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ORIGINAL ARTICLE

Influence of variants in folate metabolism genes on 6-mercaptopurine induced toxicity during treatment for childhood acute lymphocytic leukemia

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Summary

Purpose: *To analyze influence of variants in TYMS, MTHFR, SLC19A1 and DHFR genes on 6-mercaptopurine (MP) induced toxicity during maintenance phase of treatment for childhood acute lymphocytic leukemia (ALL).*

Methods: *One-hundred twenty-seven children with ALL that received maintenance therapy were involved in this study. All patients were treated according to Berlin-Frankfurt-Muenster (BFM) based protocols. Myelotoxicity and hepatotoxicity were evaluated using surrogate markers (median 6-MP dose, number of leukopenic episodes and levels of bilirubin and transaminases on each visit).*

Results: *Higher number of leukopenic episodes, as a surrogate marker of 6-MP myelotoxicity, was found in carriers of TYMS 3R3R and 3R4R genotypes (p=0.067) as well as in TYMS 3R6bp+ (28bp VNTR, 6bp indel) haplotype carriers (p=0.015). Carriers of DHFR CATAG (-680, -675, -556, -464, -317) haplotype were also found to have higher number of leukopenic episodes (p=0.070). SLC19A1 c.80A allele (p=0.079) and TYMS 2R6bp+ (5'UTR VNTR, 6bp indel) haplotype carriers (p=0.078) had fewer leukopenic episodes. No difference in genotype frequencies between the control group of volunteered blood donors and childhood ALL patients was found.*

Conclusions: *Variants in TYMS, SLC19A1 and DHFR genes are potential biomarkers of myelotoxicity and could be used for 6-MP therapy individualization in maintenance phase of childhood ALL treatment, alongside with well-established TPMT variants.*

Key words: *6-mercaptopurine myelotoxicity, acute lymphoblastic leukemia, DHFR, MTHFR, SLC19A1, TYMS*

Introduction

Childhood acute lymphoblastic leukemia (ALL) has become highly curable disease in the last few decades. Overall survival is now reported to be up to 90% in most of the developed countries [1]. Substantial increase of survival in childhood ALL was made possible by better knowledge about disease genetic make-up, risk-directed multiagent chemotherapy and improved supportive measures [2]. The maintenance phase of ALL treatment is the longest one in which we aim to minimize the possibility of disease relapse [3]. Drugs of choice for this phase of treatment are oral 6-mercaptopurine (6-MP) and methotrexate (MTX) and they are used for its entire duration [3]. Toxicities that can arise in such long period of time can be serious and troublesome, and a number of researches and studies have been undertaken to assess the options for minimizing 6-MP and MTX toxicities [3]. 6-MP exerts its effects primarily on hematopoietic tissue because the detoxification mechanism through oxidation

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is less active in this tissue, so myelotoxicity is primary concern when 6-MP is applied. Hepatotoxicity is also a concern when higher doses of 6-MP are needed to maintain desired level of myelosupression. MTX toxicity often manifest itself throughout the gastrointestinal tract.

Understanding inter-individual genetic differences in genes responsible for pharmacokinetics and pharmacodynamics of used drugs and its potential effect on drug-induced toxicities and/or therapy resistance is the primary aim of pharmacogenomics [4]. The best known example of pharmacogenomic application in pediatric ALL is the discovery of thiopurine S-methyltransferase (*TPMT*) influence on 6-MP-induced myelotoxicity [5]. However, even when doses of 6-MP are adjusted according to individual genetic profile for *TMPT*, there is still a number of patients that develop severe myelosuppression. New studies have identified other genes that have important role in 6-MP metabolism. Carriers of single nucleotide polymorphisms (SNPs) in Nudix hydrolase 15 (*NUDT15*) and inosine triphosphate pyrophosphatase (*ITPA*) genes are also prone to increased myelosuppression while on therapy with 6-MP, but those SNPas are much more frequent in East Asian than in Caucasian populations [6-8]. Protein kinase C and casein kinase substrate in neurons protein 2 (*PACSIN2*) gene variants could also contribute to myelotoxicity and gastrointestinal toxicity in childhood ALL patients [9,10].

Since maintenance phase consists of at least one more drug, a folate antagonist MTX, genes involved in the metabolism and transport of folate and MTX are significant for pharmacogenomics of this therapy phase. Polymorphisms in thymidylate synthase (*TYMS*), dihydrofolate reductase (*DHFR*), methylenetetrahydrofolate reductase (*MTHFR*) and Solute carrier family 19 (folate transporter), member 1 (*SLC19A1*) pharmacogenes that encode key enzymes and a transporter of folate pathway were proposed as biomarkers of MTX-induced toxicities [3]. For some of those genetic markers that are known to influence folate and MTX metabolism, it was shown that they can also influence 6-MP metabolism in an indirect manner [11,12]. For instance, *MTHFR* gene variant that is responsible for reduced *MTHFR* enzyme activity, reduces the synthesis of S-adenosil methionine (SAM) which is co-enzyme in *TPMT* inactivation of 6-MP. In this way, carriers of *MTHFR* variant allele could have reduced *TPMT* activity, therefore could be in greater risk for increased toxicity [13].

In this paper we aimed to investigate the possible influence of variants in *TYMS*, *MTHFR*, *SLC19A1* and *DHFR* genes on 6-MP/MTX-induced myelotoxicity and hepatotoxicity during the maintenance phase of treatment for childhood ALL.

Methods

Patients that were diagnosed and treated for childhood ALL at our institution were recruited into study from 2003 to 2013. Patients have been treated according to BFM based protocol for ALL (ALL IC-BFM 2002 or ALL IC-BFM 2009). A total number of 127 patients that had received maintenance therapy in this period was enrolled in this trial. The control group consisted of 104 volunteered blood donors. Written informed consent was obtained from each patient, from parents or legal guardians. The trial was conducted after appropriate approval was obtained from Ethics Committee of our institution.

According to abovementioned protocols, maintenance phase consists of daily oral 6-MP in a dose of 50 mg/m^2, and once a week oral MTX in a dose of 20 mg/m^2. The maintenance phase lasts between 13 and 17 months. During the entire maintenance phase, regular controls of patients' complete blood count were performed every 2 weeks and biochemistry, including transaminases and bilirubin levels, once a month. Targeted white blood cell (WBC) count while on therapy was set to be between 2.0 and 3.0×10^9/L. In case of WBC being below 2.0×10^9/L, the dose of 6-MP and/or MTX was to be reduced by 25% every week. However, if the WBC count was above 3.0×10^9/L, the dose of 6-MP and/or MTX was increased by 25% each week. If the leukopenia was severe (WBC count below 1.0×10^9/L), therapy was omitted until WBC

Table 1. Patient characteristics (n=127)

Characteristics	
Age, years	
Average	6.8
Median	5.2
Range	0.9-17.6
Characteristics	*n (%)*
Gender	
Male	76 (59.8)
Female	51 (40.2)
Immunophenotype	
B lineage	109 (85.8)
T lineage	18 (14.2)
Risk group	
Standard risk	29 (22.8)
Intermediate risk	84 (66.1)
High risk	14 (11.0)
Outcome	
CR	117 (92.1)
Relapse	8 (6.3)
LFU	2 (1.6)

CR: complete remission, LFU: lost to follow-up

rose above 2.0×10^9/L. For patients who carried *TPMT*3* variants, the starting 6-MP dose was reduced and in case of myelotoxicity, 6-MP dose was adjusted more readily than MTX dose.

All toxicities were recorded on each visit using The NCI Common Terminology Criteria for Adverse Events (CTCAE) v5.0 [14]. For each patient the following data were recorded: WBC count, transaminase and bilirubin levels, average 6-MP dose and occurrence of leukopenic episodes (periods when WBC count remains below 2.0×10^9/L). Average 6-MP dose was calculated for each patient by taking into account cumulative 6-MP dosage and the duration of maintenance therapy. For each group of ALL patients, median 6-MP dose was calculated by taking into account the average doses of 6-MP calculated for each patient. Median dose of 6-MP and number of leukopenic episodes were used as a surrogate marker of myelotoxicity, whereas levels of bilirubin and transaminases were markers of hepatotoxicity.

Demographic and clinical characteristics of ALL patients

The age of patients ranged from 0.9 to 17.6 years (median 5.2), and there were 59.8% boys. Precursor B leukemia was represented with 85.8% cases and the remaining patients had precursor T leukemia. Standard, intermediate and high risk patients were represented with 22.8%, 66.1% and 11%, respectively. Patients' basic demographic and clinical characteristics are presented in Table 1.

Detection of genetic variants

DNA of ALL patients was extracted from peripheral blood or blood smears using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Variants c.460G>A (rs1800460) and c.719A>G (rs1142345) in *TPMT* gene were detected using PCR-RFLP methodology as described previously [15]. Detection of these two variants was used to determine the presence of *TPMT*3A*, *TPMT*3B* and *TPMT*3C* alleles, which were referred to collectively as *TPMT*3* allele. Variable number of 28bp long tandem repeats in 5'UTR (rs34743033) and of 6bp long indel in 3'UTR region (rs34489327) of *TYMS* gene were analyzed using PCR amplification followed by agarose or polyacrylamide gel electrophoresis as previously described [16]. *MTHFR* c.677C>T (rs1801133) and c.1298A>C (rs1801131) variants were detected using PCR-RFLP [17] and allele-specific PCR [18], respectively. *SLC19A1* c.80G>A (rs1051266) variant was detected using allele-specific PCR [18]. Promoter region of *DHFR* gene was amplified using forward CGAAAGGAACAAGATTTTGAAGCACCC and reverse TCCTGACTCCCATTCTGATGAGGG primer and subsequently sequenced. In this region, 5 variants could be detected: -680C>A (rs442767), -675A>G (rs1643641), -556T>C (rs1650695), -464A>T (rs1650696) and -317A>G (rs408626).

Statistics

Statistical analyses were performed using IBM SPSS v.21 software. Hardy-Weinberg equilibrium for all analyzed variants was assessed using exact test [19]. For each patient, haplotype phase was estimated using EML algorithm implemented in Arlequin software [20]. Surrogate markers of maintenance response were analyzed in relation with genetic variants using dominant genetic model. Also, for each haplotype with estimated frequency above 2%, association with surrogate markers of maintenance response were analyzed (carriers of a specific haplotype were compared to all other patients who carried other haplotypes). The association of age and gender with surrogate markers of therapy toxicity were established using univariate logistic or linear regression. Multivariate analysis was employed to adjust for demographic differences between groups of patients using standard, multiple logistic or linear regression when appropriate. P value represented independent association of genetic variant. P values less than 0.05 were considered statistically significant.

Results

Maintenance therapy response

The surrogate markers of maintenance therapy tolerance and toxicity of hematopoietic tissue included median dose of 6-MP and the number of leukopenic episodes (Table 2). Specifically, lower median 6-MP dose or higher number of leukopenic episodes was indicative of higher 6-MP/MTX myelotoxicity, and *vice versa*. It should be noted that during the maintenance therapy, 6-MP dose and MTX dose are highly correlated to one another, though 6-MP dose is more readily adjusted in case of myelotoxicity, especially for carriers of non-functional *TPMT* alleles. Hepatotoxicity was assessed as previously described, and patients were divided into hepatotoxicity positive and hepatotoxicity negative group.

Table 2. Surrogate markers of maintenance therapy tolerance and toxicity

Dose of 6-MP (mg/m^2)	
Average	51.6
Median	51.5
Range	17-98.5
Patients receiving 6-MP above average dose, n (%)	62 (48.8)
Patients receiving 6-MP below average dose, n (%)	65 (51.2)
Number of leukopenic episodes	
Average	2.3 per patient Observation period
Range	0-9 per patient Observation period
Hepatotoxicity, n (%)	
Yes	65 (51.2)
No	62 (48.8)

During maintenance therapy, the median dose of 6-MP for the entire group of patients was 51.5 mg/m^2. The lowest average 6-MP dose administered to any patient was 17 mg/m^2 and the highest 98.5 mg/m^2. One quarter of patients (33 patients) had no leukopenic episodes during maintenance therapy and the remaining patients had at least one episode. The average number of leukopenic episodes was 2.3 per patient, with maximum 9 episodes in one patient. More than half of the patients (65 patients) experienced some degree of hepatotoxicity (grade 1 or higher) during the maintenance phase of therapy.

Age and gender of ALL patients were correlated with markers of maintenance therapy toxicity. The average 6-MP dose was negatively correlated with age (linear regression, p=0.008) and male gender was associated with higher average 6-MP dose (logistic regression, p=0.008). The number of leukopenic episodes were higher in girls (linear regression, p=0.019). Younger children tended to suffer more from hepatotoxicity during the maintenance phase of therapy (linear regression, p=0.001). Immunophenotype and risk group of patients were not associated with surrogate markers of maintenance therapy tolerance. Therefore, when assessing the association of genetic variants with surrogate markers of therapy toxicity, all probabilities were adjusted for demographic characteristics (age and gender), but not for clinical characteristics of ALL patients.

Genetic variants detected in ALL patients

In *TPMT* gene, *TPMT*3A* and *TPMT*3C* alleles were detected, and patients who carried either of those alleles were referred to as *TPMT*3* allele carriers. Patients who did not carry *TPMT*3* allele were assumed to be homozygous carriers of wild-type, *TPMT*1* allele. In 3'UTR region of *TYMS* gene, alleles with 2, 3 and 4 tandem repeats were detected (referred to as 2R, 3R and 4R allele, respectively). In 3'UTR region of *TYMS* gene, both deletion (referred to as 6bp- allele) and insertion (referred to as 6bp+ allele) were detected. In *MTHFR*, *SLC19A1* and *DHFR* genes a total of 8 biallelic SNPs were detected.

Association of variants in TPMT, TYMS, MTHFR, SLC19A1 and DHFR genes with maintenance therapy toxicity

Frequencies of genotypes for all investigated genes (*TPMT*, *TYMS*, *MTHFR*, *SLC19A1* and *DHFR*) conformed to Hardy-Weinberg equilibrium. For four patients genotyping was incomplete. As expected, *TPMT* variant allele carriers (4 patients, 3.3%) were treated with lower average 6-MP dose in contrast to wild type *TPMT* carriers (p=0.006) (Table 3). In this small group of patients, the number of leukopenic episodes was similar as in the group with wild type *TPMT*. In one half of *TPMT* variant carriers we observed hepatotoxicity which had the same frequency as in the group of patients

Table 3. Association of genetic variants with surrogate markers of maintenance therapy tolerance and toxicity

Genetic variant	*Genotype*	*6-MP dose*		*Number of leukopenic episodes*		*Hepatotoxicity*	
		β	*p value*	*β*	*p value*	*OR (95% CI)*	*p value*
TPMT*3	*3*3/*1*3 vs *1*1	-0.235	**0.006**	0.042	0.222	0.75 [0.10-5.66]	1.000
TYMS 28bp VNTR	2R2R/2R3R vs 3R3R/3R4R	0.006	0.822	-0.163	0.072	1.59 [0.71-3.57]	0.263
TYMS 6bp indel	$6bp^+6bp^-$ /$6bp^-6bp^-$ vs $6bp^+6bp^+$	0.009	0.899	-0.05	0.493	1.11 [0.52-2.33]	0.792
MTHFR c.677C>T	CT/TT vs CC	0.106	0.376	-0.023	0.603	1.04 [0.49-2.20]	0.915
MTHFR c.1298A>C	AC/CC vs AA	-0.071	0.961	0.013	0.250	1.14 [0.54-2.43]	0.733
SLC19A1 c.80G>A	GA/AA vs GG	0.04	0.567	-0.156	0.079	1.03 [0.45-2.38]	0.944
DHFR -680C>A	CA/AA vs CC	-0.083	0.376	-0.112	0.128	1.28 [0.60-2.74]	0.431
DHFR -675A>G	AG/GG vs AA	-0.025	0.306	-0.079	0.690	0.85 [0.40-1.81]	0.660
DHFR -556T>C	TC/CC vs TT	-0.021	0.266	-0.099	0.527	0.82 [0.38-1.84]	0.532
DHFR -464A>T	AT/TT vs AA	-0.01	0.324	-0.121	0.386	0.76 [0.36-1.62]	0.418
DHFR -317A>G	AG/GG vs AA	-0.136	0.207	0.021	0.819	1.30 [0.58-2.92]	0.444

When association of TYMS, MTHFR, SLC19A1 and DHFR genes were considered, carriers of TPMT*3 allele were excluded from analyses. Dominant genetic model was used for each genetic variant. Multivariate analysis was employed to adjust for demographic differences between groups of patients. For linear regression, standardized coefficient β was reported. For logistic regression, odds ratio (OR) with 95% confidence interval (CI) was reported. Significant associations are bolded

without *TPMT* deficiency (Supplement material, Table S1). One should bear in mind that *TPMT* genotyping was performed before maintenance therapy, and 6-MP dose was adjusted accordingly.

The association of variants in *TYMS*, *MTHFR*, *SLC19A1* and *DHFR* on maintenance therapy tolerance and toxicity was assessed only in patients who did not carry *TPMT* variant alleles in order to control for huge impact of *TPMT* variants on 6-MP toxicity, and because those patients were treated with initially reduced 6-MP doses. We assessed the dominant genetic models for all variants we studied as well as estimated haplotype association with surrogate markers of maintenance therapy toxicity (Tables 3 and 4). It was found that carriers of *TYMS* 3R3R and 3R4R genotypes tended towards higher number of leukopenic episodes in comparison with carriers of *TYMS* 2R allele (linear regression, p=0.067). Our results also showed that carriers of *SLC19A1* c.80A allele tended to have fewer leukopenic episodes (linear regression, p=0.079). No other variant showed significant association (or statistical trend) with the surrogate markers when single variant was considered.

In *TYMS*, *MTHFR* and *DHFR* genes, more than one variant was detected, so bioinformatic tools were employed to estimate the combination of variants that compose each haplotype (Supplement material, Table S2). For each gene, variants that compose a haplotype were listed in 5' – 3' direction. Our results showed that *TYMS* 3R6bp+ (28bp VNTR, 6bp indel) haplotype was associated with higher number of leukopenic episodes (linear regression, p=0.015), while *TYMS* 2R6bp+ (5'UTR VNTR, 6bp indel) haplotype carriers tended to have fewer leukopenic episodes (linear regression, p=0.078). Carriers of *DHFR* CATAA (-680, -675, -556, -464, -317) haplotype tended towards higher 6-MP average dose (linear regression, p=0.093). Carriers of *DHFR* CATAG (-680, -675, -556, -464, -317) haplotype tended towards higher number of leukopenic episodes (linear regression, p=0.070) (Table 4).

Discussion

Toxicity-related death and reduced drug exposure time, due to non-fatal toxicities remain major issues in the treatment of ALL in children [3]. As with any therapy, the goal of childhood ALL treatment is to achieve the best possible overall survival with minimal drug-induced toxicities and complications. Pharmacogenomic profiling of each child with ALL is essential for patient-tailored therapy, which represents the best option for reducing toxicities and improving survival.

In this study, besides potential pharmacogenomic markers, we also evaluated the influence of clinical and demographic characteristics of patients on maintenance therapy tolerance and most common toxicities. We observed higher rate of hepatotoxicity in younger children, probably as a

Table 4. Association of most frequent haplotypes with surrogate markers maintenance therapy tolerance and toxicity.

Haplotype	*6-MP dose*		*Number of leukopenic episodes*		*Hepatotoxicity*	
	B	*p value*	*β*	*p value*	*OR (95% CI)*	*p value*
TYMS (28bp VNTR, 6bp indel)						
2R6bp+	-0.028	0.75	-0.156	0.078	1.21 [0.55-2.65]	0.628
3R6bp+	-0.074	0.399	0.217	**0.015**	0.66 [0.31-1.43]	0.296
3R6bp-	-0.037	0.674	-0.043	0.632	0.82 [0.38-1.85]	0.608
2R6bp-	0.015	0.862	0.047	0.598	2.39 [0.67-8.51]	0.178
MTHFR (c.677, c.1298)						
CA	0.013	0.882	-0.057	0.525	0.97 [0.45-2.10]	0.939
CC	-0.076	0.390	0.028	0.759	1.12 [0.53-2.39]	0.763
TA	0.087	0.320	-0.045	0.613	0.85 [0.40-1.79]	0.667
DHFR (-680, -675, -556, -464, -317)						
AATAG	-0.025	0.776	-0.14	0.119	1.57 [0.73-3.35]	0.248
CATAA	0.146	0.093	0.144	0.109	0.72 [0.34-1.54]	0.400
CGCTA	-0.017	0.846	-0.06	0.509	0.85 [0.40-1.81]	0.670
CATAG	-0.057	0.516	0.162	0.070	1.58 [0.53-4.70]	0.408

For each haplotype, carriers of a specific haplotype were compared to all other patients who carried other haplotypes. Multivariate analysis was employed to adjust for demographic differences between groups of patients. For linear regression, standardized coefficient β was reported. For logistic regression, odds ratio (OR) with 95% confidence interval (CI) was reported. Significant associations are bolded

Table S1. Number of carriers of genetic variants in TPMT, TYMS, MTHFR, SLC19A1 and DHFR genes in relation with surrogate markers of maintenance therapy tolerance and toxicity

Genotype	*ALL patients n (%)*	*Median dose of 6-MP (mg/m²)*	*Average number of leukopenic episodes*	*Patients that suffered from hepatotoxicity, n*	*Patients that did not suffer from hepatotoxicity, n*
TPMT					
TPMT*1/TPMT*13	123 (96.9)	52.0	2.2	60	63
TPMT*3 allele carriers	4 (3.1)	38.3	3.0	2	2
TYMS 28bp VNTR					
3R3R	38 (30.9)	50.8	2.8	21	17
3R2R	53 (43.1)	49.0	2.3	26	27
2R2R	31 (25.2)	54.0	1.7	13	18
3R4R	1 (0.8)	79.5	0.0	0	1
TYMS 6bp indel					
$6bp^{+}6bp^{+}$	60 (48.8)	50.3	2.3	30	30
$6bp^{+}6bp^{-}$	52 (42.3)	52.0	2.4	26	26
$6bp^{-}6bp^{-}$	11 (8.9)	56.5	1.5	4	7
MTHFR c.677C>T					
CC	61 (49.6)	49.5	2.4	29	32
CT	50 (40.7)	53.5	2.1	23	27
TT	12 (9.8)	52.8	2.3	8	4
MTHFR c.1298A>C					
AA	58 (47.2)	52.0	2.1	21	27
AC	48 (39.0)	52.8	2.1	24	24
CC	16 (13.0)	48.0	3.0	5	11
SLC19A1 c.80G>A					
GG	33 (26.8)	51.5	2.7	16	17
GA	60 (48.8)	53.0	1.9	30	30
AA	29 (23.6)	49.5	2.5	14	15
DHFR -680C>A					
CC	52 (42.3)	53.8	2.5	27	25
CA	52 (42.3)	49.5	2.1	21	31
AA	15 (12.2)	52.0	1.5	10	5
DHFR -675A>G					
AA	63 (51.2)	53.0	2.4	29	34
AG	47 (38.2)	54.0	2.1	27	20
GG	9 (7.3)	49.5	1.9	2	7
DHFR -556T>C					
TT	62 (50.4)	53.3	2.4	28	34
TC	49 (39.8)	49.0	2.0	29	20
CC	8 (6.5)	49.5	2.1	1	7
DHFR -464A>T					
AA	61 (49.6)	53.0	2.5	27	34
AT	50 (40.7)	49.0	1.9	30	20
TT	8 (6.5)	49.5	2.1	1	7
DHFR -317A>G					
AA	38 (30.9)	54.0	2.2	20	18
AG	61 (49.6)	49.0	2.4	28	33
GG	20 (16.3)	52.0	1.5	10	10

For abbreviations see text

Table S2. Number of carriers of the most frequent haplotypes in TYMS, MTHFR, and DHFR genes in relation with surrogate markers of maintenance therapy tolerance and toxicity

Haplotype[1]		*n (%)*	*Median dose of 6-MP (mg/m²)*[2]	*Average number of leukopenic episodes*	*Patients that suffered from hepatotoxicity, n*	*Patients that did not suffer from hepatotoxicity, n*
TYMS (28bp VNTR, 6bp indel)						
2R6bp+	Non-carriers	43 (35.0)	53.5	2.67	21	22
	Carriers	80 (65.0)	51.5	2.04	42	38
3R6bp+	Non-carriers	64 (52.0)	52.3	1.78	34	30
	Carriers	59 (48.0)	49.5	2.78	29	30
3R6bp-	Non-carriers	72 (58.5)	51.8	2.29	38	34
	Carriers	51 (41.5)	52.0	2.22	25	26
2R6bp-	Non-carriers	109 (88.6)	51.5	2.24	54	55
	Carriers	14 (11.4)	53.3	2.43	9	5
MTHFR (c.677, c.1298)						
CA	Non-carriers	46 (37.7)	51.0	2.37	24	22
	Carriers	76 (62.3)	52.0	2.14	38	38
CC	Non-carriers	58 (47.5)	52.0	2.12	27	31
	Carriers	64 (52.5)	51.8	2.33	35	29
TA	Non-carriers	63 (51.6)	49.5	2.37	34	29
	Carriers	59 (48.4)	53.5	2.08	28	31
DHFR (-680, -675, -556, -464, -317)						
AATAG	Non-carriers	53 (44.5)	53.5	2.55	24	29
	Carriers	66 (55.5)	51.8	1.97	37	29
CATAA	Non-carriers	56 (47.1)	49.0	1.89	31	25
	Carriers	63 (52.9)	54.0	2.52	30	33
CGCTA	Non-carriers	65 (54.6%)	53.0	2.32	35	30
	Carriers	54 (45.4%)	49.3	2.11	26	28
CATAG	Non-carriers	102 (85.7%)	52.0	2.10	51	51
	Carriers	17 (14.3%)	50.0	3.00	10	7

[1]Only haplotypes that have estimated frequency of more than 2% are shown. [2]For each ALL patient, average 6-MP dose was calculated and median value was reported for groups of ALL patients carrying specific haplotype.

consequence of higher average 6-MP dose they received in order to maintain the desired WBC count. We also observed that boys had fewer number of leukopenic episodes than girls, even though they received higher average dose of 6-MP. This result can be explained at least to some extent by taking into account that boys have higher *TPMT* activity than girls [12]. Clinical characteristics of ALL patients like immunophenotype and risk group were not associated with surrogate markers of maintenance therapy tolerance.

Although numerous studies have been performed to evaluate the use of *TYMS*, *MTHFR*, *SLC19A1* and *DHFR* genes as pharmacogenomic predictors of MTX-induced toxicity in childhood ALL, still there is no firm evidence of their clinical significance [3]. However, interlinked metabolic pathway of MTX and 6-MP has identified "MTX-related" genes as possible markers of 6-MP-induced toxicities [3,12].

Our results suggested that, in comparison with carriers of *TYMS* 2R allele, carriers of 3R3R and 3R4R genotype had higher number of leukopenic episodes. The alleles with 3 or 4 tandem repeats are associated with higher *TYMS* activity than the allele with 2 repeats [21-23]. Therefore, carriers of 3R3R and 3R4R genotype are less likely to suffer from MTX-induced toxicity due to inherently higher *TYMS* activity, however, those patients might be more prone to 6-MP-induced myelotoxicity. Namely, *TYMS* enzyme uses folate cofactor 5,10 methylen-tetrahydrofolate necessary for synthesizing 5-methyl tetrahydrofolate and SAM cofactors, so carriers of higher expressing *TYMS* genotypes have reduced level of SAM which can lead to reduced activity of *TPMT* [11,24,25]. Therefore, patients that carry genotypes associated with higher *TYMS* activity may have reduced SAM levels, reduced *TPMT* activity and consequently higher 6-MP-induced myelotoxicity. However, certain

studies did not show correlation of *TYMS* variants with reduced *TPMT* activity [12,26,27], thus leaving our observation open for further studies.

Besides VNTR region in 5'UTR region, we also analyzed 3'UTR indel in *TYMS* gene. Presence of 6bp insertion is associated with increased level of mRNA and higher *TYMS* expression [28]. Our results suggest that patients that carry haplotype consisting of alleles 3R and 6bp+ which are associated with higher *TYMS* activity, have higher number of leukopenic episodes. Again, higher *TYMS* activity leads to reduced *TPMT* activity and consequently higher 6-MP-induced myelotoxicity (for detailed explanation see previous paragraph).

Carriers of *SLC19A1* c.80A allele were found to have fewer episodes of leukopenia in our patient group. This allele encodes a transporter which may have lower capacity to transport MTX drug [29]. Lower toxicity of hematopoietic tissue mirrored by fewer episodes of leukopenia might be direct consequence of lower intracellular MTX level. Also, lower intracellular MTX level could increase folate turnover rate leading to higher SAM levels, higher *TPMT* activity and lower 6-MP toxicity.

Our results suggested that carriers of *DHFR* CATAA (-680, -675, -556, -464, -317) haplotype might need higher average 6-MP dose. It has been shown that this haplotype, which corresponds to haplotype 1 in the study by Dulucq and colleagues have 1.5- to 2-fold higher *DHFR* expression compared to other haplotypes [30]. Higher expression of *DHFR* gene directly opposes the effects of MTX, since *DHFR* enzyme is the main target of this drug, but can be also relevant for 6-MP response. Namely, patients that carried higher expressing *DHFR* haplotype would be expected to have higher level of SAM and consequently higher *TPMT* activity, which might explain the higher average 6-MP dose they received to reach the target WBC count. Our results also suggested that carriers of *DHFR* CATAG (-680, -675, -556, -464, -317) haplotype had higher number of leukopenic episodes. This haplotype has not been previously associated with *DHFR* expression or drug response, so this result needs to be corroborated.

Therapy response and 6-MP-induced toxicities can be predicted based on *TPMT* genotype in most of the patients [3,31]. For the remainder that experience increased 6-MP-induced toxicities, other genes such as *ITPA*, *PACSIN2* and *NUDT15* can offer some explanations [3,10]. Still, there is a certain number of patients for whom explanation for increased toxicities and poor therapy response needs to be looked for elsewhere. Our results showed that other genes, namely *TYMS*, *SLC19A1* and *DHFR* can play a role in the determination of 6-MP/MTX toxicity profile, making them potential biomarkers of maintenance therapy adjustment in childhood ALL treatment. Pharmacogenetic studies based on a candidate gene approach, similar to this one, have contributed to the implementation of several pharmacogenetic markers into clinical practice. However, the era of studies based on the testing of whole genome has already started [32]. In time, pharmacogenenomic profiling will provide data that will lead to individualization of therapy, as well as to novel therapy approaches in pediatric ALL.

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Conflict of interests

The authors declare no conflict of interests.

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