

## P-GLYCOPROTEIN QUANTITATION IN ACUTE LEUKEMIA

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### ABSTRACT

Multi drug resistance(MDR) is a major problem in the treatment of cancer and hematological malignancies. This resistance is multi factorial and is the result of decreased intra cellular drug accumulation. This is partly due to the presence of a 170KD intra membranous protein termed P-glycoprotein(P-gp) that is an energy-dependent efflux pump which has increased expression on drug-resistance cells. In this study we identified the presence of P-gp by staining with Fluorescent Iso Thio Cyanate (FITC) conjugated anti P-gp in acute leukemia patients and flow cytometry in addition to performing immunophenotype analysis and French, American British (FAB) classification. Results revealed that one fifth of leukemic patients expressed P-gp and this phenotype was more prevalent in Acute Undifferentiated Leukemia(AUL) and Acute Myelogenous Leukemia (AML) than in Acute Lymphoblastic Leukemia(ALL). Other findings showed a logical relationship between this phenotype and age groups. There was not any association between P-gp+ phenotype and FAB and Immunophenotyping sub classification, but there was a linear relationship between CD34 and CD7 expression and P-gp+ phenotype. The accumulation of P-gp molecule that was stated as Mean Fluorescence Intensity (MFI) on the blasts' membrane of AUL and AML patients showed marked increase in comparison to ALL. Furthermore MFI in P-gp+ relapsed patients was much more than P-gp+ pretreatment patients.

**Keywords:** Leukemia, Drug resistance, P-glycoprotein, Flowcytometry, FAB classification, Immunophenotyping, Mean Fluorescence Intensity

### INTRODUCTION

Recent advances in cell and molecular sciences have led to better controlling of the malignant tumors

with the assistance of using cytotoxic drugs but success has not been complete due to either rapid proliferation of these malignant cells or cellular resistance, relapsing and recurrence often occurs. Cellular resistance due to cell membrane proteins that are from ABC family and consists of four groups: 1-P-gp, 2-MRP, 3-LRP, 4-a group of enzymes that are involved in glutathion metabolism and affect drug concen-

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tration within cell.<sup>(1,2,3)</sup> P-gp molecule is an end product of MDR1 gene, which twenty-five years ago was discovered by Line and Juliano.<sup>(4,5)</sup> This molecule is present normally in some tissues and its function is defined as protection of primitive precursor hematopoietic cells,<sup>(6)</sup> but in abnormal cases this phenotype is associated with a disease process such as drug resistant malignancies.<sup>(7,8)</sup>

P-gp is a 170 KD molecule and consists of 128 amino acids<sup>(9,10)</sup> with C and N-terminal directed intracellular<sup>(7,8)</sup> and has a glycosylated extra cellular component in first extra cellular loop, moreover several phosphorylation and ATP binding sites being present along molecule. These sites play a part in drug transportation into the cell.<sup>(11,12)</sup> These drug transporting parts can be detected by using monoclonal antibodies. Presence of P-gp on the surface of acute leukemia's blasts denotes some kinds of drug resistance. Clinical sig-

4-Secondary antibody against anti P-gp that conjugated with FITC cat no:f F313 from DAKO company  
5-IgG2a as Negative control(not anti human) which is P-gp isotype antibody cat no:X943 from DAKO company.

6-Formic acid 0.5%, paraformaldehyde 1% and phosphate buffer solution.

7-Centrifuge and flow cytometer (Coulter-epics xl).

8-leukocyte preparation set or Q-prep(Coulter co).

Bone marrow aspirates were collected from the patients with definite leukemia diagnosis. For FAB classification, aspirates specimens were smeared and stained by Wright method and studied by two pathologists for FAB classification and in some cases cytochemical and/or immuno cytochemical staining were done.

For immunophenotyping and P-gp determination, approximately 0.5mL heparinized BM aspiration was

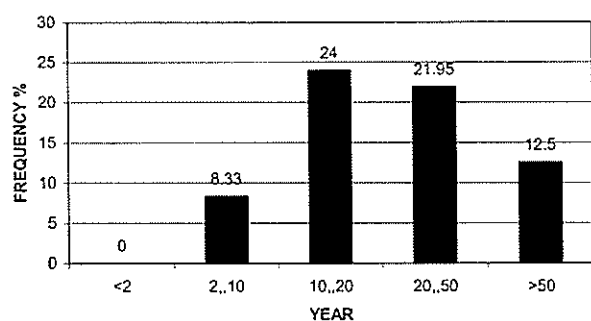


Fig. 1. P-gp distribution in age groups.

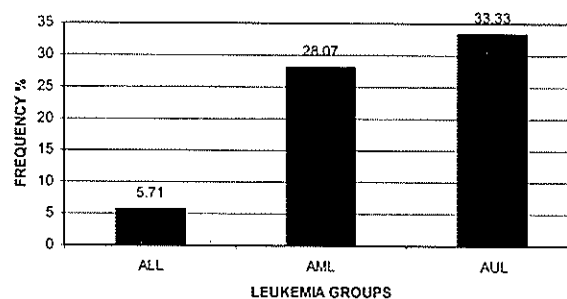


Fig. 2. P-gp distribution in Leukemic groups.

nificance of this phenotype (MDR) in ALL is not fully understood, but general agreement is for close relationship of P-gp distribution on cell membrane and some prognostic factors such as morphological features of L3, phenotype of B-ALL and leukocyte count.<sup>(13)</sup>

In AML cases, presence of P-gp+ blasts, indicates decreased survival and failor of cure rate.<sup>(7,8)</sup> This phenotype often is associated with CD34+ and CD7+ phenotype and is a cause of shorter survival.<sup>(6)</sup>

In this study, we attempt to reveal the occurrence of this phenotype in acute leukemic patients and its correlation with some other prognostic factors.

### MATERIAL AND METHODS

1-Anti coagulated bone marrow aspirates from 103 new cases of acute leukemia and 4 relapse cases.

2-RPMI 1640 culture medium.

3-Monoclonal antibody against P-gp cat no: M3523 and a series of CD markers from DAKO company that are listed subsequently.

washed twice in 3 ml RPMI1640. Pellet admixed with RPMI to adjust the cells to 1000 to 10000 per micro liter. For detection of CD markers 100 micro liter of cell suspension with 10 micro liter of antibody was incubated at 2-4°C for 30 minutes and then was introduced to leukocyte preparation set which formic acid, phosphate buffer solution and paraformaldehyde were added by the set. Then samples were ready for flow cytometric analysis. During analysis the neoplastic cells were gated, percent and count of different CD markers were determined and classified:

1-Early pre B-ALL: CD 19+, HLA DR+, CD 10+, CD 34+, CD20-

2-Pre B-ALL: CD 19+, HLA DR+, CD10+, CD 34+/-, CD20+, cIgM+

3-B-ALL: CD 19+, HLA DR+, CD 10-, CD34-, CD 20+

4-T-ALL: CD 2+, CD 7+, CD 5+, c CD 3+, TdT+

5-Poorly differentiated AML: cMPOX +, HLADR+, CD 34+, CD 33+/-, CD 13+/-, CD 7+/-.

7-Differentiated AML: MPOX+, CD 13+, CD 33+, CD 34-/-+, HLADR+

8-Promyelocytic leukemia: MPOX+, CD 13+, CD 33+, CD 14-, CD 34-, HLADR-

9-Myelomonocytic leukemia: CD 13+, CD 33+/-, CD 14+, HLADR+, NSE+

10-Erythroleukemia: Glycophorin A+, CD 71+

11-AUL: No lymphoid and myeloid markers available or present.

For P-gp measurement, DAKO procedure was followed. 100 micro liter of cell suspension was added to 10 micro liter of P-gp antibody and was incubated for 30 minutes in room temperature. After two times washing, the precipitate was dissolved in 100 micro liter PBS, then 10 micro liter of diluted secondary antibody was added to it and incubated for 30 minutes at room temperature. Finally the tubes were placed in Q-prep instrument for addition of three above mentioned solutions and then flow cytometric analysis was done, finally it revealed the percentage of cells which reacted with anti P-gp as P-gp positive cells. Non specific reactions were omitted based on reactions in negative control. Also P-gp molecule accumulation in different cases were measured that was stated as Mean Fluorescence Intensity.

We used P-gp isotype antibody (IgG2A) as negative control. The statistical procedures of data analysis were uni and bi-directional variance and regression tests.

### RESULTS

Out of 103 patients who were studied, 23% of 38 females and 19% of 65 males were P-gp+. The patients were classified in 5 age groups with one under two years of age that was not considered in bi-directional variance analysis, but different P-gp percentage in other age groups (2-10 year, 10-20 year, 20-50 year and more than 50 year) was meaningful. The greatest P-gp distribution was in 10-20 years old group (Fig. 1).

Then we classified leukemia into three groups: acute

lymphoblastic leukemia, acute myelogenous leukemia and acute undifferentiated leukemia. Thus 5.71% out of 35 ALL, 28.07% out of 57 AML and 33.33% out of 3 AUL patients showed P-gp+ phenotype. (Fig. 2). The P-gp molecule accumulation was measured by MFI. The MFI of P-gp on the surface of blasts in these three groups revealed that ALL cases blasts showed MFI with a score of 2.20, AML cases blasts showed MFI with a score of 4.34 which was twice that of P-gp+ ALL cases and finally AUL cases blasts showed MFI with a score of 17.21 that was eight times more than ALL cases (Fig. 3). The P-gp distribution prevalence in various leukemia subgroups (immunophenotype and FAB classification) were different. In ALL cases, only 2 P-gp+ phenotype were seen who were pre B ALL and one was L1 and another L2 type and 16 out of 57 AML cases were P-gp+, the prevalence of this phenotype in FAB classified AML is shown in Fig. 4. Then AML cases were grouped according to immunophenotyping criteria and 4 out of 10 poorly differentiated AML were P-gp+, out of 22 differentiated AML 8 cases were P-gp+, 1 out of 8 promyelocytic AML and 3 out of 16 myelomonocytic AML and 0 out of 1 erythroleukemia were P-gp+. Also in this study we compared P-gp distribution with CD7 and CD34 expression as poor prognostic markers. Finally P-gp molecule distribution on P-gp positive and P-gp negative pre treatment patients and P-gp+ relapsed patients were evaluated. We compared MFI of P-gp molecule in following three groups:

- 1) pre-treatment phase of patients who were P-gp- that showed MFI with a score of 0.63.
- 2) pre-treatment phase of patients who were P-gp+ that showed MFI with a score of 21.68.
- 3) patients who were P-gp+ in relapse phase that showed MFI with a score of 51.88.

### DISCUSSION

According to results, occurrence of P-gp was seen in less than one fifth of leukemic patients and it is an important factor as a prognostic parameter in agreement with previous studies.<sup>(15)</sup> We found no meaning-

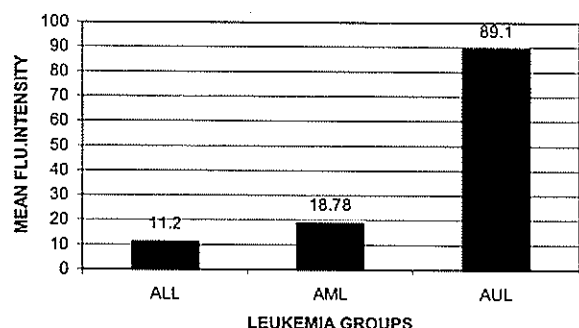


Fig. 3. Mean Fluorescent intensity in leukemic groups

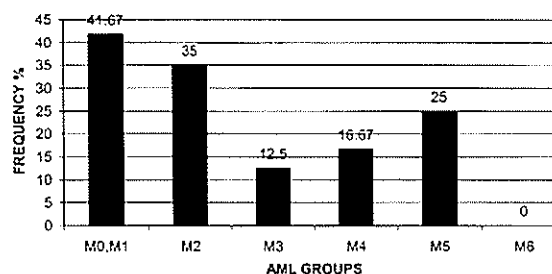


Fig. 4. P-gp distribution in AML Subgroups

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ful statistical findings within sex and P-gp expression ( $p>0.05$ ), but there were significant correlations in P-gp+ phenotype and age groups and it had more expression in 10-20 years old group ( $p<0.05$ ) that defines poorer clinical prognosis. Presence of P-gp+ phenotype on blasts of AML and AUL patients confirm poor response and unfavorable prognosis according to previous studies<sup>(6)</sup> as against ALL cases which had a low prevalence of P-gp phenotype.<sup>(17)</sup> According to present study there were not any significant correlations between various AML and ALL subtypes (FAB and immunophenotyping classification) and P-gp+ phenotypes. Although distributions of AML FAB subtypes were more prevalent in primitive and more immature subtypes, however it is not statistically significant ( $p>0.05$ ). Since CD34 and CD7 express more on immature cells and are known as poor prognostic markers. In this study linear correlation between presence of these antigens and P-gp+ phenotype ( $p<0.05$ ,  $r=0.25$ ) also indicate poor response to therapy.<sup>(6)</sup> With respect to accumulation of P-gp expression that was revealed with MFI measurement, an interesting finding was more accumulation of P-gp molecules in P-gp+ blasts in AUL and AML groups compared to ALL group. We further studied a comparison between MFI in four relapse cases and other P-gp+ patients that were studied before treatment. The results showed that P-gp molecular accumulation was several times more in P-gp+ relapse cases than P-gp+ untreated cases and this finding agreed with other studies regarding for more production of P-gp molecules in relapse phase,<sup>(15)</sup> emphasizing the determination of this phenotype before treatment, to choose better treatment protocols. Finally we propose P-gp detection as a valuable and independent parameter in predicting prognosis and clinical behavior of the disease.

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