Polymorphisms in Multidrug Resistance–Associated Protein Gene 4 is Associated with Outcome in Childhood Acute Lymphoblastic Leukemia

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Abstract

Methotrexate (MTX) and 6-mercaptopurine, important components of acute lymphoblastic leukemia (ALL) treatment, are substrates for multidrug resistance associated protein MRP4. Eight single nucleotide polymorphisms (SNP) were analyzed in MRP4 gene and four variants were identified as tagSNPs with frequency ≥ 5%. They were investigated for association with treatment responses in 275 children with ALL. The TC genotype of the regulatory T-1393C polymorphism was associated with better event free survival (EFS, p=0.02) and lower MTX plasma levels (p=0.01). The CA genotype of A934C (Lys304Asn) substitution correlated in contrast with lower EFS (p=0.02) and higher frequency of high-grade thrombocytopenia (p=0.01). Gene reporter assay showed that the promoter haplotype uniquely tagged by C-1393 allele conferred higher promoter activity in comparison to remaining haplotypes (p<0.0001). Further analyses are needed to replicate this pilot study and get closer insight into the functional effect of these polymorphisms.
Introduction

The treatment of pediatric acute lymphoblastic leukemia (ALL) has greatly improved due to the introduction of effective combination risk-adapted therapies.\textsuperscript{1,2} Nevertheless, therapy resistance in a significant number of children is still a major obstacle to successful treatment whereas intensive treatment has also important side effects. Pharmacogenetic studies identified certain genetic variations that may contribute to variability in ALL treatment responses.\textsuperscript{1,2} The drug effects depend, among other factors, on the activity and expression of multidrug resistance-related proteins (MRPs).\textsuperscript{3} MRP4 has a remarkable ability to transport a range of drugs and physiological substrates.\textsuperscript{4} MRP4 is ubiquitously expressed including high expression in the hematopoietic cells thus possibly affecting both drug intended and drug side effects in these cells.\textsuperscript{5} MRP4 affects disposition of physiological folates, but also of methotrexate (MTX) and 6-mercaptopurine (6-MP), which are key components of ALL treatment.\textsuperscript{4,6-8} MRP4 protects cells against thiopurine-induced toxicity by actively exporting thioguanine nucleotides (TGN)\textsuperscript{6}, whereas MRP4 transfected cells displayed increased resistance to MTX\textsuperscript{4,7}. Wide variation in MRP4 expression has been reported in pediatric leukemia lymphoblasts.\textsuperscript{9} This can be in part due to functional genetic polymorphisms. Here we report the analysis of MRP4 gene variations.

Patients and methods

The children enrolled in the study are 275 Caucasians diagnosed for ALL at Hospital Sainte-Justine (HSJ), Montreal, Canada, between January 1989 and December 2003. The patients underwent treatment with Dana-Farber Cancer Institute ALL Consortium protocols DFCI 87-01, 91-01, 95-01 or 2000-01.\textsuperscript{10} Patient samples were obtained after informed consent was given, in accordance with the Declaration of Helsinki, and the Ethics Committee of CHU Sainte-Justine approved the study protocol. Eight MRP4 polymorphisms in regulatory and
coding gene regions were selected from National Center for Biotechnology Information (NCBI) database\textsuperscript{11}. All selected polymorphisms were genotyped in 49 controls to estimate allele and haplotype frequency (Figure S1). Selected tagSNPs (single nucleotide polymorphism sufficient to define common haplotypes) with frequency $\geq 5\%$ (T-1393C, C-1015T, C934A and A4131C) were subsequently genotyped in 275 ALL patients. Genotyping details are given in Table S1. Survival differences for patients with different genotypes were estimated by Kaplan-Meier analysis. The hazard ratio (HR with a 95\% CI) for MRP4 variants was estimated by the Cox regression analysis, with and without inclusion of the common prognostic factors or other genetic variants (dihydrofolate reductase, DHFR, thymidylate synthase, TS, reduced folate carrier, RFC1, methylene-tetrahydrofolate reductase MTHFR, cyclin D1, CCND1 and glutathione S transferase M1, GSTM1) shown by us and others to influence the risk of relapse\textsuperscript{10,12-19} (Table S2). Toxicity on bone marrow and liver function (the appearance of toxicity and toxicity rates) was based, as previously described\textsuperscript{20}, on the results of weekly laboratory tests collected in 174 patients during consolidation and maintenance treatment. MTX plasma levels at both 36h and 48h following high dose (HD)-MTX were available for 197 patients; 267 patients had measured or conclusive MTX levels at 72h post HD-MTX. All analyses were performed by SPSS statistical package (Chicago, IL) version 13.0.

For the gene reporter experiments, haplotype-specific fragments corresponding to proximal promoter of MRP4 were amplified from genomic DNA of individuals with known genotype, and subcloned into the promoterless pGL3-basic firefly luciferase reporter vector (Promega) as previously described\textsuperscript{10}. Expression data for lymphoblastoid cells lines were taken from gene expression omnibus at NCBI\textsuperscript{21}. 
Results and discussion

Association of MRP4 genotypes with event free survival (EFS) showed that individuals with the TC-1393 genotype had better EFS and those with the CA934 genotype had reduced EFS in comparison to remaining genotypes (p=0.02, Figure 1A). When two polymorphisms were combined, three genotype groups could be distinguished with EFS decreasing from 95% to 80% and 62% (Figure 1A). Significant associations with EFS were retained in the Cox regression, when other prognostic factors or genotypes previously shown to affect EFS were included into the models (Table S2). Among event-predisposing genotypes, MRP4 CA934, DFHR haplotype *1 and TS 3R homozygosity retained significance (p ≤ 0.05). DHFR and TS, in accordance with our previous observation, result in more important EFS reduction when acting together; MRP4 leads to equally important reduction individually and combined with other genotypes (Figure S1).

Increase in the frequency of thrombocytopenia grade 3/4 was seen for individuals with MRP4 CA934 genotype (p=0.01, Figure 2A), whereas differences in MTX plasma levels were observed for MRP4 -1393 variation, being lowest in patients with TC genotype (p=0.01 for MTX retention at 72 hours post HD-MTX, Figure 2B and p=0.006 for 36h and 48h post HD-MTX levels, Figure 2C).

In this study the A allele of C934A (Lys304Asn) correlated with reduced EFS and higher incidence of high-grade thrombocytopenia. The functional impact of this polymorphism has not been clearly shown. There was no difference in protein expression in liver or in accumulation of 6-MP and antiviral agents in kidney cells in relation to the MRP4 genotypes. Gradhand et al., in contrast, showed reduction in MRP4 expression in liver samples associated with A934 allele.
Owing to the small number of patients the significance of this finding was not conclusive. We also noticed non-significant reduction in mRNA expression in lymphoblastoid cell lines (p=0.2, Figure 1B). One of possible explanation for our finding is that higher frequency of toxicity presumably associated with lower MRP4 expression would lead to more frequent drug withdrawal or dose reduction which might cause higher frequency of relapse. Indeed, relapsed ALL patients do not have higher MRP4 expression, contrasting other MRPs whose higher levels correlated with a relapse. MRP4 was also expressed at a lower level in T-cell ALL having higher resistance to treatment than B-cell ALL. The other mechanisms are likely involved as well; down-regulation of MRPs might result in decreased folate efflux thereby leading to expansion of the intracellular folate pool and antifolate resistace.

Carriers of C-1393 allele that uniquely tags promoter haplotype *C was associated with better EFS and with lower MTX plasma levels following high drug dose. Gene reporter assay showed two-fold higher promoter activity for haplotype *C, as compared to remaining haplotypes (p≤0.0001, Figure 1B). No association with basal mRNA expression in lymphoblastoid cell lines was found (Figure 1B). Several reasons might account for this discrepancy. The difference seen in vitro does not necessarily reflect an expression in vivo which is further influenced by variety of genetic and non-genetic factors. The difference in expression might exist between different cell types. MRP4 is expressed in various tissues including kidney and has affinity for many substrates. MTX levels assessed in this study likely reflect renal MRP4 transport and not the efflux from lymphocytes given that the renal excretion is the main MTX elimination route. Higher MRP4 activity in kidney associated with C-1393 allele could then explain lower MTX levels and absence of toxicity associated with this allele. MRP4 was also shown to protect cells against 6-MP hematopoietic toxicity. It would be thus important to
correlate these polymorphisms with 6-MP/TGN levels, which was not possible in this study due to the lack of 6-MP/TGN data.

Several additional non-synonymous polymorphisms were identified in MRP gene. Few of them were found to influence protein expression or drug transport. Given their low frequency (1-2%) they were not here analyzed.

In conclusion, we provided additional insight into the possible genetic modulation of treatment responses in childhood ALL. Further functional analysis and replication in independent cohort are needed to support the validity of this pilot study.

Acknowledgements

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Authorship

Contribution: MA, GS, ML, VG performed experiments; MA, CL, AM contributed to the retrieval of clinical data; ML retrieved the public data on MRP4 expression; MA, VG and MK performed the analysis, MK designed the research; MA and MK drafted the article; all authors contributed to the interpretation of data and revised the manuscript critically.

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References:


FIGURE LEGENDS

Figure 1. The impact of MRP polymorphisms on ALL outcome and transcription

A. EFS curves for TC-1393, CA934 and combined MRP genotypes. Three groups are distinguished: group 1, with TC-1393 and CC or AC934 genotypes, group 2, with TT-1393 and CC934 genotypes, and group 3, with TT-1393 and AC934 genotypes.

The genotype and the number of patients in each curve, numbers of individuals with an event (in the parenthesis), as well as the $p$ value, estimated by log-rank test for the survival differences between the patients groups, are indicated on each plot. Times to event were measured as the time between diagnosis and the event of interest; for censored cases, it corresponded to time between diagnosis and the study end-point (5-year post treatment, i.e. 84 months after diagnosis) or to the last observational period. Risk of event associated with the given genotype, expressed as univariable hazard ratio (HR) with 95% confidence interval (CI) estimated by Cox regression analysis, is indicated bellow plots.

Similar results as those shown for EFS were obtained for disease free survival, DFS ($p \leq 0.02$). A similar, but not significant trend was observed for OS for individuals with TC-1393 genotype ($p=0.07$), whereas there was no association for AC934 genotype ($p=0.4$)

B. Relative promoter activity and mRNA levels in lymphoblastoid cell lines in relation to MRP promoter haplotypes.

Relative promoter activity (mean ± standard deviation) obtained by luciferase reporter assay for MRP haplotypes *A, *B and *C are represented by dark gray, white and black bars, respectively. Empty vector is represented by light gray bar. The values are given for three different cell lines, Human placental Jeg-3, cervical cancer HeLa and hepatoma HepG2
(American Type Culture Collection, ATCC). The difference in promoter activity obtained by Student-t test (*) between haplotype *C and remaining haplotypes (*A or *B) is indicated on the plot. MRP expression derived from wide-genome expression dataset GSE1726 is presented. The mean value of expression with 95% CI, the number of individuals represented by each line and p value obtained by ANOVA for the difference of expression between indicated genotypes, taken from HapMap data are given on the plots.

**Figure 2. Hematological toxicity and MTX levels according to MRP genotypes**

**A. Relationship between MRP polymorphisms and frequency of high grade thrombocytopenia.** The presence (black bar) and absence (white bar) of at least one episode of thrombocytopenia grade 3 and/or 4 in individuals with different MRP genotype groups (upper panel) or genotypes of CA934 polymorphism (lower panel). P values for the difference between genotypes are obtained by chi-square (p1) and by Kruskal-Wallis or Mann-Whitney test (p2) for the difference in toxicity rates. Toxicity was graded using the common criteria for adverse events of National Cancer Institute. The mean number of weeks assessed per patient was 80.

**B. Relationship between MRP polymorphisms and retention of MTX in plasma at concentration higher than 1μM.** Concentration >1μM 72h following HD-MTX are indicated by black bars and concentrations ≤ 1μM are indicated by white bars in individuals with different MRP genotype groups (upper panel) or genotypes of TC-1393 polymorphism (lower panel). MTX plasma levels were measured by fluorescence polarization immunoassay (TDx Abbott Laboratories, Chicago, IL) following manufacturer instruction. P value for the difference between genotypes is obtained by chi square.
C. **Relationship between MRP polymorphisms and MTX plasma levels.** Lines represent log values of MTX levels (μM, measured at two time points following HD-MTX) in individuals with different MRP genotype (upper panel) or genotypes of TC-1393 polymorphism (lower panel). P values are obtained by repeated measures ANOVA.

In A and B, genotypes are indicated on X axis. The number of individuals represented by white and black bars is indicated above and below each plot, respectively. In C, genotype groups, genotypes and number of individuals represented by each line are indicated on the plots.

Given that the data on toxicity were available for 174 patients only, we analyzed an association between MRP4 polymorphisms and EFS in this subgroup. The same trend was observed (p=0.1 for TC-1393 and AC934 genotype, and p=0.02 for genotype groups).
Figure 1

A

<table>
<thead>
<tr>
<th>Gene</th>
<th>TC</th>
<th>CC</th>
<th>AC</th>
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<td>HR</td>
<td>0.1; 95% CI: 0.02-0.9</td>
<td>2.2; 95% CI: 1.1-4.1</td>
<td>2.7; 95% CI: 1.6-4.6</td>
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</table>

Combined genotypes

1. TC - 1393/CC or AC<sub>934</sub>
2. TT<sub>-1393</sub>CC<sub>934</sub>
3. TT<sub>-1393</sub>AC<sub>934</sub>

B

Gene reporter assay

Lymphoblastoid cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
<th>CA</th>
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<tr>
<td>Log&lt;sub&gt;2&lt;/sub&gt; (MRP4 expression)</td>
<td>81</td>
<td>6</td>
<td>81</td>
<td>6</td>
</tr>
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*p = 0.0001
Figure 2

A. Frequency of thrombocytopenia grade 3/4

B. MTX retention in plasma 72h following HD-MTX

C. MTX levels (μM) (log values)

MRP4 genotype groups

AC934, CC934

MRP4 genotypes

Post HD-MTX time (h)
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