Evaluating Frequencies of Thiopurine S-Methyl Transferase (TPMT) Variant Alleles in Israeli Ethnic Subpopulations Using DNA Analysis

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ABSTRACT: **Background:** Traditionally, medication dosage was based on clinical and demographic parameters, but drug metabolism was recently recognized as an important factor for proper dosing and prediction of side effects. Metabolic considerations are crucial when administering drugs with a narrow therapeutic index, such as those of the thioguanides family (azathioprine and 6-MP). These can cause life-threatening myelosuppression due to low activity of a critical metabolic enzyme, thiopurine S-methyl transferase. A number of single nucleotide substitutions encoding variant enzymes account for most enzyme deficiencies.

Objectives: To determine the frequency of individuals from different Israeli ethnic groups who may be at risk for drug toxicity from drugs of the thioguanide family due to enzymatic variants.

Methods: DNA analysis was performed using polymerase chain reaction methods. We tested TPMT allelic variants TPMT*3A (G460A, A719G), TPMT*3B (G460A) and TPMT*3C (A719G) in five subpopulations in Israel: mixed-origin Israeli Jews, Arabs, Druze, Jews of Kurdish extraction, and Ethiopian Jews.

Results: The Druze (P = 0.0002) and Ethiopian Jewish (P = 0.015) subpopulations had a significantly unique distribution of allelic variants compared to the rest of the Israeli population. The Druze subpopulation showed a high number of TPMT variants with decreased activity, and a homozygote for TPMT*3A/ *3A was detected. Ethiopian Jews were found to carry mainly the TPMT*3C variant, also observed in other studies of African populations.

Conclusions: It is advisable that Druze patients be tested for the TPMT enzyme before starting treatment with 6-MP or azathioprine. Such testing may also be considered for other Israeli ethnic subgroups.

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KEY WORDS: pharmacogenetics, drug metabolism, adverse effects, 6-MP, azathioprine

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TMPT = thiopurine S-methyl transferase

The accumulation of knowledge on genetic variations among populations has enabled the development of what has come to be known as "personalized medicine." Much pharmacogenomic research has been conducted to enable identification of individual patients at risk for adverse effects of drugs. This is important when considering that iatrogenic severe side effects of medications are responsible for 6–7% of all hospital admissions, prolong the average hospitalization by 2 days and cause about 100,000 deaths each year in the United States [1].

The search for pharmacogenomic markers to identify patients most likely to have increased risk of toxic effects of drugs has focused on changes in genes that code for drug-metabolizing enzymes. A change in enzymatic activity can lead to the accumulation of a drug or its metabolites which can be toxic, especially when there is a narrow therapeutic index [1]. Drugs of the thioguanides family, such as azathioprine and 6-MP, are good examples of this type of medication. They can cause life-threatening myelosuppression due to low activity of a critical metabolic enzyme, thiopurine S-methyl transferase. Those drugs are used in many medical subspecialties such as hematology (for treating acute lymphocytic leukemia), dermatology (for pemphigus vulgaris), gastroenterology (for inflammatory bowel disease) and surgery (organ transplantation).

It has long been known that treatment with these drugs might be toxic and even life-threatening because of myelosuppression. As early as 1979, it was found that leukemia patients respond differently to 6-MP. Weinshilboum and Sladek [2] studied the activity of TPMT in the erythrocytes of 298 randomly taken samples. They observed a trivalent distribution in which 88.6% had high activity, 11.1% had low activity and 0.3% had undetectable activity. From that distribution, using the Hardy-Weinberg equation, they noted the existence of an autosomal co-dominant inheritance of two alleles encoding high and low TPMT activity [2].

Subsequently, pharmacogenomic studies have revealed that some individuals are deficient in an enzyme crucial for metabolizing thioguanides, known as thiopurine S-methyl transferase. In 1996, Szumlanski et al. [3] mapped the TPMT

gene to 6p22.3 using fluorescence in situ hybridization and found that it includes 10 exons and 9 introns. They also found that in exons 7 and 10 there are single nucleotide substitutions that encode mutant enzymes. Subsequently, many other studies in different populations found over 20 genetic variants that can cause decreased TPMT activity [4,5]. Due to its ability to define deficient individuals and the potential for serious toxic side effects following the use of thioguanides, TPMT was noted by the U.S. Food and Drug Administration in 2003 as being a valid biomarker for pharmacogenomic and pharmacogenetic studies [5].

Although there are no clear-cut recommendations as to who should be tested, in clinical practice many leading institutions test TPMT activity before administering 6-MP, for example, to pediatric patients with leukemia [5,6], or to inflammatory bowel disease patients before azathioprine treatment [7]. This is because there is a strong correlation between the presence of particular variant alleles and phenotype of increased sensitivity to drug toxicity [1,4,6].

Two studies of TPMT alleles have been performed in the Israeli population. The first, conducted at Ben-Gurion University by Lowenthal and colleagues in 2000 [8], used a radioactive assay to determine the activity of TPMT in red blood cell hemolysate from 134 Jewish males. The researchers concluded that the pattern of distribution of TPMT in the Jewish population of Israel is closer to that of East Asia than of Europe and North America [8]. This study used the classic methodology of testing enzyme levels in red cells, which correlate well with the levels in leukemic blast cells [5]. However, a drawback of this type of study is that some patients received RBC transfusions before blood could be taken for enzyme testing, leading to inaccuracy of enzyme measurements [6].

An alternative is DNA testing, which is more complex. One such study, by Efrati et al. [9], was conducted in Israeli patients in the northern part of Israel, and the results were published while the current study was in progress. The investigators [9] screened three Israeli subpopulations – Jews, Moslems and Druze – for TPMT genotype using PCR restriction fragment length analysis and high-resolution melting analysis. They found that each subpopulation has a unique TPMT allelic frequency.

The terminology of these variant enzymes is based on the amino acid substitutions at particular loci in the protein [3,5]. The wild-type gene is designated TPMT *1. The designation of some of the frequent mutant alleles is as follows: the TPMT*3A allele carries two mutations, at positions 460G→A and 719A→G [10]. The TPMT *3B allele carries only the first of these mutations (460G→A) while the TPMT *3C allele carries only the second (719A→G). It is therefore difficult to distinguish compound heterozygotes of TPMT*3B/*3C, who will have very low enzymatic activity (that of a mutant homozygote since both of his/her alleles are mutated) from the heterozygote TPMT *3A/WT who will have intermediate enzymatic activity as a heterozygote, since only one copy of the gene is inactivated [3,5,10].

The present study was designed to evaluate the relative frequency of common TPMT mutations in five Israeli subpopulations: mixed-origin Israeli Jews, Arabs, Druze, Kurdish Jews, and Ethiopian Jews. The variant alleles TPMT*3A, *3B,*3C and *2 account for more than 95% of the cases in the world's literature [5,11]. We therefore chose to study these alleles with the exception of the *2 variant, which has not been found previously in Israel.

PATIENTS AND METHODS

Anonymous blood samples from 570 unrelated healthy Israelis of Caucasian Jewish, Druze, Moslem, Kurd and Ethiopian Jewish descent, as defined by the subjects themselves, were used for DNA extraction. Genomic DNA was isolated from peripheral blood leukocytes using phenol-chloroform extraction. The study of genetic polymorphisms leading to variation in drug metabolism and disposition genes was approved by the Hadassah Hospital Helsinki Committee.

PCR AND RESTRICTION ENZYMES ANALYSIS

PCR fragments were digested as reported by Oender and co-researchers [11], with some modification. Exons 7 and 10 of the TPMT gene were amplified using PCR with the following primers: TPMT7F (5′ACGCAGACGTGAGATCCTAAT-3′);

TPMT7R (5′-TGATTGAGCCACAAGCCTTA-3′); TPMT10F (5′-AATCCCTGATGTCATTCTTCATAG-3′); and TPMT10R (5′-CACATCATAATCTCCTCTCC-3′). The total reaction volume was 20 µl, consisting of 50 ng/µl DNA (2 µl), 20 ng/µl forward primer (1 µl), 20 ng/µl reverse primer (1 µl), DDW (6 µl) and 10 µl of GoTaq-Green-Master-Mix (Promega, USA) consisting of DNA polymerase, dNTPs and buffer. A hot start incubation at 95°C for 3 min, followed by 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec and a final extension step at 72°C for 7 min, was performed. PCR fragments were analyzed on agarose gels and restriction enzyme analysis was performed. The A719→G mutation in exon 10 introduces an AccI restriction enzyme cleavage site. The digestion mixture contained 0.25 µl of AccI restriction enzyme, 20 µl of PCR product and 2.25 µl 10x restriction enzyme buffer 4 in a total volume of 22.5 µl, which was incubated at 37°C for 2 hours. Similarly, the G460→A mutation in exon 7 abolishes a MwoI restriction enzyme cleavage site and was also detected using restriction enzyme digestion. The reaction mixture (23 µl) contained 0.5 µl MwoI, 20 µl of PCR product and 2.3 µl 10x restriction enzyme buffer 3, which was incubated at 60°C for 2 hours. Six microliters of the digests

RBC = red blood cell
PCR = polymerase chain reaction

were loaded on a 1.5% agarose gel and electrophoretically separated at 115 V for 30 min.

HIGH-RESOLUTION MELTING

PCR amplification was performed on DNA samples using a modification of the methods described by Efrati and co-researchers [9]. We used AmpliTaq Gold™ DNA Polymerase with the following primers:

- for G460A (variants TPMT*3A and TPMT*3B): 5′-TAGGACAAATATTGGCAAATTTGA-3′ and 5′-TTACCATTTGCGATCACCTGGATTCATGGCAAC-3′
- for A719G (variants TPMT*3A and TPMT*3C): 5′-GGTTGATGCTTTTGAAGAACG-3′ and 5′-CATCCATTACATTTTCAGGCTTT-3′ [8].

The total reaction volume was 20 µl, consisting of 50 ng/µl DNA (3 µl), 2.5 µM forward primer (3 µl), 2.5 µM reverse primer (3 µl), DDW (1.85 Ml), 0.15 µl of AmpliTaq Gold™ DNA Polymerase, 3 µl of dNTPs (0.2 mM), 2 µl buffer, 2 µl $MgCl_2$ (2.5 mM) and 2 µl SYTO®-9. A hot start incubation at 95°C for 10 min, followed by 30 cycles at 94°C for 30 sec, 60°C for 45 sec, 72°C for 30 sec and a final extension step at 72°C for 10 min, was performed. Reactions were carried out with the intercalating dye SYTO®-9 in an HRM-enabled real-time PCR, Rotor-Gene 6000 (Corbett, UK), raising the temperature by 0.02°C in each cycle.

STATISTICAL ANALYSIS

Confidence intervals, chi-square test, and Fisher's exact test were calculated using standard methods.

RESULTS

The study group comprised 570 individuals: 164 mixed-origin Caucasian Israeli Jews, 118 Arabs, 46 Druze, 73 Kurdish Jews and 169 Ethiopian Jews. They were screened for the most common TPMT low activity variants: TPMT*3A (G460A, A719G), TPMT*3B (G460A), and TPMT*3C (A719G). All of the samples were analyzed using restriction enzymes analysis. All of the mutated and some of the wild-type samples were analyzed again using high-resolution melting point analysis. No discrepancies between restriction enzyme analysis and HRM results were found. Figure 1 demonstrates a typical PCR analysis, using restriction enzymes to identify the mutation. Figure 2 demonstrates a typical HRM point analysis. The distributions of allele frequencies are shown in Table 1 and Figure 3.

The combination of variants (including WT) and their frequencies were distinctive for both the Druze ($P = 0.0002$) and the Ethiopian Jewish ($P = 0.015$) subpopulations. In contrast,

HRM = high-resolution melting

Figure 1. Restriction enzyme analysis of TPMT mutant alleles. **[A]** The G460→A mutation in exon 7 abolishes a MwoI restriction enzyme cleavage site. Thus, mutant homozygote has only one uncut fragment (418 bp), the digest (of a heterozygote sample resulted in three fragments (172, 246 and 418 bp), and the wild-type sample was completely digested in two fragments (172 and 246 bp). **[B]** PCR-amplified TPMT exon 10 was digested with restriction enzyme AccI. The A719→G transition mutation creates a new AccI restriction site. Digestion of a mutant homozygote resulted in two fragments (151, 250) and digestion of a heterozygote sample resulted in three bands (151, 250 and 401 bp), whereas the wild-type allele TPMT*1 remained undigested (401 bp)

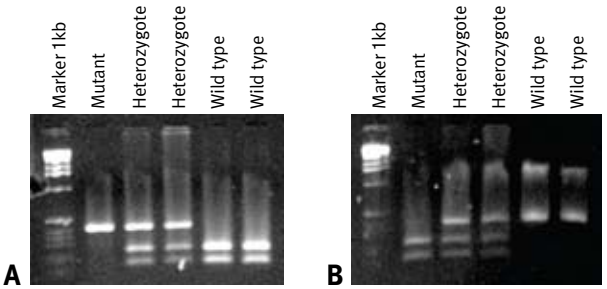

Figure 2. HRM analysis of four samples for the TPMT variant allele *3C. PCR products with the intercalating dye SYTO®-9 were melted by raising the temperature by 0.02°C in each cycle. The melting curves **[A]** are turned into normalized plots **[B]**, and then into difference plot normalized to the wild-type sample **[C]**. Genotypes are identified automatically by the software once references for wild-type, mutant homozygote and heterozygote are defined

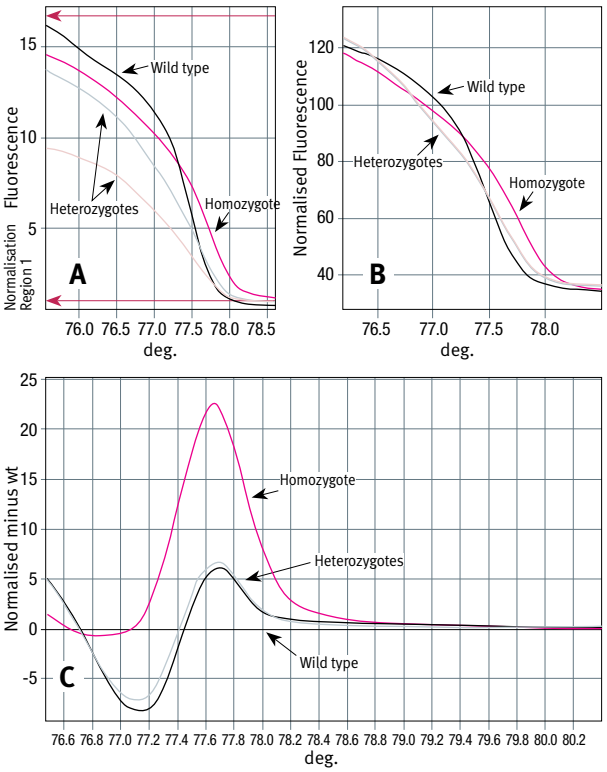

Figure 3. Distribution of the variant alleles and wild-type genotype for the different ethnic groups

Table 1. Cross-tabulation of ethnic origin and type of TPMT variant

			Genotypes				
			WT/WT	*3A/WT	*3C/WT	*3A/3A	Total
Origin	Arabs	Count	109	5	4	0	118
		% within origin	92.4%	4.2%	3.4%	0%	100%
	Jews	Count	161	2	1	0	164
		% within origin	98.2%	1.2%	0.6%	0%	100%
	Druze	Count	37	5	3	1	46
		% within origin	80.4%	10.9%	6.5%	2.2%	100%
	Kurds	Count	72	1	0	0	73
		% within origin	98.6%	1.4%	0%	0%	100%
	Ethiopian	Count	159	1	9	0	169
		% within origin	94.1%	0.6%	5.3%	0%	100%
Total		Count	538	14	17	1	570
		% within origin	94.4%	2.5%	3%	0.2%	100%

the mixed-origin Jews, Arabs and Kurdish Jews ($P = 0.1$) were not significantly distinct from each other regarding TPMT allelic distribution.

Similar to the results of Efrati et al. [9], homozygosity for a mutant allele was found only in an individual of Druze descent. Ethiopian Jews displayed a significantly higher TPMT*3C than the other ethnic subgroups.

DISCUSSION

The clinical relevance of TPMT genetic variants is well established, and it is important not only for toxicity [4,5] but for efficacy of therapy as well, as demonstrated in studies of treatment response in childhood acute lymphocytic leukemia [6,12]. TPMT has been studied at the genetic level in a variety of populations around the world [13]. It is clinically used before treatment with drugs from the thioguanides family in many leading institutions [5,6,14].

The Israeli population was studied only recently. Israeli ethnic groups are religiously distinct and intermarriage is relatively uncommon among them. It is important to specifically study Israeli ethnic subgroups since the frequency and distribution of mutant alleles may differ from those of other populations, as demonstrated in studies of thalassemia [15]. For this reason we chose to study the Kurdish Jews separately, which was not done in earlier studies in Israel. This ethnic group has previously been shown to have a unique distribution and high frequency of thalassemia alleles, differing from other subpopulations in Israel [16].

The distribution of the mutant alleles among Arabs and mixed-origin Jews was similar to that found by Efrati et al. [9]. The Kurdish Jews who were not studied by Efrati were not found to be different from Arabs or mixed-origin Caucasian Jews. We did find one *3C allele in a Jewish individual, which was not found in Efrati's study. We also studied Ethiopian Jews, who were not examined by Efrati and team [9], and found that this ethnic group had a unique distribution of mutant alleles, with a high incidence (5.3%) of carriership for the *3C allele. This variant is found less commonly in other ethnic groups in Israel and it is known to be relatively common in African populations [17].

We analyzed individuals from a different geographic region of Israel than was studied by Efrati and colleagues [9] and found some differences between our results and those previously published from Israel. This suggests that there might be a geographic variation in distribution of variant alleles in Israel even within the same ethnic groups. Such differences have been found in the geographic distribution of mutant alleles for beta-thalassemia in various regions of Israel [15]. Since the frequencies of some of the variants are low, a much larger study would be needed to confirm this apparent geographic variation with sufficient statistical significance.

The current study and that of Efrati et al. [9] clearly demonstrate that the Druze population of Israel is at high risk for bone marrow toxicity from drugs such as 6-MP and azathioprine, since the frequency of TPMT carriership for mutant alleles is much higher than in the rest of the world [19.6%]. We believe that before beginning treatment with thioguanides in this subpopulation it is mandatory to test for TPMT polymorphisms. The Druze are a relatively closed ethnic group and the high incidence of these variants may be due to founder effects, since they were demonstrated to carry several types of mutant alleles.

As for the entire Israeli population, the percentage of TPMT genetic variants is 5.6%, about half of what was described by Weinshilboum and Sladek [2]. Nonetheless, due to the high rates of consanguinity in some ethnic groups in Israel, which increase the likelihood of homozygosity for recessive alleles, we believe that testing for TPMT*3A and TPMT*3C should be considered for any Israeli patient before prescribing thioguanides.

Both the HRM and the PCR-based restriction enzyme analysis can be used to test for TPMT mutations. The HRM analysis may be superior when testing large numbers of samples. In the future, DNA chip technology may enable simultaneous testing for large numbers of even rare mutant alleles. However, the main obstacles to routine testing for TPMT alleles today

are not technical but are related to costs, availability of genetic testing, informed consent and other issues [13]. We hope that these barriers will be overcome so that patients can benefit from pharmacogenetic testing, which will enable individualization of therapy and maximize benefit, while minimizing the adverse effects of these potentially toxic agents.

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