

## PAPER DETAILS

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## Determination of P-Glycoprotein Expression by Flow Cytometry in Hematological Malignancies

### *Hematolojik Malignansilerde P-Glikoprotein Ekspresyonunun Akım Sitometri İle Belirlenmesi*

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#### ABSTRACT

**Objective:** Determination the expression of P-glycoprotein is especially problematic for normal tissues because immunological methods are limited in terms of sensitivity. We aimed to determine the expression of P-glycoprotein and CD34 by flow cytometry, and to evaluate the level of expression of P-glycoprotein and CD34 with unresponsive to treatment in patients diagnosed with hematologic malignancy.

**Methods:** Our study included fifty patients diagnosed with acute myeloblastic leukemia and acute lymphoblastic leukemia, and twenty healthy controls who were admitted to Erciyes University Hematology-Oncology Hospital. The suspended cells from bone marrow samples of patients and the peripheral blood samples of healthy people were marked with P-glycoprotein phycoerythrin and CD34 FITC or PerCP Cy 5.5; and then surface expression was measured by means of flow cytometry.

**Results:** In 6 of 30 acute myeloblastic leukemia patients P-glycoprotein and CD34 expression, in 6 of 20 acute lymphoblastic leukemia patients P-glycoprotein, in 5 of them CD34 expression were determined. A significant relation between P-glycoprotein and CD34 expressions in acute myeloblastic leukemia and acute lymphoblastic leukemia bone marrow samples was reported.

**Conclusion:** Our data indicate that flow cytometry is more reliable, precise and faster than molecular methods for measuring P-glycoprotein expression and suggests the possibility of a significant relationship between P-glycoprotein and CD34 expressions in acute myeloblastic leukemia and acute lymphoblastic leukemia bone marrow samples. The blast cells expressing CD34 on their surface along with P-glycoprotein simultaneously show that multi drug resistance 1 gene is mostly active in immature cells.

**Key words:** MDR-1 gene, AML, ALL, P-glycoprotein, flow cytometry

#### ÖZET

**Amaç:** Duyarlılık açısından sınırlı olan immünolojik yöntemler kullanılarak özellikle normal dokulardaki P-glikoprotein ekspresyonunun belirlenmesi sorun oluşturmaktadır. Bu çalışmada hematolojik malignansi tanısı almış ve tedaviye yanıt vermeyen hastalarda P-glikoprotein ve CD34 ekspresyonunun değerlendirilmesi için, P-glikoprotein and CD34 ekspresyonunun akım sitometri ile belirlenmesini amaçladık.

**Yöntemler:** Erciyes Üniversitesi Mehmet-Kemal Dedeman Hematoloji-Onkoloji Hastanesine başvuran akut miyeloblastik lösemi ile akut lenfoblastik lösemi tanısı almış 50 hasta ve yirmi kontrol çalışmaya dâhil edildi. Hastaların kemik iliği örnekleri ile kontrol grubundan alınan periferik kan örneklerinden elde edilen süspansiyon hücreler, P-glikoprotein fikoeritrin ile ve CD34 FITC ya da PerCP Cy 5.5 antikorları ile işaretilendi ve daha sonra yüzey ekspresyonları akım sitometri ile ölçüldü.

**Bulgular:** Çalışmamızda 30 akut miyeloblastik lösemi hastasının 6'sında (%20) P-glikoprotein ve CD34 ekspresyonu tespit edilirken, 20 akut lenfoblastik lösemi hastasının 6'sında (%30) P-glikoprotein, 5'inde (%25) CD34 ekspresyonu tespit edildi. Akut miyeloblastik lösemi ve akut lenfoblastik lösemi kemik iliği örneklerinde P-glikoprotein ve CD34 ekspresyonları arasında anlamlı bir ilişki olduğu görüldü.

**Sonuç:** Bulgular, akım sitometri yöntemi ile P-glikoprotein ekspresyonunun akut miyeloblastik lösemi ve akut lenfoblastik lösemi kemik iliği örneklerinde moleküler yöntemlerden daha hızlı, güvenilir ve doğru bir şekilde ölçülebildiğini, P-glikoprotein ile CD34 ekspresyonları arasında anlamlı bir ilişki olabileceğini göstermiştir. Yüzeyinde CD34 eksprese eden blast hücrelerinin aynı zamanda P-glikoprotein eksprese etmeleri hücrelerdeki çoklu ilaç direnci geninin daha çok olgunlaşmamış hücrelerde aktif olduğunu göstermektedir.

**Anahtar kelimeler:** Çoklu ilaç direnci-1 geni, AML, ALL, P-glikoprotein, akım sitometri

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## INTRODUCTION

Considerable progress has been made in the therapy of acute lymphocytic leukemia (ALL) and despite complete remission exceeding 90% in contemporary treatment series, overall survival rates of adults with ALL are within the range of 30-40% [1-3]. Similarly, there has been great progress in the treatment of acute myeloid leukemia (AML), and 70 to 80% of newly diagnosed patients have achieved complete remission. However, often associated with multiple drug resistance (MDR), relapse occurs in more than 50% of the patients [4].

P-glycoprotein (P-gp) expression, which causes the formation of MDR in the cell, can be assessed by various laboratory methods [5]. Determination expression of P-gp is especially problematic for normal tissues since immunological methods are limited in terms of sensitivity. The use of western blot methodologies which require large numbers of tumor cells to obtain sufficient protein for a reliable result is insufficient. A more focused approach using flow cytometric methodologies offers potential for the solution to this problem. Flow cytometry is a method that can be applied quickly and easily before and after chemotherapy in hematologic malignancies [6-10].

P-gp is believed to function as an energy-dependent drug efflux pump for various lipophilic xenobiotics, resulting in reduced intracellular accumulation [11]. CD34 is a member of the single-pass transmembrane sialomucin protein family and a cell-cell adhesion factor expressed in some cell types; and recent reports have demonstrated that CD34 can alter the cell adhesion, migration, and engraftment potential of hematopoietic progenitor cells in bone marrow (BM) niche [12-15]. During the differentiation of leukemia cells, while the expression of P-gp decreases, expression of CD34 increases in AML malignant cells [16-18]. However, whether CD34 levels correlate with the expression or function of P-gp remains unclear [19].

Therefore, this study was designed to determine the expression of P-gp and CD34 by flow cytometry, and to find out the expression of P-gp and CD34 with unresponsive treatment in patients with AML and ALL.

## METHODS

### Participants

The study group consisted of 50 patients who were present at Erciyes University, Mehmet Kemal Dedeman Hematology-Oncology Hospital. The patients were diagnosed with AML (16 females, 14 males) and diagnosed with ALL (10 females, 10 males). Twenty healthy controls (10 females, 10 males) were also included in the study. After they were asked for their consent, BM samples were obtained from 30 patients, and blood samples were obtained from both patients and healthy controls group. The study was approved by Ethics Committee of Erciyes University Faculty of Medicine (Decision number: 2010/65, Decision Date: 08.07.2010). The study was carried out in accordance with the principles of the Helsinki Convention on Human Rights [20].

### Preparation of Samples

Peripheral blood samples were collected in glass tubes (sample tubes with acidum citrose-dextrose solution A; Becton Dickinson, San Jose, CA, USA); and leukemic peripheral blood mononuclear cells were isolated by density gradient separation (Ficoll-Hypaque specific density 1.077; NycoMed, Oslo, Norway) immediately after sampling.

Cells were frozen without delay and stored frozen in liquid nitrogen. BM aspirate samples were washed once in pre-warmed phosphate buffered saline. Lymphocytes, monocytes, and granulocytes must exist in the cell environment to make optimal analysis possible by flow cytometry. Therefore, the erythrocytes were removed from peripheral blood and BM samples. To achieve this, 1.5 mL BD cell lysis solution was added to each of the mixture in the tubes Falcon; and mixtures were incubated in darkness for 8 minutes after they were quickly vortexed. Each tube was centrifuged for 5 minutes at 1500 rpm after the incubation for 8 minutes. Supernatant was discarded after centrifugation. 2 mL of BD cell wash solution was added to pellet on the bottom of the tubes and tubes were centrifuged for 5 minutes at 1500 rpm for the second time. Supernatant was discarded after centrifugation. Pellets were vortexed quickly, and 500 µL of BD cell washing solution was added into the tubes [21].

### Cell Analysis with Flow Cytometry

Initially, 10  $\mu$ L of phycoerythrin Mouse anti-human P-gp kit, CD45 FITC kit and CD34 FITC or PerCP Cy 5.5 kit were added to 5 ml Falcon tubes. 100  $\mu$ L BM or peripheral blood sample were added onto Antibodies. Considering the possibility of clots in BM, samples were filtered from 40  $\mu$ m diameter millipore and risk of contamination was removed. Only 100  $\mu$ L BM or peripheral blood sample were added to another 5 mL Falcon tube. The second tube was used for control purpose. Vortexing was applied to the cells to form antigen-antibody complex and to mark a fluorescently labeled antibody; and they were incubated in the darkness for 15 minutes. Suspension cells were analyzed by flow cytometry [FACSCalibur (BD Bioscience)]. The analysis was made by counting 20000 cells on the device's Celquest program. The unfixed cells were run on a BD FACSCalibur flow cytometer with forward scatter and side scatter gate set around the blast population by using pulse processing of the forward scattered light signal to gate out doublets. Phycoerythrin labeled Mouse IgG2b, kappa isotype control kit was used for positive and negative control in the study [21].

### Statistical analysis

Statistical analysis were performed using statistics programs with SPSS software version 15.0. Statistical comparisons were performed using chi-square exact method. Data were expressed as mean  $\pm$  SD for continuous variables. Statistical significance was set as  $p < 0.05$ .

### RESULTS

The control group consisted of 20 individuals of without hematologic malignancy (10 females, 10 males, mean age  $48.2 \pm 16.5$  years). The AML group

consisted of 30 individuals (16 females, 14 males, mean age  $46.1 \pm 16.6$  years). The ALL group consisted of 20 individuals (10 females, 10 males, mean age  $31.3 \pm 13.2$  years). The three groups were almost similar in terms of age and gender.

### Flow cytometric analysis of CD34 and P-gp expression

Expression of P-gp and CD34 was not observed in the samples of control group. Flow cytometric images of one subject in the control group are shown in Figure 1.

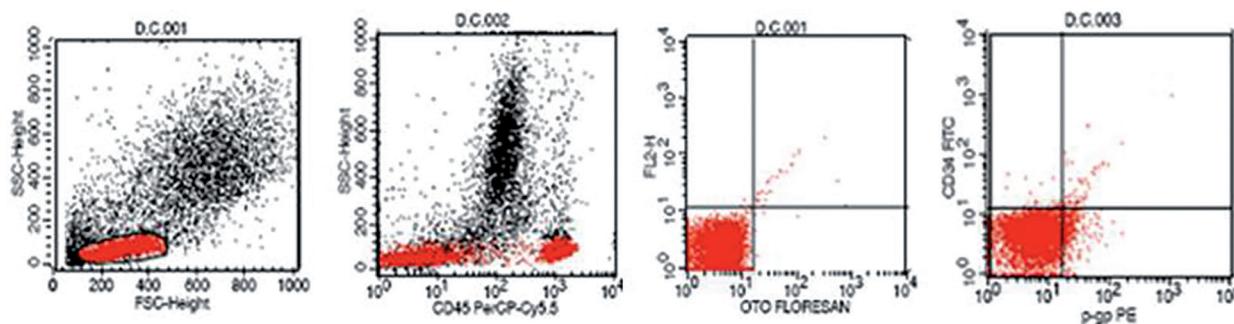
When we analyzed the BM of AML group using flow cytometry, we observed strong expression of P-gp and CD34 in 6 of 30 (20%) AML patients. P-gp and CD34 expression was not observed in 24 of 30 (80%) AML patients (Table 1). Flow cytometric images of one subject in the AML group are shown in Figure 2.

**Table 1.** Comparison of AML and ALL patients in terms of the P-gp and CD34 expression

	AML P-gp Expression		ALL P-gp Expression	
CD34 Expression	(n) P-gp (+)	(n) P-gp (-)	(n) P-gp (+)	(n) P-gp (-)
CD34 (+)	(6) 20%	(0) 0%	(5) 25%	(0) 0%
CD34 (-)	(0) 0%	(24) 80%	(1) 5%	(14) 70%

AML:  $p = 0.001$ , ALL:  $p = 0.001$ , n: number of patients

When we analyzed BM of ALL group using flow cytometry, we observed strong expression of P-gp and CD34 in 5 of 20 (25%) ALL patients. We found that there was strong expression of P-gp but there was not CD34 expression in one of 20 (5%) ALL patients. P-gp and CD34 expression was not observed in 14 of 20 (70%) ALL group (Table 1). Flow cytometric images of one subject in the ALL group are shown in Figure 3.



**Figure 1.** Expression of P-gp and CD34 in a participant of control group



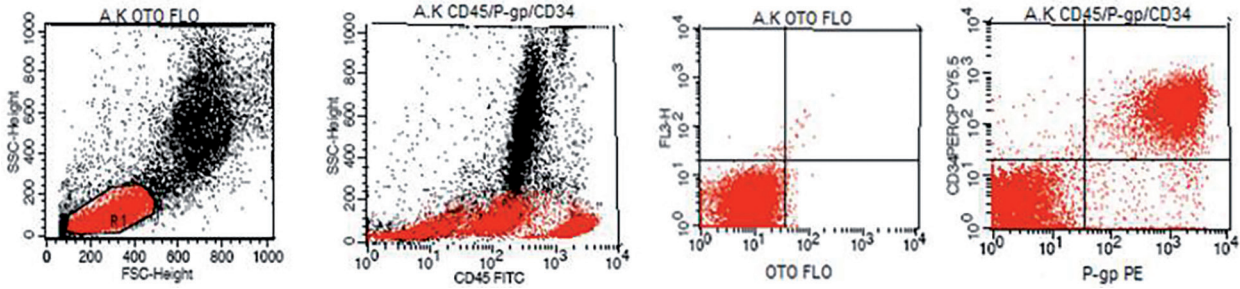


Figure 2. Expression of P-gp and CD34 in a participant of AML group

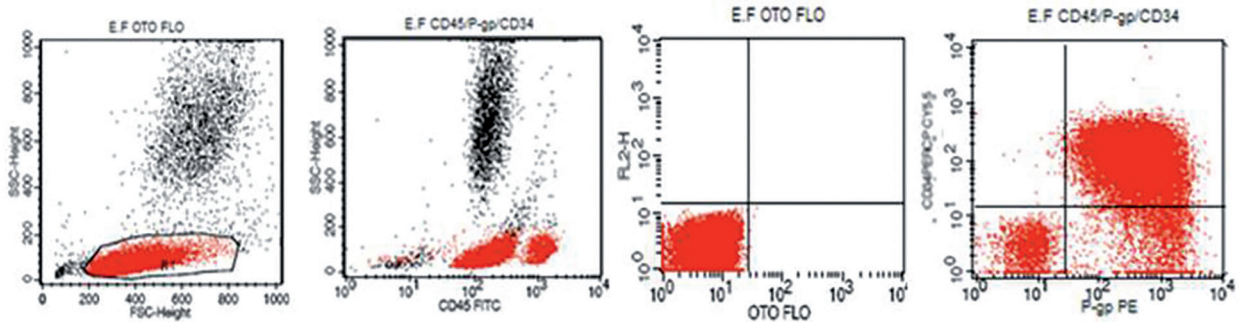


Figure 3. Expression of P-gp and CD34 in a participant of ALL group

**The distribution of P-gp and CD34 expression by gender in AML and ALL groups**

P-gp expression was observed 12.5% in females and 28.6% in males of AML patients. P-gp expression was observed 20% in females and 40% in males of ALL patients. CD34 expression was ob-

served 12.5% in females and 28.6% in males of AML patients. CD34 expression was observed 20% in females and 30% in males of ALL patients. There was not any significant difference between male and female expressions in terms of P-gp and CD34 (Table 2).

**Table 2.** The distribution of P-gp expression by gender in AML and ALL groups

AML Group			ALL Group		
P-gp Expression			P-gp Expression		
Gender (n)	(n) P-gp (+)	(n) P-gp (-)	Gender (n)	(n) P-gp (+)	(n) P-gp (-)
Female (16)	(2) 12.5%	(14) 87.5%	Female (10)	(2) 20%	(8) 80%
Male (14)	(4) 28.6%	(10) 71.4%	Male (10)	(4) 40%	(6) 60%
CD34 Expression			CD34 Expression		
Gender (n)	(n) CD34 (+)	(n) CD34 (-)	Gender (n)	(n) CD34 (+)	(n) CD34 (-)
Female (16)	(2) 12.5%	(14) 87.5%	Female (10)	(2) 20%	(8) 80%
Male(14)	(4) 28.6%	(10) 71.4%	Male (10)	(3) 30%	(7) 70%

AML: p=0.378, ALL: p=0.628, (n): Number of patients

**DISCUSSION**

Our results provide evidence of MDR as shown by positive expression of P-gp and CD34 in patients with AML and ALL. On the other hand, very low P-gp expression is observed in stem cells of normal

hematopoiesis, which is consistent with previous studies [22,23].

Many methods using the determination of P-gp, responsible for cellular MDR, have been developed that analyze the level of protein, mRNA levels and

the functional levels [24-26]. However, as a result of research conducted in several centers, Chevillard et al. recommended the use of flow cytometry for analyses of MDR in protein levels and the use of real time polymerase chain reaction method in the mRNA levels [27]. In our study, MDR-1 expressions were measured with flow cytometry using 17F9 specific monoclonal antibody for P-gp membrane surface epitopes. Both the threshold value of P-gp and the functional status of the synthesized P-gp play a role in the appearance of clinical resistance phenotype [28]. Therefore, it is necessary to determine the threshold value for P-gp positivity in the method used in the determination of P-gp. In the present study, 102 and a higher level of fluorescence intensity was evaluated as positive on forward scattered light-side scattered light screen in flow cytometry.

We found that CD34 and P-gp expression levels are consistent with each other in patients with AML. We also showed that positive expression of P-gp and CD34 in 6 (20%) of 30 patients with AML and in 5 (25%) of 20 patients with ALL receiving chemotherapy.

The positive expressions of P-gp were evaluated for the presence of drug resistance. The increase of P-gp expression accelerates the elimination of drugs which are used in the treatment of ALL and AML. Thus, cancer cells are protected from the cytotoxic effects of chemotherapeutic drugs. Kuwazuru et al. showed that 9 of 17 patients with AML and 4 of 11 patients with ALL had P-gp positive results at the initial presentation, and most P-gp positive patients did not respond to chemotherapy [29]. Pall et al. observed a significant correlation between CD34 expression and activity of P170 in AML patients. Senent et al. [30] conducted a study in patients with 82 AML by immunocytochemical method using the monoclonal antibody C219, and showed that CD34 expression were positive in 8 of P-gp positive 13 cases (62%). The researchers emphasized that P-glycoprotein expression is a reliable marker of patients first diagnosed with AML. Consistent with our study, Gruber et al. [31] stated that expression of the MDR-1 gene could be an important factor contributing to drug resistance in acute leukemia. Similarly, Boekhorst et al. [32] reported that expression of P-170 encoded by the MDR-1 should be related

to the MDR. In the present study, we also found that 24 patients were seen in the remission process. P-gp and CD34 expressions were not observed in individuals entering remission process and controls group. These findings show that the treatments were successful. El-Ghaffar et al. [33] conducted a study on patients with acute leukemia (14 AML cases and 6 ALL cases); and showed that the functional activity of MDR-1 P-gp was 71.4% in AML and 33.3% in ALL patients compared with 16.6% in normal lymphocytes. They suggested that P-gp/170 is expressed to a higher degree in leukemic cells and this is greater in relapse when compared to de novo cases and more in AML than ALL blasts.

In light of these findings, the results suggest the expression of P-gp is closely related to clinical drug resistance in acute leukemia. We also investigated the expression of P-170 encoded by the MDR-1 with flow cytometry; and we observed that P-gp was positive in patients with recurrence. The observation of MDR at a higher rate in ALL patients compared to patients with AML suggests that it may have occurred due to different morphological and phenotypic characteristics of ALL patients. There are several limitations for our study. Firstly, the small number of patients may be perceived as a limitation of the study. However, the major limitation of the study is that there was no way to evaluate the effect of individual chemotherapeutic agents that was used.

In conclusion, in terms of MDR, examination of patients diagnosed with hematologic malignancy especially in recurrent cases will shed light on chemotherapy resistance encountered in these patients. For measurement of MDR by flow cytometry, the most important advantages are the ones that make it possible to easily identify the cell clones of gated leukemic (blastic) and to make them specifically be analyzed in terms of P-gp. P-glycoprotein expression can be measured quickly by flow cytometry; and this may pave the way to more rapid implementation of alternative treatment regimens in especially in recurrent cases.

**Declaration of Conflicting Interests:** The authors declare that they have no conflict of interest.

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