International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com

Research Article

THIOPURINE METHYLTRANSFERASE AS A THERAPEUTIC INDICATOR OF PURINE ANALOGUES: A PRELIMINARY STUDY IN SOUTHERN METROPOLIS

Sadik AM[1*], Siva Prasad S[1], Guru Prasad M[1], Sasikala M[1], Manu Tandon[2], Rupa Benerjee[2] and Nageshwar Reddy D[2]
[1]Asian Health Care Foundation, [2]Asian Institute of Gastroenterology, Somajiguda, Hyderabad, Andhra Pradesh, India

Received for publication: July 21, 2013; **Revised:** August 14, 2013; **Accepted:** September 11, 2013

Abstract: The purine analogues used to treat certain chronic diseases are occasionally associated with bone marrow suppression and leucopenia. They are metabolized by Thiopurine S-methyltransferase (TPMT) to methyl mercaptopurines. Thus optimal TPMT activity of an individual plays an important role in the treatment. However a wide interindividual variation of TPMT activity may be multi-factorial including inter and intra familial genotypic variation of TPMT gene. The present study was aimed to establish the reference range of TPMT activity and correlated with genotype. 165 healthy blood donors (Male-133. Female-32) were assessed for their TPMT activity by HPLC-UV method. The mean age of study cohort was 30.4±8.7 (19-58) and 150 of them were analyzed for TPMT *2, *3B and *3C polymorphisms by PCR-direct sequencing. The range of TPMT activity was found to be wide (3.93 to 35.80 $nmolml^{-1}h^{-1}$PRBC). The mean of the TPMT activity in total population was 15.98± 7.95, in females 10.99±3.44 and in males it is 15.19±7.64. All the participants studied for TPMT gene polymorphism were found to be wild type for all major polymorphic regions. The range of TPMT activity has a wide range. The wide reference range of enzyme activity may have multi-factorial basis. The frequency of hetero and homozygous variants of screened polymorphisms in TPMT gene may be less frequent in South Indian population.

Key words Thiopurine S-methyltransferase, Thiopurine drugs, HPLC

INTRODUCTION

Immunosupressants azathioprine (AZA) and 6-mercaptopurine (6-MP), the purine analogues are used in the treatment of chronic conditions such as rheumatoid arthritis, acute non-lymphoblastic leukemia, ulcerative colitis and inflammatory bowel diseases. Indiscriminate treatment with purine analogues may lead to bone marrow suppression and leucopenia[1]. The efficacy and toxicity of these drugs mainly depends on their incorporation into the DNA as thioguanine[2]. These purine analogues are metabolized by thiopurine S-methyltransferase (TPMT) to methyl mercaptopurines. Optimal TPMT activity plays an important role in the treatment of chronic conditions, and deficiency leads to greater conversion of the drug to the active form 6-thioguanine nucleotides, which increases the risk of developing toxicity. The wide inter individual variability exhibited by thiopurine analogues depends on several factors such as drug transport, drug metabolism, cellular targets and signaling pathways. Genetic polymorphisms of enzymes involved in AZA metabolism were also demonstrated to influence the therapeutic efficacy and toxicity of this drug[3]. Homozygous variants for *TPMT* mutations were shown to have increased risk of bone marrow toxicity during treatment with AZA, but all adverse effects of drug toxicity cannot be related to a mutated *TPMT* genotype[3,4,5]. It is also reported that AZA induced bone marrow toxicity may be potentiated by drug–drug interactions which can inhibit TPMT, such as sulphasalazine[6], olsalazine[7], salicylate and other salicylic acid derivatives. The pharmacogenetic approach regarding the individual TPMT genotype and/or the assessment of TPMT activity could provide a basis for optimal dosing of purine analogues, thereby achieving a reliable immunosuppressive effect and minimizing the number of drug-induced side effects[8].

Recent studies documented 0.03% frequency of polymorphisms in Singaporean populations [22]. There is no conclusive data from South Indian population in relation to TPMT reference ranges and associated polymorphisms. Hence the present study was aimed to establish the reference range of TPMT in normal healthy people and to evaluate the presence of associated polymorphisms that may affect the TPMT activity.

MATERIALS AND METHODS

A total of 165 healthy blood donors from Asian Institute of Gastroenterology were recruited into the study. All the members were in the age group of 19-58 years and they were screened by standard laboratory investigations. All those with less than 12gm/dl of hemoglobin were excluded from the study. The study protocol was approved by the Institutional Review Board (IRB) and informed consent was taken from all the participants. A total of 7 ml of whole blood was drawn and processed for both to determine enzyme activity and genotyping. 4ml was centrifuged and erythrocytes collected were washed with two volumes

***Corresponding Author:**
Sadik Ali Mahammad,
Research Scholar (Biochemistry) ,
Asian Health Care Foundation, Asian Institute of Gastroenterology,
Somajiguda, Hyderabad, India.

of phospho-buffered saline (PBS). 1 ml of erythrocyte suspension was lysed with 4ml of distilled water and centrifuged at 13,000g for 10 min at 4°C and supernatant was stored at −80°C until analysis. 3 ml was used for DNA isolation and genotyping.

Determination of the TPMT activity:

TPMT activity in erythrocytes was determined according to the Oselin *et al.*,[9]. The assay involves extraction with acetonitrile (ACN) after incubating the lysate for one hour at 37°C in a reaction mixture containing 125µl of 0.1M NaH_2PO_4 buffer (pH 7.4), 50 µl of 1mM 6-mercaptopurine (MP) as substrate, 25µl of 40µM S-adenosyl-l-methionine and 1mM dithiothreitol and the product 6– methyl mercaptopurines (6-MMP) was detected at 290 nm (All chemicals procured from St. Louis, Sigma USA). Extraction was performed by adding 2ml of ACN (Rankem Chemicals, New Delhi, India) to pre-incubated samples. Samples were vortexed and kept at −20°C for 30min; the supernatant, organic layer (2 ml) was removed immediately and transferred to another tube. After thawing, aqueous layer was extracted again with 1ml of ACN, as described above. The collected organic layers were added and evaporated to dryness in hot air oven at the temperature of 70°C. The residue was reconstituted with 100 µl of mobile phase. The extract (40 µl) was injected into HPLC system with 515 pump, C_{18} column (150 X4.6mm) 2489 UV absorbance detector (Waters Millipore, USA). THE MOBILE PHASE USED WAS Phosphate buffer: methanol (80:20) at P^H 7.9 and the flow rate was 1.3ml/min. The retention time of 6-MMP was 6.7 ± 0.2 min. TPMT activity was expressed as nm of 6-MMP formed per milliliter of packed red blood cells per hour at 37°c incubation (nmol$ml^{-1}h^{-1}$PRBC). The chromatographic software used was empower software for analysis purpose.

TPMT Genotyping

Genotype of three major functional polymorphisms such as, TPMT *2 (Exon 3, c, G238C, Ala80Pro), *3B (Exon 5, c, G460A, Ala 154 Tyro) and *3C (Exon 8, c, A719G Tyr 240 Cys) was analyzed by PCR amplification and direct sequencing method. DNA was isolated from peripheral blood samples by using previously described method[10]. Out of 165 participants 150 of them were genotyped for their TPMT polymorphisms. The flanking sequence of polymorphic regions was amplified by using specific primers as previously described[11,12]. Primer sequence, product size and annealing temperature for different polymorphic regions are shown in Table I. DNA engine, Bio-Rad Peltier thermal cycler-200 (Bio-Rad Laboratories Ltd, Hamel Hempstead, and Hertfordshire, UK) was used for amplification. PCR reaction was carried out in a total volume of 25ul, containing 100-150ng of template DNA, 5pmol of respective primer for each polymorphic region, 1X buffer (100mM Tris HCl [pH 8.8 at 25°C], 500mM KCl, 0.8% Nonidet p40) significant amount of $Mgcl_2$, 100µM of dNTPs and 1U/µl of Taq DNA Polymerase (Fermentas Life Sciences, USA). PCR products were purified and sequenced individually on both the strand using big dye terminator cycle sequencing ready kit.

Table.1: Primer used to amplify TPMT gene polymorphic regions.

Polymorphism	Primers (5'- 3')	Product size (bp)	Annealing temp. (°C)
TPMT *2	Forward: CCTGCATGTTCTTTGAAACCCTATGAA Reverse: CTTGAGTACAGAGAGGCTTTGACCTC	507	64
TPMT *3B	Forward: GAAACGCAGACGTGAGATCC Reverse: GCCTTACACCCAGGTCTCTG	399	58
TPMT*3C	Forward: CCACCATACCCAGCTCATTT Reverse: CCTCAAAAACATGTCAGTGTGA	382	59

Statistical analysis:

The comparison of TPMT values between male and female was made using student t test and results were expressed in mean ± standard deviation. The results were analyzed using SPSS-13.0 software.

RESULTS

The blood collected from healthy donors for the analysis of the TPMT included 133 males and 32 females. The mean age of participants was 30.4±8.7 years (19-58) years. The reference range of TPMT was analyzed between the male and female as well in different age groups and also compared with the other factors such as weight and percentage of hemoglobin. Table II represents the results of distribution of the TPMT activity in studied population classified as total population, males and female groups. The mean of the TPMT activity in total population was 15.19±7.64, in females 10.99±3.44 and in males it is 15.98±7.95. The combined population study showed a bimodal distribution of TPMT activity, none of the participants showed less than 3 units. The range of TPMT activity was wide (3.93 to 35.80 nmol$ml^{-1}h^{-1}$PRBC). Maximum TPMT activity was found in age group of 19-29 years (Figure 1). TPMT genotype was assessed in 150

participants. Clinically relevant polymorphic regions such as, TPMT *2,*3B and *3C were genotyped to correlate inter individual phenotypic (enzyme activity) variation but in the present study no allelic variation was observed in any of the participants. All the three polymorphic regions were found to be wild type in all participants (Figure 2, 3 and 4).

Table.2: Distribution of TPMT activity in study population

	NUMBER	AGE (Years)	WEIGHT (kilograms)	Hemoglobin (gm/dl)	TPMT mean ($nmolml^{-1}h^{-1}PRBC$)	TPMT SD	95% confidence interval
Male	133	31.3 (19-58)	73.1 (47-114)	15.1 (13-16)	15.98 (3.93-35.80)	7.95	17.33
Female	32	26 (19-52)	52.5 (40-65)	12.3 (10.5-15.6)	10.99 (5.86-19.62)	3.44	12.18
Total	165	30.4 (19-58)	69.8 (40-114)	14.7 (10.5-16)	15.19 (3.93-35.80)	7.64	15.89

Fig 1:

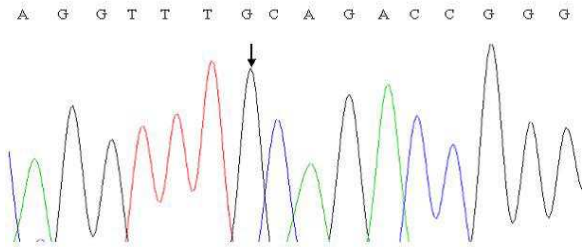
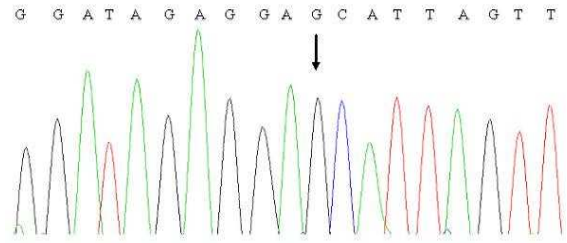
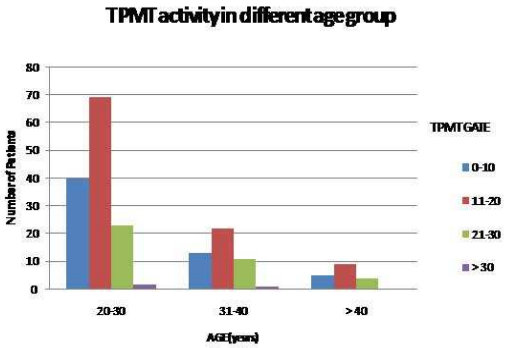


Fig 2:





DISCUSSION

The objective of the present study was to establish reference range of TPMT activity and associated polymorphisms in south Indian population. The study reports for the first time the reference range of the enzyme to be 3.93-35.8 nmol ml^{-1} h^{-1} PRBC in south Indian population. The main contribution of this report is 'the evaluation of reference range of TPMT activity' which is useful in administrating the thiopurine drugs.

The TPMT activity of the present study was found in accordance with other studies[12]. Other studies in different ethnic groups established the reference value almost similar to present study[13,14]. In contrast to the present study, low level of TPMT activity was noticed in Korean population[15].

TPMT gene exhibits signififi can't genetic polymorphism. At present, a total of 25 TPMT genetic polymorhisms, mostly SNPs, have been identifi ed. *TPMT* SNPs are, or may be associated with decreased levels of TPMT enzyme activity and thiopurine drug induced toxicity. Among these, the most common are: c.238G>C, c.460G>A and c.719A>G. There are several *TPMT* variant alleles comprising one or more SNPs. On the basis of population studies, three alleles account for more than 95 per cent of the clinically relevant TPMT variants: *TPMT**3A, *TPMT**3C and *TPMT**2, with the last of them contributing to a lesser extent8. Wild type has been designated as *TPMT**1. *TPMT**2 allele contains single c.238G>C polymorphism, *TPMT**3A allele has two polymorphisms c.460G>A and c.719A>G, while *TPMT**3C has only c.719A>G polymorphism.[24]

The TPMT genotype can predict the 1 in 300 with TPMT deficiency, who is at risk of myelo suppression when treated at standard AZA doses. The individuals with intermediate TPMT activity may experience a greater frequency of drug-induced side-effects. Genotype and phenotype (enzyme activity) correlation can, to some extent be predicted in homo and heterozygous genotypes where deficient and intermediate TPMT activity correlated respectively. However standard genotyping methods cannot predict those individuals with very high TPMT activities who may not respond to standard AZA doses[1].

A Previous study in Indian population report that Heterozygosity for TPMT *3C is 4.1% and *3A is 0.8%, where has in present study population no polymorphism is detected.[23]

About 1 in 300 Caucasians are homozygous variants for TPMT deficiency and at highest risk of life-threatening bone marrow suppression when treated with thiopurine drugs. Intermediate TPMT activity occurs in 11% of individuals and about 89% are homozygous for wild-type alleles, which result in high TPMT activity[16]. However in the present study we found neither heterozygous nor homozygous variants. All the study participants were found to be wild type for all three polymorphisms studied. Even patients with wild-type TPMT are susceptible to myelo suppression on long-term AZA therapy[8,17-20], Identification of TPMT genotype appears to be important in making the ALL treatment more effective and less toxic.[26] A previous study reported that a patient, exhibiting neutropenia on 6-MPwas observed to be G460A-homozygote, while, two Acute Lymphoblastic Leukemia (ALL) patients with side-effects exhibited wild-type alleles. Two patients showing 6-MP side-effects and responding well to the same drug at later stage also carried wild-type alleles. Thus the TPMT genotype may not be sole determining factor of treatment outcome. [25] it is also emphasized that TPMT genotyping failed to predict the majority of drug-induced neutropenias in patients on long-term AZA therapy[21].

Limitation of this study could be the genotyping of only major functional polymorphisms. The other major limitations include absence of large number of female healthy population. Screening of all the possible SNPs and studying multigenic interactions in causing immunosuppressive disorders in patients treated with regular doses of azathioprine might help in administering appropriate dosage fixation. Thus, the study evaluated and established the TPMT reference value in south Indian population and demonstrated non association of analyzed polymorphisms of TPMT gene.

ACKNOWLEDGEMENT

We duly acknowledge the financial support from Asian Healthcare Foundation.

REFERENCES

1. Clunie GPR, Lennard L. Relevance of thiopurine methyltransferase status in rheumatology patients receiving azathioprine. *Rheumatology* 2004; *43:* 13-18.

2. Elion GB. The purine pathway to chemotherapy. *Science* 1989; 244: 41-7.

3. Lennard L. TPMT in the treatment of Crohn's disease with azathioprine. *Gut 2002; 51:* 143-146.

4. Schwab M, Schaffeler E, Marx C, Fischer C, Lang T, Behrens C, Azathioprine therapy and adverse drug reactions in patients with inflammatory bowel disease; impact of thiopurine S-methyl transferase polymorphism. *Pharmacogenetics* 2002; *12* : 429-36.

5. Stocco G, Martelossi S, Barabino A, Fontana M, Lionetti P, Decorti G, et al. TPMT genotype and the use of thiopurines in pediatric inflammatory bowel disease. *Dig Liver Dis* 2005; *37* : 940-45.

6. Szumlanski C, Weinshilboum RM. Sulphasalazine inhibition of thiopurine methyltransferase: Possible mechanism for interaction with 6-mercaptopurine and azathioprine. *Br J Clin Pharmacol* 1995; *39:* 456-59.

7. Lewis LD, Benin A, Szumlanski C. Olsalazine and 6-mercaptopurine-related hematologic suppression: A possible drug–drug interaction. *Clin Pharmacol Ther* 1997; *62:* 464-75.

8. Colombel JF, Ferrari N, Debuysere H, Marteau P, Gendre JP, Bonaz B, et al. Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* 2000; *118:* 1025-30.

9. Oselin K, Anier K, Tamm R, Kallassalu K, Maeorg U. Determination of thiopurine S-methyltransferase (TPMT) activity by comparing various normalization factors: reference values for Estonian population using HPLC-UV assay. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; *834:* 77-83.

10. Miller SA, Dykes DD, Polesky H F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1998; *16:* 1215.

11. Lucia T, Silvia V, Olga G, Patricia O, Alfonso D,Myrna C. Thiopurine S -methyltransferase Gene (TMPT) polymorphisms in a Mexican population of healthy individuals and leukemic patients. *Med Oncol* 2008; 25:56-62.

12. Edna E, Lior A, Norberto K, Eli S. Distribution of *TPMT* risk alleles for thioupurine toxicity in the Israeli population. *Eur J Clin Pharmacol* 2009; 65: 257-262.

13. Tinel M, Berson A, Pessayre D, Letteron P, Cattoni MP, Horsmans Y, Pharmacogenetics of human erythrocyte thiopurine methyltransferase activity in a French population. *Br J Clin Pharmacol* 1991; *32:* 729-34.

14. Lowenthal A, Meyerstein N, Ben-Zvi Z. Thiopurine methyltransferase activity in the Jewish population of Israel. *Eur J Clin Pharmacol* 2001; *57:* 43-6.

15. Jacqz-Aigrain E, Bessa E, Medard Y, Mircheva Y, Vimer E. Thiopurine methyltransferase activity in a French population: h.p.l.c. assay conditions and effects of drugs and inhibitors. *Br J Clin Pharmaco* 1994; *38:* 1-8.

16. Jang IJ, Shin SG, Lee KH, Yim DS, Lee MS, Koo HH, et al. Erythrocyte thiopurine methyltransferase activity in a Korean population. *Br J Clin Pharmacol* 1996; *42:* 638-41.

17. Weinshilboum RM, Sladek SL. Mercaptopurine pharmacogenetics: Monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am J Human Genet* 1980; *32:* 651-62.

18. Dubinsky MC, Lamothe S, Yang HY, Targan SR, Sinnett D, Theoret Y, et al. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology* 2000; *118 :* 705-13.

19. Colombel JF,Ferrari N,Debuvsere H, Marteau P, Gendre JP, Bonaz B,et al.Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* 2000; *118 :* 1025-30.

20. Dubinsky MC, Yang HY, Hassard PV , Seidman EG, Kam LY, Abreu MT, et al. 6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease. *Gastroenterology* 2002; *122:* 904-15.

21. Naughton MA, Battaglia E, O'Brien S, Walport MJ, Botto M. Identification of thiopurine methyltransferase (TPMT) polymorphisms cannot predict myelosuppression in systemic lupuserythematosus patients taking azathioprine. *Rheumatology* 1999; *38:* 640–44.

22. Kham SK, Soh CK, Liu TC, Chan YH, Ariffin H, Tan PL, Yeoh AE. Thiopurine S- methyltransferase activity in three major Asian populations: a population-based study in Singapore. *Eur J Clin Pharmacol*. 2008; *64:*373-79.

23. Kapoor G, Maitra A; Somlata, Brahmachari V. Application of SNaPshot for analysis of thiopurine methyltransferase gene polymorphism. *Indian J Med Res.*2009; *129:*500-05.

24. Pavlovic S. TPMT gene polymorphisms: on the doorstep of personalized medicine. *Indian J Med Res*. 2009; *129:*478-80.

25. Kapoor G, Sinha R, Naithani R, Chandgothia M. Thiopurine S-methyltransferase gene polymorphism and 6-mercaptopurine dose intensity in Indian children with acute lymphoblastic leukemia. *Leuk Res*. 2010; *34:1023*-6.

26. Ashavaid TF, Raghavan R, Shah S, Kapadia A, Almel S, Desai D. TPMT and DPD polymorphisms: Efficient screening method for Indian patients considering taking Thiopurine and 5-FU drugs. *Dis Markers*. 2009; *27:*231-8.

Source of support: Asian Healthcare Foundation, India
Conflict of interest: None Declared