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Importance of pharmacogenetic markers in the methylenetetrahydrofolate reductase gene during methotrexate treatment in pediatric patients with acute lymphoblastic leukemia

Jelena Lazić [1,2,*], Nikola Kotur[3], Nada Krstovski[1,2], Lidija Dokmanović[1,2], Branka Zukić[3], Jelica Predojević-Samardžić[4], Maja Životić[5,2], Goran Milošević[1], Milica Đorić[2], Dragana Janić[1,2] and Sonja Pavlović[3]

[1] *Department of Hematology and Oncology, University Children's Hospital*, Belgrade, Serbia

[2] *School of Medicine, University of Belgrade*, Serbia

[3] *Laboratory for Molecular Biomedicine, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade*, Belgrade, Serbia

[4] *Children's Hospital, University Clinic of Banja Luka*, Republic of Srpska, Bosnia and Hercegovina

[5] *Institute of Pathology, School of Medicine, University of Belgrade*, Serbia

***Corresponding author**: lazic.jelena@gmail.com

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Abstract: Despite remarkable progress in survival of children with acute lymphoblastic leukemia (ALL) which has reached about 85%, early toxicity and relapse rate remain issues that need to to be resolved. Genetic variants are important factors influencing the metabolism of cytotoxic drugs in ALL treatment. Variants in genes coding for methotrexate (MTX)-metabolizing enzymes are under constant scientific interest due to their potential impact on drug toxicity and relapse rate. We investigated methylenetetrahydrofolate reductase (*MTHFR)* c.677C>T and *MTHFR* c.1298A>C variants as pharmacogenetic markers of MTX toxicity and predictors of relapse. The study enrolled 161 children with ALL, treated according to the current International Berlin-Frankfurt-Munster group (BFM) for diagnostics and treatment of leukemia and lymphoma protocols. Genotyping was performed using PCR-RFLP and allele-specific PCR assays. Our results revealed similar distributions of *MTHFR* c.677C>T and *MTHFR* c.1298A>C genotypes among 104 healthy individuals as compared to pediatric ALL patients. A lower incidence of early MTX toxicity was noted in the *MTHFR* c.677TT genotype (p=0.017), while *MTHFR* c.1298A>C genotypes were not associated with MTX toxicity. Carriers of any *MTHFR* c.677C>T and *MTHFR* c.1298A>C genotypes did not experience decreased overall survival (OAS) or higher relapse rates. Genetic variants in the *MTHFR* gene are not involved in leukemogenesis in pediatric ALL. The presence of the *MTHFR* c.677TT genotype was recognized as a predictive factor for decreased MTX toxicity during the intensification phase of therapy. Neither *MTHFR* c.677C>T nor *MTHFR* c.1298A>C genotypes correlated with an increased number of toxic deaths or relapse rate. Our study emphasizes the importance of implementing pharmacogenetic markers in order to optimize pediatric ALL therapy.

Key words: pharmacogenetic markers; pediatric lymphoblastic leukemia; *MTHFR* c.677; *MTHFR* c.1298

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignant disease in childhood, with a survival rate of about 85% [1]. Improvement in survival is accomplished mainly due to more precise risk stratification based on a comprehensive detection of the most frequent genetic rearrangements and the evaluation of responses to applied therapy [2]. The major issues that still compromises survival are early toxicity and relapse rate [3]. Nowadays, single nucleotide variants (SNVs) have been shown to be among the most important factors influencing the pharmacokinetics and pharmacodynamics of cytotoxic drugs [4,5].

Methotrexate (MTX) is an antifolate chemotherapeutic agent that has been widely used in the treatment of pediatric malignancies and it is the key drug in the intensification phase in therapeutic protocols for children with ALL [6]. It is well known that high-dose MTX (HD-MTX) is associated with numerous side effects, targeting almost every system and causing myelosuppression, skin toxicity, oral mucositis (OM)

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and gastrointestinal tract (GIT), liver, renal and central nervous system (CNS) toxicities.

For many years, the pharmacokinetics and pharmacodynamics of MTX were the only reliable method for detection of MTX toxicity [7]. But the interpatient variability is so great that the individual toxic response to HD-MTX cannot be predicted from these parameters alone and the genetic variants in MTX metabolic pathway are introduced to clinical practice. [8]. MTX blocks the conversion of dihydrofolates to tetrahydrofolates, the biologically active folate cofactors, increases serum homocysteine, induces a low folate level and, by affecting the intracellular folate pool, influences the activity of the enzyme methylenetetrahydrofolate reductase (MTHFR). This enzyme catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and the carbon moiety required for the conversion of homocysteine to methionine [9].

Two common variants in the *MTHFR* gene, c.677C>T and c.1298A>C lead to 30-60% reduction in MTHFR enzyme activity [10]. Patients with the c.677C>T variant have impaired remethylation of homocysteine to methionine and subsequent hyperhomocysteinemia. Although c.1298A>C has been associated with reduced *MTHFR* activity, neither the homozygous nor heterozygous state is associated with a change in homocysteine or folate levels [11]. However, it appears that individuals heterozygous for both c.677C>T and c.1298A>C have a phenotype similar to that of c.677TT homozygotes.

Several studies have shown that patients with decreased *MTHFR* activity are at an increased risk of MTX-related toxicity. HD-MTX treatment most often causes hepatic and kidney toxicity, as well as bone-marrow suppression. A number of studies reported a higher incidence of MTX toxicity, especially GI or hepatic ones among patients with the c.677T variant, while other authors did not confirm its impact on toxicity when patients were compared according to their *MTHFR* c.677C>T genotypes [12].

MTX-associated toxicity could alter the therapy response in pediatric ALL, most commonly causing therapy interruption, and thus leads to a higher number of relapses. Previous studies have shown a higher risk of relapse in ALL patients with the *MTHFR* c.677T allele, both in children and adults [13]. On the other hand, the *MTHFR* c.1298A>C variant was not associated with either altered risks of relapse or toxicity in childhood ALL [14].

This study aimed to analyze the frequency of the *MTHFR* c.677C>T and *MTHFR* c.1298A>C genetic variants and their potential role in the prediction of MTX induced toxicity and its impact on outcome.

MATERIALS AND METHODS

Patients

The study was approved by Ethics Committee of the University Children's Hospital in Belgrade. Samples for pharmacogenetic analysis were obtained from bone marrow on diagnosis with the agreement of parents by written consent. Samples from 104 healthy individuals were obtained from blood, on a voluntary basis. The study included 161 consecutive pediatric patients with *de novo* diagnosed ALL. A significant predominance of males was observed, accounting for 59.6% (96/161) of the patient group. Age ranged from 0.9 up to 17.6, median 5.5, average 7.1 years. The biological characteristics of patients in our study group, significant for risk stratification and treatment strategy, are presented in Table 1.

Diagnosis, risk stratification and treatment modalities

Diagnostic tools, risk stratification and treatment strategy were performed according to the current International Berlin-Frankfurt-Munster (BFM) group for diagnostics and treatment of leukemia and lymphoma protocols [15]. Risk stratification includes age at diagnosis, initial leukocyte count, cytogenetic and molecular genetic markers, as well as the therapy response on three time points (days 8, 15 and 33). In the standard risk group (SR), 21.1% of the children (34/161) were stratified, in the intermediate (IR) 64.6% (104/161) and in the high-risk group (HR) 14.3% (23/161). The intensification phase of therapy was divided according to initial immunophenotype and risk group into three arms: protocol mM (intravenous administration of 2 g/m^2 of MTX), protocol M (intravenous administration of 5 g/m^2 of MTX)

Table 1. Biological characteristics of patients.

Parameters		Nº (%) of pts
IPH (performed in 161 pts)	B	134 (83.2)
	T	27 (16.8)
CNS status (performed in 161 pts)	1	148 (91.9)
	2	6 (3.7)
	3	7 (4.4)
Cytogenetics (performed in 136 pts)	No mitosis	50 (36.8)
	Normal	50 (36.8)
	Hypodiploidy	1 (0.7)
	Hyperdiploidy	18 (13.2)
	One aberration	7 (5.1)
	Complex karyotype	10 (7.4)
Molecular genetics (performed in 155 pts)	BCR/ABL	10 (6.5)
	MLL/AF4	3 (1.9)
	E2A/PBX1	7 (4.5)
	TEL/AML1	34 (27.9)
	Negative	101 (65.2)
Risk groups (estimated in 161 pts)	SR	34 (21.1)
	IR	104 (64.6)
	HR	23 (14.3)

pts – patients; IPH – immunophenotype; B – B cell precursor; T – T cell precursor; 1 – no infiltration; 2 – traumatic lumbar puncture; 3 – infiltration with leukemic cells; SR – standard risk group; IR – intermediate risk group; HR – high risk group; statistical analysis – descriptive statistics

and HR blocks (intravenous administration of 5 g/m^2 of MTX plus numerous other cytotoxic drugs). Out of 85.7% of children (138/161) in the SR and IR groups, 68.3% (110/161) fulfilled the criteria to receive intensification treatment with 2 g/m^2 of MTX (protocol mM).

Evaluation of toxicity

Evaluation of toxicity was based on the Common Toxicity Criteria (CTC) of the National Cancer Institute (NCI), including: infection, skin toxicity, OM and GIT, liver, renal and neurological toxicities, grading from 0 (no toxicity) to 4 (the most severe toxic event), and is presented in Table 2. Toxicity was estimated through clinical examination, laboratory and radiological findings.

DNA analysis

Our study investigated the pharmacogenetic influence of the *MTHFR* gene in children with ALL during protocol mM, since this was the most numerous group of patients in our cohort. DNA was extracted

Table 2. Evaluation of toxicity based on the Common Toxicity Criteria (CTC) of the National Cancer Institute (NCI).

Toxicity/Grade	0	1	2	3	4
Infection	None (< 38ºC)	Mild (38-39ºC)	Pathogen not identified, IV AB (39-40ºC)	Pathogen identified, IV AB (> 40ºC < 24h)	Septic shock (> 40 ºC > 24h)
Skin	Normal	Localized erythema	Localized erythema with excoriations	Generalized erythema with hemorrhagic excoriations	Exfoliating erythema with hemorrhagic bullas
OM	None	Painless ulceration, erythema	Painful ulceration, can eat	Painful ulceration, cannot eat	TPN
GI	None	Adequate food intake, vomiting 1, diarrhea 2-3	Decreased food intake, vomiting 2-5, diarrhea 4-6	Almost no food intake, vomiting 6-10, diarrhea 7-9	TPN
Liver	Normal	TB 1.5 x N ALT/AST 2 x N	TB 1.5-3 x N ALT/AST 2.5-5 x N	TB 3-10 x N ALT/AST 5-20 x N	TB > 10 x N ALT/AST > 20 x N
Renal	Cr normal Prt none Ht none CCr> 90	Cr 1.5 x N Prt < 3 Ht - microscopic CCr 60-89	Cr 1.5-3 x N Prt 3-10 Ht - macroscopic CCr 40-59	Cr 3-6 x N Prt >10 Ht - macroscopic CCr 20-39	Cr > 6 x N Prt - NSy Ht - TS required CCr < 19
Neuro	None	Transient lethargy, mild paresthesias, decreased reflexes	Somnolence, moderate disorientation, moderate paresthesias, weakness	Somnolence, severe disorientation, hallucinations, severe paresthesias, motor deficits	Coma, seizures, paralysis

IV AB – intravenous antibiotics; TPN – total parenteral nutrition; TB – total bilirubin; ALT – alanine aminotransferase; AST – aspartate aminotransferase; Cr – creatinine; Prt – proteinuria; Ht – hematuria; CCr – creatinine clearance; NSy – nephritic syndrome; TS – transfusion; N – normal value

Table 3. Incidence of *MTHFR* c.677 and *MTHFR* c.1298 in children with ALL and healthy population.

Genotypes		Patients		Control group		Statistical significance (p)
		Sample (N°)	Frequency (%)	Sample (N°)	Frequency (%)	
MTHFR	677					
	C/C	149	47	104	51	0.5352
	C/T		41.6		33.6	0.2005
	T/T		11.4		15.4	0.3575
	1298					
	A/A	146	48.6	104	57.7	0.1585
	A/C		39.1		32.7	0.3030
	C/C		12.3		9.6	0.5028

Statistical analysis – two proportions Z-test

from blood or bone marrow samples using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The *MTHFR* c.677C>T (rs1801133) variant was detected by PCR-RFLP [16], and *MTHFR* c.1298A>C (rs1801131) variant was detected using an allele-specific PCR assay [17]. The detection of variant *MTHFR* c.677C>T was performed in 149 ALL patients and of variant *MTHFR* c.1298A>C in 146.

Statistical analysis

SPSS software package (IBM SPSS Statistics v.20) was used for statistical analysis. The chi-square test and Fisher's exact test were used to test differences in proportions. Haplotype analysis was performed using Arlequin software (version 3.5.1.3) [18]. A difference was considered statistically significant if $p<0.05$.

Table 4. Correlation of *MTHFR* c.677 and *MTHFR* c.1298 genotypes with toxicity during mM consolidation phase.

Genotypes		mM toxicity		Σ	Statistical significance (p)
		Yes	No		
MTHFR 677	C/C	31 (64.6%)	17 (35.4%)	48 (100%)	0.017
	C/T	32 (72.7%)	12 (27.3%)	44 (100%)	
	T/T	4 (33.3%)	8 (66.7%)	12 (100%)	
	Σ	67 (64.4%)	37 (35.6%)	104 (100%)	
MTHFR 1298	A/A	28 (57.1%)	21 (42.9%)	49 (100%)	0.292
	A/C	31 (72.1%)	12 (27.9%)	43 (100%)	
	C/C	5 (55.6%)	4 (44.4%)	9 (100%)	
	Σ	64 (63.4%)	37 (36.9%)	101 (100%)	

Statistical analysis – Chi-square test

RESULTS

This is the first study investigating the influence of the pharmacogenetic variants involved in the folate metabolism pathway during MTX therapy conducted in children with ALL in Serbia. The frequency of genetic variants in *MTHFR* c.677C>T was as follows: *MTHFR* c.677CC in 47%, *MTHFR* c.677CT in 41.6% and *MTHFR* c.677TT in 11.4% of patients. A similar distribution of genotypes in *MTHFR* c.1298A>C was found: *MTHFR* c.1298AA in 48.6%, *MTHFR* c.1298AC in 39.1% and *MTHFR* c.1298CC in 12.3%. Genotype frequencies between children with ALL and healthy individuals did not show a statistically significant difference, as presented in Table 3.

During MTX therapy, the most common grade 1 toxicity was infection, then OM, GIT, liver, neuro and renal toxicities. Grade 2 toxicity was most frequently noted as GIT toxicity, then OM, infection, liver, neuro and skin toxicity. Grade 3 toxicity was seen most often as OM, infection, GIT and liver toxicity. None of the patients experienced grade 4 toxicity during protocol mM.

A lower incidence of early clinical and laboratory MTX toxicity was noted only in carriers of the *MTHFR* c.677TT genotype (p=0.017). A *MTHFR* c.1298A>C variant did not influence MTX toxicity. The correlation of *MTHFR* c.677C>T and *MTHFR* c.1298A>C genetic variants and MTX toxicity is presented in Table 4. Linkage disequilibrium between *MTHFR* c.677C>T and *MTHFR* c.1298A>C was noted (D'=0.916, r^2=0.16). The variant *MTHFR* c.677C was located on the same DNA strand as the *MTHFR* c.1298C variant, with a higher than expected

frequency. Also, *MTHFR* c.677T was tied to *MTHFR* c.1298A more often than expected. The most frequent was c.[677C;1298A] haplotype (40.2%), followed by c.[677T;1298A] (30.4%), c.[677C;1298C] (28.3%) and c.[677T;1298C] haplotype (1.1%).

In our study group, OAS was 87% (140/161), while event-free survival (EFS) was 80.6% (130/161): 15% patients relapsed (24/161), 3.1% died as a result of toxic events (5/161) and two patients developed secondary malignancy (1.3%). Out of 24 relapsed patients, 11 (45.8%) are in a second complete remission (CR). Both of the children with secondary malignancy are in second CR. One of them is a carrier of the rarest c.[677T;1298C] haplotype, who experienced OM and GI toxicity during consolidation treatment and later developed acute myeloid leukemia (AML), successfully treated with allogeneic hematopoietic stem cell transplantation (allo-HSCT). The variants *MTHFR* c.677C>T and *MTHFR* c.1298A>C did not significantly influence either OAS or EFS, nor did their haplotypes. The correlation between *MTHFR* c.677C>T and *MTHFR* c.1298A>C genetic variants and OAS is presented in Table 5 and with EFS in Table 6.

DISCUSSION

Comparison of the analyzed *MTHFR* genetic variants between pediatric ALL patients and the control group did not reveal statistical significance, in contrast to a study conducted in adult patients with leukemia [19]. Another study of *MTHFR* genotypes in the Serbian population, conducted in patients with thrombophilia, also did not reveal a significant difference in genetic variants when affected and healthy individuals were compared. [20].

Further detailed analysis of the literature showed that *MTHFR* genetic variants possibly modulate the risk of ALL in adults, but not in children, as shown in our study [21]. However, a previous study investigating the leukemogenetic effect of the *MTHFR* gene in Serbian pediatric patients revealed a significant association between CT/TT genotypes and reduced risk of ALL [22]. Therefore, the role of variants in the *MTHFR* gene in susceptibility for ALL development in children needs further investigation in larger cohorts of patients.

During the intensification phase of therapy, among SR and IR patients who received 2 g/m^2 of

Table 5. Correlation of *MTHFR* c.677 and *MTHFR c.*1298 genotypes with OAS.

Genotypes		Outcome		Σ	Statistical significance (p)
		CR	Death		
MTHFR 677	C/C	61 (87.1%)	9 (12.9%)	70 (100%)	0.135
	C/T	57 (93.4%)	4 (6.6%)	61 (100%)	
	T/T	13 (76.5%)	4 (23.5%)	17 (100%)	
	Σ	131 (88.5%)	17 (11.5%)	148 (100%)	
MTHFR 1298	A/A	62 (87.3%)	9 (12.7%)	71 (100%)	0.211
	A/C	52 (92.9%)	4 (7.1%)	56 (100%)	
	C/C	14 (77.8%)	4 (22.2%)	18 (100%)	
	Σ	128 (88.3%)	17 (11.7%)	145 (100%)	

CR – complete remission (disease free); statistical analysis – Chi-square test

Table 6. Correlation of *MTHFR* c.677 and *MTHFR* c.1298 genotypes with EFS

Genotypes		Outcome				Σ	Statistical significance (p)
		CR	Relapse – second CR	Relapse - death	Toxic death		
MTHFR 677	C/C	56 (80%)	5 (7.1%)	8 (11.4%)	1 (1.4%)	70 (100%)	0.312
	C/T	53 (86.9%)	4 (6.6%)	3 (4.9%)	1 (1.6%)	61 (100%)	
	T/T	10 (58.8%)	3 (17.6%)	2 (11.8%)	2 (11.8%)	17 (100%)	
	Σ	119 (80.4%)	12 (8.1%)	13 (8.8%)	4 (2.7%)	148 (100%)	
MTHFR 1298	A/A	57 (80.3%)	5 (7%)	6 (7.5%)	3 (4.2%)	71 (100%)	0.519
	A/C	47 (83.9%)	5 (8.9%)	3 (5.4%)	1 (1.8%)	56 (100%)	
	C/C	12 (66.7%)	2 (11.1%)	4 (22.2%)	0 (0%)	18 (100%)	
	Σ	116 (80%)	12 (8.3%)	13 (8.9%)	4 (2.8%)	145 (100%)	

CR – complete remission (disease free); statistical analysis – Kruskal-Wallis test

MTX, none of the 110 children developed grade 4 toxicity, either clinical or laboratory, regardless their *MTHFR* genetic variants. Toxicities grading from 1 to 3 covered the usual side effects of MTX administration, as is frequently reported in the literature [23].

Carriers of the *MTHFR* c.677TT genotype had a lower incidence of early MTX toxicity, making the presence of this genetic variant more favorable in our group of patients. Lower MTHFR enzyme activity caused by the homozygous *MTHFR* c.677T variant leads to a reduced conversion rate of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. A higher level of 5,10-methylenetetrahydrofolat might facilitate *de novo* purine and thymidylate synthesis, which might decrease the toxic effect of MTX [24].

Considering the prediction of toxicity in children with *MTHFR* c.677TT genotype, the literature offers controversial reports. There are several studies providing results supporting the link between the *MTHFR* c.677TT genotype and increased risk of MTX [25-27]. However, Lopez-Lopez [12] questioned one of the most cited studies by D'Angelo et al. from 2011 [28] by recalculating their published results, and showed that *MTHFR* c.677TT is associated with decreased toxicity in patients receiving HD-MTX. These results are in concordance with the reports of other authors that do not find a correlation between the MTHFR c.677T variant and toxicity [29-31], or even found a small protective effect for this polymorphism [32, 33], like we have presented. Even further, a meta-analysis [12] did not indicate *MTHFR* genetic variants to be reliable markers for predictability of MTX toxicity.

In the study of Yang et al. [26], where the *MTHFR* c.677TT genotype was pointed out as an unfavorable genetic marker, patients with the *MTHFR* c.1298A>C variant showed decreased risk of MTX toxicity, while in our study group *MTHFR* c.1298A>C did not influence MTX toxicity. Similar results to ours were published by D'Angelo et al. [28], who also did not find a correlation between *MTHFR* c.1298A>C genetic variant and MTX toxicity.

In order to explain such opposing literature reports, we further analyzed the presence of different haplotypes and discovered that only three children out of 161 carried the rarest c.[677T;1298C] haplotype. Only one of them was among the children who received consolidation treatment with 2 g/m^2 of MTX and experienced OM and GI toxicity. We suspect that haplotype analysis, as well as gene-gene interactions, might explain the controversial results regarding the influence of MTHFR status in MTX toxicity [34].

The OAS and EFS in our study group is in compliance with literature reports, with 87% and 80.6%, respectively [35]. Our research did not reveal an impact of *MTHFR* c.677C>T and *MTHFR* c.1298A>C genetic variants on OAS or EFS. The same results were reached in a study with adult ALL patients [36]. Literature data point at the *MTHFR* c.677TT genotype as a cause of increased rate of relapse [37], but there are also studies supporting results similar to ours in children [38]. As for the assessment of toxicity, a probable reason for the variable conclusions on the impact of the investigated genotypes on OAS and EFS could be in unevenly detected unfavorable haplotypes. The only child with c.[677T;1298C] haplotype within the subgroup of patients receiving 2 g/m^2 of MTX developed secondary malignancy (AML), successfully achieved second remission after second-line chemotherapy and underwent allo-HSCT from a matched family donor.

The results presented herein emphasize the importance of the implementation of pharmacogenetic markers in current protocols in order to optimize pediatric ALL therapy.

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Conflict of interest disclosure: We confirm that this is an original manuscript that has not been previously published, neither in part nor whole, in any other scientific journal. We declare that there is no conflict of interest.

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