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FREQUENCY OF THIOPURINE S-METHYLTRANSFERASE MUTANT ALLELES IN INDIGENOUS AND ADMIXED GUATEMALAN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Abstract

Thiopurine S-methyltransferase (*TPMT)* polymorphisms affect the enzyme's activity and are predictive for the efficacy and toxicity of thiopurine treatment of acute lymphoblastic leukemia (ALL), autoimmune diseases and organ transplants. Because inter-ethnic differences in the distribution of these polymorphisms have been documented, we sequenced the *TMPT* gene in 95 Guatemalans, yet identified no new alleles. We also determined the frequency of the *TPMT**2, TPMT**3A, TPMT**3B* and *TPMT**3C* alleles in 270 admixed and 177 indigenous pediatric patients with ALL and healthy subjects from Guatemala using TaqMan assays and DNA sequencing. Among the 447 subjects genotyped, 10.0% of the ALL cases and 13.6% of the healthy controls were heterozygous for one of the four *TPMT* variants screened. The genotype frequencies in ALL and control populations were 0.7% and 1.7% for *TPMT**1/*2, 7.4% and 10% for *TPMT**1/*3A, 0.3% and 0% for *TPMT**1/*B, and 1.5% and 1.1% for *TPMT**1/*C, respectively (p= 0.30). No statistically significant differences between admixed and indigenous ALL (p= 0.67) or controls (p= 0.41) groups were detected; however 17% of the admixed healthy group bore one *TPMT* mutant allele and they have one of the highest reported frequencies of *TPMT* mutant allele carriers. Because of the clinical implications of these variants for therapeutic response, *TPMT* allele testing should be considered in all Guatemalan patients to reduce adverse side-effects from thiopurine drug treatments.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Keywords

TPMT gene; thiopurine therapy; childhood acute lymphoblastic leukemia; ethnic groups; Guatemala

INTRODUCTION

Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that metabolizes drugs, such as 6-mercaptopurine (6-MP), thioguanine (TG) and azathioprine (AZA), commonly used in chemotherapy and immunosuppressive therapies (8, 41). TPMT activity is a good predictive factor for the toxicity of these drugs and their effectiveness in the treatment of acute lymphocytic leukemia, autoimmune diseases and solid organ transplant. A very low or undetectable enzyme activity results in adverse pharmacokinetics and pharmacodynamics causing decreased turnover and resulting in myelosuppression, infections and secondary tumors (8, 10, 15, 39, 41, 44). It has been well established that the decreased levels of TPMT enzyme activity, is caused by single nucleotide polymorphisms (SNPs) in the *TPMT* gene. The most common alleles are *TPMT**2 (rs1800462, c.280G>A), *TPMT**3A (rs1800460, c. 460G>A and rs1142345, c.719A>G), *TPMT**3B (rs1800460, c.460G>A) and *TPMT**3C (rs1142345, c.719A>G) (1-5, 7, 9, 12, 14, 17-20, 22-25, 27, 28, 30-36, 40, 42, 43, 45-48, 51). These *TPMT* variants lead to a reduction in *TPMT* enzyme activity, and patients who are heterozygous for a *TPMT* mutant allele have an intermediate risk of hematological toxicity, whereas in *TPMT**3A homozygote subjects or compound heterozygotes the enzyme activity is very low or undetectable and the risk of toxicity is high (6, 8, 13, 20, 41, 47). Hence, independent groups have suggested a dose reduction of thiopurines or the selection of alternative therapies in mutant allele carriers to avoid side-effects that can be life-threatening due to therapy toxicity (21, 37). However, trinucleotide repeat alleles in the TPMT promoter have been described to also affect enzyme levels (38) and variants in ABCC4, a thiopurine transporter (26), and several thiopurine metabolic enzymes have also been documented to also influence thiopurine metabolism (reviewed in (11)).

Differences in *TPMT* allele frequencies among ethnic groups have been documented in the frequencies of the *TPMT* alleles with a low of 0.12% in Taiwan and 7.8% in Mexico (30, 48). The *TPMT**3A allele is the most common variant in Europeans (2-4.5%) and Latin-Americans (1.5-6.5%), whereas in Asian (0.3-1.2%) and African (1.3-7.6%) groups *TPMT**3C is the most frequent allele (1-5, 7, 9, 12, 14, 17-20, 22-25, 27, 28, 30-36, 40, 42, 43, 45-48, 51). Among Latin-American populations, *TPMT* analysis has been performed in South (Argentina, Brazil, Colombia, Chile and Bolivia) and North America (Mexico) but no data is available from Central-American (2, 7, 17, 24, 28, 31, 36, 48) populations. Hispanic populations have a very complex genetic background with high heterogeneity derived from the mixture of races during conquests, colonization, importation of slaves, and migration. Native-American, Caucasian and African contribution in different proportions explain the very diverse ethnicity of these groups (16, 29). Thus, there is a necessity to identify molecular biomarkers that could be used by clinicians as diagnostic and prognostic tools in each of these admixed populations, and to include indigenous populations in these efforts.

SUBJECTS AND METHODS

Subjects

We analyzed 447 subjects from Guatemala, 270 were children with a diagnosis of ALL and 177 were healthy adults. Oncologists at the major Pediatric Oncology Hospital (UNOP) in Guatemala City established the clinical diagnosis using standard morphologic, cytochemical and immunological criteria. After informed consent we collected saliva (Oragene, DNA-Genotek) or blood samples from all participants. Ethical approval was gained from the Medical Ethics Committee of the Francisco Marroquin School of Medicine, Guatemala. Participants identified themselves as either indigenous or Ladino (admixed), and data was collected on the place of birth and languages spoken of all parents and grandparents. Indigenous people speak one of 11 languages belonging to the Mayan linguistic group and Ladino refers to admixed (indigenous and European) populations, or European, or African descent.

DNA Sequencing and Genotyping analysis

Genomic DNA was extracted from saliva samples by using the ORAGENE Purification Kit (DNA Genotek Inc. Ontario, Ca.) according to the manufacturer's instructions. DNA purity and concentration was determined by spectrophotometry. PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3). Sequencing was performed on 95 subjects representing indigenous and non-indigenous cases, with ABI Big Dye reagents and performed on an ABI 3730XL instrument. Genotyping of the rs1142345 (exon 9) SNP was carried out by using TaqMan assay while the rs1800462 (exon 4) and rs1800460 (exon 6) variants were analyzed by sequencing. TaqMan PCR was carried out using the ABI PRISM 7900 system. PCR mix consisted of 10 ng of genomic DNA, 0.45 uM of each primer (TPMTY240CF: 5’-GAAGGTTGATGCTTTTGAAGAACGA and TPMTY240CR: 5’-ACATGTCAGTGTGTATCTATGTCTCA), 0.1 uM of each probe, 2.5 ul of TaqMan master mix (Applied Biosystems, Foster City, CA) and ddH2O up to a final volume of 5 ul. The amplification protocol included denaturing at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. The genotype of each sample was assigned automatically by measuring the allele-specific fluorescence using SDS 2.2.3 software for allelic discrimination (Applied Biosystems, Foster City, CA). A subset of random samples were genotyped in duplicate for rs1142345 and the reproducibility was 100%. To validate the TaqMan results, 3 samples of each genotype were sequenced using the forward and reverse primers (Ex-9F: 5’-GAATCCCTGATGTCATTCTTCA, Ex9R: 5’-CATTACATTTTCAGGCTTTAGCA). PCR was performed with the following conditions: an initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, and 68°C for 30 s, and a final extension at 72°C for 7 min. Sequencing was performed using an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). PCR and sequencing conditions described below were also used for the rs1800462 (exon 4) and rs1800460 (exon 6) analysis (TPMTEx-4F: 5’-CCCTCTATTTAGTCATTTGAAA, TPMYEx-4R: 5’-AAAACTTTTGTGGGGATATGG, TPMTEx-6F: 5’-GGGACGCTGCTCATCTTCT, TPMTEx6R: 5’-TTCAAACTCATAGAAGTCTAAGCTGAT).

Statistical analysis

The FINNETI algorithm (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to test Hardy-Weinberg equilibrium (HWE) for genotype distributions in both ALL cases and healthy groups. To evaluate the difference in the genotype and allele frequencies between populations, ethnic groups, or genders we used the Chi-square test (Stat-Calc program (EPIINFO 2005 V.3.2; Centers of Disease Control and Prevention, Atlanta, GA).

RESULTS

Subjects

A total of 270 children fulfilled the diagnostic criteria of ALL for this study, 186 (69%) were admixed and 84 (31%) were Indigenous; 156 (58%) were male whereas 114 (42%) were females. The healthy group consisted of 177 adults belonging to the same admixed (51%) and indigenous (49%) populations, 53 (30%) of them were males and 124 (70%) were females (Table 1). Most of the indigenous subjects speak Kaqchikel, K'iche' or Mam languages (83%)**,** whereas the rest are from Q'eqchi', Ah'chi, Q'anjob'al, Sakapulteco, Popti/ Jakalteco and Tz'utujil language groups (12%).

Genotyping

The distribution of the *TPMT* genotypes was in HWE in cases and controls, separately, and as a whole, and also when stratified by ethnicity and gender ($p > 0.05$). Genotyping analysis revealed that 10% (27/270) of ALL cases and 14% (24/177) of the healthy subjects carried one mutant allele, either *TPMT**2, *3A, *3B or *3C alleles ($p = 0.28$). A comparative analysis between admixed and indigenous populations showed no statistical differences in *TPMT* heterozygosity in either ALL or healthy groups ($p = 0.67$ and 0.41, respectively). In the admixed sample, 11% (20/186) of the ALL and 17% (15/88) of the control subjects were *TPMT* allele carriers ($p = 0.48$), whereas 8.3% (7/84) and 10% (9/87) of ALL and healthy indigenous subjects ($p = 0.74$), respectively carry at least one mutant allele. Of the 447 subjects included, only one homozygote mutant allele carrier was identified (0.2%). The *TPMT**3A allele was the most frequent *TPMT* allele detected, being found in 3.7% (20/540) of the ALL (14 admixed/6 indigenous) and 5.7% (20/354) of the control (10 admixed/9 indigenous) subjects. No statistically significant differences in the distribution of this allele between cases and controls or admixed and indigenous were observed. The *TPMT**2 and *TPMT**3C alleles were identified exclusively in admixed samples, 2 and 4 *vs.* 3 and 2 ALL patients and controls, respectively. The *TPMT**3C allele was present in admixed samples (ALL; 2.1% and healthy: 2.3%) but not in subjects from indigenous origin (Table 1). Stratification by gender did not show any statistically significant differences.

The frequency of *TPMT* mutant alleles in Guatemalan healthy subjects (7.1%) was comparable to those reported in Latin-America (Mexico: 5.3-7.8%, Bolivia: 6.5%, Argentina: 4.0%, Brazil: 4.8%, Colombia: 4.0%, Chile: 3.8%), European (3.5% - 5.5%) and some African (4-7.4%) populations. However the Guatemalan *TPMT* frequencies differ from most Asian and Middle-East ethnics groups (0.6-2.7%). As in other Latin-American (2.9 - 6.5%, except Brazil) and European (2.0 - 4.5%) populations, the *TPMT**3A was the most frequent inactive allele in Guatemala (5.6%) (Table 2).

DISCUSSION

TPMT genotypes have been proven to be an important molecular biomarker in the response prognosis of drugs currently used in the treatment of hematological malignancies, autoimmune diseases, and organ transplant (8, 41). On the basis of population studies, three alleles account for more than 95% of the clinically relevant *TPMT* variants: *TPMT**2, *TPMT**3A, *TPMT**3C and subjects who have absent or a reduced rate of enzyme activity than normal have higher circulating drug concentrations and are vulnerable to toxicity when the standard dosage is used. In single *TPMT* functional and non-functional allele carriers, the initial doses of AZA or 6-mercaptopurine should be reduced by 30-70%, whereas in homozygote non-functional allele carriers the doses of thiopurine drugs should be reduced 10-fold, or patients should receive alternative therapy (35). Therefore a simple test for *TPMT* genotypes can provide an important molecular biomarker that predicts drug response for hematological malignancies, autoimmune diseases, and organ transplantation (8, 41).

To begin to determine the distribution of alleles in Central America, we sequenced the entire *TPMT* gene in 95 Guatemalans, half of whom were indigenous peoples, and found only known alleles. We then genotyped the *TPMT*2*, *TPMT*3A*, *TPMT*3B* and *TPMT*3C* alleles in a cohort of 447 ALL and healthy subjects from Guatemala (admixed and indigenous ethnicities). The *TPMT*1/*3A* was the most common genotype found in Guatemala, and only one (0.85%) *TPMT**3A/*3A homozygote subject was identified. We did not find the compound heterozygote's *TPMT**2/*3A, *TPMT**2/*3B or *TPMT**2/*3C but our statistical power is insufficient to rule out the presence of *TPMT**3B/*3C carriers. Several studies have shown that patients who are homozygous or compound heterozygotes for TPMT mutant alleles are at a higher risk of severe bone-marrow suppression (29, 41, 49).

Comparative analysis between populations showed that Guatemalans (6.5%) exhibit a high frequency of the *TPMT**3A allele similar to that reported in Bolivia and independent studies from Mexico City (6.5%, 4.4% and 5.7%, respectively). Guatemala and Mexico both contain diverse populations; with both ancient and recent admixture between Amerindian, European and African people; and both countries have significant Mayan indigenous populations. Although the ancestral indigenous population lived in a variety of ecological circumstances in a broad swath of the Americas, anthropological and archeological studies demonstrate that the Mayans all share certain cultural features derived from the Olmecs (21). These features include Mayan hieroglyphic writing, complex calendars, and a sophisticated knowledge of astronomy. On the other hand, anthropological study shows that Mexican and Guatemalan mestizo populations are primarily a mix of Amerindian and Spanish peoples, which also could explain the high similarities between the two populations. These observations are consistent with the gene frequencies that we observe among the common polymorphisms at the TPMT locus within and between these populations.

Despite the complex composition of Guatemala populations, TPMT*C the major variant in African (1.3-3.8%) and Asian (0.3-1.0%) populations was not found in our sample. These data indicate a low intermixing of indigenous Guatemalans with African or Asian descendants, and do not support the hypothesis of a recent Asian origin in indigenous

population with an ancestral origin of the TPMT*C allele (3, 16). However a larger and more geographically diverse sample would be needed to confirm this result. On the other hand, Guatemalan admixed populations have one of the highest observed toxicity risk rates for treatments based on thiopurine drugs (17 %, see Table 1). This percentage could be an underestimate, considering that at least 20 additional mutant variants have been identified in *TPMT* (TPMT*3D, *4, *5, *6, *7, *8, *10, *23, *28, etc) and that SNPs in other genes, such as inosine triphosphate pyrophosphohydrolase (ITPA 94C>A) also affect thiopurine metabolism (3, 15, 29, 50).

In summary, this is the first study that assesses the *TPMT* variant allele frequencies in Guatemalan populations. Because at least one out of every 10 Guatemalans bears a *TPMT* mutant allele, genotyping could be performed in patients with hematological malignancies, immune diseases and organ transplants to avoid or reduce side-effects during treatments based on thiopurine drugs. Furthermore, additional studies are needed to characterize and identify polymorphisms in additional thiopurine metabolizing enzymes should be studied in Guatemalan and Central American populations.

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Table 1

Comparative analysis of the *TPMT* allele frequencies between patients and healthy individuals and by ethnicity.

	ALL cases		Total N=270	Healthy subjects		Total N=177*
	Admixed n= 176	**Indigenous n= 84**		**Admixed n= 88**	**Indigenous n= 87**	
Sex , (%)						
	59.7	53.6	57.3	22.7	37.9	31.1
Male	40.3	46.4	42.2	77.3	62.1	58.9
Female						
Genotypes, (%)						
*TPMT**1/ *TPMT* *2	1.1	0	**0.7**	3.4	0	**1.7**
*TPMT**1/ *TPMT* *3A	7.5	7.1	**7.4**	11.4	9.2	**10.2**
*TPMT**3A/ *TPMT* *3A	0	0	**0**	0	1.1	**0.56**
*TPMT**1/ *TPMT* *3B	0	1.2	**0.3**	0	0	**0**
*TPMT**1/ *TPMT* *3C	2.1	0	**1.5**	2.3	0	**1.1**
Total	10.7	8.3	**10.0**	16.9	10.3	**13.6**
P value	0.67			0.41		
Allele frequency, (%)						
TPMT *2	0.55	0	**0.035**	1.7	0	**0.8**
TPMT *3A	3.75	3.6	**3.7**	5.7	5.7	**5.7**
TPMT *3B	0	0	**0.015**	0	0	**0**
TPMT *3C	1.05	0.6	**0.75**	1.1	0	**0.6**
Total	5.4	4.2	**5.0**	8.5	5.7	**7.1**
P value	0.67			0.41		

Table 2

Worldwide distribution of the most common TMPT alleles in healthy subjects.

Region	Country	Total alleles	*2 (%)	*3A (%)	*3B (%)	*3C (%)	Deficiency alleles (%)	Reference
Central and South America	**Guatemala**	324	0.85	**5.6**	0	0.56	7 .1	**Current study**
	Colombia	280	0.4	3.6	0	0	4.0	[7]
	Argentina	294	0.7	3.1	0	0.2	4.0	[8]
	Brazil	408	2.1	1.5	0.2	1	4.8	[9]
	Bolivia	230	0	6.5	0	0	6.5	[10]
	Chile	420	0.24	2.9	0	0.71	3.8	[11]
North-America	Mexico	216 300 720	0.9 0 0.28	3.24 3.0 5.7	2.3 0.3 0.28	1.4 2.0 0.56	7.8 5.3 6.8	[12] [13] [14]
	USA Caucasian African-descendent	564 496	0.17 0.4	3.2 0.8	0 0	0.17 2.4	3.5 3.6	[15]
Europe	British	398	0.5	4.5	0	0.3	5.1	[16]
	Portugal	274	1.1	2.4	0	0.7	4.2	[17]
	France	608	0.7	3.0	0	0.4	4.1	[18]
	Sweden	1600	0.06	3.8	0.13	0.44	4.0	[19]
	Germany	2428	0.2	4.4	0	0.4	4.8	[20]
	Slovenia	388	0	4.1	0.3	0.5	4.9	[21]
	Sardinia	518	1.74	0.58	0.39	0.77	6.9	[22]
	Spain Spaniards Basque Gypsy	276 102 198	- - -	3.3 2.9 2.0	1.4 0.98 1.5	1.4 0 0	6.1 3.9 3.5	[23]
	Czech	1392	0.1	4.3	0.1	0.4	4.9	[24]
	Russia	1990	0.1	2.3	0	0.4	2.0	[25]
	Italia	1886	-	2.2	0.26	0.26	2.7	[26]
	Poland	788	0.38	3.15	0	0.13	3.7	[27]
Asia	Japan*	384 302	0 0	0 0	0 0	0.8 0.3	0.8* 0.3*	[28] [29]
	China	1404	0	0.02	0	0.9	0.92*	[30]
	Taiwan Aborigines	354 818	0	0	0	1.2 0.12	1.2* 0.12	[31]
	India	400	0	0.5	0	0.8	1.3*	[32]
	Tibet	100	0	0	0	1	1.0*	[10]
Middle-East	Turkish	212	0.9	0.9	0	0.9	2.7*	[33]
	Iran	1664 1000	2.16 0.1	1.68 0	1.62 0	0.54 2.5	6.0 2.6*	[34] [35]
	Jordania	338	0	1.2	0	0.6	0.89*	[36]

Region	Country	Total alleles	*2 (%)	*3A (%)	*3B (%)	*3C (%)	Deficiency alleles (%)	Reference
	Izrael	388	0	1.8	0	1.1	1.8*	[37]
Africa	Ghana	434	0	0	0	7.6	7.4	[16]
	Kenya	202	0	0	0	5.4	5.4	[38]
	Egypt	400	0	0.3	0	1.3	1.6*	[39]
	Mozanbique	472	0	0.2	0	3.8	4.0	[17]

* $p < 0.05$ statistical differences in the distribution of the mutant alleles among Guatemala and these populations