ONCOLOGY

Study of Expression and Functional Activity of P-GP Membrane Glycoprotein in Children with Acute Leukemia

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 141, No. 6, pp. 676-680, June, 2006
Original article submitted March 15, 2005

We studied expression and functional activity of tumor cell P-gp in children with various leukemia variants and analyzed its prognostic role in acute lymphoblastic leukemia. Functional activity of P-gp increased in acute myeloid leukemia, relapses of acute lymphoblastic leukemia (in comparison with primary disease), and in a group of patients in whom no remission was attained. The survival of patients with acute lymphoblastic leukemia was lower in cases with increased expression and function of P-gp.

Key Words: *P-gp; resistance; cytofluorometry; leukemia; prognosis*

Numerous mechanisms of the resistance of tumor cells to chemotherapeutics are known: reduced accumulation of the cytotoxic agent, modification of the target, activation of repair mechanisms, blockade of the apoptosis program, *etc.* [1]. An important role in the development of multiple drug resistance is played by intensive transport of the cytostatic from cells associated with enhanced expression of P-glycoprotein (P-gp), MRP, LRP, and BCRP [3].

P-gp is a membrane glycoprotein belonging to the ABC transporter family. This glycoprotein is encoded by MDR1 gene located on chromosome 7 (7q21) [10]. Normally P-gp is expressed on cells of the blood-brain barrier, on hemopoietic precursors, peripheral blood cells, and bronchial epithelial cells and protects cells from xenobiotics. Enhanced expression and high activity of P-gp lead to a decrease in the intracellular concentrations of some drugs used in oncology (vincristine, vinblastine, doxorubicin, daunorubicin, etoposide, *etc.*). Unfavorable effect of P-gp on drug therapy efficiency and survival of adult patients with acute myeloid leukemia (AML) was demonstrated [6,8, 9,13,14]. The data on clinical significance of P-gp for adult patients with acute lymphoblastic leukemia (ALL) and for children are contradictory [4,5,7, 11,13,14]. The role of P-gp in the formation of drug resistance of tumor cells is an important problem.

We studied expression and functional activity of P-gp in children with different types of leukemia and analyzed the prognostic role of this glycoprotein in ALL.

MATERIALS AND METHODS

The study was carried out in a group of 125 patients (1-17 years) hospitalized at Center for Children Oncology and Hematology with the diagnosis of acute leukemia. Of these, 70 patients presented with primary ALL, 19 with ALL relapses, and 36 with primary AML. Thirteen normal bone marrow specimens were included in the study. Leukemic cells and donor mononuclears were isolated from the

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0007-4888/06/1416-0727

bone marrow on a Histopaque-1077 density gradient and washed twice in RPMI-1640. All studies on leukemic cells were carried out on the day of diagnosis before chemotherapy.

The expression of P-gp on the cell surface was evaluated using FITC-labeled 17F9 specific monoclonal antibodies (Becton Dickinson) on a FACScan flow cytofluorometer. FITC-IgG1 monoclonal antibodies served as the isotype control. At least 10,000 cells per sample were analyzed using CellQuestPro software.

Functional activity of P-gp was evaluated by flow cytofluorometry by accumulation of cationic fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidasole carbocyanine iodide (JC-1; Molecular Probes) in cells. The role of P-gp in JC-1 accumulation was confirmed using cyclosporine A (specific modulator of its activity) [8].

The results were presented as medians of values varying within a range of 25-75 percentiles. The significance of differences was evaluated using Mann—Whitney test. Survival was estimated using Kaplan—Mayer test, the significance of differences was evaluated using the log-rank test. Statistical analysis was carried out using Statistica 6.0 software.

RESULTS

The density of P-gp expression on the cell membrane is low (or antibody binding is low-specific), and hence, it is impossible to evaluate glycoprotein expression adequately by using traditional parameters (percentage of positive cells and fluorescence intensity). We therefore estimated the D criterion (using Kolmogorov—Smirnov test), reflecting the differences in the distribution of cells by binding with monoclonal antibodies (17F9 and IgG1) [2,12].

The expression of P-gp by tumor cells in myeloid leukemia slightly increased in comparison with lymphoid leukemia: D criterion was 0.11 (0.07-0.21) in AML (n=36) and 0.08 (0.05-0.16) in ALL (n=70). More pronounced increase in glycoprotein expression was observed in disease relapses (n=16, D=0.19; 0.06-0.34) compared to primary ALL. No differences in glycoprotein expression in primary T and B ALL were detected (data not presented).

Apart from expression of P-gp, its functional activity is also an important parameter [2]. This is explained by poor detection of P-gp with monoclonal antibodies and high functional activity of the glycoprotein poorly expressed on cell membrane

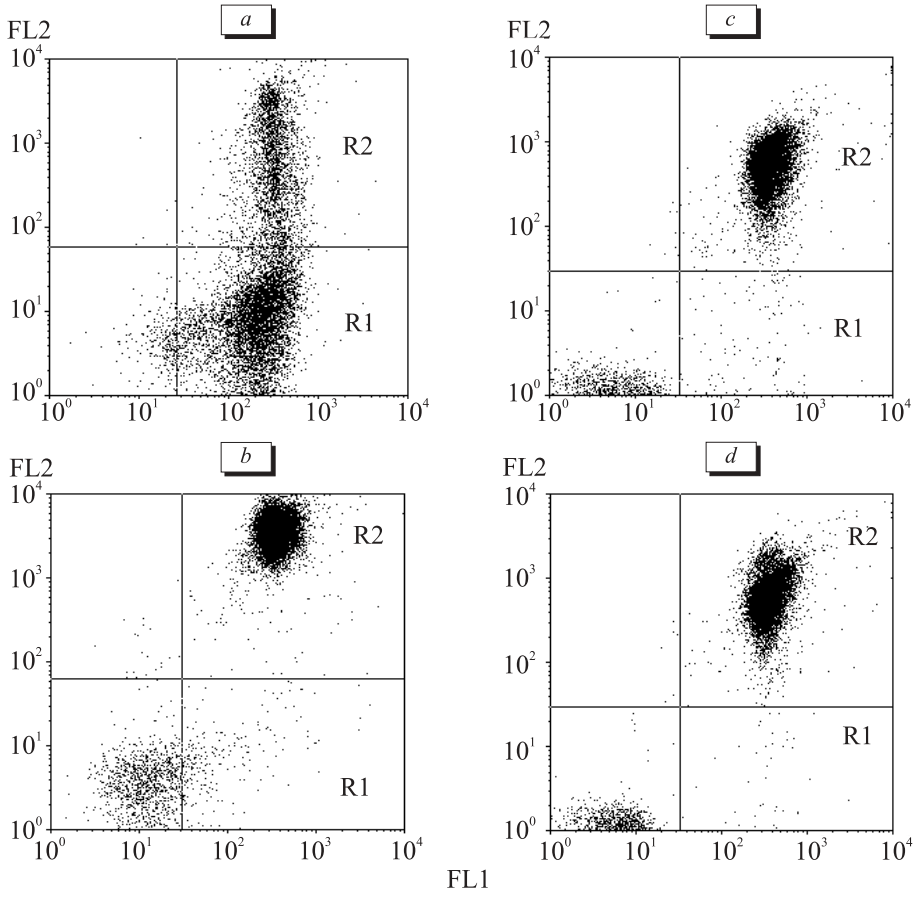

Fig. 1. Functional activity of membrane glycoprotein evaluated by flow cytofluorometry. High (*a*, *b*) and low (*c*, *d*) functional activity; *a*, *c*: JC-1 accumulation without cyclosporin A; *b*, *d*: with cyclosporin A. R1: cells with slight accumulation of JC-1; R2: cells with intensive accumulation of JC-1. FL1: fluorescence intensity at λ=527 nm (rel. units); FL2: at λ=590 nm.

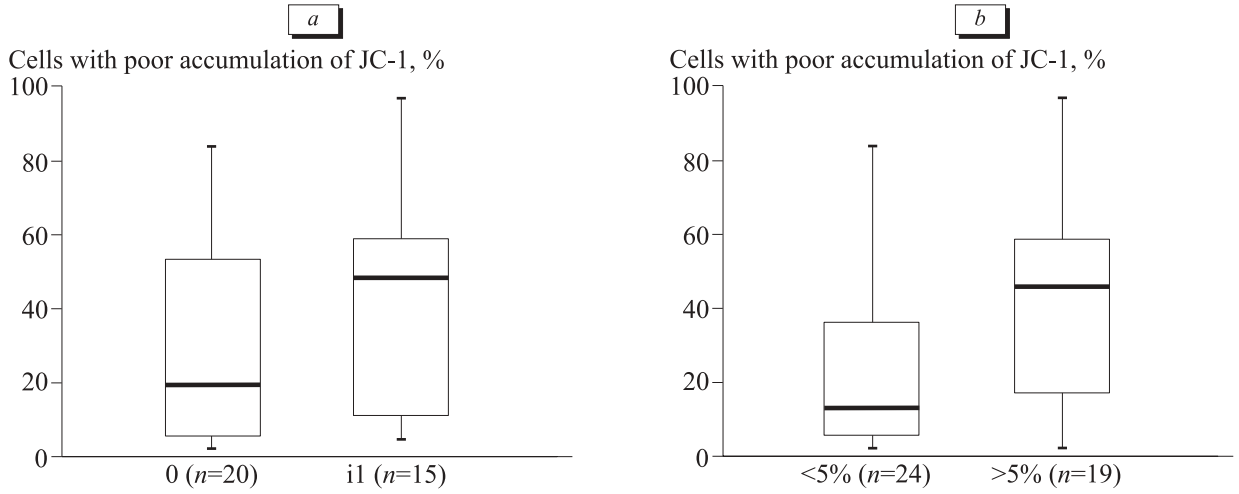

Fig. 2. Relationship between functional activity of P-gp and early response to therapy in children with acute lymphoblastic leukemia (ALL). *a*) content of blast cells in peripheral blood on day 8 of therapy; *b*) on day 15.

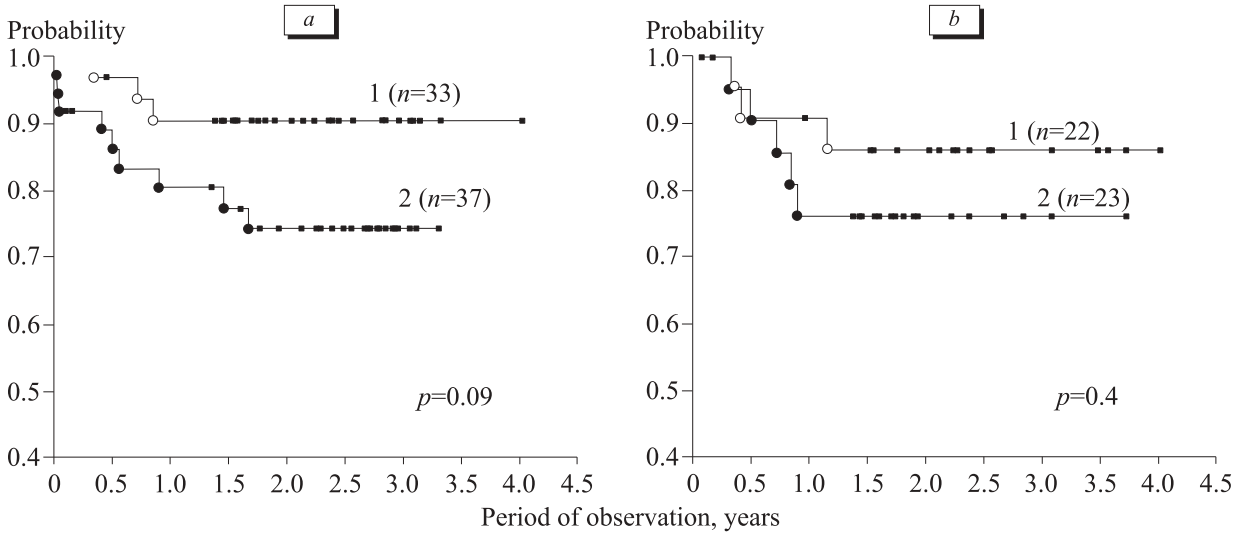

Fig. 3. Probability of 4-year uneventful survival depending on expression (*a*) and functional activity (*b*) of P-gp in children with ALL. *1*) survival at parameter<median; *2*) survival at parameter>median.

surface [9,11]. According to our data, the correlation between P-gp expression and functional activity in primary ALL is weak: Spearman's correlation coefficient was 0.29 (n=36; p=0.088).

TABLE 1. Functional Activity of P-gp in Tumor Cells in Children with Different Types of Acute Leukemia

Leukemia type	Cells with weak accumulation of JC-1, %
ALL (n=45)	23.9 (8.8-56.7)
ALL relapses (n=19)	58.0 (18.2-85.0)*
AML (n=26)	49.8 (28.0-80.0)*
Donor mononuclear cells (n=13)	40.0 (25.0-59.0)

Note. *p<0.05 compared to ALL.

P-gp function was evaluated by the efficiency of accumulation of JC-1 fluorescent probe in leukemic cells [8]. Fluorescence spectrum of this dye depends on the degree of its accumulation in cells, which results from aggregate formation at high concentrations. Accumulation of JC-1 dye (excitation wavelength 490 nm) in the cytoplasm in monomeric form is characterized by emission at 527 nm (green fluorescence), while after reaching critical concentrations JC-1 forms aggregates in mitochondria, which is associated with a shift in emission band to 590 nm (red fluorescence). Hence, cells with high functional activity of P-gp are characterized by green and weak red fluorescence (Fig. 1; R1). On the other hand, cells with low P-gp activity intensely fluoresce in the green and red spectrum bands (Fig. 1; R2). After evaluating the per-

centage of cells with poor accumulation of JC-1 (R1 region) we can quantitatively evaluate functional activity of P-gp and use this parameter for the analysis of its clinical significance.

Functional activity of membrane glycoprotein P-gp can be blocked by some compounds (verapamil, CsA and its derivatives; Fig. 1, *a*, *c*). Addition of CsA to cells with high P-gp function led to an appreciable increase in red fluorescence (Fig. 1, *b*), *i.e.* P-gp inhibits accumulation of JC-1. In cells with low functional activity of P-gp addition of CsA did not lead to appreciable changes in fluorescence intensity (Fig. 1, *d*).

In AML tumor cells exhibited higher functional activity than in ALL ($p<0.05$; Table 1). The content of cells with high functional activity of P-gp (weak accumulation of JC-1) in AML was more than 2-fold higher than in ALL. The progress of the disease was also associated with an increase in functional activity of the glycoprotein. In ALL relapses leukemic cells were characterized by higher functional activity of P-gp than in primary ALL ($p<0.05$).

In primary ALL leukemic T cells showed higher functional activity of P-gp than leukemic B cells ($p<0.05$). The percentage of cells with weak dye accumulation in T-cell ALL ($n=8$) was 37.1% (8.0-66.0), while in B-cell ALL ($n=37$) only 6.0% (1.6-28.1).

Normal hemopoietic cells also exhibited high functional activity of P-gp. Functional activity of P-gp in donor bone marrow mononuclear cells was comparable to that in AML and ALL relapses and was even higher than in ALL.

P-gp is considered to be prognostically important in acute leukemia. It was shown that in children with ALL the level of P-gp expression is not a prognostic factor for the response to chemotherapy, risk of relapses, and total survival [4,7,13,14]. On the other hand, high expression of P-gp is associated with higher incidence of relapses and lower survival of children with ALL [5].

Functional activity of P-gp in leukemic cells of patients who had no blast cells in the peripheral blood on day 8 of therapy was lower than in the patients who retained blast cells in the peripheral blood by day 8 of chemotherapy ($p=0.26$; Fig. 2, *a*). Similar regularities were detected in evaluation of therapy efficiency on day 15 (Fig. 2, *b*). Leukemic cells of patients reaching remission on day 15 of therapy (<5% blast cells in the bone marrow) were characterized by a low level of functional activity of P-gp in comparison with cells of patients who did not reach remission by this term ($p=0.05$). We detected no differences in P-gp expression in patients responding and not responding to therapy on days 8 and 15 of treatment.

For evaluation of the role of P-gp for long-term prediction in primary ALL, we analyzed the probability of uneventful survival in patients with different expression and function of this glycoprotein. The patients were divided into groups with high and low P-gp expression and function by the median value. The probability of 4-year uneventful survival in patients with high expression (Fig. 3, *a*) and function of P-gp (Fig. 3, *b*) was significantly lower than for patients with low expression and functional activity of P-gp.

Hence, we revealed an unfavorable prognostic significance of P-gp in children with ALL. Functional test showed that P-gp activity was high in patients not reaching remission, while the expression of P-gp on leukemic cells did not differ. In patients with primary ALL enhanced expression and increased function of P-gp were associated with poor survival. In prognostically unfavorable type of childhood leukemia (AML) and ALL relapses functional activity of P-gp in tumor cells was increased against the background of its slightly increased expression. Our data suggest that studies of functional activity of P-gp and thorough analysis of its expression are required in children with acute leukemia.

The study was supported by Byelorussian Foundation for Basic Research (grant No. B04-203).

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