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# DNA Flow cytometry and immunohistochemical evaluation of effusion specimens

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## Abstract

**Introduction:** Effusion is an abnormal fluid accumulation in any of the body cavities, reflects some pathology and requires rapid diagnosis. Adenocarcinomas afflicting the lung, ovary and breast frequently metastasize to the serosal cavities. Many patients affected by neoplasm come with complaints of accumulation of fluid in one of the serosal cavities. The analysis of DNA ploidy through flow cytometry (FCM) has been used to increase the accuracy and speed of analysis of effusions. Hence, the aim of this study was to assess and compare the utility of DNA flow cytometry and epithelial membrane antigen immunohistochemistry (EMA-IHC) for detection of carcinoma cells in effusion fluids with conventional cytology as bench mark.

**Materials and Methods:** 31 patients with serous effusions (28 pleural, 4 peritoneal) of various aetiologies were included in this prospective study. All the cases were analysed by DNA flow cytometry as well as by conventional cytological examination using modified Leishman stain and Papanicolaou stain, followed by EMA immunohistochemistry (IHC) on the cell blocks.

**Results:** Out of the 31 samples studied 26 were benign and 5 were malignant. Cytology and DNA FCM analysis showed sensitivity and specificity of 75% and 96.3% respectively while EMA-IHC showed sensitivity of 75% and specificity of 92.6%.

**Conclusion:** DNA flow cytometry is more valuable than EMA-IHC and a useful supplementary diagnostic modality to conventional cytology for detection of malignancy in effusion specimens.

**Keywords:** Serous effusions, Flow cytometry, DNA analysis, Cytology, Immunohistochemistry.

## Introduction

Effusion is an abnormal fluid accumulation in one or more body cavities, which reflects some pathology and requires rapid diagnosis.[1] Adenocarcinomas afflicting the lung, ovary and breast frequently metastasize to the serosal cavities.[2] Many patients neoplastic disease present with accumulation of fluid in one of the serosal cavities.

Currently, cytomorphology is gold standard for diagnosing malignant effusion. Errors in sampling, screening and interpretation confer a moderate false-negative rate to cytology reporting.[3] Some specimens make it very difficult to distinguish the malignant cells, mesothelial cells and inflammatory cells like macrophages on cytology.[2] Cytology shows an average 97.0% specificity and 58.2% sensitivity with multiple specimens increasing sensitivity.[4]

The diagnostic efficacy of cytology can be increased by performing immunohistochemistry on the cell block sections. The results of immunohistochemistry are largely dependent on the cellularity of the cell block sections.The complexity of immunostaining process is not time-efficient to make diagnosis promptly. Hence, an adjunct technique needs to be introduced that increases the diagnostic efficacy of malignant effusion samples.[4]

Over the last few decades flow cytometry is an emerging technology. DNA flow cytometry requires nuclei or single cells in a suspension of fluid. DNA specific dye is used for staining dissociated cells. DNA stoichiometry helps in binding these dyes. The stained cells are passed singly through a laser beam. Photomultiplier tube is used in flow cytometry to measure the fluorescence emitted by the cells. DNA flow cytometry is studied by many researchers to detect malignant effusions which showed variation in specificity and sensitivity. Studies carried out by Green, Griffin and Laurini et al show that this is a rapid procedure which has a high specificity (96.8%) and sensitivity (100%) for malignant effusion diagnosis due to metastatic adenocarcinoma.[5]

A large population of cells can be evaluated accurately in a small period by flow cytometry. It is a method which is applied to fresh specimens which have viable cells hence avoiding any artifacts of fixation and it could be complimentary to cytopathology.[6,7] Measurement of content of DNA is the most accepted application which allows identifying aneuploidy of cells and gives information on cell proliferation by analyzing the various cell cycle phases.[8]

Recently flow cytometry analysis of DNA aneuploidy is used to increase cytological accuracy of effusion analysis[9] and to recognize abnormal cells not identified by cytology.[10] Flow cytometry is a rapid, reproducible and sensitive method of cellular antigen detection.

In solid tumours there is good correlation between aneuploidy and neoplastic cells.[11] Flow cytometry gives positive results on basis of aneuploidy whereas cytology gives positive results by detection of abnormal morphology.[11]

Tribukait proposed a principle by which DNA ploidy determination and sample classification as diploid and aneuploidy was done.[12] DNA Index is the ratio of abnormal peak position to the diploid peak of normal lymphocytes + or - 10%. Diploid nuclear value was 1.

Immunohistochemistry is an ancillary method which is used widely in cytopathology to increase diagnostic accuracy. Epithelial Membrane Antigen (EMA) is useful in identification of carcinoma cells in serous effusion samples. Flow cytometric immunophenotyping applying an antibody

panel is used to determine patient specific data in this personalised medicine era.[13] Few studies have reported that flow cytometry is useful in cell detection with stem cell marker expression.[14]

DNA flow cytometry is a rapid and easy procedure, which provides a histogram which can be easily interpreted, and used as a supplement for the diagnosis of pleural effusions.[15] Several studies have reported DNA aneuploidy provides a diagnosis of malignant effusion.[16]

This study is conducted to identify the utility of flow cytometry in the diagnosis of malignant cells in clinical effusion specimens using DNA ploidy analysis by flow cytometry and compare the results with immunohistochemistry using EMA taking cytology as bench mark.

## Materials and Methods

Serous effusions from 31 patients were prospectively analyzed over a two year period (July 2016 to Aug 2018). The study was conducted in Dr.D.Y.Patil Medical College, Hospital and Research Centre. The hospitalized patients with pleural or peritoneal effusions were included in this study. Approval for the study was obtained from the Institute ethics committee.

All the samples were received fresh, without anticoagulant or fixatives and were processed within 24 hours. Centrifugation of the fluid was done at 3000 rpms for 5 minutes. Five to six smears were made from the sediment and stained with Leishman stain, hematoxylin and eosin stain (H&E) and Papanicolaou stain. After further centrifugation, cell block for paraffin sections was made from the cell button obtained and slides stained with H&E and IHC stain was done using anti-EMA antigen (BioGenex). Appropriate positive and negative controls were used. The cytological examination was performed on all fluid specimens and histologic examination of biopsy tissues was performed using standard hematoxylin and eosin stain (H & E), whenever possible.

Flow cytometric analysis of the effusion samples was done using the procedure given in the BD Cycle TEST PLUS DNA Reagent Kit (Becton, Dickinson & Co.; BD Biosciences). The technique of DNA analysis by flow cytometry has been previously reported.[11]

In this, the fluid samples were centrifuged and 50 microlitre pellet was transferred in tube with addition of 1ml buffer solution followed by vortex and centrifugation. The supernatant was discarded with addition of 1 ml buffer solution followed by vortex and centrifugation. The supernatant was discarded with addition of 1 ml buffer solution. The cell count was obtained with a hemocytometer using standard laboratory methods and the concentration was adjusted to $1.0 \times 10^6$ cells/mL with buffer solution. Staining was done by adding 250 microlitre of solution A and incubated for 10 mins at room temperature. 200 microlitre of solution B (trypsin inhibitor and RNase buffer) was added and incubated at room temperature for 10 mins. To this 200 microlitre of cold solution C (PI stain solution) was added and incubated in the dark or in fridge (2 -8 degrees Celsius). The sample was filtered through nylon mesh prior to FCM analysis. Peripheral blood Lymphocytes of healthy individuals were used as diploid reference control. Effusion specimens were analysed on multi parameter six colour flow cytometer (FACS-Jazz; Becton, Dickinson & Co.; USA) and DNA index (DI) was calculated as ratio of DNA quantity of testing G0/G1 cells peak/DNA quantity of control G0/G1 cells peak.

The tumour cells ploidy was calculated by ratio of the average DNA quantity of the neoplastic cells that are at the G0/G1 phase to a normal quantity of a similarly processed control sample.[17]

The results of cytological examination of the stained smears, immunohistological examination of cell blocks and FCM DNA index were divided in 3 groups. Group A consisted of malignant effusions. Group B had benign effusions. Group C consisted of effusions associated with malignancy where effusion sample was negative for malignancy, but the patient had malignancy. Cytology was considered as the gold standard for effusion diagnosis in our study.

## Results

31 patients underwent cytological examination and DNA analysis of the effusion samples. Out of 31 samples, 4 were peritoneal fluid and remaining were pleural effusions. The study group comprised of almost equal sex distribution with 16 females (51.6%) and 15 males (48.4%).

There were five malignant pleural effusions. In three cases, there was concordance between cytologic diagnosis and DNA analysis. Four pleural effusions were malignant on cytology and three of them had an abnormal DI. One of these five was initially positive for malignancy on cytology and turned out to be negative on repeat cytologic examination but expressed abnormal DNA histogram with a DI of 1.5. One pleural effusion sample was positive for malignancy on cytology, but the DI was found to be normal (Table 1).

23 patients had effusion because of benign disease. All benign effusions except five had a DNA histogram which was normal. Hypodiploid cells were found in 4 patients having pleural effusion. The DI was 0.8 in two patients while it was 0.6 in three patients. One patient having peritoneal effusion was a case of Nephrotic syndrome showing hypodiploid cells had a DI of 0.6. Cytologic examination of group B effusions including these five hypodiploid effusions was negative for malignancy (Table 2).

There were three patients who had malignancy associated effusions. These patients were diagnosed with primary malignancy in other sites, but the effusion cytological report was negative. DNA analysis result was also negative for these effusions (Table3).

Considering cytology as the gold standard, the specificity of DNA analysis was 0.96 (95% confidence values, 0.81-0.99) and the sensitivity was 0.75 (95% confidence values, 0.19-0.99). The predictive value was

0.75 (95% confidence limits, 0.29-0.96). As the sample size is small the confidence limits are wide (Table 4).

Immunohistochemistry results with EMA

Among the thirty-one effusions, five cases showed anti EMA positivity (16.1 %) and twenty-six were anti EMA negative (83.9 %). Of the 5 cases, 3 agreed with cytology and 2 agreed with abnormal DI.

Among the benign effusions and effusions associated with malignancy all except 2 were negative for anti EMA. These two cases consisted of a patient operated for benign ovarian tumor and the other was a case of renal cell carcinoma, both were negative for malignancy on cytological examination.

The specificity, sensitivity and diagnostic accuracy of EMA was 92.6%, 75% and 90.3% respectively. (Table 5)

In this study, by DNA flow cytometric analysis, a total of 4 aneuploid patterns were detected and three of these could be shown to contain malignant cells by cytologic examination and 2 also showed positive for anti EMA antibody by immunohistochemistry.

**Table 1: Results of group a patient data: Diagnosis, Cytology, EMA and DNA index**

| Group A: Malignant Effusions | | | | | |
|---|---|---|---|---|---|
| **No** | **Age/Sex** | **Diagnosis** | **Cytology** | **EMA** | **DNA Index** |
| 1. | 34/F | Breast carcinoma | + | + | 1.7 |
| 2. | 45/F | Breast carcinoma | + | - | 1.4 |
| 3. | 60/F | Breast carcinoma | - | - | 1.5 |
| 4. | 68/M | Bronchogenic squamous carcinoma | + | + | 1 |
| 5. | 61/M | Bronchogenic adenocarcinoma | + | + | 1.8 |

**Table 2: Results of Group B patient data: Diagnosis, Cytology, EMA and DNA index**

| Group B: Benign Effusions | | | | | |
|---|---|---|---|---|---|
| **No** | **Age/Sex** | **Diagnosis** | **Cytology** | **EMA** | **DNA Index** |
| 1. | 72/M | Tuberculosis | - | - | 1 |
| 2. | 28/M | Bronchiectasis | - | - | 1 |
| 3. | 18/M | Pneumonia | - | - | 1 |
| 4. | 60/F | Ovarian dermoid cyst | - | + | 1 |
| 5. | 70/F | Congestive heart failure | - | - | 0.6 |
| 6. | 32/F | *Tuberculosis | - | - | 1 |
| 7. | 10m/M | Pneumonia | - | - | 1 |
| 8. | 70/F | Rheumatoid Arthritis | - | - | 1 |
| 9. | 82/M | Congestive heart failure | - | - | 1 |
| 10. | 43/F | Pneumonia | - | - | 1 |
| 11. | 45/F | Tuberculosis | - | - | 1 |
| 12. | 60/M | *Alcoholic Cirrhosis | - | - | 1 |
| 13. | 54/F | Tuberculosis | - | - | 0.8 |
| 14. | 40/F | Tuberculosis | - | - | 1 |
| 15. | 47/M | *Pancreatitis | - | - | 1 |
| 16. | 41/M | Bronchiectasis | - | - | 1 |
| 17. | 75/F | Empyema | - | - | 0.6 |
| 18. | 44/F | *Nephrotic syndrome | - | - | 0.6 |
| 19. | 39/M | Tuberculosis | - | - | 1 |
| 20. | 60/F | Congestive heart failure | - | - | 0.8 |
| 21. | 55/F | Pneumonia | - | - | 1 |
| 22. | 61/M | Tuberculosis | - | - | 1 |
| 23. | 32/M | Bronchiectasis | - | - | 1 |

*Peritoneal effusion

**Table 3: Results of Group C patient data: Diagnosis, Cytology, EMA and DNA index**

| Group C: Benign effusion associated with malignancy | | | | | |
|---|---|---|---|---|---|
| **No** | **Age/Sex** | **Diagnosis** | **Cytology** | **EMA** | **DNA Index** |
| 1. | 31/F | Breast carcinoma | - | - | 1 |
| 2. | 59/M | Gastric carcinoma | - | - | 1 |
| 3. | 72/M | Renal cell carcinoma | - | + | 1 |

**Table 4: Comparative results of Cytology and Flow Cytometry**

| Flow Cytometry | |
|---|---|
| Sensitivity | 75% |
| Specificity | 96.3% |
| Positive Predictive Value | 75% |
| Negative Predictive Value | 96.3% |
| Diagnostic Accuracy | 93.5% |

**Table 5: Comparative results of Cytology and EMA**

| EMA | |
|---|---|
| Sensitivity | 75% |
| Specificity | 92.6% |
| Positive Predictive Value | 60% |
| Negative Predictive Value | 96.2% |
| Diagnostic Accuracy | 90.3% |

## Discussion

Serous effusion in malignancy can occur by three mechanisms; viz, hematogenous spread, obstruction of lymphatic flow or metastasis to pleura by direct extension.[18] Cytological detection of malignancy is an issue when cells are less, or they are overshadowed by reactive changes which occur in response to various stimuli.

After chemotherapy or radiotherapy these changes are more prominent. Effusion fluids with presence of cancerous cells have serious clinical implications, hence accurate diagnosis is of utmost importance.[13] The key features to identify malignant effusions in adenocarcinoma are large spheroidal three dimentional clusters which are more in breast carcinomas. The adenocarcinoma cells have vacoulated cytoplasm with eccentric nuclei. While squamous cell carcinomas are identified by the presence of keratinized cells which are round or tadpole like and have orange cytoplasm. Degenerative vacuolization of cytoplasm can be appreciated.[19] Information by cytology can be augmented using ancillary techniques including immunohistochemistry, ploidy analysis, image morphometry, chromosomal analysis, flow cytometry etc.[20] Many studies in literature have reported varying specificity and sensitivity in cytological diagnosis of malignant effusions. Specificity ranges from 96-100% and sensitivity between 22 to 81%.[21] In our study specificity and sensitivity was 96.3% and 75% respectively.

Flow cytometry can be used to determine cell cycle distribution kinetics, chromosomal analysis and cellular DNA contents. S phase fraction measurement by flow cytometry is a important prognosis predictor in some cancers.[22] Chromosomal aberrations are seen in almost all malignant neoplasms. However, the sensitivity of flow cytometry DNA analysis requires a significant abnormal quantity of DNA for its detection.[23] Sometimes the chromosomal duplications and deletions may even out result in tumour cells with normal net DNA content by flow cytometry and karyotyping quantitation but an obvious abnormal karyotype.[23]

Malignant tumours with abnormal undetectable genomes are not uncommon. The presence of neoplastic cells highly correlates with presence of aneuploidy. Therefore, presence of aneuploidy suggests malignancy in nearly all cases.[11] Aneuploidy is described as an extra discrete G0/G1 peak, different from normal G0/G1 peak with DNA index larger than 1.1 or G2M fraction 20% above of the analyzed cells.[24] DNA analysis by flow cytometry permits evaluation of greater number of cells and is accurate and rapid. The process from sampling to DNA histogram is about 20 to 30 minutes. Unlike mitotic karyotyping, this technique allows investigation of interphase cells in addition to the proliferative status of cells.[25] All these features make DNA analysis by flowcytometry a potentially attractive diagnostic tool for malignancy.

Several authors published studies using flow cytometry and immunohistochemistry markers to diagnose neoplastic cells in effusions.[26,27] There are only few studies in literature which compare flow cytometry, cytology and EMA applied together.[27] The limitation of our study is its small sample size, with scope for larger DNA flow cytometric study of malignant effusions.

Our study shows good specificity and sensitivity by DNA flow cytometry to differentiate between benign and malignant effusions and demonstrate that flow cytometry can be used as an adjunct to detect malignancy in effusion. FCM is efficient and a fast diagnostic tool. A suitable panel of flow antibodies can be used to determine additional patient specific data in this era of personalized medicine.

## Conclusion

We conclude from our study that DNA FCM is better in specificity than EMA-IHC and can be used as adjunct to conventional cytology for accurate diagnosis of malignancy in effusion samples. The advantage of flow cytometry is its accuracy and rapid analysis of the DNA content of a dense cell population. We propose that flow cytometry should be performed especially when conventional methods reveal equivocal results.

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**Conflict of Interest:** None**.**

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