The Effects of Three ABCG2 Polymorphisms on Outcome of Central Nervous System Relapses in Iranian Children With Acute Lymphoblastic Leukemia Receiving High Dose Methotrexate

Raziye Karamikhah[1], Negar Azarpira[2], Soheila Zareifar[3], Ali Dehshahri[4], Soha Namazi[5], Mohammad Hossein Anbardar[6], Iman Karimzadeh[1]

[1] *Department of Clinical Pharmacy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*

[2] *Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

[3] *Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

[4] *Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*

[5] *Department of Clinical Pharmacy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[6] *Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*

Received: 16 Jul. 2020; Accepted: 23 Dec. 2020

Abstract- Methotrexate (MTX) is the main drug for the treatment of childhood acute lymphoblastic leukemia (ALL). ABCG2 pump is the main transporter of MTX on BBB. Our aim was to investigate the possible relationship between three polymorphisms of the ABCG2 gene, and isolated CNS relapses in Iranian children with ALL receiving high dose MTX. Genotyping of three polymorphisms of the ABCG2 gene, including G34A, C376T, and C421A, was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for 56 patients. A high frequency of C376T CT genotype was observed among the patients. There was no significant association between C376T and C421 and isolated CNS relapse (P>0.05). C376T and C421A polymorphisms are not associated with isolated CNS relapse in childhood ALL.

Acta Med Iran 2021;59(3):133-141.

Keywords: Methotrexate; ATP-binding cassette transporter G2 (ABCG2); Central nervous system (CNS) relapse; Childhood acute lymphoblastic leukemia

Introduction

Relapsed ALL accounts for the fourth cause of all childhood malignant disorders. Causes of ALL treatment have been under investigation for a long time. The resistance of cancer cells to chemotherapeutic agents is a reason which impacts treatment outcome. ABCG2 overexpression is one of the resistance mechanisms (1,2).

The second member of the ABC family subgroup G (ABCG2) is located on the human 4q22 chromosome, exon 16, with 655 amino acid residues. It codes 72 KD membrane proteins (3,4). It is expressed in the luminal surface of the human micro-vessel of the endothelium. Compared to MRD1, BCRP is the main transporter of ABC in human brain microcells. In addition, its expression is higher in tumor vessels in comparison with normal neurons (3).

Higher expression of ABCG2 is reported in many hematological malignancies and solid tumors. Due to the existence of polymorphisms in this gene, the treatment outcome can be affected (5). ABCG2 is the main transporter of ABC on human brain capillary endothelial cells (3,6). It seems that the expression of BCRP in brain tumor capillaries is more than in normal conditions (3). This pump can affect agents used in the chemotherapy regimen of ALL, such as anthracyclines and MTX, and their passage across the blood-brain barrier (BBB) (5). In fact, it is the main MTX efflux pump located at the BBB (7).

CSF/plasma MTX concentration ratio is dependent on

Corresponding Author: I. Karimzadeh
Department of Clinical Pharmacy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
Tel: +98 713244128, Fax: +98 7132424126, E-mail address: karimzadehiman@yahoo.com

the existence of the pumps on BBB and single nucleotide polymorphism (SNP) within the efflux transporter genes. ABCG2 SNP creates diverse drug concentration ranges in the passage of drug through the BBB and finally in CSF (8-11). CSF MTX concentrations in children receiving HD MTX (e.g., ALL, osteosarcoma, NHL) have been shown to be highly variable and can be divided into sub-therapeutic, therapeutic, and toxic ranges (12). The sub-therapeutic levels lead to CNS relapse and toxic levels cause adverse effects such as irreversible cognitive dysfunction (13). Among variable ABCG2 polymorphisms, MTX and MTX-polyglutamates are extruded only by the wild type ABCG2 (14). MTX and anthracyclines, as the part of ALL chemotherapy regimen, are BCRP substrates. However, Kourti *et al.*, (1) reported that BCRP overexpression had no impact on treatment outcome associated with anthracyclines. In this study, we aimed to discover the relationship between three ABCG2 SNPs (G34A, C421A, and C376T) as a mechanism of drug resistance and isolated CNS relapse in Iranian children with ALL receiving high dose MTX.

Materials and Methods

Patient selection

During a 30 month period from March 2017 to August 2019, patients with ALL aged between 1-18-year-treated according to current Children's Oncology Group (COG) (15) treatment protocols at the Department of Pediatric Hematology and Oncology, Shiraz University of Medical Sciences, Iran were screened. The parents and all grandparents of each subject were of Iranian origin. This case-control study was approved by Research Ethics Committee of Shiraz University of Medical Sciences and informed consent was obtained from all individuals or their parents/guardians before including into the study. According to COG group, isolated CNS relapse is divided into early and late by a time cut-off of 18 months from initial complete remission (15). The patients were enrolled in one of the two following groups:

Control group: a group of 28 patients without medullary and extramedullary relapse at least 18 months after starting chemotherapy.

Case group: a group of 28 patients with isolated CNS relapse that occurred after achieving first complete regimen.

The following exclusion criteria were considered: Down syndrome; CNS involvement at the time of diagnosis; leptomeningeal metastases, primary CNS lymphoma, leukemic meningeal involvement; concurrent other hematological or non-hematological malignancies, and Philadelphia positive patients.

Data gathering

The data were extracted from all medical records of the patients. The available data collected from each patient were as follows: name, age, sex, laboratory data including immunophenotype (early pre-B cell, common type, pre-B cell, mature B cell, and T cell), cytogenetics (DNA index, ploidy, and translocations), lymphocyte morphology based on the FAB classification system (L1, L2, and L3), minimum residual disease (MRD) on days 0 and 29, bone marrow status at day 14 of remission induction, biochemical data (SGOT, SGPT), CBC at diagnosis (WBC count, platelet, and hemoglobin), absolute lymphocyte count (ALC) on days 7, 14, and 21.

Blood sampling, DNA extraction, and PCR protocol

Venous blood samples (3 ml) were taken from the included patients. These samples were collected in vacutainer tubes containing sodium heparin and stored at -20º C for the preparation of DNA.

Blood DNA was extracted manually by using a commercially available DNA extraction kit (Biomaxcell®, Iran) with the blood protocol.

The primers used in this investigation have been previously published (9,13). The primer sequences for genomic DNA were as follows: For G34A: forward, 5'-ATTGTCACCTAGTGTTTGC -3'; reverse, 5'-AAAAATG.TTCATGCCAGTTTCT -3´; For C421A: forward, 5´-CCTTAGTTATGTTATCTTTGTG-3´; reverse; 5´-GAAACTTCTGAATCAGAGTCAT-3´; and for C376T: forward, 5´-ATAGCATGTGTTGGAGGGAAAAA-3´; reverse, 5´-ATTGGTATCACTGTCCTTACAAG-3´.

PCR was performed in a reaction volume of 25 µl per sample in small tubes (0.2-0.5 ml volume) containing 2.5 µl of 10×PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.75 µl of 10 nM deoxynucleoside-5'- triphosphate (dNTP), 0.5 µl of each reverse and forward primer (10 pmol/µl), 0.5 µl of *Taq* DNA polymerase (5 U/µl), 9.5 µl of deionized water and 10 µl of extracted DNA. Thermal cycling conditions were as follows: denaturation: at 95º C for 5 min; 40 cycles of 45 sec at 94º C; annealing at 57.6º C (C376T, and C421A) and 59.4º C (G34A) for 1 min; elongation: 72º C for 1 min; and final extension: 72º C for 5 min. The resulting PCR products were analyzed by the gel electrophoresis on 1.5% agarose gels.

Genotyping of the variants

G34A, C421A, and C376T allele frequencies were determined for the cohort. These three polymorphisms of

ABCG2 gene were analyzed with PCR-restriction fragment length polymorphism (RFLP). The restriction enzyme for each SNP (9,13) was as follows: *BseMI* restriction enzyme for G34A; *TaaI* restriction enzyme for C421A and *RsaI* restriction enzyme for C376T. 0.3 µl of *BseMI* was diluted in a mixture containing 2 µl of buffer and 2.5 µl of sterile distilled water and 15 µl of PCR reaction mixture. The mixture was digested at 37° C for 16 hours (overnight). For *TaaI*, all the steps were the same as *BseMI*, but the digestion temperature was 65° C. The enzyme mix for *RsaI* for C376T SNPs was composed of 0.5 µl restriction enzyme, 2 µl buffer, and 15 µl PCR product, and 2.5 µl sterile distilled water. The digestion time was 16 hours at 57° C. The resulting digesting products were analyzed by gel electrophoresis on 3% agarose gels.

Statistical analysis

All statistical analysis was conducted using the SPSS software (SPSS Inc. Chicago, IL, USA, version 25, 2017). Kolmogorov-Smirnov test was applied to find out the normality of continuous data. Quantitative data were shown as either mean±SD or median (range). Descriptive data were shown as percent. Due to the non-normally distributed data, the non-parametric Mann-Whitney *U* test for quantitative data and Fisher's Exact test for descriptive data were used. To test the possible association between different demographic, clinical, paraclinical, and studied polymorphisms and risk of CNS relapse, logistic regression with odds ratio (OR) and 95% confidence interval (CI) was used. Hardy-Weinberg test by Arlequin software (version 3.11, Bern, Swiss) was exploited to determine the alleles distribution. $P>0.05$ in this test indicates no deviation in genotypes and alleles' distributions over different generations in a population.

Results

Demographic data and clinical characteristics of patients

Fifty six patients were recruited into this study. Their mean±SD age was 11.83±5.55 years. Regarding gender, 67.86% were male. More than two-thirds (76.79%) of the patients had B-cell immunophenotype. The most frequent (94.64%) lymphocyte morphology was L2. According to the risk-based therapy, 69.65% of the cohort were categorized as standard risk. One-third of the study population had a leukocyte count above 50,000 cells/µl. None of the patients with T cell ALL had mediastinal mass at the time of diagnosis. In addition, no case of hepatosplenomegaly and/or lymphadenopathy at diagnosis was detected in the cohort. Except for age and MRD on day 0, there was no significant difference between various demographic, clinical, paraclinical features of patients in the case and control groups (Table 1).

Frequencies of G34A, C376T, and C421A in the study population

The frequencies of these three SNPs were evaluated in ALL population. G34A was only detected as G/G genotype. For C376T polymorphism, the frequencies of C/T and T/T genotypes were 80.35% (n=45) and 12.5% (n=7), respectively. In contrast, for C421A, most of the patients were of the C/C genotype 91.07% (n=51). The frequency of C/A genotype and A/A genotype was 3.57% (n=2) and 5.35% (n=3), respectively. The frequency of variant allele of C421A (421A) was 7.14%.

Frequencies of G34A, C376T, and C421A in control and isolated CNS relapse groups

The comparison of three genotypes of these polymorphisms between control and relapsed groups is shown in Table 2. All the patients had the G/G genotype for G34A polymorphism. Therefore, comparing the frequency of this polymorphism between case and control groups was not statistically feasible. Since all the patients had G/G genotype, the Hardy-Weinberg law was not applicable here. For C376T, although not statistically significant, there was a higher distribution of C/C genotype in the relapsed groups. C allele tended to have a higher frequency in the relapsed group ($P>0.05$). For C421A, there was no significant difference between the two groups ($P>0.05$). Both C376T and C421A polymorphisms were not in the Hardy-Weinberg equilibrium ($P<0.05$). These findings were also demonstrated after adjusting C376T as well as C421A polymorphisms by demographic and paraclinical variables, including age at the time of study entering or disease diagnosis and MRD0 in the multivariate logistic regression analysis. Considering the fact that all the cohort were wild type regarding G34A polymorphism, entering this SNP into the multivariate model was not possible (Table 3).

Assessment of the relationship between C421A and C376T SNPs and isolated CNS relapse

Among the case group, CNS relapses in 13 and 15 patients were classified as early and late, respectively. There was not a significant association between C421A or C376T polymorphisms and type of CNS relapse (early versus late) (Table 4).

The possible association between prognostic factors of childhood ALL C376T and C421A SNPs

Univariate logistic regression was done to determine the relationship between these prognostic factors of childhood ALL population and C421A. There was no statistically significant association between C421A and the considered prognostic factors in childhood ALL (P>0.05). In contrast, there was only a statistically significant association between C376T and immunophenotype (P=0.020).

Table 1. Demographic, clinical, and paraclinical characteristics of the study population at the diagnosis of acute lymphoblastic leukemia (n=56)

Characteristics		Control group (n=28)	Case group (n=28)	*P*
Age at the time of disease diagnosis, years (median [range])		8.5 (2.25-9.28)	10 (2.38-20.46)	0.001>†
Age at the time of study entering, years (mean±SD)		8.78 ± 3.57	15.47 ± 5.29	0.001>
Sex (%)	**Male**	21 (75)	17 (60.71)	0.415
	Female	7 (25)	11 (39.28)	
Immunophenotype (%)	**B- lineage**	22 (78.57)	4 (14.28)	0.211
	Common type	-	18 (64.28)	
	Pre - B	3 (10.71)	-	
	T- lineage	3 (10.71)	4 (14.28)	
	Not known	-	2 (7.14)	
Risk-group (%)	**Standard risk groups**	20 (71.42)	19 (67.85)	0.376
	High risk groups	8 (28.57)	5 (17.85)	
	Not known	-	4 (14.28)	
Lymphocyte morphology (%)	**L1**	-	2 (7.14)	0.491
	L2	28 (100)	25 (89.28)	
	L3	-	1 (3.57)	
MRD, median (range)	**On day 0**	90.00 (6.00-92.00)	80.00 (3.00-90.00)	0.046†
	On day 7	14.00 (1.00-60.00)	6.50 (1.00-45.00)	0.664†
	On day 29	3.00 (1.00-70.30)	3.00 (0.01-10.00)	0.578†
Day -14 bone marrow response (%)	**M1**	18 (64.28)	15 (53.57)	0.249
	M2	2 (7.14)	4 (14.28)	
	M3	3 (10.71)	5 (17.85)	
	Unknown	5 (17.85)	4 (14.28)	
Absolute lymphocyte count (ALC) median (range)	**On day 7**	935.00 (25.60-4902.00)	623.00 (1.00-42987.00)	0.649†
	On day 14	357.00 (4.00-3478.00)	414.00 (00.00-3660.00)	0.826†
	On day 21	576.00 (56.00-4355.00)	980.50 (00.00-9476.00)	0.173†
SGOT, median (range)		29.00 (70.00-940.00)	42.00 (9.00-330.00)	0.727†
SGPT, median (range)		26.00 (8.00-1094.00)	40.00 (19.00-147.00)	0.702†
Initial WBC count × 10^3 (µL), median (range)		7.10 (1.60-500.00)	8.5 (1.1-155)	
< 10,000 (%)		18 (64.28)	16 (57.14)	0.862†
10,000- 49,000 (%)		7 (25.00)	6 (21.42)	
> 50,000 (%)		3 (10.71)	6 (21.42)	
Platelet × 10^3 (µL), median (range)		95.00 (12.5-490.00)	151.00 (6.00-399.00)	
< 20,000 (%)		6 (21.7)	7 (25.00)	0.796†
20,000- 99,000 (%)		9 (30.4)	6 (21.42)	
> 100,000 (%)		13 (47.8)	15 (53.57)	
Hemoglobin (g/dl), median (range)		9.30 (1.3-96.00)	9.05 (3.6-76)	
< 7 (%)		6 (21.42)	8 (28.57)	0.937†
7- 11 (%)		16 (57.14)	12 (42.85)	
> 11 (%)		6 (21.42)	8 (28.57)	

†: Mann-Whitney U Test is used

Table 2. Comparison of G34A, C376T, and C421A genotypes between case and control groups

Polymorphism		Control group (n=28)	Case group (n=28)	*P*
G34A (%)	GG (Wild type)	28 (100)	28 (100)	NA
	GA (Heterozygous)	--	--	
	AA (Homozygous)	--	--	
C376T (%)	CC (Wild type)	--	4 (14.28)	0.131
	CT (Heterozygous)	25 (89.28)	20 (71.42)	
	TT (Homozygous)	3 (10.71)	4 (14.28)	
C421A (%)	CC (Wild type)	26 (92.85)	25 (89.28)	0.611
	CA (Heterozygous)	2 (7.14)	2 (7.14)	
	AA (Heterozygous)	--	1 (3.57)	

Table 3. Multivariate logistic regression model to assess the possible association between a number of demographic and paraclinical variables along with C376T as well as C421A polymorphisms and risk of CNS relapse in the study population.

Variable	*P*	OR	95% C.I.	
			Lower	Upper
Age at the time of study entering	0.233	1.000	1.000	1.001
Age at the time of disease diagnosis	0.063	1.264	0.988	1.616
MRD0	0.125	0.970	0.934	1.008
C376Tpolymorhism	1.000	--	0.000	--
C421A polymorphism	0.188	0.114	0.004	2.903

Tables 4. The comparison between the frequency of C376T and C421A genotypes and the association with early and late isolated CNS relapse

Polymorphism		Isolated CNS relapse		*P*
		Early (n= 15)	Late (n= 13)	
C376T (%)	CC (Wild type)	2 (13.33)	2 (1.18)	1.000
	CT (Heterozygous)	11 (73.33)	9 (69.23)	
	TT (Homozygous)	2 (1.33)	2 (1.18)	
C421A (%)	CC (Wild type)	14 (93.33)	11 (84.61)	1.000
	CA (Heterozygous)	1 (6.66)	1 (7.69)	
	AA (Homozygous)	0	1 (7.69)	

Discussion

This study aimed to evaluate three non-synonymous ABCG2 polymorphisms (G34A, C376T, and C421A) and their effect on the outcome of isolated CNS relapse in children with ALL receiving HD MTX. Our results showed that 376T and 421A polymorphisms had no significant association with isolated CNS relapse. V12M (34G>A, rs2231137) is one of the most common ABCG2 SNPs. This polymorphism is very variable among different ethnic groups. The protein expression level of this SNP is the same as that in the wild type or only with a slight decrease (9). The V12M SNP has its maximum frequency in the Mexican-Indian population at 90%, followed by specific islanders (60%) and southeastern Asians (45%) (16). Similar to Neibudek *et al*., (17) study on the contribution of ABCG2 G34A and C421A polymorphisms to multiple myeloma susceptibility in the Polish population, all patients in our study were homozygous for the wild type allele of G34A (G/G).

Q141K (421 C>A, rs2231142) is the most studied polymorphism of ABCG2 because of its relation with gout development. In most cases of in vitro and in vivo studies on the effects of Q141K SNP on ABCG2 pump function, a significant reduction of the overall protein expression has been demonstrated. Some studies showed that a reduction in ABCG2 transport function is due to reduced ATPase activity (16). This polymorphism occurs with varying frequencies among different ethnic groups with higher rates in Chinese (34.2-35%) and Japanese (26.6-35%) populations (8,16). Our study demonstrates the dominancy of the C/C genotype related to the Q141K polymorphism.

The non-synonymous SNP, Q126X (376C >T, rs72552713), is a rare polymorphism of ABCG2. This polymorphism results in the early introduction of a stop

codon, leading to autolytic degradation of RNA and finally not expressing membrane proteins (7,16). To date, this has been most frequently reported in Korean and Japanese populations with the frequency of 1.8% and 0.9-2.4%, respectively (16,18). Relevant studies on other populations, such as Caucasians and African-Americans, show no Q126X polymorphism in such populations (7, 16). In our study, we found that 52.70% of our patients had at least one variant allele of this polymorphism.

Overexpression of transporter BCRP is one of the multidrug resistance mechanisms in cancer therapy. Higher levels of ABCG2 is significantly expressed in relapsed childhood ALL (19). Recent studies suggest that ABCG2 strongly affects MTX disposition (5,10,20). In addition to the gene expression level, ABCG2 SNPs change pharmacokinetic feature of MTX and lead to inter-individual differences in treatment response (10,20). This change in MTX pharmacokinetics is significantly related to ALL prognosis (21). MTX systemic exposure is a strong and independent factor in ALL treatment. In fact, higher systemic clearance of MTX resulting in lower plasma and thereafter CSF concentrations, is significantly related to higher rate of early relapse (22).

Stanulla *et al*., (23) reported that there was no significant association between two SNPs within the coding region of the GSTPs (I105V and A104V) and risk of CNS relapse. The investigated SNP within the MDR1 gene located at position 3435 had no significant association with risk of CNS relapse. Although the patients with GSTP1 V105V genotype in intermediate- and high-risk groups (higher treatment intensity) had significantly reduced risk of CNS relapse. The T allele polymorphism in the MDR1 gene had the same relationship with protection from CNS relapse in intermediate- and high-risk groups (23). The author mentioned that intrathecal drugs and methotrexate and 6-mercaptopurine are not the substrates for P-gp. As a mechanism of drug resistance, P-gp efflux activity can affect the outcome of leukemia therapy and the incidence of CNS relapse (23). The other study by Stanulla *et al*., (24) on tumor necrosis factor-alpha (TNF-α) and lymphotoxin-alpha (LT-α) genetic polymorphisms demonstrated that there was no significant relationship between TNF-α and LT-α genotypes and the time to relapse or the site of ALL relapse (isolated bone marrow relapse; combined and isolated CNS relapse; combined and isolated testis relapse) (24).

The results of the Rocha *et al*., study (25) showed in the patients who were categorized as high risk, all those carrying at least one VDR FokI T allele relapsed in CNS. So, the VDR FokI T allele was significantly associated with an increased risk of relapse. Among these, patients with the VDR intron 8 GG genotype had a significantly higher risk of relapse (25). VDR intron 8 G>A and VDR FokI T>C loci exhibited a significant correlation with CNS relapse (25). This correlation can be explained by the VDR FokI T allele, and the VDR intron 8 GG genotype was associated with P-gp overexpression. The elevated expression of P-gp in the BBB may lead to decreased CNS penetration and accumulation and increased biliary excretion of anti-leukemic agents (25). Evaluation of the patients categorized as low risk showed that only two patients had CNS relapse. The presence of TYMS 3/3 genotype in these two patients was significantly associated with an increased risk of CNS relapse. The author stated that this genotype could negatively impact methotrexate as the major drug in CNS prophylaxis (25).

The results of Ansari *et al*., (26) study showed a significant association of MRP3 189A polymorphism with CNS relapse. Higher MRP3 189A allele was associated with MTX efflux and then higher drug resistance. It may be due that the expression of MRP3 may limit brain uptake of drugs (26).

Nine studies have specifically investigated different clinical aspects of ABCG2 gene polymorphism in the setting of pediatric ALL. In this regards, Zhai *et al*., (27) reported that Chinese children with ALL carrying the 421C allele showed lower pretreatment WBC counts ($<20\times10^9$/l). Carrying the 34A allele was significantly associated with improved survival. In this study, there was a significant association between the G34A allele and some prognostic factors of ALL (pretreatment WBC count ($>100\times10^9$/l), post-treatment peripheral leukemic cell count $>1000\times10^9$/l on day 8, bone marrow leukemic cells >25% on day 15 and 5-25% on day 33) (27).

Semsei *et al*., (28) found that the distribution of different genotypes of BCRP SNPs (G34A and C421A) did not differ between patients and controls. In other words, these SNPs did not increase susceptibility to ALL in the Hungarian children. In this study, there was also no significant relationship between G34A as well as C421A polymorphisms and prognostic factors of ALL, including age at diagnosis, sex, immunophenotype, risk group, relapse, leukemia related death, and also hyper-ploidy (28). Also, the studies have shown that there was no significant association between ABCG2 (C421A) and MTX toxicity (including hematopoietic, hepatic, mucositis, myelosuppression, and episodes of elevated LDH) (8,29-33), MTX plasma concentration at different hours of injection (8,29-31) in Spanish, Japanese, and Egyptian populations with ALL, and hepatotoxicity or

MTX concentration at 48 hours in Japanese children with ALL or malignant lymphoma (29).

ABCG2 C421A was associated with decreased BCRP protein expression with no effect on MTX transport and increased risk of encephalopathy in Egypt's child population with ALL (8). Finally, Gervasini *et al.*, reported that there was no significant association between ABCG2 C421A polymorphism and myelosuppression, incidence of hematological toxicity, episodes of elevated LDH, and higher sensitivity to maintenance chemotherapy in Spanish pediatrics with ALL under MTX and 6-mercaptopurine maintenance chemotherapy (32). The results of these studies suggested that the intracellular concentration of MTX is less affected by the ABCG2 C421A variant (33).

In contrast to the above studies, in adult ALL, the ABCG2 G421T variant was associated with reduced risk of ALL in the Han Chinese population. As a transporter for folates and antifolates, ABCG2 G421A is associated with decreased BCRP protein expression leading to decrease folate efflux from the cells and maintain a high folate concentration in the cells (34).

Our negative results can be explained by a number of issues. First, because MTX systemic exposure (Cp steady state) is an independent and strong variable related to prognosis (35) and ABCG2 does not have any effect on MTX plasma concentrations (8,29-31), ABCG2 may not be a useful marker of prediction or prognosis in pediatric ALL. Second, ABCG2 strongly affects MTX disposition (5,10,20), but MTX is the substrate for both ABCB1 and ABCG2 transporters, thus the proven ABCB1-ABCG2 synergistic action of MTX distribution on BBB may be the dominant factor (20,36). Third, by risk stratification of newly diagnosed children with ALL and using HD-MTX in our cohort, the possible effects of studied polymorphisms on MTX pharmacokinetics and clinical outcome may be either omitted or minimized. Fourth, HD MTX overcomes drug resistance in early CNS relapse in wild-type patients. However, patients may relapse after discontinuation of therapy. This may be due to the resistant cells that survive from the initial chemotherapy. Fifth, mechanisms of drug resistance to MTX rather than ABCG2 polymorphisms exists and they may contribute in clinical outcome of ALL patients under HD MTX. Finally, the low sample size of our cohort may preclude us in achieving any significant association between studied polymorphisms and CNS relapse.

Regarding the strengths of our study, to our knowledge, this is the first investigation on genetic polymorphisms of ABCG2 in Iranian pediatric ALL patients. In addition, this is the first study assessing the relationship between the ABCG2 SNPs, as an efflux pump, and risk as well as the type of CNS relapse in the setting of pediatric ALL.

The present study had a number of limitations as follows: 1) isolated CNS relapse is rare, and therefore the resulting sample size would turn out too small, we conducted a retrospective analysis. On the other hand, we did not have access to the records of some patients in the relapsed group because they had been referred to our center only after isolated CNS relapse; 2) due to small sample size, the calculated statistical power of this study (52%) appears not to be adequate, and the possibility of type II error cannot be ruled out; 3) MTX levels in the plasma, as well as CSF and their ratio, were not determined and its association with ABCG2 polymorphisms and clinical outcome were not investigated. Although, in a subgroup of the adult ALL population in the study done by Erdilyi *et al.*, (36), there was no significant relationship between G34A and C421A and CSF MTX concentrations (36). The absence of an ABCG2 pump is related to increased MTX AUC and its toxicity (28). It also increases the brain tissue entry of MTX and subsequently cellular concentration of the drug, resulting in an increased CSF/plasma concentration ratio (20). Also, due to IT MTX injection in these patients, the drug is retained longer in the brain tissue space; and 4) direct DNA sequencing of PCR products was not performed to confirm the results of PCR-RFLP due to financial problems.

In conclusion, our results suggested that three studied ABCG2 polymorphisms, including G34A, C421A, and C376T, had no significant association with risk as well as the type of CNS relapse in pediatric patients with ALL receiving HD MTX. Assessing the above polymorphisms along with other genetic SNP of MTX pharmacokinetics steps (e.g., transporting, elimination) and concurrently measuring the plasma as well as CSF level of MTX in a larger sample size seem crucial to draw a clearer conclusion about this topic.

References

1. Kourti M, Vavatsi N, Gombakis N, Sidi V, Tzimagiorgis G, Papageorgiou T, et al. expression of multidrug resistance 1 (MDR1), multidrug resistance-related protein 1 (MRP1), lung resistance protein (LRP), and breast cancer resistance protein (BCRP) genes and clinical outcome in childhood acute lymphoblastic leukemia. Int J Hematol 2007;86:166-73.
2. Plasschaert S, van der Kolk D, de Bont E, Kamps W, Morisaki K, Bates S, et al. The role of breast cancer

resistance protein in acute lymphoblastic leukemia. Clin Cancer Res 2003;9:5171-7.
3. Eyal S, Hsiao P, Unadkat J. Drug interactions at the blood-brain barrier. Fact or fantasy? Pharmacol ther 2009;123:80-104.
4. Vasiliou V, Vasiliou K, Nebert D. Human ATP-binding cassette (ABC) transporter family. Hum Genomics 2009;3:281-90.
5. Natarajan K, Xie Y, Baer M, Ross D. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. Biochem Pharmacol 2012;83:1084-103.
6. Couraud PO, Sandrine S. Blood- brain barrier models for investigating CNS pathologies. In: Pasquanlini R, Wadih A, eds. Protein Discovery Technologies. United States: CRC Press, 2009:62.
7. Kobayashi D, Ieiri I, Hirota T, Takane H, Maegawa S, Kigawa J. Functional assessment of ABCG2 (BCRP) gene polymorphisms to protein expression in human placenta. Drug Metab Dispos 2005;33:94-101.
8. El Mesallamy HOE, Rashed WM, Hamdy NM, Hamdy N. High-dose methotrexate in Egyptian pediatric acute lymphoblastic leukemia: the impact of ABCG2 C421A genetic polymorphism on plasma levels, what is next? J Cancer Res Clin Oncol 2014;140:1359-65.
9. Imai Y, Nakane M, Kage K, Tsukahara S, Ishikawa E, Tsuruo T. C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low- drug resistance. Mol Cancer Ther 2002;1:611-6.
10. Mimeto H, Kato R, Ito A, Kasamatsu S, Ikegami Y, Kii I. A functional study on polymorphism of the ATP-binding cassette transporter ABCG2: critical role of arginine-482 in methotrexate transport. Biochem J 2003;373:767-74.
11. Sharifi M, Bahoush G, Zaker F, Ansari S, Arjmandi Rafsanjani K, Sharafi H. Association of -24CT, 1249GA, and 3972CT ABCC2 gene polymorphisms with methotrexate serum levels and toxic side effects in children with acute lymphoblastic leukemia. Pedit Hematol Onc 2014;31:169-77.
12. Niemann A, Muhlisch J, Fruhwald M, Gerß J, Hempel G, Boos J. Therapeutic Drug Monitoring of Methotrexate in Cerebrospinal Fluid After Systemic High-Dose Infusion in Children: Can the Burden of Intrathecal Methotrexate be Reduced? Ther Drug Monit 2010;32:467-75.
13. Packer RJ, Schiff D. Neuro-oncology. Chichester, West Sussex: Chichester, West Sussex:Wiley-Blackwell, 2012.
14. You G, Morris M. Drug transporters: molecular characterization & role in drug disposition. Hoboken, NJ: Wiley-interscience, 2007.
15. Lanzkowsky P, Lipton JM, Fish JD. Lanskowsky's manual of pediatric hematology and oncology. 6th ed. Amsterdam: Elsevier Science, 2016.
16. Heyes N, Kapoor P, Kerr ID. Polymorphisms of the multidrug pump ABCG2: a systematic review of their effect on protein expression, function and drug pharmacokineticd. Drug Metab Dispos 2018;46:1886-99.
17. Niebudek K, Balcerczak E, Mirowski M, Pietrrzak J, Zawadzka I, Nawrocka MZ. The contribution of ABCG2 G34A and C421A polymorphisms to multiple myeloma susceptility. Onco Targets and Therapy 2019;12:1655-60.
18. Kim HS, Sunwoo YE, Ryu JY, Kang HJ, Jung HE, Song IS, et al. The effect of ABCG2 V12M, Q141K and Q126X, known functional variants in vitro, on the disposition of lamivudine. Br J Clin Pharmacol 2007;64:645-54.
19. Mehrvar N, Rezvany MR, Abolghasemi H, Akbari ME, SaberyNejad J, Mehrvar A, et al. Gene expression of ABCG1, ABCG2, and ABCB1 and their role in Iranian pediatric pateints with acute lymphoblastic leukemia's recurrence. Int J Cancer Manag 2019;12:e93300.
20. Sane R, Wu SP, Zhang R, Gallo JM. The effect of ABCG2 and ABCC4 on the pharmacokinetics of methotrexate in the brain. Drug Metab Dispos 2014;42:537-40.
21. Borsi JD, Moe PJ. Systemic clearance of methotrexate in the prognosis of acute lymphoblastic leukemia in children. Cancer 1987;60:3020-4.
22. Rodman JH, Sunderland M, Kavanagh RL, Ochs J, Yalowich J, Evans WE, et al. Pharmacokinetcs of continuous infusion of methotrexate and teniposide in pediatric cancer patients. Cancer Res 1990;50:4267-71.
23. Stanulla M, Schaeffeler E, Arens S, Rathmann A, Schrauder A, Welte K, et al. GSTP1 and MDR1 genotypes and central nervous system relapse in childhood acute lymphoblastic leukemia. Int J Hematol 2005;81:39-44.
24. Stanulla M, Schrauder A, Welte K, Schrappe M. Tumor necrosis factor and lymphotoxin-alpha genetic polymorphisms and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case-control study of patients treated with BFM. BMC Blood Disord 2001;1:2.
25. Rocha JCC, Cheng C, Liu W, Kishi S, Das S, Cook EH, et al. Pharmacogenetics of outcome in children with acute lymphoblastic leukemia. Blood 2005;105:4752-8.
26. Ansari M, Sauty G, Labuda M, VGagne' V, JRousseau J, Moghrabi A, et al. polymorphism in multidrug resistance-associated protein gene 3 is associated with outcomes in childhood acute lymphoblastic leukemia. Pharmacogenomics J 2012;12:386-94.
27. Zhai X, Wang H, Zhu X, Miao H, Qian X, Li J, et al. Gene polymorphisms of ABC transporters are associated with clinical outcomes in children with acute lymphoblastic leukemia. Arch Med Sci 2012;8:659-71.
28. Semsei AF, Erdélyi DJ, Ungvári I, Kámory E, Csókay B,

Andrikovics H, et al. Association of some rare haplotypes and genotype combinations in the MDR1 gene with childhood acute lymphoblastic leukaemia. Leuk Res 2008;32:1214-20.

29. Imanishi H, Okamura N, Yagi MN, Moriya Y, Nakamura T, Hayakawa A, et al. Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukemia and malignant lymphoma. J Hum Genet 2007;52:166-71.
30. Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, Piñan MA, Garcia-Miguel P, Navajas A, et al. Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia. Pediatr Blood Cancer 2011;57:612-9.
31. Suthandiram S, Gan GG, Zain SM, Bee PC, Lian LH, Chang KM, et al. Effect of polymorphisms within methotrexate pathway genes on methotrexate toxicity and plasma levels in adults with hematological malignancies. Pharmacogenomics 2014;15:1479-94.
32. Gervasini G, de Murillo SG, Jiménez M, de la Maya MD, Vagace JM. Effect of polymorphisms in transporter genes on dosing, efficacy and toxicity of maintenance therapy in children with acute lymphoblastic leukemia. Gene 2017;628:72-7.
33. 33. Horinouchi M, Yagi M, Imanishi H, Mori T, Yanai T, Hayakawa A, et al. ssociation of genetic polymorphisms with hepatotoxicity in patients with childhood acute lymphoblastic leukemia or lymphoma. Pediatr Hematol Oncol 2010;27:344-54.
34. Yang L, Liu L, Wang J, Qiu L, Mi Y, Ma X, et al. Polymorphisms in folate-related genes: impact on risk of adult acute lymphoblastic leukemia rather than pediatric in Han Chinese. Leuk Lymphoma 2011;52:1770-6.
35. Mc Lead LH, Evans WE. Clinical pharmacodynamics of anticancer drugs. In: Derendorf H, Guenther H, eds. Handbook of pharmacokinetic/pharmacodynamic correlation. Boca Raton, Florida: CRC Press LLC, 1995:398.
36. Erdilyi DJ, Kámory E, Csókay B, Andrikovics H, Tordai A, Kiss C, et al. Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy. Pharmacogenomics J 2008;8:321-7.