves the diagnostic dilemma in the cytomorphology which are otherwise indistinguishable and show overlap.

Data sharing statement provided by the authors is available with the full text of this article at jemds.com.

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