

Original Research Article

Immunohistochemical Study on Cell Block of Ascitic Fluid to Ascertain the Etiology

Dr. Sucharita Sarkar¹, Dr. Manisha Sarkar¹, Prof. Asim Kr Manna¹, Dr.Soma Sabui¹, Dr. Mou Das¹, Prof. Diptendra Kr Sarkar².

¹Department of Pathology, I.P.G.M.E&R, Kolkata, West Bengal, India.

²Department of General Surgery, I.P.G.M.E&R, Kolkata, West Bengal, India.

***Corresponding author**

Dr. Sucharita Sarkar

Email: sucharitasarkar78@yahoo.com

Abstract: Cytological examination of body fluids is important for the diagnosis, staging and assessment of prognosis in malignant lesions. Based on morphologic features alone, cytological differentiation of reactive mesothelial cells from adenocarcinoma is found to be difficult. Therefore, ancillary studies often are performed to assist in the differential diagnosis. We have done this study to evaluate the validity of combined approach of routine cytological staining methods and cell block techniques along with immunohistochemistry on cell block sections in differentiating adenocarcinoma cells from reactive mesothelial cells. The study was conducted in a tertiary care hospital over the period of two years, September 2014 to August 2016. Ten millilitres of 250 fresh ascitic fluid samples from patients who were taken for conventional cytological examination. Suspicious samples are processed to cell block. Immunostaining with Calretinin, Cytokeratin7, Cytokeratin20 done for differentiating adenocarcinoma cells from reactive mesothelial cells & metastatic adenocarcinoma. Chi-Square Test proved increased diagnostic yield. There is 3.3% more cases are diagnosed as malignant after histological study on cell block. So, there will be no necessity to give a diagnosis of suspicious of malignancy or equivocal for malignancy. We had studied histology in 55 cases, out of which 44(80%) cases yields superior diagnosis after cyto-histological & immunohistochemical correlation. We recommend combined use of cytology on conventional smears with cell block & immunohistochemistry for suspicious or malignant ascitic cases to raise diagnostic accuracy as much as possible.

Keywords: Ascitic fluid; Cell block; calretinin; CK7

INTRODUCTION

The body cavities are lined by a single layered flat mesothelial cells called serosa[1]. Interestingly, mesothelial cells are derived from mesoderm but they demonstrate many morphological and biological features of epithelial cells[2]. Ascites is the pathologic accumulation of fluid within the peritoneal cavity[3]. Moreover, mesothelial cells react variously to different sorts of both external & internal acute and chronic stimuli, undergo hyperplasia & hypertrophy and significant nuclear atypia, called 'Reactive' mesothelial cell leading to misdiagnosis of a malignancy [4].

In 85% cases, cirrhosis is most common cause of ascites [1], followed by tumors (10%) & other benign or non-neoplastic lesions like heart failure, acute

sororities, pancreatitis, tuberculosis etc. On the other hand, malignant effusion results from peritoneal carcinomatosis either from primary peritoneal malignancies such as mesothelioma, gastric or colonic adenocarcinoma or metastatic malignancy from breast or lung carcinoma or melanoma [5]. Adenocarcinomas are the most common neoplasm [2]. The most common occult primary tumor presenting with malignant effusion are intestinal and pancreatic adenocarcinoma in men and ovarian cancer in women [6] once an effusion fluid is positive for malignant cells, signifies spread of disease beyond the organ of origin and is associated with significant therapeutic and prognostic implications [7]. Ascitic fluid cytology is a simple and useful procedure since long time associated with high specificity false positive diagnosis occurs in less than

1% of cases [8]. The false-positive, false negative & suspicious diagnosis are caused by mesothelial cell in the setting of pulmonary infraction, tuberculosis, post chemotherapy, acute pancreatitis, ovarian fibroma & cirrhosis [9]. In conventional cytology discrimination of the reactive mesothelial cells and malignant cells is one of the important diagnostic problems. Availability of cell blocks is helpful in these setting & also provides scope for performing immunohistochemistry [10].

For our study we use few sensitive immunomarkers that will aid to establish diagnosis in resource limited setup. Aims and objectives of the study was:

1. Study of ascitic fluid cytology for the presence or absence of malignant epithelial cells.
2. To assess the utility of cell block preparation in increasing the sensitivity of cytodagnosis specially in case of malignant ascitis.
3. Effectiveness of immunohistochemistry on cell block sections in differentiating metastatic adenocarcinoma from reactive mesothelial cells.

MATERIAL AND METHOD

The study was conducted in the department of pathology of a tertiary care hospital with 250 ascitic fluid samples collected from 250 patients attending the medicine, G&O OPD or admitted indoor were included in this study. Fluid containing clotted blood was excluded from this study.

Ten millilitres of each fresh ascitic fluid sample was divided into two equal parts of 5ml each. One part was subjected to the conventional smear cytology technique and the other part for the cell block technique. At first, this 5 ml fluid was centrifuged at 2500 rpm for 15 minutes. From centrifuge deposits H & E and papanicolaou stained smear prepared. Smears are examined for presence or absence of malignant cells using morphological criteria including cellularity, arrangement of cells, nuclear and cytoplasmic details were put together and used for the categorization of the fluid specimen [11].

The following points were noted in smear:

a) Specimen cellularity [12].

The cellular material in conventional smear is considered to be

-mild when there are 5-50 nucleated cells per high power field,

-moderate when there are 50-200 cells per high power field

-marked when there are >200 cells per high power.

b) Type of predominant cells.

c) Characteristics of cytoplasm.

d) Nuclear characteristics-nuclear membrane, chromatin pattern, presence of nucleoli, mitotic figures.

e) Smear background.

A categorical diagnosis of each case was given-

Benign - Scanty cellularity

-Acute inflammatory infiltrate rich

-Lymphocyte rich suspicious for malignancy malignant.

Thereafter, in diagnostically difficult cases (which are categorised as Suspicious for malignancy and malignant on conventional H&E, papanicolaou stained smears), cell blocks were prepared. Special stain like PAS stain and immunohistochemical test (Calretinin, CK7 & CK20) were performed in the cell block sections to confirm the diagnosis.

The corresponding cell blocks were cut 3-5 micrometer thick sections & examined. The cellularity of in the cellblock is considered as Scanty when there were 5-200 nucleated cells per high power field; Adequate for diagnosis when 200-1000 cells per high power field & High when there were >1000 cells per high power field.¹² Poly-L-Lysine (1:10 dilution) coated slides were processed for immunostaining with primary antibody against Calretinin, cytokeratin7 (CK7), cytokeratin20 (CK20).

RESULT & ANALYSIS

In our study majority of patient were in age group of 50-60 years (35.6%) followed by above sixty age group (28%). Least commonly affected age group first & second decade (0.8%). Male: Female ratio was found to be 1.8:1. (Diag-1) In case of patient presented with malignant ascites the M: F ratio was 0.4:1. Samples received with history of suspicious malignancy had male: female ratio of 0.8:1. Most patient presented with ascities with or without cirrhosis(64.8%), next with clinical suspicion of malignant ascities (12.4%). [Table-1] Total 9.2% of fluid samples were received as malignant ascites under investigation out of which 32% belong to fifth decade of life. Patient presented with non neoplastic diseases consists of 64.8% with cirrhosis, 9.2% presented with CRF, and 4.4% with CCF. Total 21.6% samples were processed under clinic-radiological suspicion of malignancy. [Table-1] Mesothelial cells are found to be the predominant cells in most of the cytological smears (40%) and 8.4% of smears showed presence of

malignant cells. Others show inflammatory cells as predominant cell. [Table-2]

The sensitivity of ascitic fluid cytology alone was found to be 74. 2% in our study and the specificity was found to be 89.4%. Table 3 shows Comparison of diagnostic efficacy between Cytology & Cell block method. There is 3.3% more cases are diagnosed as malignant after histopathological study on cell block. Table 4 is a cross table shows that Pearson Chi-Square test value .000(<0.5) & Likelihood Ratio 260.46.

Diagnostic efficacy for identifying malignant ascities is significantly increased after cell block study.

For identifying primary site of metastatic carcinoma, we use cytokeratin 7 & cytokeratin 20. Out of total 27 cases of metastatic adenocarcinoma, 40% were positive for CK7 out of which 8 cases were from ovarian carcinoma including papillary serous type. 48% cases show CK20 positivity, mostly were from adenocarcinoma colon. Two (7.4%) cases were positive for both CK7 & CK20 and 5 cases were negative for both the markers. [Table-5]

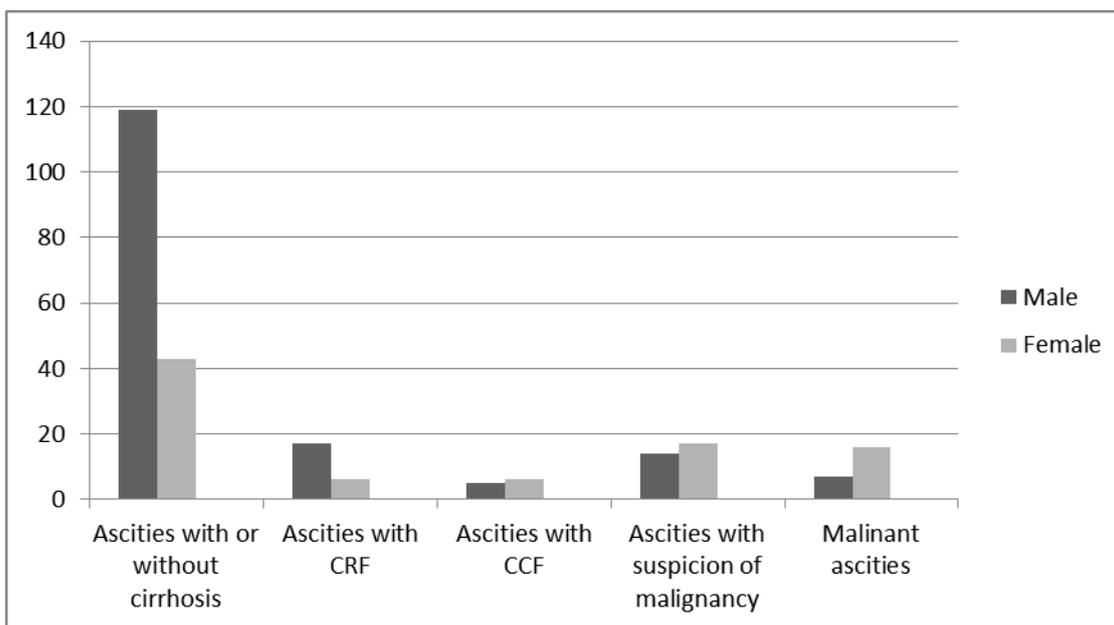


Fig-1: Bar diagram showing distribution of cases according to sex

Table-1: Showing the clinico-radiological diagnosis of patients with ascites

Clinical-radiological diagnosis	No. Of Cases (n=250)	Percentage
(A) Cirrhosis of liver with ascites	152	58.8%
(B) Ascites with chronic renal failure	30	12%
(C) Ascites with congestive cardiac failure	15	6%
(F) Ascites with suspicion of malignancy	30	12%
(G) Malignant ascites under investigation	23	9.2%
Total	250	100

Table-2: Showing the distribution of predominant cells in cytological smear

Predominant cells	Ascities negative for malignant cell (n=196)	Suspicious for malignancy(n=31)	Malignant ascities (n=23)
Mesothelial cells	101	1	0
Lymphocytes & inflammatory	95	1	0
Malignant cells	0	0	21
RMC/ malignant cells	0	29	2

Table-3: Comparison of diagnostic efficacy between Cytology & Cell block method

Features	Cytological Diagnosis		diagnosis after cellblock	
	No	Percentage	No	Percentage
Benign	196	78.4%	217	87.5%
Suspicious	31	12.4%	0	0%
Malignant	23	9.2%	31	12.5%
Total	250(100%)		248 (100%)*	

*Diagnosis could not be possible in two cases

Table-4: maligncyto * maligblock crosstabulation count

		MALIGBLOCK			Total
		MALIGNANT	NONMALIGNANT	NOT DONE	
MALIGNCYTO	MALIGNANT	21	1	2	24
	NON MALIGNANT	1	0	194	195
	SUSPICIOUS	10	21	0	31
Total		32	22	196	250
Chi-Square Tests					
	Value	Df	Asymptotic Significance (2-sided)		
Pearson Chi-Square	327.319 ^a	4	.000		
Likelihood Ratio	260.466	4	.000		
Linear-by-Linear Association	.733	1	.392		
N of Valid Cases	250				

a. 4 cells (44.4%) have expected count less than five. The minimum expected count is 2.11.

Table-5: Correlation of cytological & histological diagnosis with combined CK7/CK20 and Calretinin reactivity

Cytological & histological diagnosis	No. Of cases	CK7 Positivity	CK20 positivity	Calretinin
Reactive mesothelial cells	21	0	0	21
Adenocarcinoma	27	11	13	0
Total	48	11	13	21

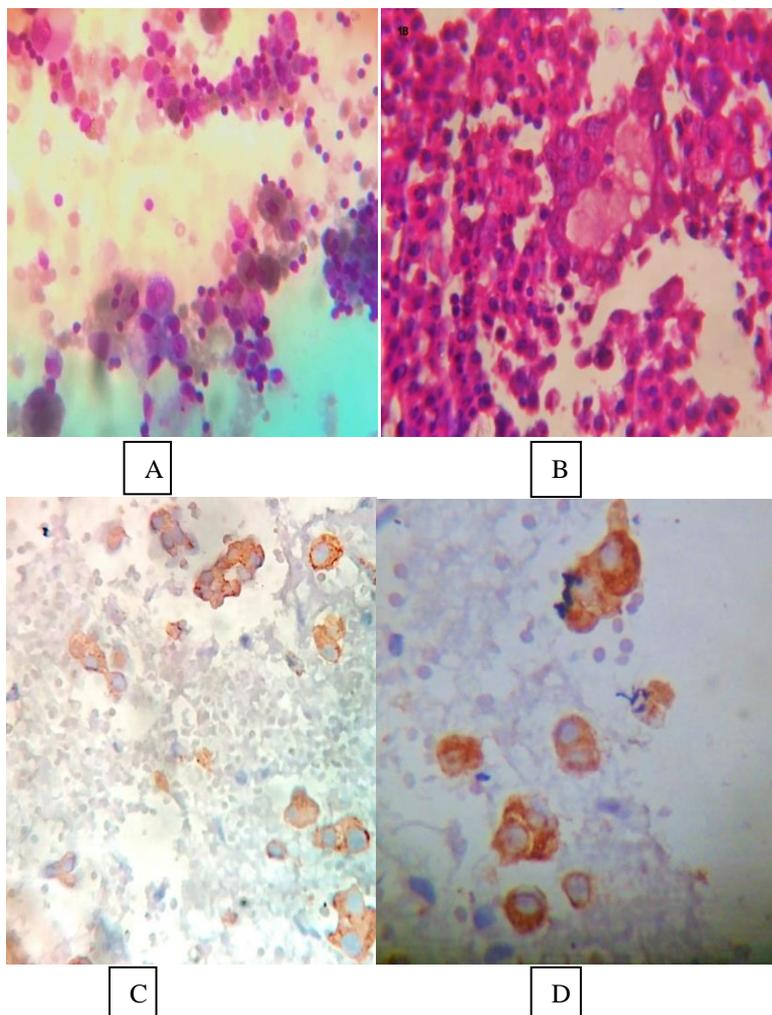


Fig-A: Conventional cytos smear with suspicion of malignancy (H&E;400X).
Fig-B: Cellblock preparation showing adenocarcinoma deposit (H&E;400X).
Fig-C: CK 20 positivity on cellblock (400X).
Fig-D: CK7 positivity on cellblock (400X).

DISCUSSION

The cytological examination of serous fluid has diagnostic, therapeutic and prognostic implications since long time. Lucke and Klebs first demonstrated atypical cells in ascetic fluid in 1867 [13]. In case of malignant lesions, it also helps in the staging of these lesions. The malignant cells in serous fluids were almost always suggestive of metastatic tumours, as primary malignancies were usually rare. Primary malignancy if present, the tumour cells were usually found in clusters & numerous [14]. The examination of body fluids for the presence of malignant cells has been accepted as a routine laboratory procedure, not only for

the detection of unsuspected cancers, but also for the detection of metastasis of an unknown primary origin. Ascitic fluid has a much higher rate of detecting malignant cells specially bloody fluid is highly suspicious of malignancy [15]. In our study, hemorrhagic aspirates were noted in most of cases with malignant effusions and cellularity was also high.

We have taken 250 ascitic fluid samples from 250 patients with ascites reporting in outdoor or admitted indoor. Most patients were in the age group of 51-60 years (35.6%) followed by above 60 age group. Bar Diagram-1 shows distribution of cases according to

sex. In a previous study done by Bodal V K maximum number of cases (26.8%) were in the age group of 41-50 years (fifth decade) followed by 20.8% in sixth decade [16]. In our study maximum number of cases presented with ascities with or without cirrhosis(64.8%), followed by patient presented with clinical suspicion of malignant ascities (12.4%). 9.2% of fluid samples were received as malignant ascites under investigation. In another similar study done by Dowerah and Das et al commonest cause of ascites was found to be Liver cirrhosis accounting for 40% of cases and suspicious cases of malignancy accounted for 11% [17].

Luse and Reagan reported that maximum number of cases of non-malignant effusion were of pleural followed by peritoneal whereas least number of pericardial effusion. According to them the majority of the cases were due to underlying congestive heart failure comprising 18% of all cases, followed by cirrhosis and tuberculosis comprising 11% and 8% on cytology [18]. In our study overall Male : Female ratio is 1.8:1. In case of malignant ascites the ratio is 0.4:1 and 0.8:1 for suspicious of malignancy cases. This is in contrast with a study done by Bhanvadia VM. et al where they found a female predominance [11].

Mesothelial cells are found to be most predominant cell in most of the smear (40% of cytological smear) and 8.4 % of smear showed presence of malignant cells. Other shows lymphocytes & inflammatory cells as predominant cell. (Table 2) Smears showing malignant cells or suspicious cells were mostly from adenocarcinoma (60%) including 1.6% papillary serous adenocarcinoma of ovary, 1.4% undifferentiated carcinoma, 8% Non-hodgkin lymphoma, 0.4% of Small round cell tumour and spindle cell tumor. Bhanvadia VM. *et al* also found most of the malignant neoplasm in ascitic fluids were derived from adenocarcinoma of ovarian tumours [11].

The sensitivity of cytological diagnosis of ascitic fluid alone was found to be 74. 2% and specificity was 89.4% in our study. After doing cell block preparation we had almost 100% sensitivity & specificity. (Table- 3) A study conducted by R O S Karoo *et al* found 60% sensitivity of ascitic cytology with 100% specificity [19]. However, reactive mesothelial cells poses a great deal of diagnostic problem in conventional smear. Abundance of inflammatory cells and a relative paucity of cells of interest contribute to the considerable difficulties for a conclusive diagnosis. The reactive mesothelial cells are

found in a number of non-neoplastic condition like hepatic cirrhosis, allergic pleurisy, polyarteritis, pulmonary infarcts, congestive cardiac failure and in long standing effusions. These benign conditions also presented with reactive changes such as cytomegaly, multinucleation, mitotic figures and a high N/C ratio. Another limitation of the conventional cytological examination of effusions is that it has a sensitivity of only 40–70% for detecting the presence of malignant diseases. The important causes are the overcrowding of the cells, cell loss and difference in processing methods & artifacts caused by poor fixation, preparation, or staining techniques [20].

In this study, we use cell block method especially for difficult cases to improve the accuracy of diagnosis particularly in those cases which either showing malignant cells and suspicious/reactive mesothelial cells. Cell blocks prepared from residual fluid provide opportunity to see the tissue architecture which aid in diagnosis. Cell Block represent an aggregate of exfoliated cells from serous fluids. The technique is very cost effective, reproducible and safe [14]. Availability of the serial sections made from the serous fluids for the further histological examination, special staining, immunohistochemistry is also unique feature of this technique [14]. The diagnosis of malignancy could be made more reliable by examination of three dimensional cell clusters [14], details of morphological features in their proper perspective i.e., the presence of the nucleoli and the acinar structures, cell balls or distinct papillary architectures. The presence of mucin within the malignant cells is an evidence of glandular epithelium [21] of well differentiated adenocarcinomas originating from the lung, ovary or gastrointestinal tract [14]. There were some disadvantages with the cellblock technique such as more time consuming when it is compared to the conventional smears and the loss of tissue during the processing. Centrifugation artefacts may form rosettes or pseudoacini & may cause a misdiagnosis [14]. We had used 10% alcohol-formalin as a fixative which was found to be simple and inexpensive for cell block and it could be processed without any special training or special instruments. Formalin causes cross linking of protein molecules and the gel structure formed could not be dissolved in processing, thus minimizing the tissue loss [22].

There is 3.3% more cases diagnosed as malignant after histopathological study on cell block. (Table-3) So, there will be no necessity to give a diagnosis of suspicious of malignancy or equivocal for malignancy. We had studied histology in 55 cases, out

of which 44(80%) cases yields superior diagnosis after cyto-histological & immunohistochemical correlation. Pearson Chi-Square test value is .000(<0.5) & Likelihood Ratio is 260.466. Diagnostic efficacy for identifying malignant ascitis is significantly increased after cell block study. This finding is corroborative with study done by Shivakumarswamy Udasimath et al & Khan N *et al* [14, 23]. Out of 31 suspicious for malignancy cases on cytology 33% were diagnosed as metastatic adenocarcinoma & 67% were actually reactive mesothelial cells mimicking malignant cells. Thus histological study done by cell block also reduces chance of false positive cases & hereby increases accuracy of effusion study. We have found that out of 33 cases of malignant ascitis, 70% were from metastatic adenocarcinoma and 12% from papillary serous carcinoma metastazing from ovary. NHL was 6%, 3% was squamous cell ca, spindle cell ca & undifferentiated ca each. These findings can be correlated with previous studies [14,24,25].

We used a panel of antibodies that was helpful for differentiating between adenocarcinoma and reactive mesothelial cells in suspicious for malignant cases. We included calretinin, a marker that recognizes mesothelial cells with 100% sensitivity & specificity. Out of total 27 cases of metastatic adenocarcinoma, 40% were positive for CK7. Of them 8 cases were from metastatic ovarian carcinoma including papillary serous type. CK7 is known to label several types of normal and neoplastic glandular epithelia; carcinomas from the GI tract and from stomach. 48% cases show CK20 positivity, mostly were from metastatic colon carcinoma and also from mucinous ovarian tumour. Two (7.4%) cases were positive for both CK7 & CK20 indicating a primary cancer from the G I malignancies other than colon, most probably from pancreas. Five cases were negative for both the adenocarcinoma markers, possible causes include carcinoma of adrenal cortex or prostate [26]. Similar studies were also done by Esteban JM *et al* [27]. Nance KV *et al* [28] to improve cytomorphological diagnosis of serous effusions. In our study, we use combination of three easily available markers so that we can give more conclusive diagnosis reducing the cost of diagnosis. Use of cell block technique eliminated the suspicious for malignancy category giving more definitive diagnosis and shows additional increase in diagnostic yield with subsequent immunohistochemistry. Positive results, identification of primary site in malignant ascitic fluids and further typing will have an obvious influence on patient management as most of the cases it can also predict the prognosis.

We recommend combined use of cytology on conventional smears along with cell block & immunohistochemistry for suspicious or malignant ascitis cases to raise diagnostic accuracy as much as possible. In resource limited areas, as in developing countries like India this approach can be the investigation of choice that helps in diagnosis & provide valuable information to clinician's earlier than other costly investigation just by performing cell block technique in residual fluid specimen. This step by step approach is also cheap & effective with use of a panel, which included markers that recognizes adenocarcinoma as well as cells of mesothelial origin, making the differentiation between metastatic adenocarcinoma and reactive mesothelial cells easy. Limitations of our study are -only ascitic fluids were taken, not other type of effusion fluids were included.

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