European Journal of Molecular & Clinical Medicine

ISSN 2515-8260 Volume 09, Issue 04, 2022

ORIGINAL RESEARCH

IMPORTANCE OF CELL BLOCK WITH IMMUNOCYTOCHEMICAL EVALUATION IN CARCINOMATOUS PERITONEAL EFFUSIONS OF UNKNOWN PRIMARY IN FEMALES

Priya Singh[1], Sachin Kumar[2], Mohd. Anwar[3], Sharique Ahmad[4], Anurag Gupta[5], Alina Kazmi[6]

[1]Assistant Professor, Department of Pathology, T.S.Misra Medical College and Hospital, Lucknow, Uttar Pradesh, India

[2]Senior Resident, Department of Pulmonary and critical care Medicine, King George's Medical University, Lucknow, Uttar Pradesh, India

[3]Senior Resident, Department of Transfusion Medicine, SGPGI, Lucknow, Uttar Pradesh, India

[4]Professor, Department of Pathology, Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh. India

[5]Professor, Dr RMLIMS, Uttar Pradesh, India

[6]Junior Resident, Department of Pathology, Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh. India

Corresponding Author:

Prof. (Dr) Sharique Ahmad, Department of Pathology, Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh. India

ABSTRACT

Background: Diagnosis of carcinomatous cells in peritoneal and pleural effusions is important for staging procedures and resulting therapeutic decisions. Various methods are available like routine smears, cell blocks, liquid based cytology etc. for cytological diagnosis. Difficulties are often faced to detect site of primary in carcinomatous effusions due to overlap in the morphologic features from various sites. Various ancillary studies have been used to increase the diagnostic accuracy of cytology. Immunocytochemical analysis is the most commonly used "special" technique and often involves the use of a panel of antibodies. Neoplasms of breast, lung is most common cause of carcinomatous pleural effusion in females and males respectively. Most common primary neoplasms causing carcinomatous peritoneal effusions in females are carcinomas of ovary and in males common sites of carcinomatous effusion include colon, rectum and stomach. The aim of this study is to immunocytochemically evaluate carcinomatous peritoneal effusions in females using cell block.

Materials and Methods: Study included 850 cases of effusion in one and a half years of which 50 (5.9 %) were positive for malignancy. Most patients were in age group of 40-

60 years, Cell blocks were made of effusion fluids and immunocytochemical markers Calretenin,CK 7, CDX-2,WT-1,PAX-8 were used.
Results: 44 cell blocks (88%) were adequate in terms of cellularity and malignant cells. Out of 44 , 38 were peritoneal fluids. 35/38 (92%) were concluded as ovarian primary, 03/38 (8%) as gall bladder primary. All the cases showed positivity for CK7, none of them showed positivity for Calretenin ruling out possibility of mesothelioma. We concluded the positivity of WT-1 in 18 out of 35 cases (51.42 %) with a sensitivity of 51.4 %, specificity of 100 %, PPV of 100 %, NPV of 34.6 %. PAX-8 was positive in 25 out of 35 cases (71.43 %) with a sensitivity of 71.43 %, specificity of 100 %, PPV of 100 %,NP V of 47.37 %. On combining WT-1 and PAX-8 the sensitivity increased to 74.29 %, specificity of 100 %, PPV of 100 %, NPV of 50 %.
Conclusion: PAX-8 is more sensitive marker than WT-1 for peritoneal effusion with ovarian primary. Sensitivity further increases on combining both the markers. CDX-2 were positive in all the 3/3 cases of Gastrointestinal tract with Sensitivity, specificity, PPV, NPV of 100 %.
Keywords: Effusion, Cell Block, WT-1, PAX-8, ICC.

INTRODUCTION

Diagnosis of carcinomatous cells in peritoneal and pleural effusions is important for staging procedures and resulting therapeutic decisions. Various methods are available like routine smears, cellblocks, liquid based cytology etc. for cytological diagnosis. Paired-box genes (PAX) encode a family of nine well-characterized paired-box transcription factors (PAX1-9), which are important for embryogenesis and disease.[2] PAX proteins have been implicated as regulators of organogenesis and key factors in maintaining pluripotency of stem cell populations during development. Among these PAX genes, PAX8 has been hypothesized to be crucial in determining cell fate during the development of the thyroid, kidney, brain, eyes and Müllerian system and regulates expression of the Wilms' tumor suppressor gene (WT1).[3-6]PAX8 has been found to be expressed at high levels in ovarian carcinomas and serous effusions, indicating that PAX8 detection may prove useful in the clinic.[7] WT-1 has proven useful in the diagnosis of ovarian serous carcinoma. This can present as diagnostic pitfall in effusion cytology, because mesothelial cells can demonstrate immunoreactivity for WT-1. PAX-8 has a superior edge over WT-1 as it does not stain mesothelial cells and has shown very high sensitivity and specificity for ovarian carcinoma.[14] The studies in the literature for CDX-2 have proven that it is a highly specific and sensitive marker for colorectal origin in diagnosing adenocarcinoma as well as neuroendocrine tumours of small intestine and large intestine.[8,9,10]

MATERIALS & METHODS

The present study was conducted in the Department of Pathology of RMLIMS, Lucknow, from January 2016 to June 2017.This was a prospective observational study for diagnostic test evaluation performed at this Institute. A total of 38 malignant peritoneal effusion female cases were studied. Only Female patients with carcinomatous peritoneal effusions were included with preserved cell morphology. Criteria for adequacy of cell block were on the basis of adequate amount and cellularity. Malignant fluids with inadequate cellularity, poor

preservation and inadequate amount were not included. Effusion fluid samples which were received in the cytology laboratory of Department of Pathology of Dr. Ram Manohar Lohia Institute of Medical sciences, Lucknow were analyzed in the following manner:

Clinical and radiological Assessment: Adequate clinical and radiological details shall be documented including age, sex, duration of symptoms, presenting clinical features, past history and radiological findings. Staging and any site of distant metastasis was noted.

Cytological Assessment: Cytology evaluation of Ascitic fluids for malignancy with the help of cytocentrifuge smears. Positive for malignancy effusions were processed for cell block preparation.

Cytocentrifuge method:

- Label 2 cytoslides with cytopathology (CP number).
- Insert cytoslides into a slide holder, then the filter followed by funnel.
- Place specimens into funnel drop- wise, 2 to 3 drops are usually sufficient.
- Set the cytotek at 1000 rpm for 5 minutes.
- Cytocentrifuge smears were made and stained with H and E and PAP stains , further adequacy was checked and effusion fluid were processed for cell block.

Processing of sample collected for cell block in our study

- Sample is centrifuged in a 10 ml disposable centrifuge tube at 4,000 rpm for 6 minutes to create 1 or more cell pellets (1 pellet in most cases.)
- Nathan alcohol formalin substitute (NAFS) is added to spun- down cell pellets after the supernatant is discarded.
- After a minimum of 45 minutes, the sample is re-centrifuged.
- The cell pellets are wrapped in filter paper, placed in cassette, and stored in 10 % formalin.
- Above cell pellets were processed in the automatic tissue processor using a 13 hour processing schedule. Cell pellets were embedded in paraffin to prepare cell blocks.
- Cell blocks were sectioned at 3 µm thickness to perform H&E staining and immunohistochemistry.
- NAFS consists of a 1:9 solution of 40 % formaldehyde and 100% ethanol. The fixative must be prepare fresh and used immediately because formalin is capable of oxidizing to formic acid.

Immunohistochemistry

Commercially available antibodies were used for various Immunocytochemical markers. [Table 1]

Immunocytochemistry evaluation:

1. Tissue section and Deparaffinization: 2 - 5 µ section were cut and fixed at 56°c for maximum 90 minutes. Deparaffinized in Xylene and rehydrated through graded alcohol 100%, 70 %, 50% followed by distilled water.
2. Peroxidase Treatment: Section were treated with 50 ml methanol + 1.5 ml H_2O_2 for 30 minutes to block endogenous peroxidase activity followed by 3 gentle washes in tris buffered saline TBS (pH 7.4) for 5 minutes.
3. Unmasking of antigenic sites by heat antigen method: Deparaffinized sections were immersed in acidic or alkaline ph as per requirement for antigen retrieval and were kept

on Pascal pressure system. Section was then allowed to cool at room temperature followed by 3 gentle washes in TBS for 5 minutes.

4. Protein Block: Sections were treated with protein blocking reagents for 10 minutes to reduce non – specific binding of primary antibody.
5. Primary antibody was applied Slides were wiped off excess buffer and incubated one hour with primary antibody at room temperature in a moist chamber for 1 hour incubation. Slides were rinsed with Tris buffer thrice.
6. Enzyme conjugate: Polymeric HRP was applied for 30 minutes by 3 washes in Tris Buffer for 5 minutes each.
7. Substrate chromogen Solution: Concentrated diaminobenzidine solution (DAB) was diluted with substrate buffer (950 µL of substrate buffer + 50 µL of DAB) was diluted DAB solution was added to smears for 5 to 7 minutes followed by Tris buffer washing.
8. Counter stain: Sections were dipped in 10 % hematoxylin for 2 minutes and washed in distilled water, dehydrated with alcohol, cleaned in xylene and mounted With DPX.

Table: 1

Markers	**Clone**	**Source**	**Dilution**
PAX – 8	BC12	Biocare	Ready to use
WT – 1	6F – H2	DAKO	Ready to use
CDX-2	DAK-CDX2	DAKO	Ready to use
CK 7	OV – TL 12/30	DAKO	Ready to use
Calretenin	DAK – calret 1	DAKO	Ready to use

[Table 1]: Various IHC markers used in the study with their clone, staining for PAX-8, WT-1, CDX-2, CK-7, Calretenin was evaluated and scored by two pathologists (A.G and P.S). Nuclear staining in more than 5% of the malignant cells was considered a positive result for the markers.

Statistical analysis: of results with calculation of sensitivity, specificity, positive predictive value and negative predictive value. Categorical variables were presented in number and percentage (%) and continuous variables as mean ± SD and median. A p value of < 0.05 will be considered statistically significant. The data was entered in Ms Excel spreadsheet and analysis will be done using statistical package for Social Sciences (SPSS) version 21.0.

RESULTS

The present study was carried out in department of pathology at Dr. RMLIMS, Lucknow. The aim of this study is to immunocytochemically evaluate carcinomatous peritoneal effusions in females using cell block. A total of 850 effusions were received in on department during the duration of one and half years. 50 were positive for malignancy. [Table 2] 35 were peritoneal effusions, Cell blocks were made.

Table: 2

Effusion	**Frequency**	**Percentage**
Positive for Malignant cells	50	6%
Negative for Malignant cell	800	94%
Total	850	100%

[Table 2]: Majority of serous fluid came for cytological examination were negative for malignancy (800/850=94%), (50/850=6%) of fluid were positive for malignant cells.

Table: 3

Age Distribution	**Frequency**	**Percentage**
25 – 40	**3**	**8%**
40 – 60	**30**	**79%**
>60	**5**	**13%**

[Table 3]: In our study we included female effusion only. The highest number of malignancy was seen between 40 – 60 years of age (79%).

Diagnostic test evaluation:

WT-1

Table: 4

WT-1	**Frequency**	**%**
Positive	18	51.42
Negative	17	48.6
TOTAL	35	100

[Table 4]: WT-1 was positive in 18 out of 35 cases (51.42%) cases where is negative in 17 out of 35 cases (48.6 %).

Table: 5

ICC MAKER	**Sensitivity**	**Specificity**	**PPV**	**NPV**
WT-1	**51.4%**	**100%**	**100%**	**34.6%**

[Table 5]: WT-1 showed the sensitivity of 51.4 %, specificity of 100%, PPV of 100%, NPV of 34.6%

PAX-8:

Table: 6

PAX-8	**Frequency**	**%**
Positive	25	71.4
Negative	10	28.6
TOTAL	35	100

[Table 6]: PAX -8 was positive in total 25 cases of 35 effusions in ovary with positive percentage of 71.4%

Table: 7

ICC Maker	Sensitivity	Specificity	PPV	NPV
PAX-8	**71.45%**	**100%**	**100%**	**47.37%**

[Table 7]: PAX-8 showed sensitivity of 71.45%, SP of 100%, PPV of 100%, and NPV of 47.37%.

Table: 8

ICC Markers	Sensitivity	Specificity	PPV	NPV
WT and PAX-8	**74.29**	**100**	**100**	**50**

[Table 8]: On combing WT-1 and PAX-8, the sensitivity incubates to 74.29% and specificity was 100%, PPV of 100% and NPV of 50%. Intensity of expression of WT-1 and PAX-8 in ovary.

Table: 9

	WT-1				PAX-8			
Intensity Percentage	0	+1	+2	+3	0	+1	+2	
	17	4	10	4	10	7	11	7
	48.57	11.42	28.57	11.42	28.57	20	31.42	31.4

[Table 9]: O – Negative, +1 – Week stain, + 2 – Moderate stain, +3 – Strong stain. PAX-8 showed moderate to strong expression in 62.84% of cases in comparison to WT-1 which showed in 39.99% of cases. Further percentage of no staining was for more in WT-1 (48.47%) in comparison to PAX-8 (28.57%).

CDX-2

CDX-2 is a highly sensitive and specific marker for gastrointestinal tract carcinoma. In our study all the three cases of GIT were positive for CDX-2.CK-7 was positive in all cases whereas Calretenin was negative in all cases ruling out mesothelioma**.**

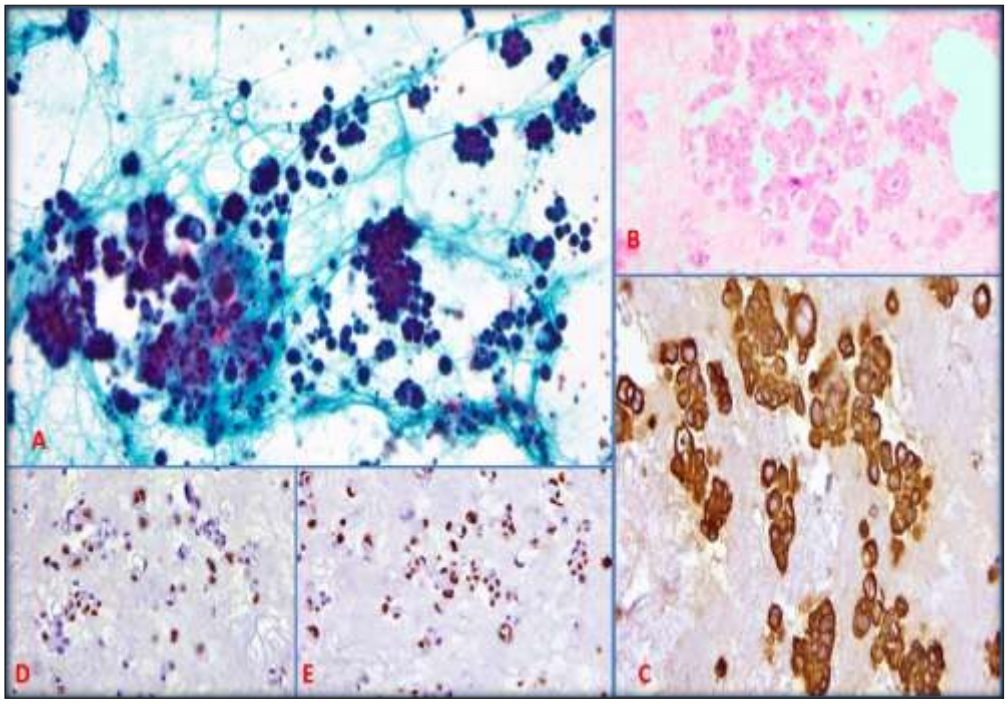

Figure: 1. (A, B) Papillaroid Clusters of malignant epithelial cells from a metastatic papillary serous adenocarcinoma ovary. (H&E smear & CB, x 200), (C) section shows strong membrane immunoreactivity for CK7 on ICC (hematoxylin counter stain, x 200), (D) section shows strong (+3) nuclear immunoreactivity for WT1 on ICC (hematoxylin counter stain, x 200), (E) section shows strong (+3) nuclear immunoreactivity for paired box gene 8 (PAX8) on ICC (hematoxylin counter stain, x 200)

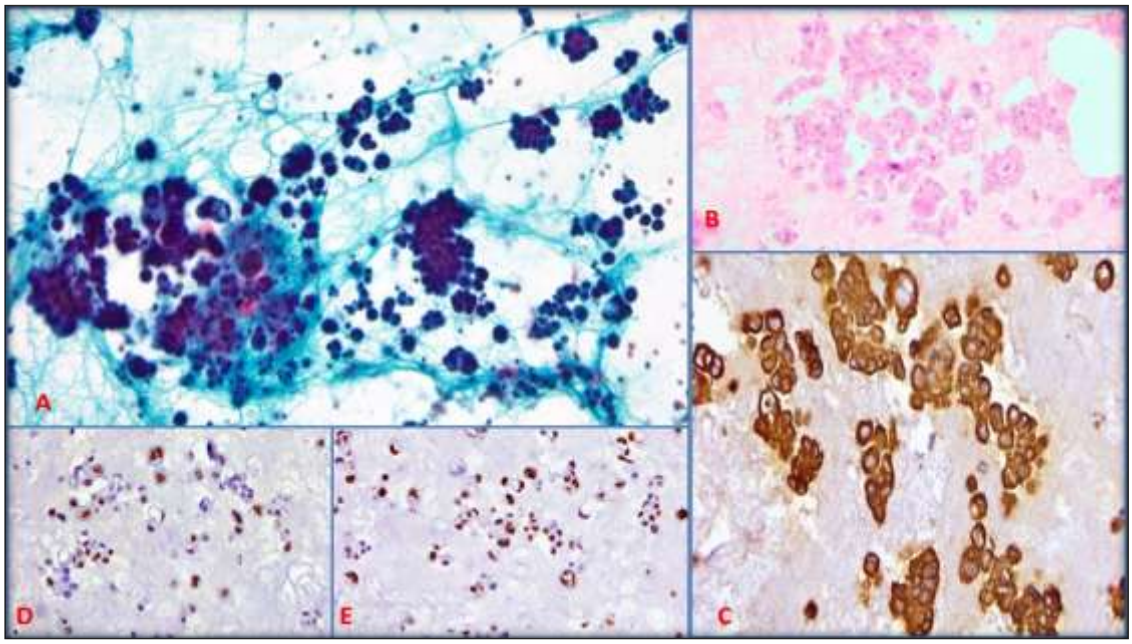

Figure: 2. (A, B) Papillaroid Clusters of malignant epithelial cells from a metastatic papillary serous adenocarcinoma ovary. (H&E smear & CB, x 200), (C) section shows strong membrane immunoreactivity for CK7 on ICC (hematoxylin counter stain, x 200), (D) section shows strong negative immunoreactivity for WT1 on ICC (hematoxylin

counter stain, x 200), (E) section shows moderate (+2) nuclear immunoreactivity for paired box gene 8 (PAX8) on ICC (hematoxylin counter stain, x 100)

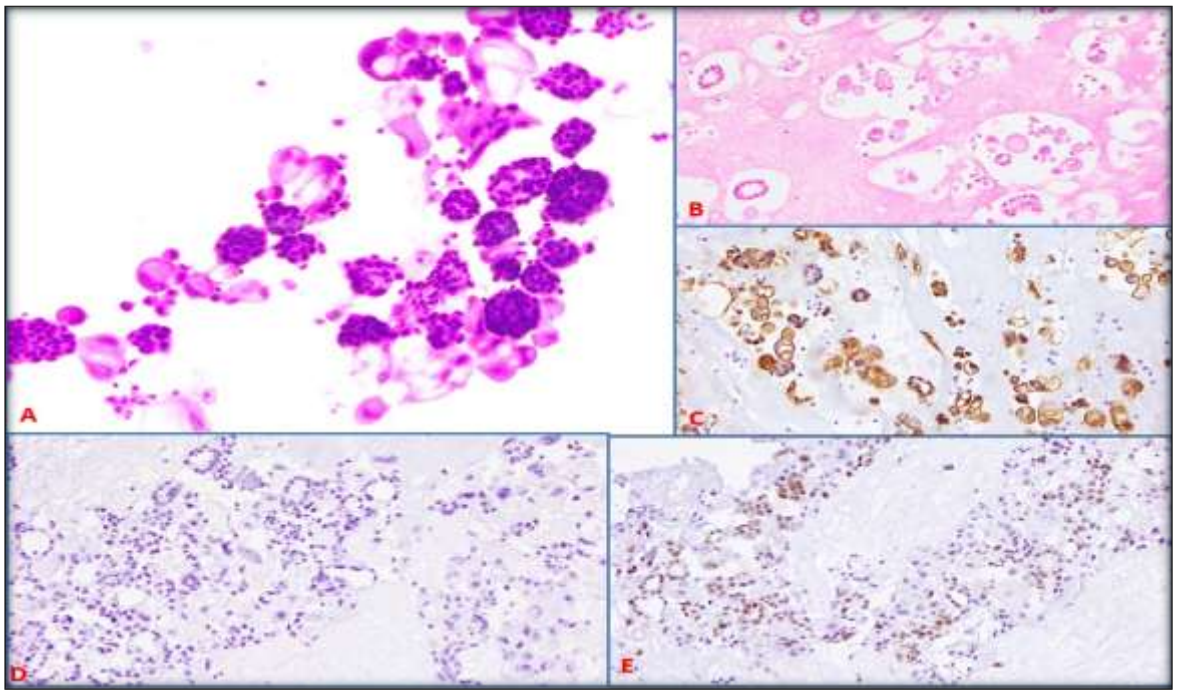

Figure: 3. (A , B) Clusters and singly scattered cells of malignant epithelial cells with ntracytoplasmic vacuoles from a metastatic invasive ductal carcinoma breast. (H&E smear & CB, x 200), (C) section shows strong menbrane immunoreactivity for CK7 on ICC (hematoxylin counter stain, x 200), (D) section shows negative immunoreactivity for mammaglobin on ICC (hematoxylin counter stain, x 200), (E) section shows strong nuclear immunoreactivity for GATA-3 on ICC (hematoxylin counter stain, x 200)

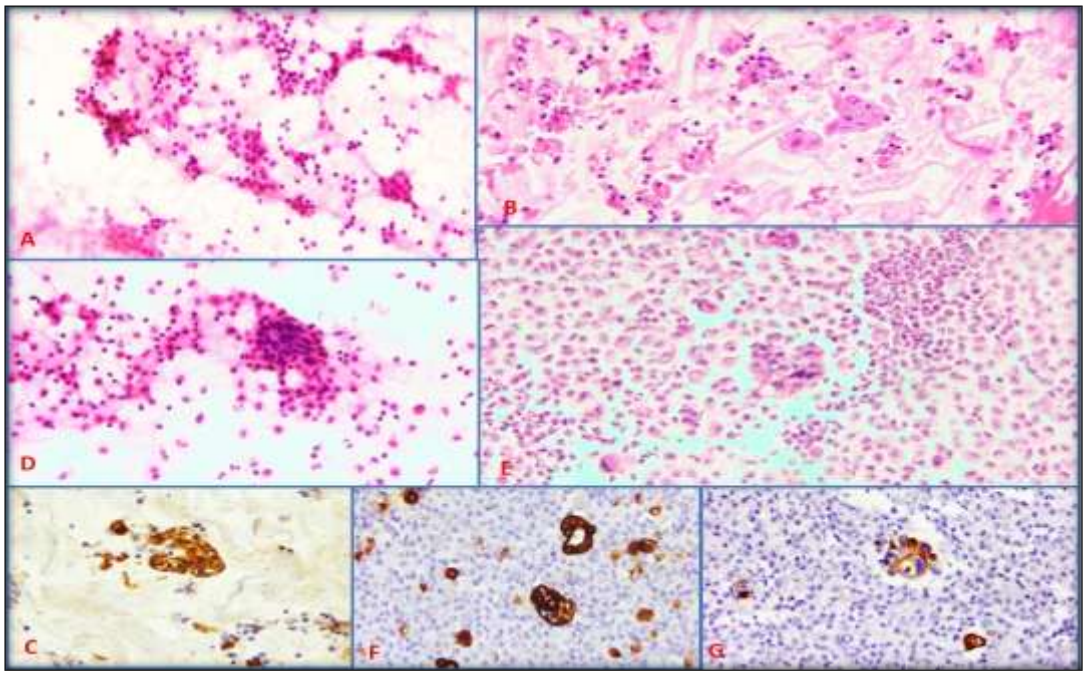

Figure: 4. (A, D) 2 Cases with clusters and singly scattered cells of malignant epithelial cells from metastatic adenocarcinoma gall bladder. (H&E x 200), (B, E,) 2 cases with

clusters of malignant epithelial cells from metastatic adenocarcinoma gall bladder. (H&E CB, x 200) (C) Section from 1st case show membrane immunoreactivity for CK7 on ICC (hematoxylin counter stain, x 200), (FG) section from 2rd case show membrane immunoreactivity for CK7 and CK20 (hematoxylin counter stain, x 200)

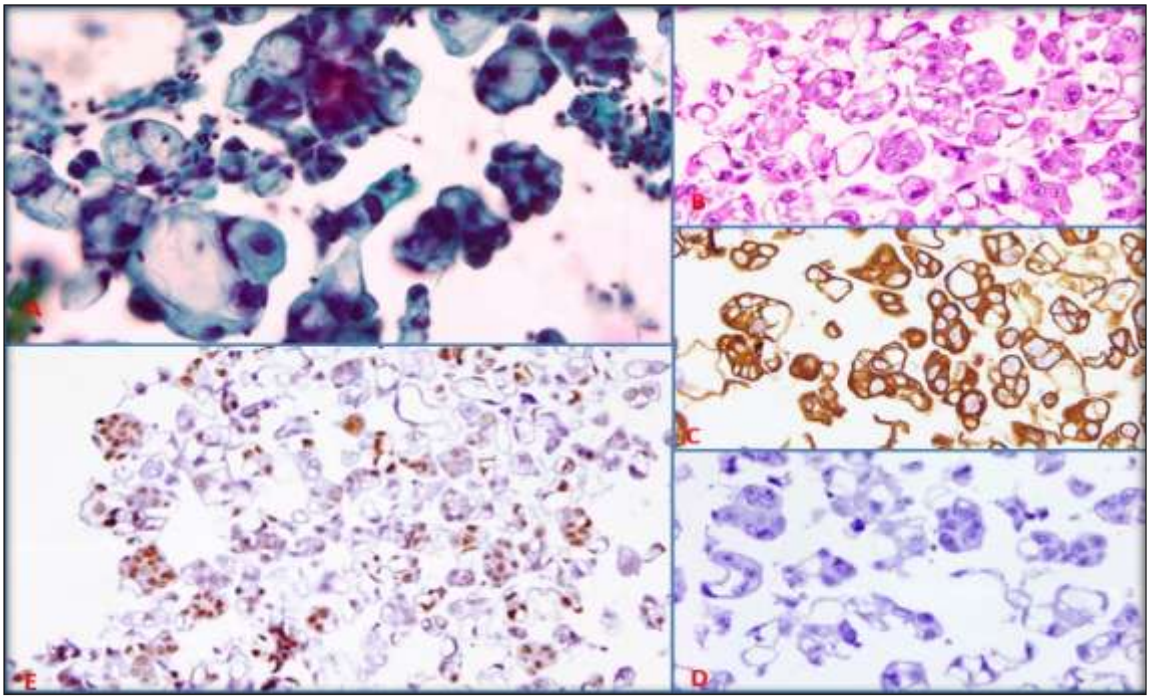

Figure: 5. (A, B) Clusters and singly scattered cells of malignant epithelial cells with intracytoplasmic vacuoles from a metastatic adenocarcinoma stomach. (H&E smear & CB, x 200), (C) section shows strong membrane immunoreactivity for CK7 on ICC (hematoxylin counter stain, x 200), (D) section shows negative immunoreactivity for CK20 on ICC (hematoxylin counter stain, x 200), (E) section shows strong nuclear immunoreactivity for CDX2 on ICC (hematoxylin counter stain, x 100).

DISCUSSION

Cytological examination of serous effusion has been accepted and a positive diagnosis often considered as definitive diagnosis. It helps for staging and prognosis of the patients for malignancy.[11] Cell block (CB) method provides better architecture , morphological feature between reactive mesothelial cells and malignant cells and ancillary studies, immunocytochemistry are superior in the diagnosis workup of effusion cytology.[12] considering the above known fact we planned a study to analyze the efficacy of PAX-8,WT-1 and CDX-2 in carcinomatous effusion among females. A Total of 850 effusions were received in our department in the duration of one and a half years of which 800 were neither negative for malignancy nor inadequate and 50 were positive for malignancy (6%). A Study by Gupta S et al showed similar percentage of positive effusion , they analyzed 11,562 effusion samples respectively for cytopathological examination over a 10 year period were analyzed of which 836 (7.3%) were malignant.[13] In Our study we had 38 cases of carcinomatous peritoneal effusion, all of them were carcinomatous and more specifically adenocarcinoma. Ebru Cakir et al in there study noted that carcinoma most commonly seen in

malignant effusion was adenocarcinoma though they also reported malignant mesothelial (3.8%) small cell carcinomas, (3.5%) hematolymphoid malignancies and (2.7%) squamous cell carcinoma. Hematolymphoid malignancies included non – Hodgkin lymphoma. In our study cases were skewed and all of them were adenocarcinoma. The reason of skewing might be short period of study, less number of samples and more of solid tumours being treated in the hospital due to absence of clinical hematologists. In Our study the most common malignancy causing carcinomatous peritoneal effusion was ovary (35/38). Few previous studies have reported that most common primary neoplasm causing malignant peritoneal effusion was carcinomas of ovary. Malignant cells were associated with serous and endometrioid carcinomas more oftenly than with carcinomas of other types.[14-15]

WT-1 in our study showed 51.4 % sensitivity, 100% specificity, PPV of 100%, NPV of 34.6% for ovarian malignancies. Studies for WT- 1 in the past have demonstrated that WT1 helps in typing primary surface epithelial ovarian tumour, serous and transitional cells carcinomas being positive while other subtypes such as mucinous, clear cell and Brenner tumors and negative. Satoshi ohno et al demonstrated that WT1 gene is highly expressed in hematological malignancy and solid tumors, including endometrial cancer.

In our study PAX-8 turned out to be the best marker of ovarian primary with a high sensitivity (71.43%) and specificity (100%). Sensitivity of Pax-8 was far than that of WT – 1 and CK – 7, Yue Wang et al studied that PAX8 is a sensitive marker to diagnose the carcinomas of ovarian origin. Using WT – 1 and PAX – 8 together for carcinomatous effusion, the sensitivity further increased to 74.29 % and specificity remained with 100%, with PPV of 100% and NPV of 50%. In our study cases of gastrointestinal tract carcinoma (3/3) showed immunoreactivity for CDX-2 with sensitivity and specificity of 100 %.

As demonstrated by Patricia et CDX-2 was expressed in virtually 100 % percent of colorectal adenocarcinoma and in approximately one-half of gastric adenocarcinoma. They also found that CDX-2 was expressed even at higher frequency in the intestinal type adenocarcinoma subset, and in approximately one –third of pancreatobiliary tract carcinomas.[16]

The studies in the literature for CDX-2 have proven that it is a highly specific and sensitive marker for colorectal origin in diagnosing adenocarcinoma as well as neuroendocrine tumours of small intestine and large intestine.[8,9,10]

CONCLUSION

GATA-3 and PAX-8 have shown good sensitivity and very high specificity for breast and ovarian malignancy respectively. However other markers CK7/CK20, CDX-2, WT-1 etc. have also shown their contribution in delineating various carcinomatous causes of effusion. Few studies published in the literature have demonstrated that GATA-3 is more sensitive marker than mammoglobin, GCDFP-15 in diagnosing breast malignancies.WT-1 has proven useful in the diagnosis of ovarian serous carcinoma. This can present as diagnostic pitfall in effusion cytology, because mesothelial cells can demonstrate immunoreactivity for WT-1.PAX-8 had a superior edge over WT-1 as it does not stain mesothelial cells and has shown very high sensitivity and specificity for ovarian carcinoma. Therefore, we incorporated GATA 3 and PAX 8 in our study, with focus on breast and ovarian carcinomatous effusions in females.

REFERENCES

1. Marel M, Stastny B, Melínová L, Svandová E, Light RW. Diagnosis of pleural effusions. Experience with clinical studies, 1986 to 1990. Chest. 1995 Jun;107(6):1598-603.
2. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR. Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene. 2003 Sep 11;22(39):7989-97.
3. Siehl JM, Reinwald M, Heufelder K, Menssen HD, Keilholz U, Thiel E.. Expression of Wilms' tumor gene 1 at different stages of acute myeloid leukemia and analysis of its major splice variants. Ann Hematol 83: 745-750
4. Nonaka D, Chiriboga L, Soslow RA. Expression of pax8 as a useful marker in distinguishing ovarian carcinomas from mammary carcinomas. Am J Surg Pathol. 2008 Oct;32(10):1566-71. doi: 10.1097/PAS.0b013e31816d71ad. PMID: 18724243.
5. Mittag J, Winterhager E, Bauer K, Grümmer R, Congenital Hypothyroid Female Pax8-Deficient Mice Are Infertile Despite Thyroid Hormone Replacement Therapy, Endocrinology, Volume 148, Issue 2, 1 February 2007, Pages 719–725
6. Dehbi M, Pelletier J. PAX8-mediated activation of the wt1 tumor suppressor gene. EMBO J. 1996 Aug 15;15(16):4297-306. PMID: 8861958; PMCID: PMC452155.
7. Xiang L, Kong B. PAX8 is a novel marker for differentiating between various types of tumor, particularly ovarian epithelial carcinomas. Oncol Lett. 2013 Mar;5(3):735-738. doi: 10.3892/ol.2013.1121. Epub 2013 Jan 10. PMID: 23425942; PMCID: PMC3576179.
8. Bayrak, R., Haltas, H. & Yenidunya, S. The value of CDX2 and cytokeratins 7 and 20 expression in differentiating colorectal adenocarcinomas from extraintestinal gastrointestinal adenocarcinomas: cytokeratin 7-/20+ phenotype is more specific than CDX2 antibody. Diagn Pathol 7, 9 (2012).
9. Erickson LA, Papouchado B, Dimashkieh H, Zhang S, Nakamura N, Lloyd RV. Cdx2 as a marker for neuroendocrine tumors of unknown primary sites. Endocr Pathol. 2004 Fall;15(3):247-52. doi: 10.1385/ep:15:3:247. PMID: 15640551.
10. Furrukh M, Burney I, Qureshi A, Lakhtakia R (2015) Cancer of Unknown Primary Site: Not All is Lost!. J Clin Diagn Res 3:115. doi:10.4172/2376-0311.1000115
11. Pal S, Murmu D, Goswami B. ASCITIC FLUID CYTOLOGY IN SUSPECTED MALIGNANT EFFUSIONS WITH SPECIAL EMPHASIS ON CELL BLOCK PREPARATION. Journal of Evolution of Medical and Dental Sciences. 4. 10488-10493. 10.14260/jemds/2015/1511.
12. Jain D, Mathur SR, Iyer VK. Cell blocks in cytopathology: a review of preparative methods, utility in diagnosis and role in ancillary studies. Cytopathology. 2014 Dec;25(6):356-71. doi: 10.1111/cyt.12174. Epub 2014 Aug 11. PMID: 25113785.
13. Gupta, S, Sodhani, P, Jain S. Cytomorphological profile of neoplastic effusions: An audit of 10 years with emphasis on uncommonly encountered malignancies. Journal of cancer research and therapeutics. 8. 602-9. 10.4103/0973-1482.106574.
14. McKnight R, Cohen C, Siddiqui MT. Utility of paired box gene 8 (PAX8) expression in fluid and fine-needle aspiration cytology: an immunohistochemical study of metastatic ovarian serous carcinoma. Cancer Cytopathol. 2010 Oct 25;118(5):298-302.

15. Yoshimura S, Scully RE, Taft PD, Herrington JB. Peritoneal fluid cytology in patients with ovarian cancer. Gynecol Oncol. 1984 Feb;17(2):161-7
16. Patricia L. Kandalaft, MD; Allen M. Gown, MD. Practical Applications in Immunohistochemistry Carcinomas of Unknown Primary Site. Arch Pathol Lab Med. 2016;140:508–523; doi: 10.5858/ arpa.2015-0173-CP.