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Quantitative and Functional Assay of MDR1/P170-mediated MDR in Ascites Cells of Patients with Ovarian Cancer

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Abstract. *Background: MDR1-associated P-glycoprotein-dependent multidrug resistance is a common cause of chemotherapy failure in patients with advanced ovarian cancer. Here, we describe a clinical method for simultaneously assessing the expression and function of the MDR1/Pgp in tumour cells from ascites of patients with malignant ovarian carcinoma. Materials and Methods: Cells from ascites from 35 patients were collected. The expression and function of Pgp were detected by flow cytometry. For functional study, rhodamine 123 was used. Results: Using the Pgp- specific UIC2 and MM6.15 antibodies, we demonstrated the presence of Pgp in 10-79 % (38.9±20, 7; n=35) of the CA125-positive cell subpopulations. The results of the functional assay showed strong correlation with the level of Pgp expression ($r=0.976$; $p=3.2x10^{-5}$). Conclusion: Direct detection of the expression level and function of MDR1/Pgp in the ascites provide useful information for the more efficient treatment of malignant diseases by proper adjustment of the chemotherapeutic protocol.*

The second most frequent gynaecological malignancy is ovarian carcinoma (1). Ovarian cancer spreads early in the disease to the peritoneal cavity. The tumour cells often remain confined to the peritoneum and an *en bloc* resection of the tumour is usually not possible (2). Therefore, surgery combined with chemotherapy is the most accepted approach in treating ovarian cancer at later stages. The major problem of chemotherapeutic cancer treatment is intrinsic or acquired multidrug resistance (MDR) (3-5). Several types of drug resistance mechanisms have already been identified (6-10), but the overexpression of P-glycoprotein (Pgp) encoded by the MDR1 gene (3, 6, 7, 10, 11) is the most frequent reason for therapy failure. P-glycoprotein expression correlates with unfavourable prognosis and is suggested as a marker for chemotherapy resistance in advanced ovarian cancer (11). The detection of the expression level and function of MDR1/Pgp should provide useful information for the more efficient treatment of malignant diseases by properly adjusting the chemotherapeutic protocol. Karaszi *et al.* (6) reported on different approaches to detect MDR1. These approaches have been evaluated by several authors and positive correlation has been found between P-glycoprotein expression and pump activity (6, 12). Pgp function can be monitored by measuring the cellular uptake of different fluorescent dyes. Rhodamine 123 (R-123) is regarded to be a Pgp-specific probe since R-123 is not very efficiently effluxed by MRP (8). Assessments of the classical MDR phenotype in ovarian carcinoma had been made using solid biopsy samples (4), but none of them have been applied to such a complex system as ascites fluid. There are many factors involved in the formation of ascites in malignancies (13) including cytokines, direct lymphatic blockade, tumour neovascularization, *etc.* In the case of ovarian cancer, it is a sign of peritoneal implantation / dissemination and a bad prognostic factor (14). It is usually associated with resistance to the ongoing or previously applied chemotherapeutic protocol. Consequently, there is a need for the detection of Pgp expression in cells derived from the ascites fluid of patient with ovarian cancer. Flow cytometry is the most suitable diagnostic cell analysis technique to precisely identify the various subtypes of cells or the presence of expressed proteins in the cell membrane (15). We describe a flow cytometric method that provides a tool for the quantitative estimation of the expression with parallel measurement of the function of the Pgp multidrug transporters in tumour cells from ascites of patients with malignant ovarian cancer.

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Key Words: P-glycoprotein, multidrug resistance, ascites, ovarian cancer, functional assay.

Materials and Methods

Patients and samples. This study was carried out between June 2001 and June 2004 at the Department of Obstetrics and Gynaecology and the Department of Biophysics and Cell Biology, University of Debrecen, Hungary. The protocol was approved by the Ethical Committee of the University. Ascites from 35 patients (18 to 75 years old) with diagnosed and histologically proven serous or endometrioid adenocarcinomas were used. The patients received a combination of chemotherapies for ovarian cancer. In the vast majority of the patients (30/35) the samples were taken after numerous cycles of chemotherapies. All patients were screened and found positive for the CA125 ovarian carcinoma marker using the Hoffmann-LaRoche (Basel, Switzerland) routine serum test. Ca125 values are considered to be positive above 25 IU. In our patients, they varied between 52 and 6074 IU with an average±SD of 1295±1614, median 691 IU.

Reagents. All of the applied chemicals were of analytical or spectroscopic grade. Propidium iodide (PI) and rhodamine 123 were obtained from Molecular Probes (Eugene, OR, USA). D-glucose, RNase, bovine serum albumin (BSA), digitonin, cyclosporin A and inorganic chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS) contained: 140 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4 and 3 mM NaH_2PO_4 at pH 7.3.

Cells. The mouse fibroblast cell line NIH 3T3 and its derivative stably transfected with the human MDR1 gene, NIH3T3 MDR1 G185 and human peripheral lymphocytes were used. Cell viability was more than 90%, as assessed by the exclusion of trypan blue.

Separation of cells from ascites. One hundred ml ascites drained upon therapeutic puncture was centrifuged at room temperature for 10 min at 800 x g. The pellet was washed in PBS and centrifuged for 5 min at 800 x g. The pellet was resuspended in 3 ml standard ammonium chloride lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1 mM EDTA, pH 7.4) to remove the red blood cells. Lysis was terminated after 10 min by adding 10 ml PBS to the solution. The cells were washed again in PBS and the pellet was resuspended in PBS containing 1% bovine serum albumin and 5 mM D glucose. Viability was tested using the standard PI/FDA test (16). One part of the cell suspension was directly used for analysis of Pgp function, another part was fixed with 1% formaldehyde and kept at 4˚C before immunofluorescent staining took place, and a third part was fixed with ethanol for DNA analysis. Smears made from the cell suspension for cytological analysis were fixed in methanol and stained with either Haematoxylin-Eosin, or Giemsa.

Flow cytometry. A Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with two (Ar ion and He-Ne) lasers was used to determine fluorescence intensities.

Rhodamine 123 uptake. Rhodamine 123 (R-123) uptake was measured in PBS solution containing 5 mM glucose. One million cells in 1 ml PBS were preincubated at 37˚C for 10 min in the presence or absence of 20 μM cyclosporin A. After this process, 2 μM R-123 was added to the cells and further incubation for 30 min took place, after which the cells were washed twice with ice-cold PBS and kept on ice until measurement. The mean of flow cytometric fluorescence intensity histograms was determined. $R_{R\text{-}123}$ values were calculated as the ratio of the mean fluorescence histogram of R-123-labelled cells after CSA treatment to the same parameter without CSA treatment.

DNA analysis. Cells fixed in 70% cold ethanol and kept at -4˚C before use were pelleted and 10^6 cells were resuspended in 1 ml PBS and treated with RNAse (100 μg/ml) for 30 min at 37˚C, followed by propidium iodide (50 μg/ml) staining. The proliferation index was calculated from the fraction of cells in the different phases of the cell cycle as $[(S+G_2/M)/(G_0/G_1+S+G_2/M)] \times 100\%$ (17).

Flow cytometric Pgp detection. For immunofluorescent labelling, the Pgp-specific antibodies UIC2, prepared from hybridoma purchased from ATCC and MM6.15 (18, 19), kindly provided by M. Cianfriglia (Laboratorio di Immunologia, Instituto Superiore di Sanità, Rome Italy), were used. Cells were incubated with a saturating concentration of MM6.15 or UIC2 antibodies in PBS and 1% bovine serum albumin at 1 x 10^7 cells/ml for 40 min on ice. For visualization, rabbit anti-mouse IgG2a fluorescein isothiocyanate conjugate secondary antibody (RAMIG-FITC, F/P=4.3 from Sigma) was used. Irrelevant mouse IgG2a at the same concentration as specific antibodies was used for isotypic control.

Ovarian cancer cell detection. Ovarian cancer cells were identified by the use of CA125 monoclonal antibody, which recognizes the tumour-associated CA125 antigen that is highly expressed on the ovarian tumour cell surface, with little or no reactivity with normal tissues. Samples (cells separated from ascites and HPBL and JY human lymphoid cells as negative controls) were washed with PBS before antibody labelling. 10^6 cells were labelled first with mouse anti-human CA125 IgG1 (Serotec Ltd, Kidlington, Oxford, UK) in 100 μl commercially available prediluted mAb solution. Indirect immunofluorescent staining was carried out with Alexa488-GAMIG (1 μg/100 μl PBS, for 40 min).

Pgp detection by confocal microscopy. Cells were labelled in suspension with MM6.15-FITC monoclonal antibody against Pgp and sedimented onto poly-L-lysine-coated coverslips. A Zeiss LSM 510 confocal laser-scanning microscope was used for the measurements. Excitation of FITC was with the 488 nm line of an Ar ion laser, while PI was excited with a 543 nm He-Ne laser in multitrack mode. Detection of fluorescence was through a 505-550 nm band pass filter in the case of FITC, and a 560-615 nm band pass filter was used for PI fluorescence. 512 x 512 pixel images were obtained in extended focus mode, through a 63 x NA=1.4

Data analysis. Every experiment was repeated at least three times, and the mean and S.D. values were calculated. A $p=0.01$ value was considered significant using Student's *t*-test (two tail). The relationship between the various measured parameters was determined from the Pearson's product moment correlation coefficient.

Results

The ascites of patients contained various quantities of cells in the range of 0.2-10 x 10^5/ml, averaging 2.88±3.2 x 10^5 (n=35). Using the lysis and cell separation technique described in *Materials and Methods*, the viability of cells gained from ascites was higher than 90%.

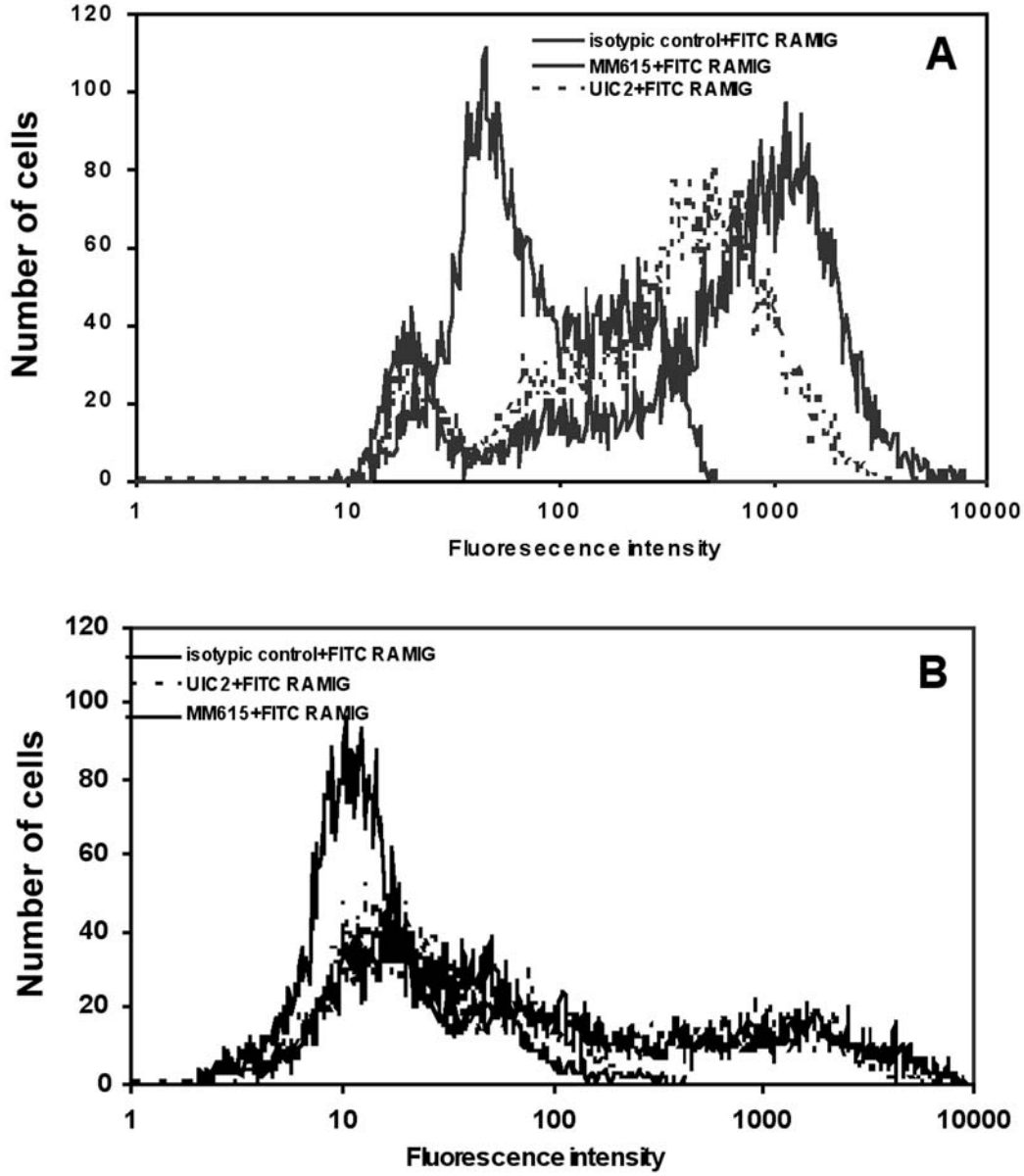

Figure 1. *Fluorescence histograms of ascites cells (A:patient No. 8, B:patient No. 26.) labelled with Pgp-specific antibodies.*

Expression of Pgp protein in cells derived from ascites. Two cell subpopulations from ascites showed CA125 positivity and they were also characterised by their forward scatter signal. These two populations were then used to determine the level of Pgp expression. The two negative controls, the HPBL and JY human B lymphoid cells, did not show any CA125 positivity. The fluorescence intensity of the gated cells incubated with anti-Pgp antibody was compared to that of the cells incubated with isotypic control. The Pgp-positive cell population ($\%_{MM6.15}$ Pgp^+ and $\%_{UIC2}$ Pgp^+) was identified as the percentage of cells characterized by fluorescence intensities higher than the isotypic control. The mean fluorescence intensity of the histogram of specifically-labelled cells was normalized to that of the isotypic control to generate a signal to background ratio, R, which varied between 2.0 and 18 for the MM6.15 (mean ± SD 8.6±4.3; n=35), and between 1.5 and 15 for the UIC2 (mean ± SD 6.6±2.7; n=11). The $\%_{MM6.15}$ Pgp^+ populations varied between 10 and 79 % (mean ± SD 38.9±20.7; n=35) and the $\%_{UIC2}$ Pgp^+ between 14 and 70 % (mean ± SD 42.5±16.7; n=11). A very strong correlation was found between the R values of parallel samples labelled with MM6.15 and UIC2 (r=0.924; p=4.8 x

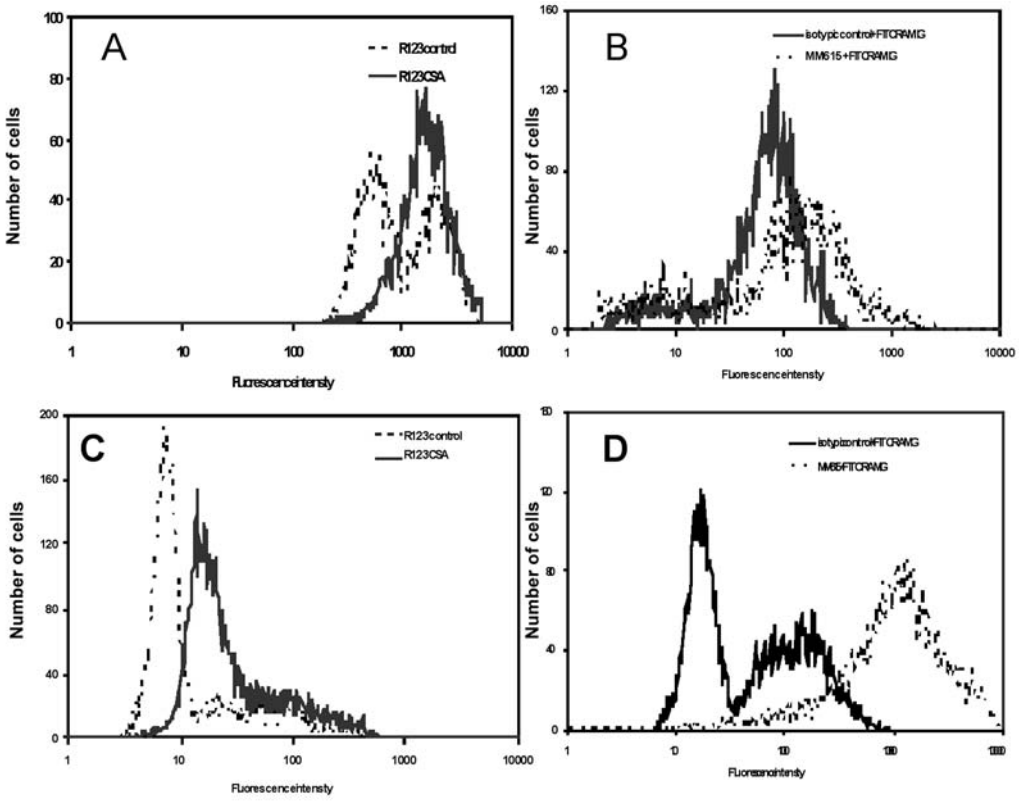

Figure 2. *Parallel flow cytometric analysis of Pgp expression and function of cells from ascites. A, C: fluorescence histograms of cells incubated with R-123 in the presence and absence of CSA. B, D: fluorescence histograms of cells labelled with isotypic control and MM6.15 antibody followed by secondary FITC-RAMIG. A, B: patient No. 21.; C, D: patient No. 17.*

10^{-5}). Similarly, a very strong correlation was found between the proportion of MM6.15-positive and that of UIC2-positive cells in the samples (r=0.992; $p=2.13 \times 10^{-9}$). Figure 1 shows the fluorescence histograms of two representative samples for the isotypic control, the MM6.15 and the UIC2 labelling.

Functional study of Pgp pump. The function of the Pgp pump in the cells derived from the ascites of the patients and that of MDR^+ and MDR^- cell lines was determined by R-123 substrate uptake of the cells in the presence and absence of the Pgp pump blocker cyclosporin A (CSA). CSA treatment increased the R-123 uptake of the MDR^+ cells, while it did not modify the uptake of MDR^- cells. The results of the functional test are summarized in Table I. For positive control of Pgp expression and function the NIH3T3 MDR1 G185, while for negative controls the NIH3T3, JY and HPBL cells were used. The ratio of R-123 uptake of CSA-treated and untreated samples was 8.1±3 in the case of NIH3T3 MDR1 G185 cells (n=3). The original NIH3T3 showed a significantly lower drug uptake ratio (1.1±0.05, n=3, $p<0.0001$). RUIC2 was 75±22, 1.08±0.1, 1.1±0.05 and 1.02±0.1 for the NIH3T3 MDR1 G185, NIH3T3, JY and HPBL cells, respectively (n=3). The drug uptake of control human peripheral lymphocytes and JY cells, which do not express the Pgp pump, also did not change significantly in the presence and absence of CSA. Within the ascites samples, subpopulations could be observed on the basis of substrate accumulation. The ratio of R-123 uptake of CSA-treated and native cells was between 1.3 and 2.8 for the total investigated cell population derived from ascites (n=8), but among various subpopulations of individual samples this ratio varied between 1 and 5. Figure 2A shows the R-123 uptake of a representative ascites sample. As can be seen on the histograms, about 60% of the cells expressed the Pgp pump, while in about 40% of the whole population it did not exist or function. After blocking the Pgp pump with CSA, the substrate uptake of the Pgp-positive cells grew to the level of the Pgp-negative ones. The result of the functional assay correlated well with the level of Pgp expression (Figure 2B). Figure 2C shows the R-123 fluorescence histogram of a representative ascites sample in the presence and absence of CSA, while in Figure 2D, the fluorescence histogram of the cells labelled with the specific MM6.15 antibody and that of the control sample is presented ($R_{MM6.15}=18$). Very strong correlation was found between the R-123 uptake and the MM6.15 positivity (%) of the samples examined (r=0.976; $p=3.2 \times 10^{-5}$). In Figure 3, the transmission light microscopic

Table I. *Assay of Pgp function in cells from ascites. Parallel samples were labelled with MM6.15 Pgp-specific antibody and R-123, a substrate of the pump in PBS containing 1% of BSA. The $R_{MM6.15}$ value is calculated as the mean of the fluorescence histogram for specifically-labelled cells normalized to that of the isotypic control. R_{R-123} values are calculated as the ratio of the mean fluorescence histogram of R-123-labelled cells after CSA treatment to the same parameter without CSA treatment. The Pgp-positive cell population ($\%_{MM6.15}$ Pgp^+ and $\%_{R-123}$ Pgp^+) was identified as the percentage of cells characterized by fluorescence intensities ($R_{MM6.15}$) higher than the isotypic control upon MM6.15 labelling, or by R_{R-123} lower than after CSA treatment, respectively.*

Patient No.	Cell conc. x10^5/ml	$R_{MM6.15}$	$\%_{MM6.15}$ Pgp^+ cells	RR-123	$\%_{R-123}$ Pgp^+ cells	PI^+ %
15	0.1	9	28	1.57	20	10
16	0.4	6	43	1.4	30	8
17	5.5	18	76	2.1	70	5
18	0.5	14	47	2.6	40	7
19	1.0	17	21	2.0	20	3
21	1.2	8	58	2.8	60	3
22	0.7	11	67	1.3	60	10
23	3.7	16	89	2.5	70	10

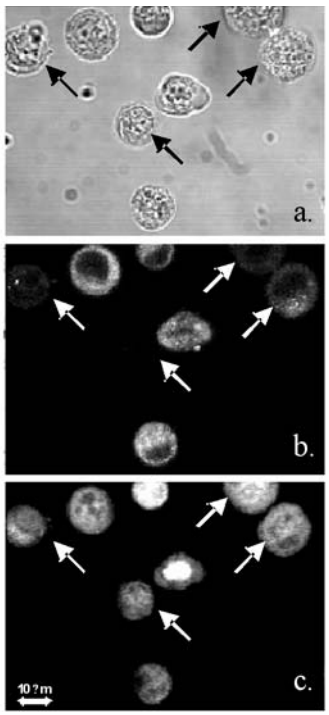

Figure 3. *Transmission light microscopic image of cells from the same sample (a), confocal fluorescent microscopic scan of the FITC-MM6.15-labelled cells (b), and PI-stained nuclei of the same cells (c).*

image of cells from this sample (a), the confocal fluorescent microscopic scan after MM6.15 labelling (b), and the image after staining the nuclei with PI (c) can be seen. The cells marked by arrows on the transmission scan are the ones that were not labelled by Pgp-specific antibodies. The proliferation index of the cells of 21 patients were determined. The mean and SD was 31.15±9.6 and no correlation was found between the proliferation index and Pgp expression level of the cells.

Discussion

The detection and characterization of multidrug-resistant cells in tumour samples is a critical clinical diagnostic parameter. Flow cytometry is the most suitable technique for fluorescent measurement of the presence and function of Pgp in living cells. Pgp function can be monitored by measuring the cellular uptake of fluorescent MDR1 substrates such as rhodamine 123 (12). Several fluorescence assay methods have been worked out to discriminate between drug-resistant and drug-sensitive cells (6, 8, 9, 12), but none of them deals with cells derived from malignant ascites.

In this paper, we present the results of the first clinical study of the level of expression and function of multidrug transporters in cells isolated from ascites of patients with ovarian cancer. The results showed varying rates of Pgp expression, the percentage of Pgp-positive cells being between 10 to 79% in the samples from different patients. Holló *et al.* (9) reported on the functional and immunological detection of human MDR/Pgp and MRP1. They used antibodies against external and internal epitopes for immunofluorescent labelling of Pgp and came to the conclusion that the anti-MDR monoclonal antibodies (UIC2 and MKR16) specific for the external epitope were superior in sensitivity to antibodies recognizing internal epitopes. In our experiments, we also used antibodies reacting with external epitopes, namely UIC2 and MM6.15. We compared the sensitivity and reproducibility of the quantitative flow cytometric detection of Pgp using these two antibodies and did not find significant differences between them. In spite of the broad range of Pgp expression among the samples from various patients, the rate of expression determined for each sample using one or the other antibody was statistically identical.

By determining the proliferation index and relating that to Pgp expression or function it became obvious that the sample to sample and subpopulation-related variation of protein expression or function is not a result of the different proliferative state of cells derived from the ascites.

We demonstrated the expression of Pgp in the membrane of cells derived from ascites using single cell fluorescence digital imaging. While single cell imaging is time consuming, flow cytometry based on fluorescent markers binding extracellularly provides reliable results supported by good statistics. Therefore, although single cell imaging is also a possible method to detect Pgp expression in cells from ascites, we recommend it as a useful additional method in special selected cases. In our study, we found excellent correlation between multidrug resistance

activity and Pgp protein expression level using CSA-inhibitable R-123 extrusion and the extracellular immunostaining. In general, R-123 is a substrate for Pgp, MRP1 and BCRP. However, it seems plausible that the increased expression of Pgp detected by immunofluorescence is the main cause of CsA inhibitable R-123 extrusion in the functional assay. It is also supported by the facts that R-123 can be effluxed only by cells expressing mutant BCRP with glycine or threonine instead of arginine at position 482 (20) and R-123 is transported approx. 10 times less by MRP1 than by Pgp (8, 21).

All of our patients had been treated with chemotherapeutic drugs before the collection of ascites samples. Baekeland *et al*. (11) reported that the presence of Pgp could be found in 47% of the patients prior to chemotherapy and they considered the presence of it as a negative prognostic sign. They also report that a part of the patients became Pgp-positive due to chemotherapeutic treatment. They found a statistically significant positive correlation between Pgp positivity and the presence of ascites ($p=0.013$).

We suggest that the quantitative and functional method to detect MDR1/P170- mediated multidrug resistance in cells derived from ascites of malignant ovarian cancer patients can provide useful information for the more efficient treatment of the disease by proper adjustment of the chemotherapy protocol.

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