Thiopurine S-methyltransferase alleles, *TPMT*2*, **3B* and **3C*, and genotype frequencies in an Indian population

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Abstract. Thiopurine S-methyltransferase (TPMT) catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds including thiopurine drugs such as 6-mercaptopurine, 6-thioguanine and azathioprine. TPMT activity exhibits genetic variation and shows tri-modal distribution with 89-94% of individuals possessing high activity, 6-11% intermediate activity and approximately 0.3% low activity. Patients with intermediate or deficient TPMT activity exposed to thiopurine drugs show severe hematopoietic toxicity. Three single nucleotide polymorphisms (SNPs) in *TPMT* (NM_000367.2:c.238G>C, NM_000367.2:c.460G>A and NM_000367.2:c.719A>G) define the most prevalent mutant alleles associated with loss of catalytic activity reported in several populations. The present study investigated, for the first time, the frequency distribution of these three SNPs of *TPMT*, their alleles and genotypes in a Southern Indian population. Peripheral blood was obtained from 326 individuals of a Southern Indian population, and genomic DNA was isolated from total peripheral white blood cells. The genotypes at the polymorphic loci were determined by allele-specific polymerase chain reaction, restriction fragment length polymorphism and confirmatory DNA sequencing. The estimated genotype frequency for homozygous *TPMT*1/*1* was 97.24%, for heterozygous *TPMT*1/*2* and *TPMT*1/*3B,* 0.61% each, and for heterozygous *TPMT*1/*3C,* 1.53%. The frequency of heterozygous mutants in the studied Indian population was 2.76%. This study demonstrated significant variations in *TPMT* gene polymorphisms in an Indian population in relation to other human populations and may help to predict both clinical efficacy and drug toxicity of thiopurine drugs.

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Introduction

Thiopurine S-methyltransferase [TPMT: EC 2.1.1.67 (MIM 187680)] is a cytoplasmic enzyme present in both prokaryotes and eukaryotes. TPMT, first described by Remy (1), catalyzes the S-methylation of aromatic compounds including thiopurine drugs such as 6-mercaptopurine, 6-thioguanine and azathiopurine (2,3). Thiopurines are extensively used as chemotherapeutic agents in patients with neoplasia and autoimmune disorders. The prodrugs, thiopurines, are converted intracellularly to a thioguanine nucleotide form which exerts a cytotoxic effect. The first step in the intracellular activation of mercaptopurine involves being catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HPRT), which yields thioinosine monophosphate (TIMP). Subsequently, TIMP is converted to thioguanine triphosphate nucleotides, which are incorporated into DNA. Excessive accumulation of thioguanine (TGN), a cytotoxic compound, induces severe cytotoxicity. Fig. 1 illustrates the drug metabolic pathway for thiopurine drugs by TPMT and the subsequent conversion into an inactive substance thereby decreasing the amount of prodrug available for active TGN (2).

The level of TPMT activity in human tissue is controlled by genetic polymorphisms responsible for individual differences in thiopurine toxicity and therapeutic efficacy (4-6). Several studies have reported that patients with low or undetectable TPMT activity are at high risk for severe toxicity when treated with conventional dosages of thiopurine drugs, while similar doses may be adequate to treat patients with high levels of enzyme activity (5,6). Due to these important clinical consequences, it is suggested that patients be screened for *TPMT* genotype to determine the enzyme activity before thiopurine therapy. The active gene for the enzyme is 34 kb in length, consisting of 10 exons, and is localized at 6q22.3 (4,7). The *TPMT* pseudogene has also been reported and mapped to human chromosome band 18q21.1 (8).

TPMT enzyme activity shows trimodal distribution with 89-94% of individuals possessing high activity, 6-11% intermediate activity and 0.3% low activity. The wild-type allele for high TPMT activity has been designated *TPMT*1.* Several variant alleles for *TPMT* (*TPMT*2* to *TPMT*20*) have been reported in many ethnic groups. A list of known alleles of the *TPMT* gene is presented in Table I, with the type of variations and the loci. Among these, four mutant alleles namely

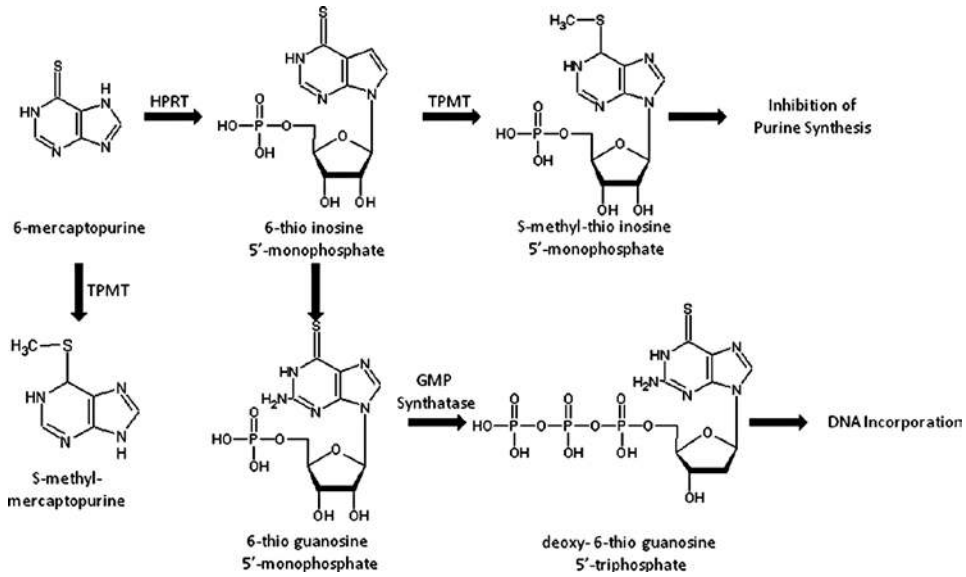

Figure 1. Pathway of thiopurine metabolism. TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

*TPMT*2*, *TPMT*3A*, *TPMT*3B* and *TPMT*3C* have been identified as responsible for enzyme deficiency in several populations. Another sixteen allelic variants, *TPMT*5* to *TPMT*20* (Table I), have also been suggested to be associated with deficient TPMT activity (9-11).

The first identified variant allele, *TPMT*2,* contains a transversion c.238G>C, leading to substitution of p.Ala80Pro residue. It is a rare allele reported to be found in European Caucasians and African-Americans (12-14). The *TPMT*3A* mutant allele contains two nucleotide transitions, c.460G>A and c.719A>G, in the open reading frame, leading to the substitution of amino acids p.Ala154Thr and p.Tyr240Cys, respectively, and are found in African-American, European Caucasians and Southwest Asians. It is the most common allele among the European Caucasian population (13,15). *TPMT*3B,* that contains the transition c.460G>A, is a common allele in Caucasian populations. *TPMT*3C* contains transversion c.719A>G and is the most prevalent allele among the Chinese population. However, no similar data are available on *TPMT* polymorphisms in Indian populations. Identifying the most prevalent *TPMT* allele in Indian populations could facilitate the deployment of rapid DNA-based assays for patients before they are subjected to thiopurine drug therapy. Thus, the aim of the study was to determine the frequency of *TPMT* variant alleles in an Indian population in comparison to other populations. The present study focused on the detection of signature alleles for the *TPMT* gene, *TPMT*2*, **3A*, **3B* and **3C* by using allele-specific (mutation-specific) oligonucleotide polymerase chain reaction (ASO-PCR), polymerase chain reaction, followed by restriction fragment length polymorphism (PCR-RFLP) analysis and by confirmatory DNA sequencing of the loci.

Materials and methods

Sample collection. Participants for the study were recruited randomly from among the population of Southern India. A total of 326 (176 males; 150 females) unrelated healthy

Table I. List of currently known mutant alleles of the thiopurine S-methyltransferase (*TPMT*) gene.

Allele	Nucleotide change	Amino acid change	Exon position
*TPMT*1*	Wild	Wild	
*TPMT*1A*	C178T	Silent	1
*TPMT*1S*	T474C	Silent	6
*TPMT*2*	G238C	Ala80Pro	5
*TPMT*3A*	G460A	Ala154Thr	7
	A719G	Try240Thr	10
*TPMT*3B*	G460A	Ala154Thr	7
*TPMT*3C*	A719G	Try240Thr	10
*TPMT*3D*	G292T	Glu98-Stop	5
	G460A	Ala154Thr	7
	A719G	Try240Thr	10
*TPMT*4*	G-A	Splice junction intron 9/exon 10	
*TPMT*5*	T146C	Leu49Ser	4
*TPMT*6*	A539T	Tyr180phe	8
*TPMT*7*	T681G	His227Glu	10
*TPMT*8*	G644A	Arg215His	10
*TPMT*9*	A356C	Lys119Thy	5
*TPMT*10*	G430C	Gly144Arg	7
*TPMT*11*	G395A	Cys132Thy	6
*TPMT*12*	C374T	Ser125Leu	6
*TPMT*13*	A83T	Glu28Val	3
*TPMT*14*	A1G	Met1Val	3
*TPMT*15*	G-A	Splice junction intron 7/exon 8	
*TPMT*16*	G488A	Arg163His	7
*TPMT*17*	C124G	Gln42Glu	3
*TPMT*18*	G211A	Gly71Arg	4
*TPMT*19*	A356C	Lys122Thr	5
*TPMT*20*	G106C	Gly36Ser	3

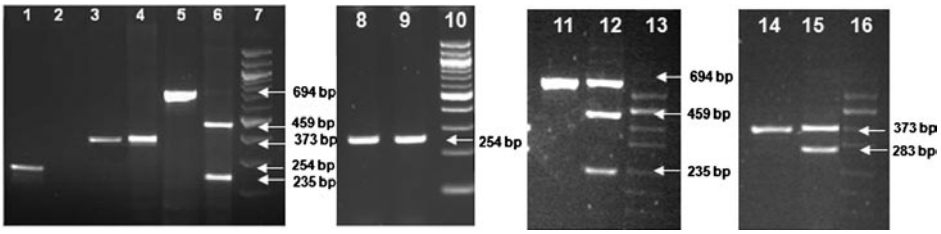

Figure 2. Images of PCR amplicons containing SNP loci of *TPMT*, NM_000367.2:c.238G>C, NM_000367.2:c.460G>A and NM_000367.2:c.719A>G, and their restriction fragments after agarose gel electrophoresis. All the lanes in the consecutive gel images are numbered sequentially from 1 to 16. Sizes of the DNA fragments are indicated by arrows shown in the image of 1.5% agarose gel electrophoresis. Lanes 7, 10, 13 and 16 show DNA size markers; 7 and 11 are 100-bp DNA ladder, and lanes 13 and 16 are pUC/*Hin*d III DNA ladder. Lanes 1-6 show the wild-type alleles of the three *TPMT* polymorphisms (NM_000367.2:c.238G>C, NM_000367.2:c.719A>G and NM_000367.2:c.460G>A) and their restriction enzyme digestion patterns; lane 1 shows the allele-specific amplicon representing the wild-type c.238G allele; lane 2, which is blank, corresponds to the mutant allele-specific reaction; lanes 3 and 4 represent PCR amplicon with c.719A allele and the *Acc*I restriction enzyme-treated and undigested PCR amplicons, respectively; lanes 5 and 6 show the wild-type c.450G PCR amplicon and its *Mwo*I restriction endonuclease digested fragments of sizes 459 and 235 bp, respectively. Lanes 8-16 show the gel images of the PCR amplicons with mutant alleles at the three *TPMT* SNP loci, NM_000367.2:c.238G>C, NM_000367.2:c.460G>A and NM_000367.2:c.719A>G, respectively. Lanes 8 and 9 show allele-specific PCR amplicons of 254 bp from a heterozygote for the c.238G>C polymorphism. Lane 11 shows PCR amplicon of the heterozygous c.450G>A and the *Mwo*I digested fragments of the amplicon giving DNA fragments of 559 and 235 bp, respectively, along with the wild-type undigested fragment of size 694 bp (lane 12). Lane 14 depicts the PCR amplicon of a heterozygote of c.719A>G SNP and the *Acc*I restriction enzyme-treated undigested mutant allele, and the PCR fragments with wild-type allele digested to fragments of 283 bp and a smaller one of 90 bp that is not shown in the gel (lane 15).

Indian individuals were recruited, with a mean age of 31.4 years (range, 18-69 years). Venous blood (4 ml) was obtained from each participant in an EDTA vacutainer. The study was approved by the Institutional Ethics Committee of Manipal University as per the guidelines of the Indian Council of Medical Research, and written informed consent was obtained from all participants.

Isolation of genomic DNA and polymerase chain reaction. Genomic DNA was isolated from all the samples collected by the standard phenol-chloroform extraction method (16), and the three major *TPMT* polymorphisms were genotyped in each sample. The genotypes of the *TPMT* gene were analyzed for the three SNP loci, NM_000367.2:c.238G>C, NM_000367.2:c.460G>A and NM_000367.2:c.719A>G, to determine the frequencies of *TPMT*2*, **3A*, **3B* and **3C* alleles and their genotypes. Genotyping of the NM_000367.2:c.238G>C locus was performed by ASO-PCR and those of NM_000367.2:c.460G>A and NM_000367.2:c.719A>G loci were performed by polymerase chain reaction, followed by PCR-RFLP. All PCR reactions were performed with 30-50 ng of DNA in 25 μl of the total reaction mixture containing 0.15 mM dNTPs, 20 pmol of each primer and 1 unit of Taq DNA polymerase.

Allele-specific oligonucleotide polymerase chain reaction for NM_000367.2:c.238G>C. The NM_000367.2:c.238G>C polymorphism was analyzed by ASO-PCR to test for the presence of the *TPMT*2* allele. DNA was amplified using a common reverse primer, 5'-TAAATAGGAACCATCGGAC AC-3' and forward primer with the single allele discriminating base at their 3' end used in a separate wild-type allele specific or mutant allele specific PCR reaction was either a) 5'-GTA TGATTTTATGCAGGTTTG-3' or b) 5'-GTATGATTTTATG CAGGTTTC-3', respectively, as previously reported. PCR thermal cycling conditions were: i) an initial 5-min denaturation at 94˚C; ii) 34 cycles at 94˚C (30 sec), 56˚C (30 sec) and 72˚C (60 sec); and iii) a final extension for 5 min at 72˚C. Amplified 254-bp PCR product was visualized by electrophoresis using 2% of agarose gel containing ethidium bromide (0.5 μg/ml).

*Genotyping of TPMT*3B and TPMT*3C by polymerase chain reaction-restriction fragment length polymorphism.* The *TPMT*3B* (NM_000367.2:c.460G>A) polymorphism was genotyped by employing PCR-RFLP. The forward primer for PCR was 5'-AGGCAGCTAGGGAAAAAGAAGGTG-3' and reverse primer 5'-CAAGCCTTATAGCCTTACACCCAGG-3'. PCR thermal cycling conditions were: i) an initial 5-min denaturation at 94˚C; ii) 34 cycles at 94˚C (30 sec), 62˚C (30 sec) and 72˚C (60 sec); and iii) a final extension for 5 min at 72˚C. The 694-bp PCR amplicon containing c.460G>A was digested with the restriction enzyme *Mwo*I, and the DNA fragments were separated on a 2% agarose gel to discriminate the alleles. The wild-type allele contains the *Mwo*I restriction site; however, the restriction site is absent in the mutant allele.

The analysis of the *TPMT*3C* polymorphism was performed by PCR-RFLP. DNA was amplified using the forward primer 5'-GAGACAGAGTTTCACCATCTTGG-3' and reverse primer 5'-CAGGCTTTAGCATAATTTTCAAT TCCTC-3'. PCR thermal cycling conditions were: i) an initial 5-min denaturation at 94˚C; ii) 34 cycles at 94˚C (30 sec), 62˚C (30 sec) and 72˚C (60 sec); and iii) a final extension for 5 min at 72˚C. The 373-bp PCR amplicon containing c.719A>G was digested with the restriction enzyme *Acc*I, and the DNA fragments were separated on a 2% agarose gel to discriminate the alleles. The mutant type allele introduces an *Acc*I restriction site which is absent in the wild-type allele.

DNA sequencing. The target *TPMT* PCR amplicons were sequenced in both forward and reverse directions for all the available genotypes for *TPMT*2*, **3B* and **3C*. Amplified PCR products were purified after eluting the fragments from the agarose gel using the standard isopropanol precipitation method, followed by direct sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction Mix Kit v3.1

Table II. Distribution of genotypes and alleles of thiopurine S-methyltransferase, 238G>C, 460G>A and 719A>G single nucleotide polymorphisms[a], in a Southern Indian population, tested for Hardy-Weinberg equilibrium.

Locus	Genotype counts (Frequency %)			Allele counts (Frequency %)		P-value[b]
	GG	GC	CC	G	C	
c.238G>C (rs1800462)	324 (99.39)	2 (0.61)	0	650 (99.69)	2 (0.31)	0.96
	GG	GA	AA	G	A	
c.460G>A (rs1800460)	324 (99.39)	2 (0.61)	0	650 (99.69)	2 (0.31)	0.96
	AA	AG	GG	A	G	
c.719A>G (rs1142345)	321 (98.47)	5 (1.53)	0	647 (99.23)	5 (0.77)	0.89

[a]Ref. seq: NM_000367.2, based on cDNA. [b]P<0.05 is considered significant (Chi-square test for Hardy-Weinberg equilibrium).

Table III. Functional variants of thiopurine S-methyltransferase (*TPMT*) gene alleles and their distribution in a Southern Indian population with their 95% confidence interval (CI).

Type of allele	No. of alleles	Percentage of allele	95% CI
*TPMT*1*	643	98.62	97.7-99.5
*TPMT*2*	2	0.31	0.0-0.7
*TPMT*3A*	0	0.00	-
*TPMT*3B*	2	0.31	0.0-0.7
*TPMT*3C*	5	0.76	0.1-1.4
Total	652	100.00	

(ABI, Foster City, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed in an ABI, 3130 genetic analyzer, and the electropherograms of the DNA sequences in sense and antisense directions were analyzed for detection of mutations using the Bioinformatics Tools and Human Genome Nucleotide Reference Sequence NT_007592.14.

Statistical analysis. Subjects were genotyped, and allele frequencies were calculated by counting the alleles. The 95% confidence interval (CI) was calculated for all proportions of alleles and genotypes. The Chi-square test was performed to test for deviation from Hardy-Weinberg equilibrium for each locus.

Results

Our study determined the allele and genotype frequencies of the three *TPMT* gene polymorphisms, c.238G>C, c.460G>A and c.719A>G in a sample group of 326 individuals drawn from a Southern Indian population. Agarose gel electrophoresis images representative of genotyping experiment results by ASO-PCR and PCR-RFLP are depicted in Fig. 2. A summary of the results of allele and genotype frequencies at the three loci (c.238G>C, c.460G>A and c.719A>G) are presented with their Chi-square test P-values in Table II. The allele and genotype frequencies involving *TPMT*1*, **2*, **3A*, **3B* and **3C* alleles in the population sample were also calculated with 95% CI and are presented in Tables III and IV, respectively.

Of the 326 healthy Indian participants investigated in this study, 317 were found to be homozygous for *TPMT*1/*1* (97.24%; 95% CI, 95.5-99.0). Two participants were heterozygous for the alleles *TPMT*1/*2* (0.61%; 95% CI, 0-1.5), 2 were heterozygous for alleles *TPMT*1/*3B* (0.61%; 95% CI, 0-1.5) and 5 were heterozygous for alleles *TPMT*1/*3C* (1.53%; 95% CI, 0.2-2.9) among all the individuals of the group. The *TPMT*3A* allele was not found in individuals from the investigated population in this study, and no individual was found homozygous for mutant alleles (Table IV). The allele and genotype frequencies did not show any deviation from Hardy-Weinberg equilibrium as shown by the Chi-square test results (χ^2=0.06; P=0.8).

Discussion

TPMT catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine and 6-thioguanine to form inactive metabolites, a pathway that competes with xanthine oxidase-mediated conversion of 6-MP to the inactive 6-thiouric acid and HPRT-mediated conversion of 6-MP into the active 6-thioguanine nucleotide. Patients with inherited low levels of TPMT enzyme activity are at increased risk for thiopurine-induced toxicity when treated with thiopurine drugs such as 6-mercaptopurine, 6-thioguanine and azathioprine; and those with inherited higher levels of enzyme activity may be treated inadequately. Since these drugs have a narrow therapeutic window, toxicities such as life-threatening myelosuppression assume great importance. The *TPMT* genetic polymorphism represents a good example of the well established clinical

Table IV. Distribution of genotype frequencies of thiopurine S-methyltransferase (*TPMT*) gene functional variants (involving *TPMT*1*, **2*, **3A*, **3B* and **3C* alleles) in a Southern Indian population and their Chi-square test result for Hardy-Weinberg equilibrium.

Genotype	No. of samples	Frequency (%)	95% CI
Distribution of genotypes for individual loci			
*TPMT*1/TPMT*1*	317	97.24	95.5-99.0
*TPMT*1/TPMT*2*	2	0.61	0.0-1.5
*TPMT*1/TPMT*3A*	0	0.00	0
*TPMT*1/TPMT*3B*	2	0.61	0.0-1.5
*TPMT*1/TPMT*3C*	5	1.53	0.2-2.9
Total	326	100.00	
Distribution of genotypes for all loci			
Homozygous wild (Total)	317	97.24	95.5-99.0
Heterozygous mutant (Total)	9	2.76	0.98-4.5
Homozygous mutant	0	0.00	0
Total	326	100.00	

Result of the Chi-square test for Hardy-Weinberg equilibrium for wild-type vs. all mutants[a], χ^2=0.06; P=0.8. [a]P<0.05 is considered significant, or P<0.05 is not consistent with Hardy-Weinberg equilibrium. CI, confidence interval.

Table V. Frequencies (%) of thiopurine S-methyltransferase (*TPMT)*2*, **3A*, **3B* and **3C* alleles reported in different populations of the world.

Population	No. of participants (n)	*TPMT*2*	*TPMT*3A*	*TPMT*3B*	*TPMT*3C*	Reference
Indians	326	0.61	0.0	0.61	1.53	Present study
African American	248	0.40	0.8	N/D	2.40	(9)
American Caucasian	282	0.20	3.2	N/D	0.20	(9)
Brazilian	306	0.80	2.0	N/D	2.50	(26)
Kenya	101	0.00	0.0	0.00	5.40	(27)
British Caucasian	199	0.50	4.5	0.00	0.30	(28)
South West Asian	99	0.00	1.0	0.00	0.00	(28)
Egyptian	200	0.00	0.3	0.00	1.30	(30)
French Caucasian	469	3.00	0.7	N/D	0.40	(31)
Italian	103	0.50	3.9	N/D	1.00	(32)
Norwegian Caucasian	66	0.00	3.4	N/D	0.30	(35)
Saami	194	0.00	0.0	N/D	3.30	(35)
Polish	358	0.40	2.7	0.00	0.10	(37)
South East Asian	300	0.00	0.0	0.00	1.00	(38)
Swedish	800	0.10	3.8	0.10	0.40	(39)
Thai	200	0.00	0.0	0.00	5.00	(40)

N/D, not determined.

importance of pharmacogenetic variation of a drug-metabolizing enzyme. The prevalence of various *TPMT* alleles in the Caucasian population is reported to be 0.3% which occur as homozygous mutants, 11% which occur as heterozygous and 89% as homozygous wild-types (17,18). Homozygous-mutated individuals are at increased risk for potentially life-threatening toxicity when exposed to standard doses of thiopurine drugs, while heterozygous carriers are advised to begin therapy with 50-60% of the recommended dose (19). The molecular basis of *TPMT* deficiency is well understood, where *TPMT*2*, **3A*, **3B* and **3C* are the most common variants detected in more than 80% of individuals with low or

intermediate TPMT activity (20,21). The frequencies of these alleles in various populations of the world that are available in published literature have been summarized in Table V.

To date, no studies have been carried out in the Southern Indian population to determine the frequencies of the *TPMT* alleles that are important in thiopurine drugs pharmacogenomics. In this study, we did not include an analysis for other alleles including rare variants such as *TPMT*3D*, **4* and **20*. The promoter polymorphisms were also ignored due to their minor effect on the modulation of TPMT activity compared with variation in the open reading frame. In this study, the *TPMT*2* and **3B* variants were found at a low allele frequency of 0.31% for each. No *TPMT*3A* allele was observed in the subjects studied that appeared to be significant. However, the absence of the *TPMT*3A* allele in this population needs to be confirmed through study of an appropriately large sample of the population. The most predominant mutant allele found in the Southern Indian population in this study was the *TPMT*3C* allele with a frequency of 0.76%, representing 55.6% of the mutant alleles investigated. Therefore, a test for this mutation alone in this population may prospectively identify approximately half the proportion of individuals at risk of hematopoietic toxicity in the event of administration of a regular dose of thiopurine drugs. From an anthropological point of view, the *TPMT*3C* allele was found in a Chinese (22) and in a Ghanaian population (23). It was the first *TPMT* mutation to arise in humans, and this mutation is also found in Caucasians, either alone in the *TPMT*3C* allele or in combination with the *TPMT*3B* allele in the form of *TPMT*3A* (24). It has been postulated that the ancestral *TPMT*3C* allele founded prior to the divergence of African and non-African populations was likely to have evolved to contain a second mutation giving rise to the *TPMT*3A* allele which predominated in American and European Caucasians. The presence of the *TPMT*3A* allele in African-Americans may be explained on the basis of genome integration due to interethnic admixture (9,25,26). Inhibitors of the activity of the enzyme may also have adverse consequences for carriers of these mutant alleles, whether homozygous or heterozygous. Furthermore, it should be noted that the true significance of this human genetic variation may be better explained in relation to the S-methylation of some endogenous substrates yet to be identified, as opposed to the metabolism of any group of drugs (41).

Our study was the first major study designed to analyze the genetic polymorphisms of *TPMT* in 326 randomly selected unrelated South Indian subjects from different locations in this region of the country representing the heterogeneous ethnic background of the population. Our study established the frequencies of the *TPMT* alleles. Overall, 2.76% of the Indian subjects were found to be heterozygous variants for *TPMT* alleles. Conformity for Hardy-Weinberg equilibrium for the loci suggest that no significant genotyping error was involved (Table II), that allele-based genetic effects may not have been biased and that the mutations are not relatively recent. Comparison of the mutation frequencies obtained in this study with those reported in different ethnic groups shows considerable difference between the Indian and other populations (Table V). The present study delineates more data on variations in the *TPMT* gene in Indian populations. Identification of such variant allele frequencies may help to predict both clinical efficacy and drug toxicity of thiopurine drugs in Indian populations and to introduce appropriate genetic screening protocols.

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