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Evaluation of Epithelial Membrane Antigen and Vimentin on cell block of body fluids for indistinguishable cytomorphology

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Abstract

Background: Cell blocks are useful when conventional cytology fails in interpretation of the overlaps of the reactive atypical mesothelial cells and infiltrate of low-grade adenocarcinoma cells of unknown and known primaries. This distinction is sought for the staging of the tumor as well as appropriate therapeutic interpretation. Cytomorphological differences between aforesaid two conditions even on cell block may fail. Immunocyto Che Mistry performed on such cell blocks may help in knowing the mesothelial versus epithelial histogenesis of the cells. EMA and vimentin have been studied for their usefulness in resolving the dilemma of interpretation, the present study is carried out in the background of such a dicey interpretative situation of cytomorphology where immunocytochemistry may support cytomorphologic interpretation.

Objectives

1. To compare the results of conventional smear diagnosis with cell block preparations of effusions;

2. The role of IHC of EMA and vimentin at distinction of well-differentiated adenocarcinoma from benign atypical reactive mesothelial cells on cell block.

Material and Methods: Sixty patients divided in three groups of twenty cases each of adenocarcinoma, Benign reactive mesothelial cells and overlap cytomorphology (low grade adenocarcinoma versus reactive atypical mesothelial cells) were included. The three tier diagnostic methods were applied conventional cytology, cell block and ICC.ICC was performed by standard protocol. The comparisons were made of the results.

Results: The comparison of cytodiagnosis between conventional cytology and cell block for their sensitivity were 100%. EMA had 100% sensitivity and specificity for adenocarcinoma (group 1 and 3) while vimentin had 100% sensitivity and specificity in reactive mesothelial cells (group 2 and 3).

Conclusion: The ICC panel of EMA andvimentincould confirm the infiltration of malignant epithelial cells in twelve cases of known primary and additionally diagnose six more cases of infiltration of adenocarcinoma with overlapping cytomorphological patterns. The use of ICC panel of EMA and vimentin on cell block is advised when light microscopy fails to interpret overlapping cytomorphology with nuclear atypia.

Keywords: Adenocarcinoma, Reactive mesothelial cells, immunocytochemistry, EMA, Vimentin.

Introduction

Conventional cytology of body effusions contributes immensely to the diagnostic process. However non representation or the difficulties of interpretation or cytomorphological overlaps are limiting factors for conventional cytology. Another limiting factor with conventional cytology is the cell concentrations.[1] The cell block preparations of the effusions provide a supplementary as well as auxiliary method to overcome these limitations. However, there remain a good number of effusions that could still face the diagnostic dilemmas due to overlap cytomorphology.

ICC performed on cell block in characterization of cells is relatively new technique with several advantages. There are studies which have highlighted ICC over cell block preparations of effusions in resolving diagnostic dilemmas of overlap cytomorphologies. [2, 3, 4, 5]

One such troublesome and fairly confronted difficulty in cytology of effusion is to distinguish between reactive atypical mesothelial cells from that of low-grade adenocarcinoma cells. [6, 7, 8, 9,10] This limitation arises because of nuclear atypism and progressive loss of natural gland formation or loss of intercellular windows. The consultant pathologists often find it difficult distinguishing these two conditions more so in known or even unknown primary.

EMA and vimentin have been studied for their sensitivity and specificity to know origin of cells as glandular epithelial and mesothelial respectively. [2, 3, 4, 5] However, studies on these antigenic expressions are limited in literature.

There exists a gap of understanding in selection of ICC panel in determining the origin of the cells as an epithelial versus mesothelial. Therefore, more such number of studies are required that would through their results suggests a simple and workable ICC panel in the resource limited cytopathology laboratories at for resolving this overlap diagnostic dilemma.

The present study aims at resolving the morphologically indistinguishable situations of reactive atypical mesothelial cells versus low grade adenocarcinoma cells with following objectives;

1) To compare the results of conventional smear diagnosis with cell block preparations of effusions; 2) The role of IHC for EMA and vimentin at distinction of well-differentiated adenocarcinoma from benign atypical reactive mesothelial cells on cell block

Material and Methods

Our study was prospectivecross-sectionalstudy carried out in duration of one year from August 2019 to August 2020, in Division of Cytopathology of Department of Pathology. The study included 60 body fluids with the following distribution; pleural fluid – 19, peritoneal fluid – 36, pericardial fluid – 1 and fluid from pouch of Douglas- 3. Demographic, clinical details and known primaries were recorded of available cases. These 60 body fluids were segregated for comparable dispositions in three groups.

Group 1

(Control group of adenocarcinomas) included 20 cases of adenocarcinoma diagnosed by conventional cytology irrespective of origin of fluid);

Group 2

(Control group of benign mesothelial cell reaction) included the fluids which were cytodiagnosed as benign mesothelial cell hyperplasia/ reaction on conventional cytology; and

Group 3

(Study group of overlapping cytomorphology) included the cytomorphology indistinguishable and with overlapping features for low grade adenocarcinoma versus benign atypical mesothelial cell reaction.

Cytology samples were processed by conventional cytology methods. The smears were prepared of the fluid samples by cytocentrifugation. These smears were kept fixed and unfixed to be stained by Papanicolaou and Hematoxylin and eosin stain and Geimsa stain respectively. [11, 12]

Cell block of the fluid samples were prepared byThrombo Plast in – plasma method as described previously in the literature. [12, 13, 14]

Immunocytochemistrywasperformed on 60 cell blocks belonging to all three groups. ICC for EMA and vimentin were detected by commercially available antibodies (Brand- DAKO^{TM}, Glostrup Denmark). The cell blocks were immuno stained by standard methods as meant for routine paraffin embedded tissue blocks. [2, 3, 4, 5]

The interpretation for positive EMA and vimentin were performed by appreciating brown granular staining pattern with parallel run controls for EMA and vimentin. The results of immunostaining were recorded similar to criteria's applying for histological sections as described in the studies of Murugan et al, Vrinda A et al, Farnaz H et al, NehaNautial et al, Goldstein NS et al. [2, 3, 4, 5, 15] The comparisons for consistency of the finding in group 1 and 2 were recorded for ICC results so also the findings in group 3 with overlap cytomorphologies.

Statistical analysis of the data was done using the IBM-SPSS software version 20.0. Sensitivity, specificity, negativepredictive value, positive predictive value and value of significance were carried out through the comparisons of the results in all three groups. The utility of ICC for EMA and vimentin on cell block in group 3 was statically evaluated.

Results

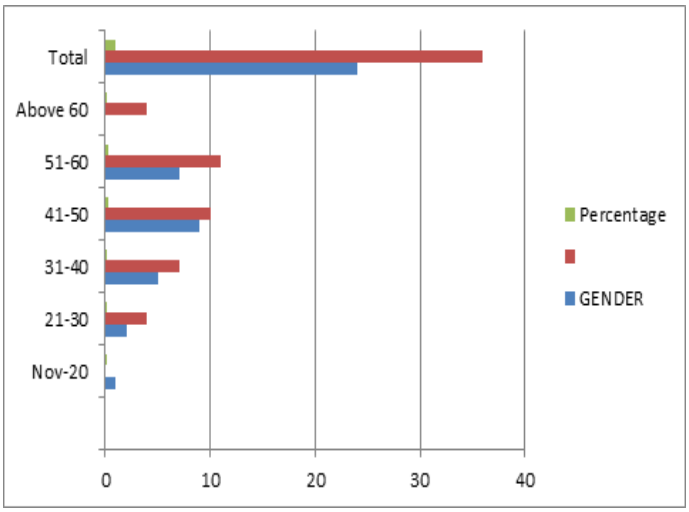
A total of 60 patients who had body effusions were included in the study. All the groups included 20 patients each having effusion types of pleural, pericardial, peritoneal and fluid from pouch of Douglas. The

breakup of age and gender distribution and there percentage is shown in table 1.

Table 1: Age wise distribution of cases.

Age range	Gender		Percentage
	Male	Female	
11-20	01	00	1.6%
21-30	02	04	10%
31-40	05	07	20%
41-50	09	10	31.6%
51-60	07	11	30%
Above 60	00	04	6.6%
Total	24	36	100%

Graph 1:



There were 36 female and 24 male (M: F - 2:3). The highest number of effusions was observed to be in age range of 41-50(19) cases followed by 51-60 (18) cases. The age range of 41-60 has maximum cytodiagnosis of malignant effusion (16 cases).

The cases of benign mesothelial reaction showed a pan age distribution. The cases of overlapping cytomorphologicalfeatures were also distributed irrespective of their age ranges, but were predominantly belong to female gender. The youngest patient was 20yrsmale while the oldest one was 84-year female.The 20-year male was belonging to group 2 of benign mesothelial cell reaction.

There were 12 patients in grp 3 who were known to carry primary of adenocarcinoma.

The samples of these patients were send to cytologic examination in evaluation of metastasis. The remaining patient of this group had no known primary tumor on the clinical, radiological and lab investigations.

The distribution of type of fluid in group 1, 2 and 3 is depicted in table 2.

Table 2: Site and source of fluid in Group 1,2 and 3 patients

Type of fluids	Group 1(n=20)	Group 2 (n20)	Group 3(n=20)	Percentage
Pleural fluids	05	07	08	20 (33.3%)
Peritoneal fluids	12	13	11	36 (60%)
Pericardial fluids	01	00	00	01 (1.6%)
Fluid from pouch of Douglas	02	00	01	03 (5%)

The peritoneal fluids were 36 in number and were distributed along all three groups. The pleural fluids too were distributed in all groups.

The total of 20 cases with known primary sites of malignancy was observed with the split of eight cases in group 1 and twelve cases in group 3.

The distribution of cases for the sites of malignancy of group 1 and 3 is shown in table 3.

Table 3: Depicts the distribution of known primary in group 1,2 and 3

Groups	Sites of known primary						
	Ovary	Lung	Gastro-intestinal	Breast	Gall Bladder	Endometrium	Pancreas
Group 1	3	1	1	1	1	1	-
Group 2	-	-	-	-	-	-	-
Group 3	4	2	2	1	1	1	1

The ovary was the commonest site of primary malignant neoplasm followed by primaries in lung, GIT, and breast.

The peritoneal fluid sample predominated over other sources of fluids in Group 1. This group included eight patients with known primary and twelve patients were newly diagnosed as adenocarcinoma. In group 2, maximum cases of the fluids submitted for diagnostic screening were peritoneal for sources. This group had no known primaries and was sent as the samples in exclusion of malignancies as a routine patient workup protocol for ascites.

The maximum number of cases in group 3 was samples of peritoneal origin (eleven cases). Twelve cases were of known primary malignant diagnosis but did not show overwhelming cytomorphological features of malignancy of adenocarcinoma cells in the sample processed on conventional cytology as well as on cell block studies. Therefore, these cases were included in the study group even though these cases had a known primary. The remaining cases showed the cellular atypia to the extent that confused for morphology indistinguishable from atypical mesothelial cells versus low grade adenocarcinoma cells.

Conventional Cytology and cell block

Group 1 and group 2 cases diagnosed on conventional cytology were confirmed on cell block studies. No mismatches of the diagnosis were observed in group 1 and group 2 when compared even for the subtype of adenocarcinoma. Therefore, the sensitivity, specificity positive predictive value and negative predictive value in Group 1 and Group 2 by conventional cytology when compared with cell block diagnosis were 100%. However the group 3 cases which had indistinguishable morphology on conventional cytology to categorize it either as reactive atypical mesothelial cells or cells of low grade adenocarcinoma still persisted even on cell block studies. This group necessitates the evaluation further on immunohistochemistry on the sections of the cell block as neither the cell type nor the morphological nuclear atypia prompted any definite diagnosis.

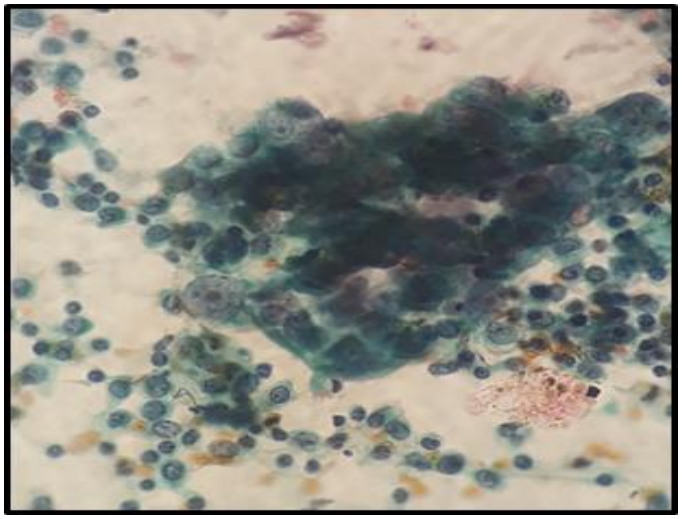


Figure 1: (40x) Photomicrograph showing cluster of Adenocarcinoma cells within asciticfluid in conventional smear preparation stained by pap stain.

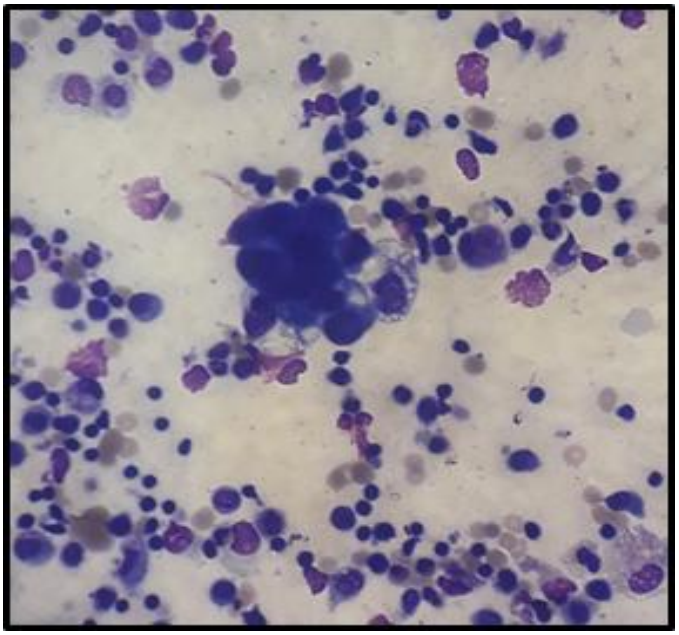


Figure 2: (40x) Photomicrograph showing cell ball of Adenocarcinoma cells within pleural fluid in conventional smear preparation stained by MGG stain.

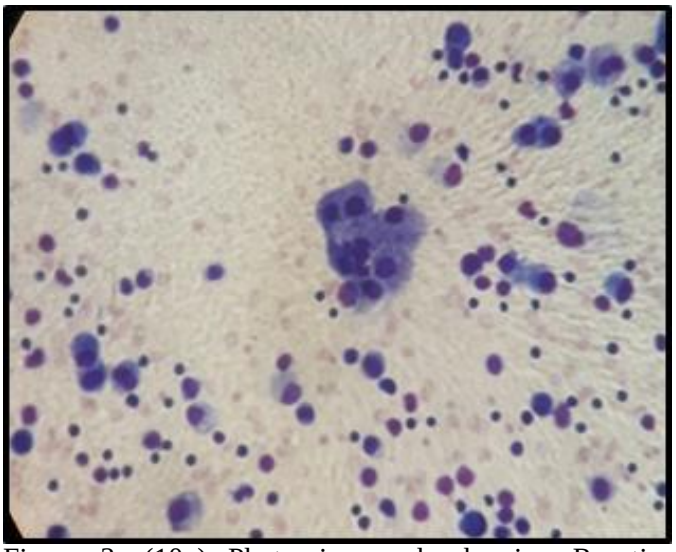


Figure 3: (10x) Photomicrograph showing Reactive mesothelial cells within pleural fluid in conventional smear preparation stained by MGG stain.

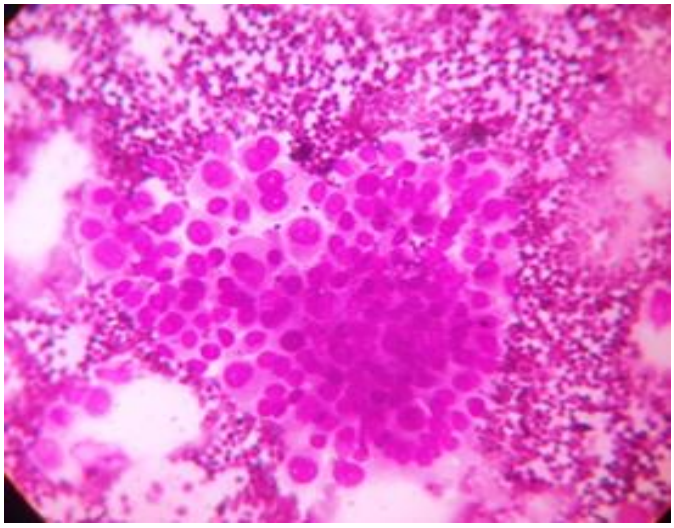


Figure 4: (40x) Photomicrograph showingReactive Atypical Mesothelial Cells in Conventional smear stained by MGG Stain Immunohistochemistry

All the cases of group 1, group 2, and group 3 underwent immunocytochemistry for EMA and vimentin. The results of ICC of EMA and vimentin are tabulated in table 4.

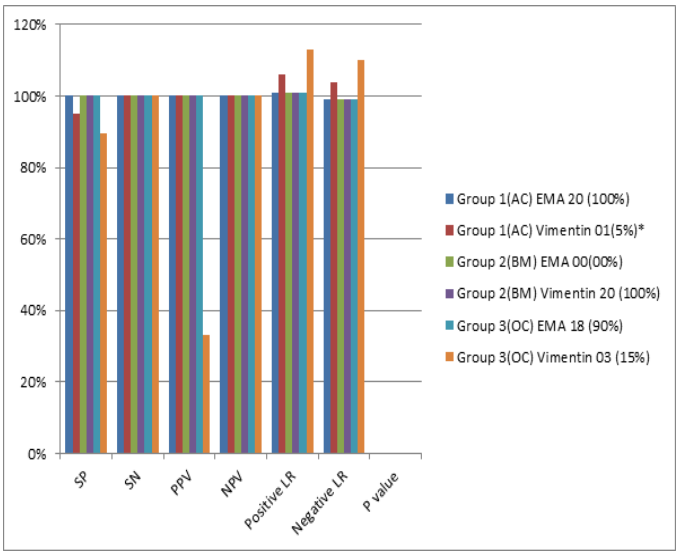
Table 4: The results of immunohistochemistry

Groups	Group 1(AC)		Group 2(BM)		Group 3(OC)	
	EMA	Vimentin	EMA	Vimentin	EMA	Vimentin
n=20	20 (100%)	01(5%) *	00(00%)	20 (100%)	18 (90%)	03 (15%)
SP	100%	95%	100%	100%	100%	89.47%
SN	100%	100%	100%	100%	100%	100%
PPV	100%	100%	100%	100%	100%	33.3%

NPV	100%	100%	100%	100%	100%	100%
Positive LR	1.01	1.06	1.01	1.01	1.01	1.13
Negative LR	0.99	1.04	0.99	0.99	0.99	1.10
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

AC-Adenocarcinoma; BM – Benign mesothelial reaction; OC – Overlapping Cytomorphology,
SN-Sensitivity, SP – Specificity, PPV- Positive Predictive Value,NPV- Negative Predictive Value,
LR- Likelihood, * dual population of cell is indicated, P < 0.001 significant

Graph 2:



The interpretation of IHC for EMA and vimentin performed on cell block sections were distinct for the features and were similar to Histopathological sections. The observations for ICC for EMA and vimentin were distinct for interpretation in group 1 and 2.

EMA was positive in all cases of group 1 suggesting the high sensitivity and specificity of it with low grade carcinoma cells. A single case showed significant no. of mesothelial cells which showed positivity for vimentin in addition to EMA.

The results of EMA in group 2 were negative, therefore the observation was made that EMA and vimentin can separate the adenocarcinoma cells from that of reactive mesothelial cells with specificity, sensitivity, positive predictive value and negative predictive value at 100%.

Group 3 of overlapping cytomorphology revealed distinct EMA immune staining in twelve cases where primary were known. EMA was strongly positive in six more cases of group 3 with unknown primary, which suggested the epithelial character of infiltrating cells offering six additional diagnoses of infiltrate of adenocarcinoma.

There were two cases which had negative EMA immune staining but showed strongly positive Vimentin immune staining thus suggesting the mesothelial origin of the cells.

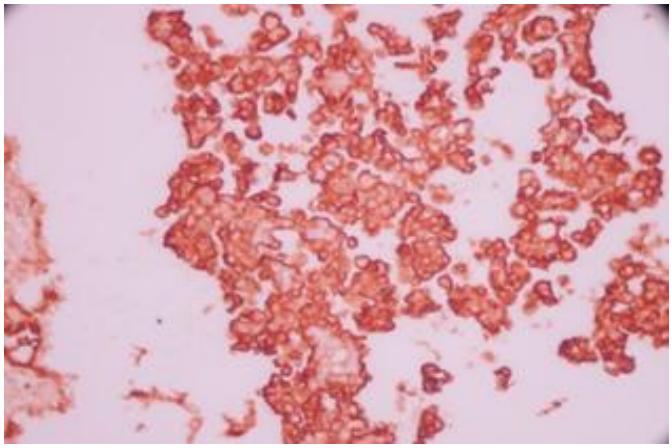


Figure 5: (40x) EMA: Corresponding cell block of adenocar cinoma, malignant cells showing membranous and cytoplasmic positivity.

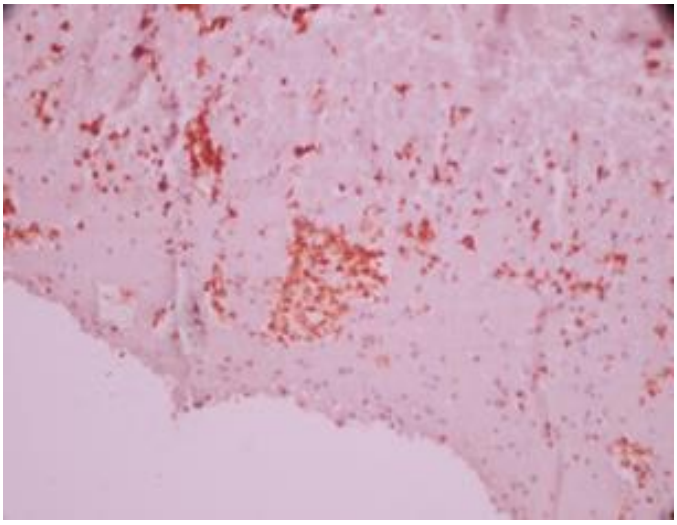


Figure6: (40x) Vimentin: Corresponding cell block of reactive mesothelial cells displaying positive stain.

The combinations of EMA and vimentinimmunostaining resolve the confusion in twelve and six cases of known and unknown primaries for infiltrate of adenocarcinoma respectively.

The p value drawn for EMA and vimentin in group 1, 2 and 3 were significant for immunostaining of EMA and vimentin. The likelihood positive ratio associated of EMA and vimentin for adenocarcinoma and mesothelial cells was 1.01 and 1.13 respectively in group 3.

Discussion

Our study made an observation for the situations of reactive atypical mesothelial cells in body fluids cytologically posing the problem for its differentiation from low grade adenocarcinoma cells. This is an inadvertent situation for pathologists as an erroneous diagnosis of the interpretation of these cells may lead to wrong options of treatment. [2-5, 16-24]

It has been observed that the overlap cytomorphology may occur in all body fluids submitted for cytological examination but more for peritoneal and pleural fluids. The similar observations for the fluid types frequently encountering the problem of interpretation in overlap cytomorphology have also been supported by few studies. [2, 4, 5, 18, 19, 21, 22, 23]

Several studies coated the high values of sensitivity and specificity in diagnosis of adenocarcinoma in the body fluids by conventional cytologic methods duly supported by cell block diagnosis. [2, 4,5, 8, 9, 16-23] Our study made a similar observation that the infiltrate of adenocarcinoma is one of the common malignant diagnoses made at examination of body fluids.

The cell block remains the diagnostic choice when conventional cytology fails. [4, 5, 16, 18-24] Cell block has an ability to enhance the diagnostic capacity of exfoliative cytology by 5 to 30%. [4, 5, 16, 19, 21, 22, 24] This study did not find the specific advantage for the samples recruited in the study. However, this study has observed that the cell blocks of fluid limit the area of the microscopy and gives an advantage at microscopy. This microscopy on the cell block can be carried out in much similar way as that of histopathological section of the tissue.

The cell blocks of the fluids are ideal to be utilized for ICC. [2-6, 8-10, 16, 18-24] This added advantage have made the cell blocks, a favorite investigative modality often being used in the situations where successful distinction between reactive atypical mesothelial cells from that of cells of low-grade adenocarcinoma is not possible on conventional cytology by submitting its section for immunocytochemistry. The ICC on cell blocks is credited for interpretable results that can be utilized in above situations. [2-5, 16, 18, 19, 21-24]

The following immunocytochemistry panel were used to distinguish reactive atypical benign mesothelial cells from that of well differentiated/borderline adenocarcinoma cells on cell block preparations by various authors; EMA, CEA, E-cadherins, calretinin, desmin and vimentinimmunomarkers were utilized by

Murugan et al[2] ; EMA, CK7, Calretinin, vimentin, CA-125, were used by Shukla et al[21] ; desmin, EMA, Ki67 were used by Farnaz et al [3] ; calretinin and EMA by Aggarwal et al[4] ; calretinin and EMA by Nautial et al[5] ; calretinin and E- cadherin by Kitazume et al[6] ; E-cadherin, CEA, MOC-31, calretinin by Su et al[7] ; CEA, CK, EMA and fibronectin by Lee et al[8] ; EMA, CEA by Keith et al[9] ; EMA, CEA, CK and vimentin by Kim et al[10]. By reviewing the panels viewed by above authors and their results our study concluded that the antibodies for vimentin and EMA are useful at distinction of reactive atypical mesothelial cells from well differentiated adenocarcinoma cells when cytomorphologic overlaps are uninterpretable.

The study of Murugan etal [2] used the panel of EMA, CEA, E-cadherins, calretinin, desmin and vimentin for distinguishing cells of reactive mesothelium and adenocarcinoma and has found EMA as a best single marker for adenocarcinoma, with 100% sensitivity and 92.31% specificity. The study ofAggarwal et al [4] used the panel of calretinin and EMA and has found that 100% cases showed positivity with EMA and only 6.25% of reactive mesothelial cells showed positivity with EMA. The study of Nautial et al [5] used the panel of calretinin and EMA and has found that EMA had a sensitivity of 91.89%, specificity of 100% and accuracy 94.8%. The study of Lee etal [8] used the panel of CEA, CK, EMA and fibronectin and has found that CEA and EMA were present in 89% and 86% of carcinoma cases respectively. The study of Kim et al [10] used the panel of CEA, CK, EMA and has found that sensitivities of stain for adenocarcinoma were 89% in EMA and 25% in vimentin. The sensitivities of stain for reactive mesothelial cells were 19% in EMA and 75% in vimentin.

The present study have found that, the combination of EMA and vimentin for their ICC on the cell block preparation of fluids works well at differentiating the cells in all three study groups.This ICC panel when applied to study group 3 has specificity -100%, sensitivity -100%, PPV -100%, NPV - 100%, Positive likelihood ratio -1.01, Negative likelihood ratio - 0.99 and P value -0.0001, Significant for EMA.Thevimentinimmunostaining was found to have specificity - 89.47%, sensitivity - 100%, PPV - 33.3%, NPV - 100%, Positive likelihood ratio - 1.13, Negative likelihood ratio - 1.10 and P value -0.0001, Significant. For control group (group 2) EMA ICC has following results specificity - 95%, sensitivity - 100%, PPV - 100%, NPV - 100%, Positive likelihood ratio - 1.06, Negative likelihood ratio - 1.04 and P value - 0.0001, Significant. The vimentin ICC showed the specificity - 100%, sensitivity - 100%, PPV - 100%, NPV - 100%, Positive likelihood ratio -1.01, Negative likelihood ratio - 0.99 and P value - 0.0001, Significant. For control group (group 1) EMA ICC has following results specificity - 100%, sensitivity - 100%, PPV - 100%, NPV - 100%, Positive likelihood ratio - 1.01, Negative likelihood ratio - 0.99, P value - 0.0001, Significant.The vimentin ICC showed the specificity - 95%, sensitivity - 100%, PPV - 100%, NPV - 100%, Positive likelihood ratio - 1.06, Negative likelihood ratio - 1.04 and P value - 0.0001, Significant.

The studies of Murugan et al [2], Kim et al [10] has advised to include the combined panel of EMA and vimentin in the algorithm of diagnostic evaluation of body fluids where indistinguishable and overlapping cytomorpho logies exist for cells of reactive benign atypical mesothelial cells and adenocarcinoma cells. The present study is in agreement for placement of

immunocytochemistry panel of EMA and vimentin in diagnostic algorithm in situation of overlapping cytomorpho logies as it could bring about eighteen additional cases resulting in appropriate treatment protocols and helped out at staging of the disease. The immunocytochemistry panel of EMA and vimentin when positive for results have extended the search for primary six cases in our study. The similar advantage of immunocytochemistry of EMA and vimentin being used on the cell block at revealing unknown primary have also been mentioned in the studies of Murugan et al [2]and Kim et al. [10]

Conclusion

The study concludes from its results that conventional cytology of the fluids in detecting the infiltration of the malignant cells or establishing the benign character of effusions such as mesothelial cell reaction is indisputable and the results of it runs parallel to the Cell block preparation. The panel of EMA and Vimentin served to distinguish and confirm eighteen cases of adenocarcinoma and two cases of atypical mesothelial hyperplasia in a cytomorphological group 3 of overlapping indistinguishable atypical cytomorphological patterns, where conventional cytology and cell block failed. Therefore, the panel of EMA and vimentin is a problem solver when used together where light microscopy on conventional cytology fails to distinguish the atypia belonging to well differentiated epithelial malignancy and atypical reactive mesothelial cells. It is advisable to put EMA and vimentin in diagnostic algorithm where conventional cytology fails.

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