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.

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

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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 specialties 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 thioguaninides, 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 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’-TGATTGAGCCACAAGCCCTTA-3’); TPMT10F (5’-AATCCCTGTAGTCATTCTTGATG-3’); and TPMT10R (5’-CACATCATAATCTCCCTCC-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 Accl restriction enzyme cleavage site. The digestion mixture contained 0.25 μl of Accl 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...
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 μl), 0.15 μl of AmpliTaq Gold™ DNA Polymerase, 3 μl of dNTPs (0.2 mM), 2 μl buffer, 2 μl MgCl2 (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. 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 Accl. The A719+G transition mutation creates a new Accl 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.

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,
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 also 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 order to 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 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.

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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**References**


**Capsule**

**HPV16 E2 is an immediate early marker of viral infection, preceding E7 expression in precursor structures of cervical carcinoma**

The viral E2 gene product plays a crucial role in the human papillomavirus (HPV) vegetative cycle by regulating both transcription and replication of the viral genome. E2 is a transcriptional repressor of the E6 and E7 viral oncoproteins for HPV types 16 and 18, which are involved in cervical cancers. Using new polyclonal antibodies against the HPV16 E2 protein, Xue et al. showed that E2 is expressed at various precursor stages of cervical carcinoma by immunohistochemistry on paraffin-embedded clinical samples. E2 was found to be highly expressed in the nuclei and cytoplasm of cells forming the intermediate and upper layers of cervical intraepithelial neoplasia (CIN). The authors could show that the expressions of E2 and p16INK4a (surrogate marker for oncogenic E7 expression) were exclusive in most of the cases, thus implying that E2 is not expressed together with high levels of E7. Moreover, they found that E2 is expressed in a subset of columnar cells adjacent to the CIN. The researchers could show that expression of E2 is topologically distinct from the proliferation markers p63 and Ki67, whereas it coincides with the expression of cytokeratin K13, a marker of squamous cell differentiation. Expression of E2 also topologically coincides with episcopal amplification of viral genomes in the upper layers of CIN1. These in vivo data thus validate previous assumptions of the crucial role of E2 in the early steps of HPV infection and of its negative link with expression of the viral E6 and E7 oncoproteins.

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Eitan Israeli

“Would the boy you were be proud of the man you are?”

Laurence J. Peter (1919-1990), Canadian educator and “hierarchiologist,” best known for the formulation of the Peter Principle