Evaluation of Flow Cytometric Immunophenotyping and DNA Analysis for Detection of Malignant Cells in Serosal Cavity Fluids

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The serosal cavities are frequent sites of tumor metastasis. The distinction between carcinoma cells, inflammatory cells, and reactive or malignant mesothelial cells can be difficult in cytology. Multicolor flow cytometry (FCM) provides the opportunity to evaluate multiple antigens simultaneously, making it possible to characterize various cell populations. In this study, we aimed to assess the diagnostic accuracy of FCM immunophenotyping and DNA in comparison with serum tumor markers and classic cytology for detection of malignant cells in pleural and ascitic fluids. One hundred and nineteen samples of body cavity fluids were analyzed. Immunophenotyping was performed by four-color immunofluorescent staining using monoclonal antibodies against Ber-EP4, cytokeratin, CD3, and CD45. The DNA analysis by FCM was also performed. In addition, serum CA19-9, CEA, AFP, and CA125 were analyzed. Ber-EP4 marker had the highest sensitivity (73%) and specificity (95.5%) in the detection of carcinoma cells in serous fluid and correlated with cytology in most of cases (73%). The mean of DI differed statistically in patients with malignant effusions than in benign one. DI showed no difference in fluids due to infiltration of malignant epithelial cells or hematopoitic malignancy or due to hepatocellular carcinoma developing in cirrhotic liver. Thus, flow cytometry appears to aid not only in the detection of malignant cells but also in the characterization of cell type. On the other hand, although DNA ploidy examination had better sensitivity; it had no advantage over conventional cytopathological examination in identification of malignant cells. Diagn. Cytopathol. 2009;37:498–504. © 2009 Wiley-Liss, Inc.

Key Words: flow cytometry; Ber-Ep4; DNA analysis; serosal fluids

The serosal cavities are frequent sites of tumor metastasis, with adenocarcinomas of the lung, breast, and ovary being the most frequent primary sites. They are also the site of origin of several tumors, including primary peritoneal carcinoma and malignant mesothelioma. The cytological distinction between carcinoma cells, inflammatory cells, and reactive or malignant mesothelial cells can be extremely difficult in cytology.[1] Several protein markers have been studied including CA 125,[2] α-fetoprotein (AFP), CA19-9,[3] and carcino-embryonic antigen (CEA).[4,5] None is specific enough for cancer diagnosis because a variety of normal tissues and benign tumors express these markers.[6,7]

Immunohistochemistry is the most widely used ancillary method and has been shown to increase the overall diagnostic accuracy in many studies.[8,9] However, flow cytometry (FCM) immunophenotyping is more rapid, reproducible, and sensitive method for detecting cellular antigens. Multicolor FCM provides the opportunity to evaluate multiple antigens simultaneously, making it possible to characterize various cell populations in a more precise manner.[1]

Cytokeratins (CK) are intermediate filaments expressed in benign and malignant mesothelial cells.[10] This antibody is claimed to give reactivity in most epithelioid, mesotheliomas, and squamous cell carcinomas, and in all adenocarcinomas. It has been suggested as a sensitive "positive"

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mesothelioma marker, although its specificity is not very good.[11] On the other hand, Ber-Ep4 is a monoclonal antibody that recognizes a glycoprotein epitope present in most epithelial cells, but absent in mesothelial cells.[12]

Flow cytometry analysis of DNA content has been used by several investigators for the detection of aneuploid malignant cells in body cavity fluids.[13,14] Some reported that DNA flow cytometry was in general less sensitive than cytology for the detection of malignant cells and a higher percentage of false-positive cases were seen by FCM.[15,16] Others reported increasing sensitivity and specificity.[17]

Diagnostic algorithms were developed based on tumor marker measurements in effusions.[18] For instance, high levels of these tumor markers are now considered indicative for more invasive diagnostic procedures to determine the presence of malignant disease whereas low levels suggest benign disease. Research on diagnostic techniques of suspected malignant effusions has focused on the question whether laboratory measurements could replace invasive and time-consuming procedures. The measurement of tumor products or substances in serum and effusions could be a solution for this problem.[19]

The present study aimed to assess the diagnostic accuracy of FCM immunophenotyping and DNA in comparison with serum tumor markers and classic cytology for detection of malignant cells in pleural and ascitic fluids.

Materials and Methods

One hundred and nineteen samples of body cavity fluids were analyzed, 72 peritoneal fluids and 47 pleural effusions. There were 56 female and 63 male patients. The mean age was 53.36 ± 13.12 years (range, 18–80). The diagnosis of malignant ascites was made in 23 patients, and in the remaining 49 the ascites was due to cirrhosis (28), hepatocellular carcinoma developing in cirrhotic liver (17), and ascites due to tuberculosis (4). In the malignant ascites group; 18 patients had adenocarcinoma (10 with ovarian origin, six from GIT, and two metastasized from breast cancer) and in five the ascites was secondary to hematopoitic malignancy; three due to lymphoma, one due to chronic myeloid leukemia and one caused by multiple myeloma.

As regards the pleural samples, the diagnosis of malignant pleural effusions was made in 26 patients and in the remaining 21; the effusions were due to tuberculosis (12), pneumonia (4), cirrhosis (4), and undiagnosed (1). In the malignant pleural group, 14 patients had bronchogenic adenocarcinoma, eight had bronchogenic squamous cell carcinoma, three had small cell cancer of the lung, and one had malignant mesothelioma (as confirmed by brochoscopic biopsies and video assisted thoracoscopic biopsies).

The diagnosis for peritoneal and pleural malignancy was established through a biopsy. In the group with benign ascites, the diagnosis of cirrhosis was established by clinical-echographic-laboratorial data.[20] Informed consent was obtained from all patients and the medical ethical committee of Assiut University approved this study.

Paracentesis was performed according to well-established techniques.[21]

Samples Preparation

Two samples of 10 ml each were collected, one for cytology and the other for flow cytometry. Cells in the sample for flow cytometry were counted by hemocytometer, a minimum of 1 ml containing at least one cell/ 10 μl was needed. Fresh nonfixed samples were centrifuged at 100*g* for 10 min. The supernatant was discarded and the cell pellet washed with phosphate buffered saline (PBS), and then by hemolysite (Becton Dickinson) if the samples were contaminated with Red Blood Cells. Two hundred micro liters of rabbit Ig (Dako cytomation X0903, Glostrup, Denmark) were added to 200 μl of cell suspension.

Hundred micro liters of cell suspension were incubated with 10 μl of PE-labeled anti-cytokeratin (pan-reactive, ck4,5,6,8,10,13,18, Exbio, Prahy, Czech Republic), anti-CD3 (CyQ), and anti-CD45 (APC) antibodies (IQ product, Groningen, The Netherlands) for 20 minutes at 4°C protected from light. For the negative control isotype, matched mouse IgG antibodies were used. After washing with PBS, 100 μl of IQ Starfiqs fixation reagent (IQ product, Groningen, The Netherlands) were added and incubated for 15 minutes at room temperature. Ten micro liters of FITC-labeled monoclonal mouse antihuman epithelial antigen (clone Ber-EP4, Dako cytomation, Glostrup, Denmark) and 100 μl of IQ Starfiqs permeabilization reagent were then added and incubated for 15 minutes at room temperature. The cells were washed, resuspended in 100 μl PBS and analyzed directly by FCM.

Another 500 μl of the cell suspension were prepared for DNA analysis by Cycle Test Plus DNA reagent Kit (Becton Dickinson).

FCM Immunophenotyping Analysis

The flow cytometer (FACSCaliber; Becton Dickinson, San Jose, CA) was calibrated using CaliBRITTE beads (Becton Dickinson) for four-color flow cytometer setup. Data acquisition and analysis was performed using CellQuest software (Becton Dickinson).

Immunophenotyping was performed by four-color immunofluorescent staining using fluorescence-labeled monoclonal antibodies, directed against the following mixes:

1. Isotypic controls.
2. Cytoplasmic Ber-EP4/cytokeratin/CD3/CD45.

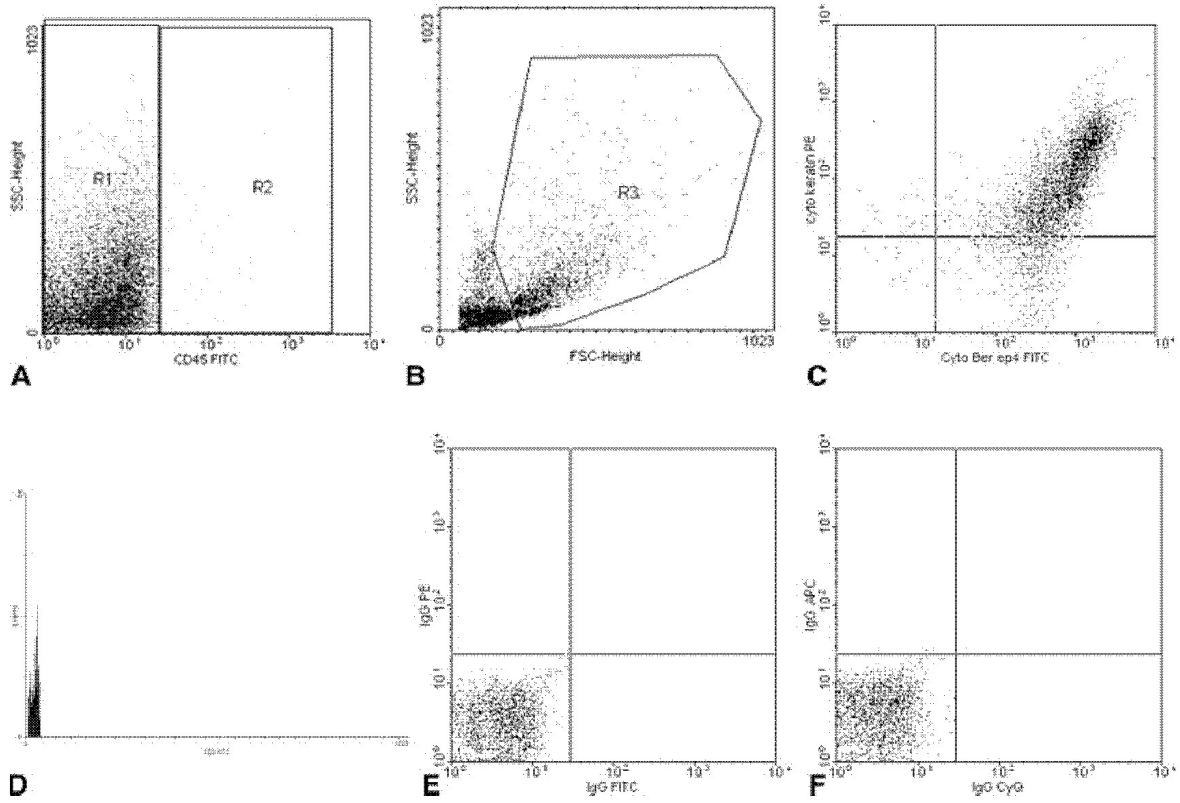

Fig. 1. (**A**) Dot plot of CD45 versus SSC showing distinct population negative for CD45 (R1) and R2 is drawn around positive population. (**B**) Cells in R1 are expressed on a dot plot combining FSC and SSC, and back gating was used to exclude debris and R3 is drawn around cells with high SSC and FSC values. (**C**) Dot plot of cytokeratin versus Ber-EP4 (gated on R3) showing positive both epithelial markers. (**D**) Histogram of CD3 shows negative reaction. (**E** and **F**) The isotypic controls.

The minimum number of cells required for the analysis were 10,000. Cells were expressed on a scatter diagram combining SSC with CD45 APC fluorescence. A region (R1) was drawn around a clear-cut population negative for CD45 and another one (R2) around CD45 positive cells. Cells in R1 were further expressed on a dot plot combining FSC and SSC, and back gating was used to exclude debris, by drawing R3 around cells with high SSC and FSC values. Quadrant cursors were set by using isotypic negative controls. Positive threshold was 20% for all markers. Cell populations were interpreted as immunereactive for a given antibody only when there is unequivocal separation from the negative control [lymphocytes (CD3+ve)] in the case of epithelial markers (Fig. 1).

Flow Cytometry DNA Analysis

The DNA analysis by FCM was performed using ModFit software (Becton Dickinson). DNA Quality Control Particles were used to set the voltages and check instrument resolution and linearity on the FacsCalibar FCM. G0/G1 peak of nuclei with DNA Index of 1.0 was recorded at channel 40. The tumour cells' ploidy was determined by the average DNA quantity of the cells of a neoplastic population that are at G0/G1 phase compared with a normal quantity of a similarly processed control sample. This rate can be determined by the following equation[22]: DNA index (DI) = DNA quantity of the testing G0/G1 cells' peak / DNA quantity of the standard G0/G1 cells' peak. The DNA histograms were classified as diploid or aneuploid based on the DNA quantity related to the normal control (Fig. 2). DNA aneuploidy was defined as the presence of two different peaks of the G0/G1 phase in the histogram. A histogram was considered diploid when the DI was equal to 1.0 and anueploid when <0.95 or >1.05. Coefficient of variation of the G0/G1 peaks in most of the samples was less than 3%.

Serum Tumor Markers

Serum samples were collected for tumor markers analysis. CA19-9 and CA125 were done for all patients, AFP was

done for patients with ascites and CEA was done for patients with pleural effusion. The tumor marker levels were quantified by the sandwich assay using Elecsys 1010 (Roche, Mannheim, Germany), cut off values were assessed as manufacturers kit instructions.

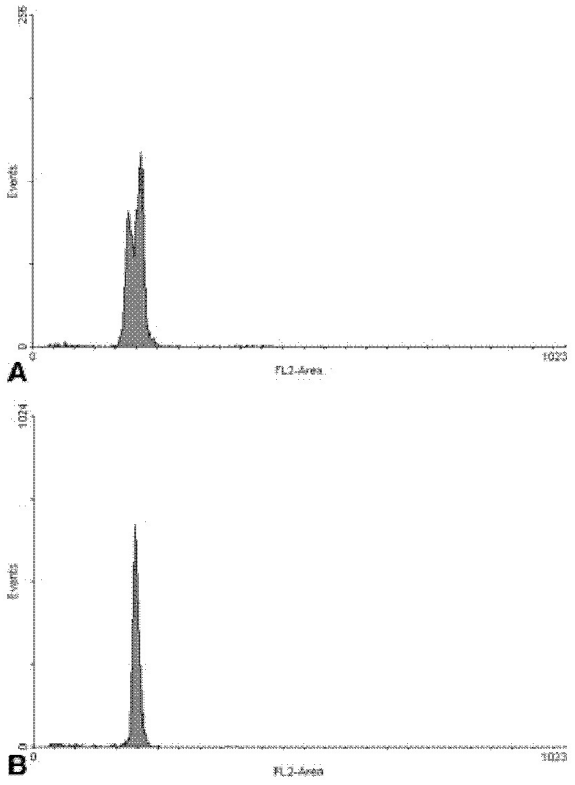

Fig. 2. Data from the two peritoneal fluid specimens, (**A**) shows aneuploid DNA histogram, (**B**) shows DNA ploidy histogram.

Statistical Analysis

The sensitivity, specificity, positive predictive values, negative predictive values, and accuracy were calculated in relation to the definitive pathological diagnosis after exclusion of patients with hepatocellular carcinoma developing in cirrhotic liver. The Student's *t* test was used for comparison of the DI for all patients.

Results

The specimens were classified into three groups (Table I): group I (malignant effusions), group II (effusions due to benign causes), and group III (hepatocellular carcinoma developing in cirrhotic liver). The efficiency of the used parameters namely cytology, cytokeratin, Ber-EP4, DNA ploidy, CA19.9, CA125, AFP, CEA in the diagnosis of various groups is presented in Table II.

In the malignant effusions group (group I) there were 24/37 aneuploid samples (64.9%); 22 in epithelial types and two in hematological types. The third group showed more or less the same percentage (63.6%). On the other hand, 29.7% (11/37) of the benign group (group II) were aneuploid as shown in Table II.

From Table II sensitivity, specificity, positive and negative predictive values, and accuracy of the tests are calculated. The malignant cytology or positive results for other markers in malignant effusions group were considered as true positive and those in benign group were considered as false-positive. The benign cytology and negative results for other markers in malignant group were considered as false-negative and those in benign group were considered as true negative. The sensitivity, specificity, positive and negative predictive values, and accuracy of the tests are shown in Table III.

Table I. The Distribution of 119 Cell Serosal Fluid Specimens into Three Studied Groups

Groups	*Diagnosis*	*No*
Group I: Malignant effusions		49
(a) Epithelial type		44
Ascitic fluid (18)	Ovarian Cancer	10
	GIT cancer	6
	Breast cancer	2
Pleural fluid (26)	Lung cancer	26
(b) Non epithelial (Hematopoitic)		
Ascitic fluid (5)	Lymphoma	3
	CML	1
	Multiple Myeloma	1
Group II: Effusions due to benign causes (Control group)		
Ascitic fluid (32)	LC	28
	TB peritonitis	4
Pleural fluid (21)	TB	12
	Pneumonic effusion	4
	effusion secondary to LC	4
	Undiagnosed	1
Group III: hepatocellular carcinoma developing in cirrhotic liver		17

LC, Liver cirrhosis; TB, Tuberculosis.

Table II. The Frequency of Results of Various Tests for Serosal Fluid Specimens

	Cytology			*Cytokeratin*			*Ber-EP4*			*DNA*			*CA19-9*			*CA125*			*AFP**			*CEA**		
	M	*B*	*No*	+	−	*No*	+	−	*No*	*A*	*D*	*No*	+	−	*No*	+	−	*No*	+	−	*No*	+	−	*No*
Malignant effusions																								
Epithelial	28	16	44	40	4	44	30	6	36	22	10	32	17	23	40	26	5	31	0	18	18	19	3	22
Hematopoitic	0	5	5	2	3	5	0	5	5	2	3	5	0	5	5	4	1	5	0	5	5	–	–	–
Benign effusions	3	50	53	18	31	49	2	43	45	11	26	37	18	30	48	40	8	48	2	30	32	6	14	20
Hepatocellular carcinoma developing in cirrhotic liver	0	17	17	7	10	17	3	14	17	7	4	11	9	8	17	12	4	16	10	7	17	–	–	–

M, malignant; B: benign; No, number of the specimens; +, positive; −, negative; A, aneuploid; D, diploid.
*AFP was done for patients with ascites and CEA was done for patients with pleural effusion only.

Table III. Comparison Between the Different Methods for Diagnosis of Malignant Serosal Fluids

The test	*Sensitivity (%)*	*Specificity (%)*	*+ve predictive value (%)*	*−ve predictive value (%)*	*Accuracy (%)*
Cytology	57	94	90	70	76
Cytokeratin	85	63	70	79	74.5
Ber EP4	73	95.5	94	80	82.5
Ploidy	65	70	68.5	67	67.5
CA19.9	35	62.5	40	57	51
CA125	85	16	63	60	59
CEA*	86	70	76	82	79

*CEA was done for patients with pleural effusion only.

Table IV. DNA Index for the Three Studied Groups

Groups	*DNA index*
Group I: Malignant effusions	1.21 ± 0.19
(a) Epithelial type	1.15 ± 0.07
(b) Nonepithelial (Hematopoitic)	1.13 ± 0.08
Group II: Effusions due to benign causes (Control group)	1.09 ± 0.07
Group III: Hepatocellular carcinoma developing in cirrhotic liver	1.46 ± 0.24

Values are presented as mean ± SE.

There was concordance between positive results of cytology and Ber-EP4 (73%) and between cytokeratin and Ber-EP4 (77.9%). The concordance between cytology and DNA ploidy was in 49.4% of cases and between Ber-EP4 and DNA ploidy in 55% of cases.

The mean of DI differed statistically in patients with and without malignant effusions, being 1.2 ± 0.19 (SE) and 1.09 ± 0.07, respectively ($P = 0.02$). There was no difference between the means of DI in malignant ascites due to adenocarcinoma and malignant ascites secondary to hematopoitic malignancy; 1.15 ± 0.07 and 1.13 ± 0.08, respectively ($P = 0.7$). There was no statistically significant difference between the mean of DI in malignant effusions and ascites with hepatocellular carcinoma developing in cirrhotic liver; 1.21 ± 0.04 and 1.46 ± 0.24, respectively ($P = 0.3$), Table IV.

Discussion

The cytopathological examination of the sediment of effusion has been the gold standard for detecting the presence of neoplasms for many years.[23] It generally presents a low sensitivity and a high specificity.[15] The increased sensitivity observed in some studies is obtained with a decrease in specificity, which is far from ideal.[24,25] In the present study, conventional cytopathological examination produced a sensitivity of 57% and a specificity of 94%, in accordance with many other studies.[15,23,24]

The evaluation of tumor markers in serum and fluid has been proposed as an alternative way of establishing a diagnosis of malignant effusions. CEA was found to be the best single tumor marker in pleural fluid.[26] However, in our study the specificity of CEA serum level was lower than that of cytology.

CA125 was used to monitor the course of epithelial ovarian cancer. It has been reported that liver cirrhosis is associated with elevated serum CA125,[27,28] which was attributed to its secretion by normal mesothelia.[29] This may be the explanation of the very low specificity for malignant diagnosis in our results.

The oligosaccharide of the CA 19.9 (sialo-lacto-*N*-fucopentose-II) epitope is related with Lewis a (Le^a) blood group antigen. At least 3% of the population is genotypically Le^a negative and their tumors cannot express CA-19.9. Therefore, the maximum sensitivity of this test would be 97%.[30] In our study, the sensitivity of CA19.9 was only 35% with higher specificity (62.5%). Some studies showed higher sensitivity and specificity as Kuralay et al. who reported a specificity of 97% and sensitivity of 90%, this might be attributed to monitoring CA-19.9 in the fluid not in the serum. However, they stated that this

tumor marker alone is insufficient to discriminate malignant from benign.[31]

Several studies have used immunocytochemical markers to diagnose malignant cells in body fluids. Friedman et al.,[32] reported that 100% of malignant mesotheliomas stained positive for cytokeratin and negative for the CEA, B72.3, Leu-M1, and Ber-EP4 markers. They also stained positively for the vimentin, EMA, and CA125 in 75, 75, and 25% of the cases, respectively. Adenocarcinomas were more likely to stain positively with B72.3, Ber-EP4, and CEA and negatively for vimentin. As FCM is a method that can evaluate a great number of cells in a short time with quantitative evaluation, and it could be complementary to the cytopathological examination,[33–36] we decided to study two of these markers (cytokeratin and Ber-EP4) in combination with CD45 and CD3 by multiparametric FCM which can detect and differentiate malignant cells. We found that Ber-EP4 immunophenotyping had the highest sensitivity and specificity in the detection of carcinoma cells in serous effusions and correlated with cytology in most of cases (73%). This is in concordance with Risberg et al.,[37] who examined 49 serous effusions and peritoneal washings for malignant cells using monoclonal antibodies against CD45, Ber-EP4, and N-cadherin by both ICC and flow analysis and Davidson et al.,[38] who compared immunophenotyping by flow analysis with morphological and immunocytochemical staining in 92 effusions. They reported that expression of Ber-EP4, B72.3, AH6, and HB-TN was suggestive of malignancy and that expression of these four markers would support the morphologic diagnosis with high certainty.

Immunophenotyping using FCM is a sensitive and rapid method of detecting cellular surface antigens in cytological material. It facilitates the evaluation of cell populations using simultaneous staining analysis, thus making it possible to characterize various cell types in a more precise manner.[37]

One of FCM accepted applications is the measurement of DNA content, which allows the identification of cell populations with abnormal quantities of DNA (aneuploidy) and provides information on the cellular proliferate activity by analyzing the distribution of cells in their different phases of the cell cycle.[34,36] The analysis of the DNA aneuploidy through FCM has been used in an attempt to increase the accuracy of the analysis of effusions by cytology; it is able to identify abnormal cell populations not recognized by conventional cytopathological examination.[39,40] However, the greatest limitation to the widespread application of this method is the lack of concordance among the published studies.[41] In our study, DNA aneuploidy was more sensitive in malignant cell detection than cytology but much lower in specificity. The results of both were the same in 49.4% of cases only. This is in concordance with Both et al.[41] study. We observed that, the mean of DI differed statistically in patients with malignant effusions than in control group. This is in accordance with other studies.[15,40] Nevertheless, the majority of published studies did not perform that comparison.[16,42,43] On the other hand, some authors did not explore the DI average and just refer to the FCM results in terms of aneuploid or diploid.[44–48] We also observed that DI shows no difference in effusions due to infiltration of malignant epithelial cells or hematopoitic malignancy or due to hepatocellular carcinoma developing in cirrhotic liver. Also, in our study, there was a difference between DI in effusions due to benign causes and ascites due to hepatocellular carcinoma developing in cirrhotic liver though not statistically significant. This means that in spite of being very helpful for the detection of malignancy, DI is not helpful in differentiating the type of malignancy.

Conclusions

Multiparametric flow cytometry of ascites and pleural effusion specimens may be a valuable tool for rapid identification of malignant cells by immunophenotyping using both cytokeratine and Ber-EP4 in combination with cytomorphology. Flow cytometry appears to aid not only in the detection of malignant cells, but also in the characterization of cell type. The Ber-EP4 appears to be the most accurate marker for carcinoma cells. On the other hand, although DNA ploidy examination has better sensitivity; it has no advantage over conventional cytopathological examination in the identification of malignant cells.

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