Original Article

Common Polymorphism's Analysis of Thiopurine S-Methyltransferase *(TPMT)* in Iranian Population

Mehdi Azad, M.Sc.[1], Saeid Kaviani, Ph.D.[1]*, Masoud Soleimani, Ph.D.[1], Mehrdad Noruzinia, Ph.D.[1], Abbas Hajfathali, M.D.[2]

1. Hematology Department, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Hematology Department, Taleghani Hospital, Shahid Beheshti Medical Sciences University, Tehran, Iran

** Corresponding Address: P.O.Box: 14115-199, Hematology Department, School of Medical Sciences*
Tarbiat Modares University, Tehran, Iran
Email: kavianis@modares.ac.ir

Abstract

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Objective: Thiopurine S-methyltransferase (*TPMT)* catalyses the S-methylation of thiopurine drugs. Low activity phenotypes are correlated with several mutations in the *TPMT* gene and adverse drug reactions. The molecular basis for dissimilar enzymatic activity of *TPMT* has been established in Caucasians, African-Americans and Southwest Asians, but it remains to be elucidated in Iranian population. Until present, no study on Iranian population has been performed on the known alleles of *TPMT*. The aim of this study was to investigate the frequencies of four of the most common variants of this gene.

Materials and Methods: This study was conducted during 2007 at the Department of Hematology, Tarbiat Modares University, Tehran, Iran. Using PCR-RFLP and allele specific PCR techniques, allelic variants of the *TPMT* gene *TPMT**2(G238C), *TPMT**3B (G460A), *TPMT**3C (A719G) and *TPMT**3A (G460A and A719G) were genotyped in a normal population of 127 Iranians.

Results: In this study *TPMT**2 showed a prevalence of 7.08%. *TPMT**3C and *3A were found in 2.47% and 2.18% of the samples, respectively. *TPMT**3B variant was not detected in Iranian subjects. 112 out of 127 participants showed homozygote wild type allele.

Conclusion: This study is the first to analyze *TPMT* allele frequencies in a sample of Iranian population and indicates that *TPMT**2 is the most common allele (7.08%) in this population. These results can help to organize national pretreatment strategies in patients with acute lympho blastic leukemia (ALL) or other diseases requiring thiopurine medication in their standard therapy.

Keywords: Thiopurine S-methyltransferase, Polymorphism Genetic, Pharmaco Genetics

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Introduction

Drug metabolizing enzymes participate in the neutralization of xenobiotics and biotransformation of these drugs. Polymorphisms in genes coding drug-metabolizing enzymes can alter the activity of the enzymes for their substrates (1). The anti-cancer prodrugs, 6-mercaptopurine (6-MP) and azathioprine (AZA), are metabolized by thiopurine S-methyltransferase (*TPMT)* and widely used to treat several diseases such as childhood Acute Lymphoblastic Leukemia (ALL), autoimmune hepatitis, myasthenia gravis and rheumatoid arthritis (2, 3).

Thiopurine S-methyltransferase (*TPMT*, MIM# 187680) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds like 6-Mercaptopurine (6MP) (4). The *TPMT* gene is localized on chromosome 6p22.3 and consists of 10 exons. *TPMT* hypo activity is inherited as an autosomal co-dominant trait that occurs in 1/300 of general population, also it appears as homozygosity in some polymorphisms causing complete enzyme inactivity. About 10% of individuals have intermediate activity because of heterozygosity (5, 6). Some polymorphisms of *TPMT* have been shown to interfere with normal optimum activity of this enzyme and cause AZA and 6-MP toxicity.

To avoid hematotoxicity associated with *TPMT*-deficiency, phenotyping and genotyping tests should precede along with thiopurine therapy. *TPMT* molecular pharmacogenetic studies resulted in the discovery of a series of variant alleles (containing single nucleotide polymorphisms; SNPs) associated with significantly decreased levels of *TPMT* activity (3). High *TPMT* activity, results in a greater production of inactive methylated metabolites, which reduces therapeutic efficacy. Con-

versely, low *TPMT* activity leads to accumulation of thioguanine nucleotides (TGNs) that confers higher therapeutic efficacy, but is associated with increased risk of severe myelotoxicity (2, 5).

To date, at least 23 single nucleotide polymorphisms in the *TPMT* gene (*TPMT**2-*23) have been identified which are associated with decreased or absent *TPMT* activity as compared to *TPMT**1, the wild-type allele (7, 8)

These alleles include *TPMT**2 (G238C), *TPMT**3A (G460A and A719G) and *TPMT**3C (A719G). They are accounted for more than 80% of *TPMT* gene polymorphisms in populations. *TPMT**2 that contains a G/C transition at position 238 is a rare allele in European Caucasians and African-Americans (9, 10). *TPMT**3A that contains two nucleotide transitions, G/A at position 460 and A/G at position 719, is the most prevalent allele in European Caucasians and also found in African-Americans and Southwest Asians (11, 12). *TPMT*3*Cthat contains an A/G at position 719 is found in European Caucasians, African-Americans and as the most prevalent allele in Chinese subjects. Testing for *TPMT* alleles is used to establish individualized doses of thiopurines such as 6-MP and AZA in patients with ALL and also while using steroids, antidepressants, benzodiazepines, immunosuppressive agents and macrolide antibiotics.

TPMT genotyping assays have been facilitated by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) (9), denaturing high performance liquid chromatography (HPLC) (13), sequencing (14) and melting-curve analysis (15).

Previously, Radiochemical assay was used to measure the enzyme activity (16); but more recently, methods based on HPLC have been reported (17, 18). All these methods are based on in vitro conversion of 6-mercaptopurine to 6-methylmercaptopurine or 6-thioguanine to 6-methylthioguanine, using S-adenosyl-L-methionine as the methyl donor. In these HPLC assays, the product of the enzymatic reaction is extracted by liquid–liquid or solid-phase extraction and is measured by HPLC with ultraviolet (UV) or fluorescence detection.

The general advantage of phenotyping is a reliable detection of an individual's enzyme activity. However, *TPMT* enzymatic activity may be influenced by disease status (e.g. impaired renal function) which making enzyme activity detection less reliable than genetic analysis.

There has been no published study on *TPMT* allele frequencies in Iranian Population. In this study, we investigated allele frequencies of the most prevalent alleles (i.e. *TPMT**2, *TPMT**3A, *TPMT**3B and *TPMT**3C) in Iranian population.

Materials and Methods

The study was performed from April 2007till December 2007, in Tarbiat Modares University, Tehran, Iran. This research was pre-approved by the ethical committee of Tarbiat Modares University. 2ml peripheral blood in ethylene diamine tetra acetic acid (EDTA) (0.5 mM) was obtained from 127 unrelated Iranian volunteers in Tehran's Shariati Hospital. Personal and family history was unremarkable. Genomic DNA was extracted from peripheral blood using DNG plus kit (Cinnagen Inc., Tehran. Iran). Primer design and restriction enzyme analysis was performed according to previous studies with minor modifications (19). In brief, an Allele-Specific PCR was used for analysis of the G238C mutation (*TPMT**2). DNA was amplified with 0.20 mM primers. P2C (5' -TAA ATA GGA ACC ATC GGA CAC-3')(reverse) and either P2W (5' - GTA TGA TTT TAT GCA GGT TTG-3') or P2M (5' -GTA TGA TTT TAT GCA GGT TTC-3') (forward) were used in the wild-type specific or mutant specific reactions, respectively. PCR ingredients were unchanged according to Yates et al (19). PCR amplification consisted of an initial denaturing step at 94ºC for 5 minutes followed by 35 cycles of denaturing at 94ºC for 30 sec, annealing at 57ºC for 30 sec and extension at 72ºC for 1 minutes. The final extension step was 72ºC for 5 minutes. All these steps were carried out in an Eppendorf Thermocycler (master cycler gradient). 254 bp PCR products were separated on a 1.5% agarose gel (Fig.1)

PCR-RFLP was set up to analyze G460A (*TPMT**3B) and A719G (*TPMT**3C) point mutations. A 694-bp fragment containing nucleotide 460 was amplified with 0.20 mM P460F (5' -AGG CAG CTA GGG AAA AAG AAA GGT G-3') and P460R (5' -CAA GCC TTA TAG CCT TAC ACC CAG G-3'). PCR amplification consisted of an initial denaturing step at 94ºC for 5 minutes followed by 33 cycles of denaturing at 94ºC for 30 seconds, annealing at 62ºC for 30 seconds and extension at 72ºC for 30 seconds. The final extension step was performed at 72ºC for 5 minutes.

In wild type alleles MwoI digestion will yield 2 fragments of 443bp and 251 bp long (Fig 1).

To analyze A719G polymorphism, a 373-bp fragment containing nucleotide 719 was amplified with 0.20 mM P719F (5' -GAG ACA GAG TTT CAC CAT CTT GG-3') and P719R (5' - CAG GCT TTA GCA TAA TTT TCA ATT CCT C-3'). PCR reaction consisted of an initial denaturing step at 94ºC for 5 minutes followed by 33 cycles of denaturing at 94ºC for 30 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 1 minutes.

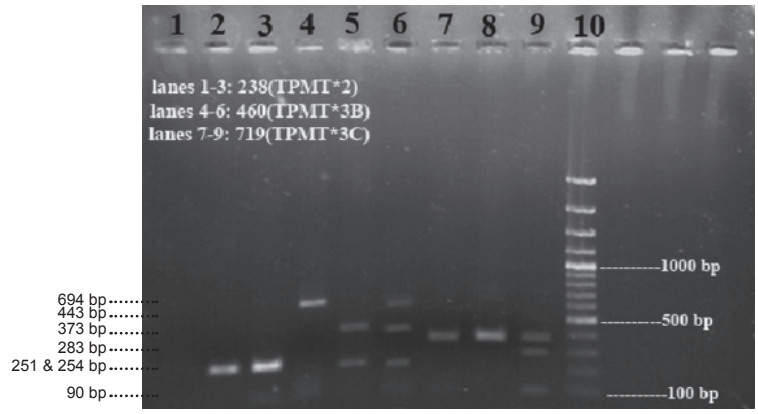

Fig 1: Electrophoresis patterns corresponding to migration of different alleles of TPMT revealed by PCR-RFLP and Allele Specific PCR (100 bp ladder – lane 10) on a 1.5% agarose gel. Lane 1 shows negative control. Lanes 2 and 3 show 254 bp PCR product corresponding to TPMT*2 polymorphism with wild type and mutation primers (wild type-lane 2 and mutant allele-lane 3). Lane 4 shows wild type and undigested fragment of 694 bp corresponding to TPMT*3B, Mwo I digestion of wild- type fragments yields fragments of 443 and 251 bp. Lane 5 shows two fragments of 443 and 251 bp and wild type TPMT allele (nucleotide 460). Lane 6 corresponds to heterozygote TPMT*3B polymorphism and shows three fragments of 694, 443 and 251 bp after Mwo I digestion. Lane 7 PCR product corresponding to allele TPMP*3C with 373 bp, Acc I digestion will not cut the wild type sequence. Lane 8 shows only a 373 bp fragment corresponding to wild type TPMT allele. Lane 9 shows three fragments of 373, 283 and 90 bp corresponding to heterozygote TPMT*3C polymorphism.

The final extension step was performed at 72ºC for 5 minutes.

In presence of mutation, AccI digestion will yield two fragments of 283 and 90 bp (Fig 1) (20). Samples with one deficient allele (*TPMT**1/*2, *1/*3C, *1/*3B, *1/*3A) were genotyped as heterozygous, and samples with two deficient alleles (*TPMT**2/*3C, *2/*3B, *3C/*3B, *2/*3A etc.) were genotyped as homozygous. The samples that carried both G460A and A719G mutations were named *TPMT* *3A.

Results

In this study, *TPMT* genotypes of the most prevalent mutant alleles (*TPMT**2, *TPMT**3A, *TPMT**3B and *TPMT**3C) were determined for Iranian normal subjects (Table 1).

11.8% (15 out of 127) of the samples carried at least one *TPMT* polymorphism. The individuals who carried none of these variants were named as *TPMT**1.

One hundred and twelve (88%) of the 127 subjects did not have any variation at nucleotide positions 238; 460 and 719 (i.e., G238C, G460A, A719G.), and carried the *TPMT**1/*1 genotype.

Eight individuals carried single nucleotide polymorphism G238C in heterozygote state, thus named as carriers of *TPMT**2. One sample showed *TPMT**2 allele in homozygote state. Four samples carried polymorphism A719G indicating to have mutant allele *TPMT**3C. Two samples carried both G460A and A719G mutations and were named *3A. No sample was found to carry polymorphism G460A alone (Table 2).

Table 1: Demographic data of 127 healthy Iranian subjects

Allele	SNP position	Aminoacid substitution	N	Frequency (%) (n = 254)
TPMT*1	Wild type		238	93.70
TPMT*2	238G>C	Ala80Pro	10	3.94
TPMT*3A	460G>A and 719A>G	Ala154Thr and Tyr240Cyc	2	0.79
TPMT*3B	460G>A	Ala154Thr	0	0
TPMT*3C	719A>G	Tyr240Cyc	4	1.57
Total			254	100

Table 2: Allelic frequencies of TPMT variants in a sample of 127 Iranian subjects (N= No. of alleles)

Demographic parameters	Iranian population
Number of subjects	127
Age range	5-70
Gender (female/male)	47/80
***TPMT* variants detected (n)**	*1/*2 = 8 *2/*2 = 1 *1/*3C = 4 *1/*3A = 2

Discussion

The molecular basis for low *TPMT* activity has been more elucidated with identification of the wild-type (WT) allele *TPMT*1* and three nonsynonymous single-nucleotide polymorphisms accounting for the majority of mutant alleles which lead to almost 50-fold variation in enzyme activity between individuals (14, 21). Patients with low or intermediate enzyme activity are at a higher risk to develop severe hematopoietic toxicity after receiving standard doses of thiopurine medications (22). Further epidemiologic studies has shown ethnic variance in frequency and allele prevalence(7).

So far, a high degree of concordance has been shown between *TPMT* genotype and phenotype in studied populations (19, 23). Based on these studies, it is suggested that both *TPMT* activity measurement and genotyping methods can be used for at risk individuals (24). However, genotypic analysis may be more accurate for determining the actual *TPMT* levels. For example, we know that heterozygous patients have intermediate activity and homozygous patients have low activity, but variability in enzymatic activity is seen between these groups (2, 3).

TPMT deficiency, inherited as an autosomal trait, causes severe hematopoietic toxicity in patients under standard dosages of mercaptopurine or azathioprine.

Table 3. Frequencies of TPMT variants in different populations

Population	N	*2	*3A	*3C	Ref
French	382	-	5.7	0.8	[6] {McLeod, 2002 #279}
British Caucasian	398	0.5	4.5	0.3	[14] {Collie-Duguid, 1999 #354}
Italian	412	0.4	3.9	1	[23] {Rossi, 2001 #301}
Norwegian	132	-	3.4	0.3	[30] {Loennechen, 2001 #294}
Saami Norwegian	388	-	0	3.3	[30] {Loennechen, 2001 #294}
Kenyan	202	0	0	5.4	[3] {McLeod, 1999 #336}
Ghanaian	434	0	0	7.6	[15] {Ameyaw, 1999 #357}
African-Americans	496	0.4	0.8	2.4	[31] {Hon, 1999 #356}
Caucasian-American	564	0.2	3.2	0.2	[31] {Hon, 1999 #356}
Chinese	400	0	0	3	[32] {Kham, 2002 #268}
Indian	400	-	0	2.3	[32] {Kham, 2002 #268}
Malay	400	-	0.5	0.8	[32] {Kham, 2002 #268}
Japanese	384	0	0	0.8	[22] {Hiratsuka, 2000 #309}
Egyptian	400	-	0	0.3	[33] {Hamdy, 2003 #231}
Kazak	654	0	0.3	0.9	[34] {Wei, 2005 #123}
German Caucasian	2428	0.2	4.4	0.4	{Schaeffeler, 2004 #176}
Brazilian	408	2.2	1.5	1	[26] {Boson, 2003 #230}
South-east Asian	698	0	0	1	[29] {Chang, 2002 #281}
Turkish	296	2	1	1.4	[27] {Tumer, 2007 #34}
Argentinean	-	0.7	3.1	0	[35] {Larovere, 2003 #226}
Bolivian	-	0	1	0	[36] {Lu, 2005 #127}
Swedish	-	0	3.7	0.4	[37] {Haglund, 2004 #199}
Thai	400	0	0	9	[38] {Srimartpirom, 2004 #179}
Mexican	218	0.9	3.2	1.4	[39] {Taja-Chayeb, 2008 #16}
Iranian	254	3.93	0.87	1.57	Present study

N =number of alleles

In absence of presymptomatic genetic analysis of such polymorphisms, the majority of at risk patients are identified only after a period of severe toxicity.

Genetic analysis helps to identify individuals even families with a higher cancer risk which permits specific treatment tailored for each individual and family group (25).

We found an overall frequency of *TPMT* alleles of 11.8% in the study of Iranian subpopulation. *TPMT**2 and *3 alleles are the most common mutant alleles in Caucasians (26). *TPMT**2 is the most prevalent mutation in some other nations like as Brazilian and Turkish populations (27, 28).

On the other hand, the most common genetic variant in American and European Caucasians is *TPMT**3A (7, 29). *TPMT**3C allele (A719G) with a frequency of 1.0% has been previously shown in several other ethnic groups including the Sámi, Kenyans, Ghanaians, African- American and Asians (11, 12, 30, 31). Finally, *TPMT**3B(G460A), the very uncommon allele, was not found in our subjects as in concordance to its very low prevalence in other populations (11, 14, 19) (Table 3).

In this study, four of the most prevalent *TPMT* mutant alleles, *TPMT**2, *TPMT**3A, *TPMT**3B and *TPMT**3C were genotyped in Iranian subjects. However, additional rare *TPMT* mutant alleles (*TPMT**3D,*4,*5,*6,*7,*8, and *10-*22) which have been identified recently need to be verified in Iranian Population (7-10). Polymorphisms in the 5'-flanking promoter region of *TMPT* gene have also been identified due to a variable number of tandem repeats (VNTR) with three kinds of motif (A, B, and C) differing by the length of the unit core and nucleotide sequence. However, a recent study did not show any impact on enzyme activity in patients with one of these VNTR alleles in two populations (32). Further studies are needed to sequence the open reading frame and promoter region of *TPMT* gene for novel mutations in Iranians.

Conclusion

This is the first study to elucidate the genetic basis of *TPMT* enzyme deficiency in Iranian population. Unfortunately, today no enzymatic or genetic assay is being done for patients with ALL in Iran. We hope that our results show the presence of genetic causes of hypoactivity in this population and help to provide genetic strategies to analyze these patients before beginning anticancer therapies. Further studies with more participants and analyzing more *TPMT* alleles, will be needed to establish nation wide pretreatment strategies among patients.

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