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Association of TPMT (rs1800460) Gene Polymorphism with Childhood Acute Lymphoblastic Leukemia in a Population from Guilan, Iran

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant transformation and proliferation of lymphoid progenitor cells in bone marrow and blood, which is mainly found in children. Thiopurine methyltransferase (TPMT) is a thiopurine drug metabolizer enzyme that is prescribed for the treatment of ALL. Several single nucleotide polymorphisms in the *TPMT* gene have been reported to be associated with the decreased and deficient activity of the enzyme, which disrupts thiopurine drug metabolization and results in severe hematopoietic toxicity, which could be fatal in the patients. Since genetic screening before the thiopurine drug treatment of patients could be helpful for dosage optimization and efficient chemotherapy, this study was conducted to evaluate the association of G>A 146 *TPMT* gene polymorphism (rs1800460) with ALL susceptibility in an Iranian childhood population from the province of Guilan. This case-control study was performed on 400 individuals including 200 patients and 200 healthy children. Allele-specific PCR (AS-PCR) was applied to genotype the polymorphism of the *TPMT* gene. We found a significant difference in genotype distributions of G>A 146 *TPMT* polymorphism between patients and controls (p= 0.0001). Our results showed that individuals with the GA genotype had significantly increased risk of ALL (OR = 3.66, %95CI = 1.87-7.17, p = 0.0001), whereas individuals with the AA genotype were not associated with the increased risk of ALL (p= 0.2). This study may provide useful information for early diagnosis and an optimized strategy for the treatment of patients. However, more studies must perform for further characterization of this issue.

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Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common hematologic and childhood malignancy which forms up about 25% of all childhood cancer cases (Reisi *et al*., 2009). The ALL results from the malignant transformation and uncontrolled proliferation of lymphoid progenitor cells, especially in bone marrow and blood. About 80 % of ALL incidents observed in children; while the second peak occurs around the age of 50. The major symptoms of the disease include fever, weight loss, night sweats, easy bleeding or bruising, fatigue, lymphadenopathy, splenomegaly, or hepatomegaly (Terwilliger and Abdul-Hay, 2017). The standard protocols of the ALL treatment involve a daily oral administration of thiopurine drugs including, 6-mercaptopurine (6-MP), 6-thioguanine, and azathioprine (Abaji and Krajinovic, 2017). Thiopurine drugs are all

inactivated prodrugs which need to be converted into thioguanine nucleotides (TGN) to exert their effects on the target cells (McLeod *et al*., 2000). Thiopurine methyltransferase (TPMT) is a cytosolic enzyme responsible for S-methylation and thus, metabolization of thiopurine drugs (McLeod *et al*., 2000). Previous studies revealed that any deficiency related to the TPMT causes accumulation of TGN in Red Blood Cells (RBCs) resulting in severe hematopoietic toxicity which could be fatal in the patients (McLeod *et al*., 2000). In patients with decreased TPMT activity, the therapeutic dose of thiopurine drugs is decreased by 10-15 folds, while in case of complete TMPT deficiency the thiopurine drugs are avoided (McLeod *et al*., 2000).

Genetic variation in the *TPMT* gene is considered as the major factor affecting enzyme activity. *TPMT* gene is located on chromosome 6 and its alleles are inherited from both parents (Hindorf and Appell, 2012). Generally, several single nucleotide polymorphisms in the TMPT encoding alleles have been reported to decline the enzyme activity (McLeod *et al*., 2000). Although 20 polymorphisms in *TPMT* gene were identified so far, three mutant alleles including *TPMT**2 (238 G>C), *TPMT**3A (460 G>A, and 719 A>G) and TPMT*3C (719 A>G), account for the majority of mutant alleles of this gene. In the case of heterozygous inheritance of mutant alleles, the individuals show intermediate TPMT activity, while homozygote state usually causes no detectable functionality of the enzyme (McLeod *et al*., 2000; Kapoor *et al*., 2010). The rs1800460 single nucleotide polymorphism (SNP), which involves a substitution G/A at 460 bp at exon 7 of the *TPMT* gene, results in p.Ala154Thr variation and so remarkable decreased activity of the TPMT enzyme. This SNP in combination with a 719 A>G SNP accounts for the majority of *TPMT* gene polymorphisms in the studied populations (McLeod *et al*., 2000; Murugesan *et al*., 2010).

Genotyping of the *TPMT* gene before thiopurine therapy is an efficient strategy for the dosage optimization and thus efficient treatment of ALL patients (McLeod *et al*., 2000). Therefore, the current work was conducted to determine the frequency of 460 G>A (rs1800460) polymorphism in the *TPMT* gene among Iranian children from the province of Guilan. Also, the association between 460 G>A polymorphism with the risk of ALL was investigated.

Materials and Methods

Studied population

In this case-control study, the prevalence of the rs1800460 polymorphism in the *TPMT* gene among the children with ALL (200 cases) and healthy children (200 controls) was investigated. The ALL in the patients was confirmed by expert physicians and the patients were receiving chemotherapy. All the children were in an age range of 3-11 years and case and control individuals were matched based on their ethnicity, gender, and age. The informed consent was obtained from the subjects before participation in this study and Ethics approval was obtained from the Guilan University of Medical Science.

Genotyping

The genotyping of the rs1800460 polymorphism among the studied population was investigated using AS-PCR assay.

Peripheral blood samples (3 ml) from healthy and patient children were collected in tubes containing ethylene diamine tetraacetate (EDTA). The extraction of genomic DNA was performed using a DNA extraction kit (Gene Pajohan Co, Iran) according to the manual. Agarose gel electrophoresis was used to evaluate the quality of the extracted DNA. Allele-specific primers were designed by the Oligo 7 software (Table 1A).

The PCR assay performed to amplify wild type and mutant alleles of the *TPMT* gene. Table 1B provides the details of thermal cycling for amplification of wild type (G) and mutant (A) alleles of the *TPMT* gene. Finally, the PCR products electrophoresed on 2% agarose gel, and the results assessed as follows: the presence of one 246 bp band as wild type allele and presence of 435 bp band as an indicator for the mutant allele. Depending on the presence of either or both alleles, the individuals considered heterozygote and homozygote, respectively for the mutant allele.

Table 1. Sequences of the primers and PCR program.

A) Sequences of the primers					
Allele	**Primer**	**Sequence (5'→3')**	**Length (nt)**	**TM (°C)**	**GC (%)**
Wild type allele (G)	Forward	GACATGATTTGGGATAGAGGAG	22	55.9	45.5
	Reverse	CACCCAGGTCTCTGTAGTCA	20	57.3	55
Mutated allele (A)	Forward	GACATGATTTGGGATAGAGGAA	22	53.1	45
	Reverse	CAATACCTGGAATATGCAAG	20	53.5	40.9
B) PCR program					
Stage	**Temperature (°C)**	**Time (Sec)**	**Cycle**		
Initial denaturation	94	300	1		
denaturation	94	60			
annealing	56	45	30		
extension	72	60			
final extension	72	300	1		

Statistical analyses

Statistical analyses including chi-square, Odds-ratios (OR), and 95% confidence interval (CI) were performed using MedCalc software (version 12.1.4) to evaluate the association between the ALL risk with the genotype and frequency. The *p*-value of less than 0.05 was regarded as statistically significant.

Results

Genotyping of rs1800460 SNP was performed by using AS-PCR assay using allele-specific primers. In general, three genotypes as GG (wild-type homozygote), GA (heterozygote), and AA (mutant homozygote) were observed in control and patient groups.

According to the results, the frequency of G and A- alleles in the patient group were 59.25 % and 40.75 %, respectively; whereas in controls the G and A alleles had a frequency of 57.0 % and 43.0 %, respectively. Given the *p*-value = 0.72 for the allelic frequency analyses, there was no significant difference between healthy and patient children for this parameter.

Also, our results showed that among 200 patient children, 62 children (31%) had wild-type homozygote genotype GG, while 112 patients (56%) showed heterozygote mutant genotype GA, and 26 children (13%) were homozygote for the mutant allele (AA). On the other hand, in the control group the prevalence of wild type GG genotype was 46% 9 (n= 92), followed by GA genotype with the prevalence of 22.5 % (n= 45), and AA genotype (31.5%) (Table 2). Fig. 1 shows the amplification of A and G alleles of *TPMT* gene among the studied population.

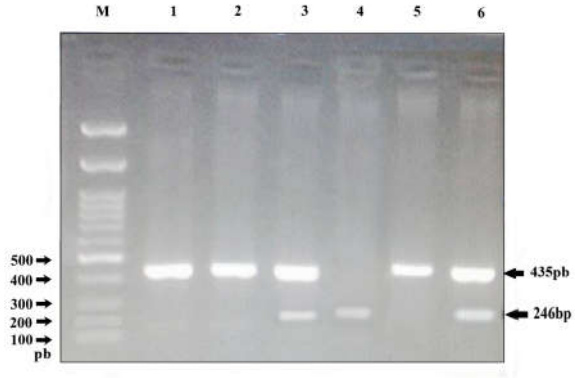

Fig. 1. Genotyping of the rs1800460 SNP using AS-PCR. Presence of a single 246 bp band as homozygous wild type genotype, a single 435 bp band as homozygous mutant genotype, two bands of 246 and 435 bp as heterozygote genotype. Lane M;100 bp DNA ladder, 1-6 amplified DNA samples.

There was a significant association between 460 G>A polymorphism of the *TPMT* gene and heterozygote GA genotype in ALL patients (*p*=0.0001). Due to the OR value > 1, the association of heterozygote genotype with the increased risk of ALL was noticed, while no significant association between the 460 G>A polymorphism of the *TPMT* gene and homozygote genotypes was found (*p*=0.2). (Table 3).

Discussion

Acute leukemia is the most common malignancy in children (Bomken and Vormoor, 2009; Terwilliger and Abdul-Hay, 2017). The disease usually appears with symptoms such as fatigue, fever, bleeding, infection, generalized lymphadenopathy, bone pain, and

hepatosplenomegaly. Also, inflammation of the central nervous system (CNS) and testis may be observed at the time of diagnosis or the relapse of the disease (Kaatsch, 2010; Lacour *et al*., 2010). Based on the literature, only 10 percent of childhood leukemia cases are clinically and epidemiologically justifiable, and the etiologic agent of 90% of cases remains unknown. Similar to other cancers, it seems that childhood leukemia is a multifactorial disease in which both environmental and genetic factors are involved (de Sousa *et al*., 2015; Jin *et al*., 2016).

Table 2. Genotype and allele frequency rs188460 polymorphism of the *TPMT* gene among the studied population.

	Genotype frequency n (%)			**Allele frequency n (%)**	
Polymorphism	GG	GA	AA	G	A
Controls	92 (46)	45 (22.5)	63 (31.5)	229 (57.3)	171 (42.7)
patients	62 (31.0)	112 (56.0)	26 (13)	236 (59.0)	164 (41.0)
	χ^2= 25.05, p= 0.0001			χ^2= 0.124, p= 0.72	

Table 3. Association of rs1800460 polymorphism of the *TPMT* gene with ALL risk

Genotype	Controls n (%)	Cases n (%)	OR	95% CI	*p*-value
GG	92 (46.0)	63 (31.0)	1.00	Reference	-
GA	45 (22.5)	112 (56.0)	3.66	1.87-7.17	0.0001*
AA	63 (31.5)	26 (13.0)	0.57	0.24-1.34	0.2

*statistically significant ($p < 0.05$)

Thiopurine drugs are commonly used for the treatment of ALL, particularly in the 2-3 years of maintenance therapy. Inside the body, these drugs are converted to TGN, which exerts their cytotoxicity against cancer cells by incorporation into the DNA and RNA molecules (Terwilliger and Abdul-Hay, 2017).

The proper metabolization of thiopurine drugs is crucially important to minimize the side effects of them. In other words, failure in proper drug metabolization causes accumulation of very high concentrations of TGN in RBCs which could be fatal in patients (Terwilliger and Abdul-Hay, 2017).

Thiopurine methyltransferase is an enzyme that transfers the heterocyclic sulfhydryl and aromatic compounds such as 6-mercaptopurine (Evans, 2004) and is involved with the metabolization of thiopurine drugs. Several gene polymorphisms are contributed to the reduced and/or deficient activity of the TPMT enzyme. Among the studied populations to date, the majority (90%) with wild type alleles have natural enzyme activity. The people with the heterozygous inheritance of the mutant allele show decreased activity of the enzyme (with a prevalence of 10%), while 0.3 % of populations with the homozygous inheritance of the mutant allele have no functional TPMT enzyme. Patients with low or moderate enzyme activity are exposed to the increased risk of hematopoietic suppression of bone marrow following receiving thiopurine drug medications (Schwab *et al*., 2002; Kim *et al*., 2010) and need to receive a lower dosage of the drugs (10-15 folds) or avoid thiopurine drug medications (Terwilliger and Abdul-Hay, 2017). Thus, a reliable screening method before thiopurine drug therapy is very helpful.

Previous studies reported genotyping of the *TPMT* gene as a reliable screening method for prediction of the toxicity of thiopurine drugs and dosage optimization (Kapoor *et al*., 2009, Kapoor *et al*., 2010). Several SNPs in the *TPMT* gene have been reported. Various studies reported the different prevalence of *TPMT* polymorphisms in different populations. Thus, in this study, the prevalence of rs1800460 SNP in the *TPMT* gene among patients with ALL and healthy children was investigated.

This study showed that the frequency of G and A-alleles in patient and control groups were not significantly different. However, analysis of genotype frequency showed a significantly higher prevalence of the heterozygous state of G>A 460 SNP among the patients, indicating a significantly increased risk of ALL with the heterozygous inheritance of the mutation.

Previous studies on the populations from different countries have been performed. Tantawy *et al*. (2017) reported the prevalence of 4.5 % for G>A 146 *TPMT* gene polymorphism

(rs1800460) in British Caucasians and a prevalence of 5.7 % among French Caucasians. Also, investigating the prevalence of TPMT gene polymorphism in a Turkish population revealed a prevalence of 5.8 % for the most common *TPMT* SNPs, with a prevalence of 1.7 % for G>A 146 *TPMT* gene polymorphism (Akin *et al.*, 2018). In another study on the Turkish children, Albayrak *et al*. (2011), reported a prevalence of 3.4 % for the *TPMT*3A* variant, as the most prevalent variant of the *TPMT* gene.

The prevalence of the *TPMT* gene polymorphism in Iranian children has rarely been investigated. In the only study performed on the *TMPT* gene polymorphism, Azad *et al*. (2009) reported the prevalence of 11.8 for the four *TPMT* polymorphisms in an Iranian subpopulation which was lower than our results. The difference between different studies contributes to the different population size and ethnicity of individuals.

In conclusion, an association between the heterozygote genotype of re1800460 SNP with increased risk of ALL in patients from Guilan, Iran was observed, while no significant difference for the frequency of mutant and wild type alleles was noticed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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ارتباط بین پلی‌مورفیسم ژن *TPMT* (rs1800460)با لوکیمی لمفوبلاستیک حاد کودکان در یک جمعیت از استان گیلان، ایران

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چکیده

لوسمی لنفوبلاستیک حاد (ALL) یک ترانسفورماسیون بدخیم و تکثیر سلولهای پیش ساز لنفوئید در مغز استخوان است که عمدتا در کودکان مشاهده می شود. تیوپورین متیل ترانسفراز (TPMT) یک آنزیم متابولیزه کننده داروی تیوپورین است که برای درمان ALL تجویز می شود. گزارش شده است که چند پلی مورفیسم تک نوکلئوتیدی در ژن TPMT با کاهش و نقص فعالیت آنزیم همراه است، که متابولیسم داروی تیوپورین را مختل می کند و منجر به سمیت شدید خونی می شود و می تواند در بیماران کشنده باشد. از آنجا که غربالگری ژنتیکی قبل از درمان دارویی تیوپورین در بیماران می تواند برای بهینه سازی دوز دارو و کارآمدی شیمی درمانی مؤثر باشد، این مطالعه با هدف بررسی ارتباط پلی مورفیسم ژن TPMT (rs1800460) G> A 146 با استعداد ابتلا در یک جمعیت کودکان ایرانی در استان گیلان انجام شد. این مطالعه مورد- شاهدی بر روی ۴۰۰ فرد شامل ۲۰۰ بیمار و ۲۰۰ کودک سالم انجام شد. از PCR اختصاصی آلل (Allele-specific polymerase chain reaction)برای ژنوتیپ چند شکلی ژن TPMT استفاده شد. در توزیع ژنوتیپ پلی مورفیسم TPMT 146 G> A بین بیماران و افراد کنترل تفاوت معنی داری مشاهده شد ($P = 0.0001$) . نتایج نشان داد که افراد با ژنوتیپ GA به طور معنی داری خطر ابتلا به لوسمی لنفوبلاستیک حاد را دارند ($OR = 3.66$, %95CI = 1.87-7.17, $p = 0.0001$) ، در حالی که افراد با ژنوتیپ AA با افزایش ریسک لوسمی لنفوبلاستیک حاد همراه نبودند ($P = 0.2$) . این مطالعه ممکن است اطلاعات مفیدی را برای تشخیص زودهنگام و یک استراتژی بهینه را برای درمان بیماران فراهم کند. با این حال باید مطالعات بیشتری برای روشن شدن این موضوع صورت بگیرد.

واژگان کلیدی: لوسمی لنفوبلاستیک حاد؛ سرطان خون کودکان؛ پلی مورفیسم TPMT، تیوپورین متیل ترانسفراز

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