Thiopurine methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including mercaptopurine (MP), thioguanine (TG), and azathioprine (AZA). These thiopurine medications are currently used to treat many diseases, including cancer.

Application of SNaPshot for analysis of thiopurine methyltransferase gene polymorphism

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Background & objectives: Mercaptopurine, azathioprine, and thioguanine, used as antineoplastic agents and immunosuppressants are catabolized by thiopurine methyltransferase (TPMT) enzyme, which exhibits genetic polymorphism. Genotyping patients and the population to which the patients belong, is important for effective treatment and reducing toxicity. There is a need for faster methods for genotyping. Hence the present study was planned to test the application of SNaPshot technique for analysis of the three common TPMT alleles: TPMT*2, TPMT*3A, and TPMT*3C in DNA from healthy Indian volunteers as well as to apply the method on cDNA samples obtained from children with acute lymphoblastic leukaemia (ALL).

Method: A total of 120 healthy volunteers and 25 patients were analysed by multiplexed SNaPshot reaction. Genomic DNA was the template for most of the analyses, but additionally the cDNA synthesized for translocation detection was used as the template in case of patients with ALL. The results of SNaPshot reaction were confirmed by direct sequencing.

Results: The TPMT genotype could be reliably identified by SNaPshot analysis in multiplex reactions both in genomic DNA samples and cDNA. The overall frequency of the three common polymorphisms was observed to be 4.9 per cent, arising from heterozygosity for TPMT*3C (4.1%) and TPMT*3A (0.8%).

Interpretation & conclusion: SNaPshot method for TPMT polymorphism analysis works faster with the potential for high throughput. By simultaneously interrogating the genotype at multiple sites, the method can provide future opportunity to multiplex, though multiplexing has not been done in the present analysis. Heterozygosity for TPMT*3C (119 A>G) was detected in 4.1 per cent of the study population and no homozygosity was observed. Our results indicated that TPMT*3C was the most common polymorphism in Indian population, while TPMT3*A, associated with the absence of catalytic activity of TPMT, was very rare.

Key words: ALL - Indian population - polymorphism - SNaPshot - TPMT

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autoimmune disease and as immunosuppressants after organ transplantation. The human TPMT gene exhibits single nucleotide polymorphism, and TPMT activity is inherited as an autosomal codominant trait. Patients with TPMT deficiency accumulate higher levels of thioguanine nucleotides in erythrocytes if they receive standard doses of mercaptopurine or azathioprine. This accumulation of nucleotides usually leads to severe haematopoietic toxicity and possibly death. The TPMT enzyme catalyzes S-methylation of mercaptopurine to methylmercaptopurine, which is the inactive form. Patients inheriting TPMT deficiency have a pronounced risk of potentially life-threatening haematopoietic toxicity when treated with conventional doses of these medications and patients with heterozygosity at the TPMT locus have lower activity and an intermediate risk of toxicity.

TGN concentration is correlated with both efficacy and toxicity of thiopurines. Three out of 19 polymorphisms known in the TPMT gene account for 80-95 per cent of the intermediate or low activity of the enzyme in Caucasians. In view of the reported ethnic differences in the incidence of the various single nucleotide polymorphisms (SNPs), it is important not only to evaluate the genotype of TPMT alleles of the patients but also the general frequency of the alleles / SNPs in the population, to optimize the effective drug dosage to maximize beneficial effects and avert severe haematological toxicity.

The methods presently utilized for analysis of TPMT polymorphism involve amplification of relevant fragment by PCR followed by restriction fragment length polymorphism (RFLP) analysis. In these protocols both the steps of analysis are carried out in separate reactions for each polymorphism. In order to enhance the robustness and throughput of genotyping the TPMT polymorphisms in the present study, SNaPshot was used. This single tube technique is based on PCR amplification of the desired region from genomic DNA, followed by a single base extension of the interrogating oligonucleotide primer.

In this study the application of SNaPshot technique was tested on a small scale of multiplex for analysis of the three common TPMT alleles in Indian population. The wild-type has been designated as TPMT*1, the 3 alleles tested include TPMT*2 allele that contains a single transversion at 238 G>C, TPMT*3A allele that contains two nucleotide transition polymorphisms 460 G>A and 719 A>G, and TPMT*3C allele that contains only a single transition polymorphism 719 A>G. This method is generally used for genotyping of genomic DNA samples but in this study it was also used to genotype patient cDNA samples, thus successfully extending the scope of the application of this technique.

Material & Methods

The study population consisted of 120 genomic DNA samples (from our laboratory at Dr B.R. Ambedkar Center for Biomedical Research and Institute of Genomics and Integrative Biology, New Delhi) from normal healthy volunteers belonging to various regions of India, and cDNA samples from 25 children with ALL (Rajiv Gandhi Cancer Institute and Research Centre, Delhi). Informed consent from the patients/their parents was obtained as per the guidelines of the institutional ethics committee of the Rajiv Gandhi Cancer Institute and Research Centre, Delhi.

DNA was extracted from peripheral blood samples of healthy volunteers using the Genomic DNA Mini Kit (Geneaid, India) as per manufacturer’s instructions. The cDNA was synthesized using M-MuLv reverse transcriptase (Fermentas International Inc, USA), and RNA was extracted from bone marrow cells of patients using QIAmp RNA Blood Mini Kit (Qiagen, Germany), according to company’s manual.

Three TPMT alleles- TPMT*2, TPMT*3A, and TPMT*3C were analysed. The regions spanning these polymorphisms were amplified by PCR as described by Kham et al with a newly designed oligonucleotide primer for TPMT*2 (G238C) (TPMT*-2F: TCTTTGAAACCCTATGAACCTG). The primer sequences for analysis of cDNA samples are listed in Table I. Oligonucleotides were synthesized at Microsynth GmbH (Balgach, Sweden). The cycling for amplification consisted of denaturation at 94°C and extension at 72°C each for 1 min and PCR was carried out for 30-35 cycles. The reannealing temperature varied for each primer (Table I). The amplified products were pooled for each sample and purified by digestion with 0.5 U of Exonuclease I and 0.5 U of Shrimp Alkaline Phosphatase (both from Amersham Biosciences, Piscataway, USA) at 37°C for 2 h to remove unutilized primers and dNTPs and subsequently the enzymes were heat inactivated by incubation at 85°C for 15 min.

SNaPshot analysis of TPMT polymorphism was performed at The Centre for Genomic Applications (TCGA, New Delhi, India). Briefly, oligonucleotide
primers for SNaPshot were designed with the help of Primer Express software (Applied Biosystems, Foster City, USA) and Mfold (mfold web server [http://www.bioinfo.rpi.edu/applications/mfold] to interrogate each locus and were located in 5’ or 3’ region flanking the respective polymorphisms (primer sequences, Table II). The size difference of a minimum of 6 bases between the primers was created by adding tail sequences of poly A or GATC to the 5’ end of the primers.

Genotyping was performed with SNaPshot Multiplex kit (Applied Biosystems, Foster City, USA). Briefly, about 0.5 pmole of purified ampicon was added to 2 µl of SNaPshot Ready Reaction Mix and 2 pmoles of each SNaPshot oligonucleotide primer. The reactions were carried out for 30 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec in a PCR 9700 instrument (Applied Biosystems, Foster City, USA) and digested with Shrimp alkaline phosphatase to dephosphorylate unincorporated fluorescent ddNTPs. The SNaPshot reaction products were analyzed in 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) by mixing 1 µl of SNaPshot product with 0.5 µl of GS120 Liz Size Standard and 9.5 µl of Hi-Di Formamide (Applied Biosystems, Foster City, USA) for each sample and denaturing the injection mix at 95°C for 5 min prior to analysis using POP7 polymer (Applied Biosystems, Foster City, USA) in E5 dyset. Subsequent automated genotype analysis of the data was performed with GeneMapper 3.5 (Applied Biosystems, Foster City, USA). DNA sequencing was performed for selected samples at The Center for Genomic Applications (TCGA, New Delhi, India) or Labindia Instruments Pvt. Ltd (New Delhi, India) to confirm the genotype data obtained.

### Results

A total of 120 genomic DNA samples from healthy individuals were analysed for three variant TPMT alleles by SNaPshot method (Table II). The representative data are shown in the Fig. As an initial step, the PCR products from a random selection of eight samples were sequenced to confirm the specificity of the primers (Fig. b), these data were also used as a reference to evaluate the fidelity of the SNaPshot method. A typical SNaPshot data is shown in Fig. d and e.

The genotype frequencies were in Hardy Weinberg equilibrium. No polymorphism was detected at the TPMT*2 position (nucleotide 238 on mRNA) in our study population. The TPMT*3A variant was observed in only one sample (0.8%) which was heterozygous for both the G460C and A719G locus. Heterozygosity for TPMT*3C (719 A>G) was detected in 4.1 per cent of the study population and no homozygosity was observed. Hence the observed overall frequency of the three SNPs i.e., three TPMT genotype was 4.9 per cent, all of which are heterozygous variant genotypes (Table III).

In addition, cDNA samples of 25 children with ALL were also analyzed for the three TPMT alleles. The primers used were mapping across exons to ensure amplification from cDNA only. Only one sample
revealed the TPMT*3C heterozygous variant. The remaining samples had the wild type allele associated with high TPMT activity at all the three sites.

**Discussion**

Identifying patients having high risk of developing toxicity with mercaptopurine is important as toxicity gives undue morbidity and mortality. In modern leukaemia therapy identifying patients deficient in TPMT activity is crucial to make the therapy more effective, less toxic and reduce life-threatening haematological complications. A combination of allele specific PCR, and RFLP analysis of PCR amplicons are conventionally used to detect TPMT polymorphism. The main advantage of SNaPshot over the conventional RFLP method is its up-scalability, as a high through-output platform for interrogating SNPs with the possible automation of data analysis offering additional benefit. Further, the restriction endonuclease digestion based methods are highly sensitive to the purity and concentration of substrate DNA. This can result in partial or complete inhibition and incomplete digestion of the DNA. All these can result in substantial limitation of throughput and accuracy of sample analysis.

In the present study, the application of the multiplexed fluorescent-labelled deoxy nucleotides based single base extension/termination of oligonucleotides by SNaPshot analysis was carried out on 120 samples as a test of the applicability of the method for pharmacogenomic analysis. Further the application of the method to cDNA samples was attempted. Many of the laboratories engaged in molecular diagnosis of childhood ALL often have patient bone marrow cDNA

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**Table III.** Summary of the analysis of TPMT gene polymorphism in Indian population

<table>
<thead>
<tr>
<th>SNP</th>
<th>Heterozygote (%)</th>
<th>Homozygote (%)</th>
<th>Wild type (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMT*2 (238G&gt;C)</td>
<td>GC=0</td>
<td>CC=0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>TPMT*3A (460 G&gt;A &amp; 719A&gt;G)</td>
<td>CT+AG=1</td>
<td>0</td>
<td>99.2</td>
<td>0.8</td>
</tr>
<tr>
<td>TPMT*3C (719A&gt;G)</td>
<td>AG=5</td>
<td>GG=0</td>
<td>95.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Total frequency of TPMT polymorphisms is 4.9%, among 120 healthy volunteers. *TPMT*3A (460 G>A and 719 A>G) was observed in only one DNA sample.

Fig. Polymorphism analysis at TPMT gene by SnaPshot method. The data are for sample #G15 for TPMT 3C allele. (a) Amplicon obtained with primers TPMT 3C F and R which includes two products of the same size and sequence except for a difference in one nucleotide: (b) partial profile of direct sequencing of the amplicon where both cytosine and thymine are read at one position: (c) schematic representation of the SNaPshot reaction containing chain terminating deoxy nucleotide triphosphates tagged with different fluorochromes: (d) alignment of the primer on the denatured template derived from the two alleles, ddGTP is incorporated in one while ddATP is incorporated in the other: (e) representation of the SNaPshot data obtained. In this example the sample is heterozygous at this locus, having C/T polymorphism.
samples collected for initial leukaemia work-up and disease stratification. These may simultaneously be utilized to obtain TPMT genotype, for decision on appropriate therapeutic dosage of the drug. Our results suggested this as a novel and successful additional application that could be used with cDNA samples. However, using genomic DNA PCR clearly remains a cheaper and simpler approach.

We analyzed three of the most common polymorphisms in the exons of TPMT gene and compared our data with that from other populations. The overall frequency of SNPs in the three variant TPMT alleles arising out of the three polymorphisms, was 4.9 per cent, lower than that reported in Caucasians population. Kham et al reported ethnic variations in a multiethnic Asian population in Singapore. The frequency we observed was higher than that reported by them among the predominant Tamil-Indian migrant population in Singapore (*3A, 0.5%; *3C, 0.8%), British South West Asians (2%) and similar to Thai population.

Among the 25 children with ALL, one child had TPMT*3C heterozygous allele and the remaining had the wild type allele, associated with high enzyme activity. Identification of TPMT enzyme deficient individuals not only helps reduce the risk of potentially fatal haematopoietic toxicity in them, but also correctly identifies thiopurines as the drug which requires dose reduction, if toxicity is observed during a multidrug therapy. This method may have significant implication on the efficacy of antileukaemia protocol and relapse-free survival.

In summary, our findings demonstrated SNaPshot analysis as a novel method for pharmacogenetic screening of TPMTI genotypes not only on genomic DNA samples but also on cDNA samples. We also report an interestingly higher overall incidence of TPMT*3C genotype in the Indian population, having significant implication on the dosage of drug used for treatment.

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