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Changes in the *MDR1* gene expression after short-term ex vivo therapy with prednisolone have prognostic impact in childhood acute lymphoblastic leukemia

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Abstract Multidrug resistance 1 (*MDR1*) gene expression determined by real-time polymerase chain reaction and results of rhodamine assay were analyzed at diagnosis and after 3 days of ex vivo therapy with prednisolone in 36 pediatric patients with acute lymphoblastic leukemia (ALL). Only 62% patients with de novo ALL had significant decrease of *MDR1* expression. These patients had over twofold lower rhodamine retention in the presence of cyclosporine A on day 3 than others and had better probability of disease-free survival. In this study, we have shown that changes in the expression of *MDR1* gene after short-term incubation of lymphoblasts with prednisolone may have prognostic value in pediatric de novo ALL patients.

Keywords Childhood ALL · Ex vivo therapy with prednisolone · *MDR1* expression · Real-time PCR

Introduction

The resistance of tumor cells to cytotoxic drugs is a major obstacle to successful therapy of hematological malignancies. One of the most important determinants of treatment response is the overexpression of the multidrug resistance

gene multidrug resistance 1 (*MDR1*), encoding transmembrane protein P-glycoprotein (PGP). The main function of PGP is reduction of drug accumulation inside the cells by active transport of the drug outside the cell membrane [1, 2]. Activity of PGP was shown to have prognostic significance in acute myeloid leukemia in adults [3] and, to lesser extent, in children [4]. On the other hand, relationship between the *MDR1* gene expression and childhood acute lymphoblastic leukemia (ALL) is still a matter of controversy. Some authors do while others do not find expression of this gene to be clinically significant in children with ALL [5–8].

Glucocorticoids are one of the most important drugs used in treatment of childhood ALL, and prednisolone is most often included in induction therapy. The initial response to 1-week prednisolone therapy is regarded as one of the strongest prognostic factor in childhood ALL [9]. Subgroup of patients with ALL, such as infants, are more resistant to prednisolone than older children with ALL [10]. However, no correlation between expression of *MDR1* gene and cytotoxicity of prednisolone in pediatric ALL was found [11, 12].

Our approach to this subject was to perform *MDR1* expression analysis in two time points. Thus, the objective of the study was the analysis of changes in the *MDR1* gene expression in children with ALL at the diagnosis and after short-term ex vivo therapy with prednisolone.

Material and methods

Patients

A total number of 36 patients with ALL aged 0.1–16.8 years, treated in the Department of Pediatric Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus

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University, Bydgoszcz, between September 2005 and May 2007, were qualified for the study. The research protocol was approved by the Ethics Commission of the Collegium Medicum. With respect to phase of the disease, 29 patients were included at first diagnosis (de novo ALL), and seven at relapse (relapsed ALL). Median follow-up for all de novo patients was 1.8 years. Newly diagnosed patients were treated according to intercontinental protocol ALL-IC-2002, while relapsed patients with ALL-BFM-REZ-2002 protocol.

Design of the study

Freshly isolated leukemic cells obtained from bone marrow were isolated on Ficoll gradient and placed in the culture flask for 72 h for ex vivo therapy with prednisolone (Jelfa, Jelenia Gora, Poland) with a final concentration of 50 µg/ml. Only leukemic samples with the percentage of leukemic blasts at least 90% in the sample after Ficoll isolation and with percentage of leukemic blasts at least 70% in the sample after 3 days of culture were qualified for the study. Cells were maintained in Roswell Memorial Park Institute 1640 medium (Sigma), supplemented with 2 mM glutamine and 20% fetal bovine serum. Culture medium was supplemented with 100 U/ml penicillin (Polfa Tarchomin, Poland), 100 µg/ml streptomycin (Polfa Tarchomin, Poland), 200 µg/ml gentamicin (Krka, Slovenia), and 0.125 µg/ml amphotericin B. The culture was carried out in conditions of 5% CO₂, 37°C, and 95% humidity.

For each experiment, 8 ml of suspension containing two million leukemic cells per 1 ml was used. Before (day 0) and after the incubation (day 3), the following assays were performed: quantitative analysis of the *MDR1* gene expression by real-time polymerase chain reaction (PCR), rhodamine 123 (Rh123) retention in leukemic blasts with and without the presence of cyclosporine A (CsA; Sandimmun Neoral, Novartis Pharma AG, Basel, Switzerland). PGP expression was analyzed at diagnosis by flow cytometry. Ex vivo cytotoxicity of prednisolone was estimated simultaneously by the MTT assay.

Real-time PCR conditions and relative quantification of analysis

Total RNA was extracted from bone marrow cells using a TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations. RNA yield and purity were determined spectrophotometrically. First-strand complementary DNA (cDNA) was synthesized using 1 µg of total RNA, random hexamers, and SuperScriptIII reverse transcriptase according to the manufacturer's instructions (Invitrogen).

The messenger RNA expression levels of *MDR1* and the endogenous housekeeping glyceraldehyde 3-phosphate de-

hydrogenase (*GAPDH*) gene as a reference were quantified by real-time PCR technique. Reactions were performed in a Rotor Gene 6000 (Corbett, Sydney, Australia) using 2.5 µl cDNA as template and SYBR Green I fluorescent dye as described in the instruction manual for DyNAmo HS SYBR Green qPCR kit (Finnzymes, Finland). The expression of *MDR1* and *GAPDH* genes in duplicates of all cDNA samples were analyzed in the same PCR run. The primer sequences were as follows: *MDR1* 5'-CCC ATC ATT GCA ATA GCA GG-3' forward and 5'-GTT CAA ACT TCT GCT CCT GA-3' reverse; *GAPDH* 5'-CGG GAA GCT TGT GAT CAA TGG-3' forward and 5'-GGC AGT GAT GGC ATG GAC TG-3' reverse. Thermal cycle conditions consisted of an initial denaturation step of 95°C for 5 min, followed by 45 repetitive cycles of 95°C for 45 s, 60°C for 25 s, and 72°C for 35 s, with a final extension of 72°C for 7 min.

Relative quantification of the *MDR1* gene expression on day 3 vs 0 was evaluated using the comparative $2^{-\Delta\Delta C_t}$ method [13]. C_t value is the PCR cycle number at which the accumulated fluorescence signal in each reaction crosses a threshold above background. The ΔC_t value was calculated by subtracting the *GAPDH* C_t value of sample from its respective *MDR1* C_t value. The mean ΔC_t was calculated from the two independently calculated ΔC_t s for duplicates of all cDNA samples. Calculation of $\Delta\Delta C_t$ was performed using *MDR1* on day 0 sample mean ΔC_t value, as an arbitrary constant, to subtract from *MDR1* on day 3 sample mean ΔC_t value. Difference in expression of the *MDR1* gene on day 3 compared to day 0 for each leukemic sample was determined by the $2^{-\Delta\Delta C_t}$ value.

At least 50% decrease ($2^{-\Delta\Delta C_t} \leq 0.50$) or increase ($2^{-\Delta\Delta C_t} \geq 1.50$) of *MDR1* relative expression on day 3 in comparison to initial expression on day 0 was defined as important change of *MDR1* expression in incubated leukemic cells.

The relative *MDR1* gene expression level to *GAPDH* for each cDNA sample before incubation with prednisolone was calculated using the following equation:

$$\text{relative } MDR1 \text{ expression} = 2^{-\Delta C_t}$$

Multidrug resistance protein expression

The expression of intracellular epitopes of multidrug resistance protein : PGP (clone JSB-1, Alexis Biochemicals, Lausanne, Switzerland) was analyzed by flow cytometry on diagnosis. The value of protein expression was presented as the mean fluorescence intensity, corrected by expression of respective isotype controls. Negative control was CCRF-CEM cell line, while positive control was adenocarcinoma LoVo-Dx cell line.

Retention of rhodamine 123 without/with cyclosporine A

Rh123 is a lipophilic fluorescent stain, accumulating in mitochondria of living cells. Rh123 is an artificial substrate actively effluxed from the cells by multidrug resistance protein PGP, serving as a measure of PGP function (but not its expression). Rh123 (Sigma, St Louis, MO, USA) solution at concentration of 200 ng/ml was used. CsA is an inhibitor of PGP function in vitro and in vivo. Retention of Rh123 was tested without and with the presence of cyclosporine A at concentration of 2 μ M. Cells were incubated with Rh123 \pm CsA for 30 min, followed by flow cytometry analysis.

Cytotoxicity assay

Cellular drug resistance to prednisolone was tested by means of the MTT assay (prednisolone concentration range 0.007–250 μ g/ml) as described elsewhere [14]. The LC50 value, defined as the concentration of prednisolone that was lethal to 50% of tested cells, was calculated from the dose–response curve and was used as a measure for in vitro prednisolone resistance in each sample.

Statistical analysis

Statistical analysis was done for non-categorical variables by means of the Mann–Whitney *U* or Wilcoxon matched-pair tests. Associations between categorical variables were analyzed using chi-squared test or Fisher exact test. Correlations were measured by Spearman's coefficient. Median survival and probability of disease-free survival (pDFS) were determined by Kaplan–Meier method.

Results

Relative quantification of the *MDR1* gene expression by real-time PCR

The relative expression of the *MDR1* in comparison to reference *GAPDH* gene in samples of leukemic cells ($n=36$) before incubation with prednisolone, as estimated by $2^{-(\Delta C_t)}$ value, displayed range between 37,640.55 and 0.01. In one out of 29 patients with de novo ALL, high (37,640.55) relative *MDR1* expression was found. In 19 (66%) patients, low relative *MDR1* expression (range 0.48–0.15) and in nine (31%) very low (range 0.10–0.01) expression was revealed. Among seven relapsed ALL patients, in six (86%) low (range 0.46–0.14) while in one patient very low (0.02) relative of *MDR1* vs *GAPDH* expression was observed.

Relative difference in *MDR1* expression after 3 days of incubation of leukemic cells with prednisolone, when

compared with day 0, and calculated by $2^{-\Delta\Delta C_t}$ method, ranged from 0.01 to 1.49. Downregulation (range 0.01–0.99) and upregulation (range 1.01–1.49) of *MDR1* expression were observed in 31 (86%) and five (14%) patients, respectively. The important decrease of expression (range 0.01–0.50) in 18/29 (62%) patients with de novo and in three of seven (43%) with relapsed ALL was revealed, while 11/29 (38%) patients with de novo and four of seven (57%) with relapsed ALL had an insignificant *MDR1* expression change (range 0.51–1.49; Fig. 1).

For the purpose of further analysis, de novo ALL patients were divided into two groups: group A with decrease of *MDR1* expression of at least 50% ($2^{-\Delta\Delta C_t} \leq 0.50$) and group B with changes below 50% ($2^{-\Delta\Delta C_t}$ between 0.51 and 1.49). Both groups did not differ neither in their characteristics nor in their response to initial therapy (Table 1).

PGP expression

No correlation was found between PGP expression at diagnosis and the relative difference in expression of *MDR1* between days 3 and 0 in both groups A and B of de novo ALL patients.

Retention of rhodamine 123 without/with cyclosporine A

Retention of Rh123 was higher in the presence of cyclosporine A (PGP inhibitor) than in its absence both on day 0 ($p<0.001$) and day 3 ($p<0.001$) in all patients, which indicates inhibiting PGP function by the presence of

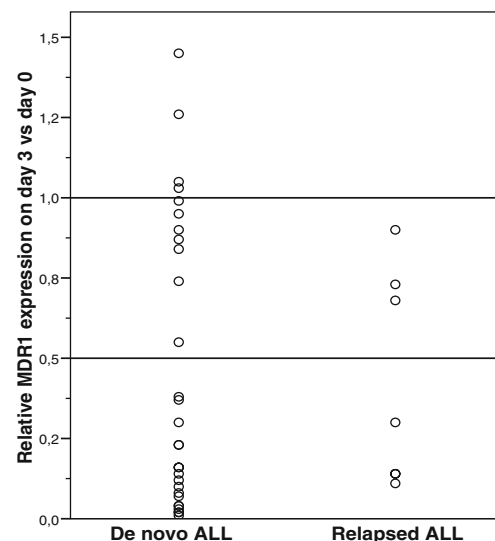


Fig. 1 Relative changes of the *MDR1* gene expression quantified by $2^{-\Delta\Delta C_t}$ method after 3 days of leukemic cells incubation with prednisolone. Two horizontal lines indicate: $2^{-\Delta\Delta C_t}=1$, no differences; $2^{-\Delta\Delta C_t}=0.50$, 50% decrease of expression

Table 1 Characteristics of de novo ALL patients with respect to radical (group A) and unimportant changes (group B) of *MDR1* expression after 3 days of leukemic cells incubation with prednisolone

| Patient characteristics | Group A | Group B | <i>p</i> value |
|---|---------|---------|--------------------------|
| Number of patients | 18 | 11 | |
| Gender (male/female) | 12/6 | 7/4 | 0.979, chi-squared test |
| Age: median and range (years) | | | |
| <1 year | 1 | 1 | 0.979, chi-squared test |
| 1–6 years | 10 | 6 | |
| 6–10 years | 4 | 2 | |
| >10 years | 3 | 2 | |
| Immunophenotype common/T/pro-B | 13/3/2 | 8/2/1 | 0.999, Fisher exact test |
| Initial leukocytosis (range) | | | |
| <10,000 per liter | 6 | 2 | 0.166, chi-squared test |
| <50,000 per liter | 8 | 2 | |
| <100,000 per liter | 2 | 4 | |
| >100,000 per liter | 2 | 3 | |
| <i>BCR-ABL</i> (positive/negative) | 1 | 1 | 0.999, Fisher exact test |
| <i>TEL-AML</i> (positive/negative) | 4 | 2 | 0.999, Fisher exact test |
| Poor steroid responders | 1 | 1 | 0.999, Fisher exact test |
| Bone marrow response by day 15 (M1 vs M2/M3) | 12 vs 6 | 5 vs 6 | 0.438, chi-squared test |
| Bone marrow response by day 33 (M1 vs M2/M3; remission induction) | 15 vs 2 | 5 vs 4 | 0.137, chi-squared test |

CsA. De novo ALL patients with an important decrease of *MDR1* expression (group A) had over twofold lower Rh123 retention in the presence of CsA on day 3 than those in group B (relative retention 5.1 vs 11.1, $p=0.011$; Fig. 2). These differences were also observed at day 0; however, it

did not reach statistical significance (relative retention 33.7 vs 45.6, $p=0.109$). Thus, the PGP function after in vitro 3-day therapy with prednisolone was blocked by CsA stronger in group B than in group A, indicating lower PGP activity in patients from group A.

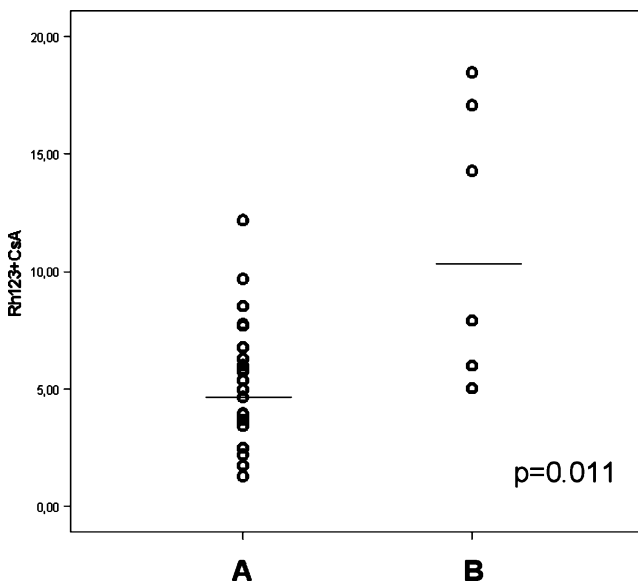


Fig. 2 Rhodamine (Rh123) retention in the presence of cyclosporine A (CsA) on day 3 in de novo ALL patients: with radical decrease (group A) and unimportant change (group B) of *MDR1* relative expression. The lines indicate median values of retention (given in flow cytometry arbitrary units [AU])

In vitro cytotoxicity of prednisolone

The in vitro drug resistance to prednisolone in relapsed ALL was higher than in de novo ALL patients. Median in vitro cytotoxic concentration (LC50) of prednisolone in relapsed ALL was 160.8 $\mu\text{g/ml}$ (range: 10.3–219.8), while in de novo patients it was 3.9 $\mu\text{g/ml}$ (range 0.084 to >250), $p=0.006$ (Mann–Whitney *U* test). No correlation between in vitro prednisolone cytotoxicity and *MDR1* gene or PGP protein expression was observed.

Probability of disease-free survival

De novo ALL patients from group A with high decrease of *MDR1* expression on day 3 had better probability of disease-free survival than group B patients ($\text{pDFS}=0.900\pm0.095$ vs 0.545 ± 0.150 , $p=0.005$, log-rank test). Median disease-free survival was 37.6 months (95%CI=33.1–42.1 months) for patients from group A, while for patients from group B it was 15.5 months (95%CI=8.7–22.3 months).

Out of seven relapsed ALL patients, three showed high decrease of the *MDR1* gene expression on day 3 and four unimportant changes of the expression. Two in the former

subgroup and none in the latter subgroup remained disease free during follow-up.

Discussion

The expression level of the *MDR1* gene in childhood ALL remains an object of many studies using various models and methods. Some authors did not detect expression of the gene in leukemic cells [15], while others found low level of *MDR1* expression in majority of analyzed cases [1, 12, 16, 17]. Our findings related to *MDR1* expression before therapy of ALL are in concordance with the latter results. In the group of 29 patients with de novo ALL, 66% showed low relative expression of *MDR1* versus reference gene and 31% very low, estimated by $2^{-\Delta\Delta Ct}$ value. Only one patient disclosed high *MDR1* relative expression. On the other hand, among relapsed ALL patients ($n=7$), the low relative *MDR1* expression was shown in most (six of seven, i.e., 86%) patients, and only in one child was it very low. These results confirmed the observations of other authors that in young patients with relapsed ALL expression of the *MDR1* gene is more often detectable and in some patients it is higher than at diagnosis of ALL [1, 12, 18, 19].

Moreover, our study showed that in the group of relapsed ALL, an important (over 50%) relative decrease of *MDR1* expression after short-term in vitro treatment of leukemic cells with prednisolone occurred in lower number of patients (43%) than in de novo ALL (62%). This possibly might be one of the factors contributing to drug resistance in relapsed ALL patients.

PGP expression was low in most ALL patients. In most cases, it has decreased after 72 h of ex vivo therapy with prednisolone. In the same period of time, Rh123 retention has also decreased. In the presence of cyclosporine A, Rh123 retention was higher both before and after prednisolone therapy. An increase of Rh123 retention in the presence of cyclosporine A, in spite of low PGP expression, might be caused by a relatively good activity of PGP—inhibited by cyclosporine A or presence of other proteins with similar properties or non-specific accumulation. Three days of culture with prednisolone could cause induction of PGP activity. A decrease of basic Rh123 retention after 3 days of prednisolone ex vivo treatment might also suggest an induction of other proteins responsible for efflux of xenobiotics. These changes in Rh123 retention indicate that multidrug resistance proteins might show good activity, with their expression being low. On the other hand, dying cells gather more Rh123, without its efflux.

Relapsed pediatric ALL is obviously more resistant to therapy than de novo ALL. This is caused by emerging development of mechanisms of cellular drug resistance,

including cytogenetic changes in leukemic cells, upregulation of multidrug resistance proteins, increased drug efflux, changes in topoisomerase II structure, increased activity of glutathione transferases, resistance to apoptosis, and specific mechanisms related to resistance to single drugs [20, 21]. Overexpression of transmembrane drug transporters, such as PGP, might have an important relevance in ALL, especially in coexistence of other mechanisms of drug resistance. This is more likely to be seen in adults [22, 23] than in children with ALL [24, 25]. It is regarded that PGP expression and function might have prognostic relevance in T-lineage ALL [24] and probably in relapsed ALL patients [23].

Umeda et al. [26] showed that long-term medication for benign chronic disorders by various non-cytotoxic drugs (such as non-steroid anti-inflammatory drugs, antiulcer agents, antituberculosis agents, herb medicines, new quinolones, hemorrhoidal suppositories, laxatives, progestagens, coronary vasodilators, prednisolone, or protease inhibitors) before primary chemotherapy for acute leukemia increased significantly the expression of PGP in peripheral blood leukemic blasts of patients with leukemia and this increased expression was associated with a poorer remission rate. This may be one of the causes of higher PGP expression in relapsed patients and relative refractoriness to therapy [26].

Out of 29 de novo ALL patients, 18 had decrease of the *MDR1* gene expression of at least 50% on days 3 versus 0, determined by relative $2^{-\Delta\Delta Ct}$ value. We have shown that pDFS was significantly higher in this group of patients. The other newly diagnosed 11 patients had changes in *MDR1* expression, after 3 days of ex vivo therapy with prednisolone, of lower than 50%, either increase or decrease. That group of patients had significantly worse pDFS.

Since this is a small study, appropriate validation strategies should be taken into account. The results should be confirmed in a larger study with a longer follow-up.

In summary, we have shown that short-term ex vivo therapy of lymphoblasts with prednisolone induces changes in the expression of *MDR1* gene, as tested by real-time PCR technique and calculated by $2^{-\Delta\Delta Ct}$ relative quantification method. With this respect, changes in *MDR1* gene expression during short-term ex vivo therapy with prednisolone may have prognostic value in pediatric de novo ALL patients, while other mechanisms contribute to prednisolone resistance and worse response to combined anticancer chemotherapy in relapsed patients.

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Conflicts of interest All authors declare no conflicts of interest relevant to this study.

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