Pyrosequencing of TPMT Alleles in a General Swedish Population and in Patients with Inflammatory Bowel Disease

Sofie Haglund,1,3†* Malin Lindqvist,2† Sven Almer,3 Curt Peterson,2 and Jan Taipalensuu1

Background: Interindividual differences in therapeutic efficacy in patients treated with thiopurines might be explained by the presence of thiopurine S-methyltransferase (TPMT) alleles that encode for reduced TPMT enzymatic activity. It is therefore of value to know an individual’s inherent capacity to express TPMT.

Method: We developed a pyrosequencing method to detect 10 single-nucleotide polymorphisms (SNPs) in TPMT. A Swedish population (n = 800) was examined for TPMT*3A, TPMT*3B, TPMT*3C, and TPMT*2. Patients with inflammatory bowel disease (n = 24) and healthy volunteers (n = 6), selected on the basis of TPMT enzymatic activity, were investigated for all 10 SNPs to determine the relationship between TPMT genotype and phenotype.

Results: In the general population we identified the following genotypes with nonfunctional alleles: TPMT*1/*3A (*3A allelic frequency, 3.75%), TPMT*1/*3C (*3C allelic frequency, 0.44%), TPMT*1/*3B (*3B allelic frequency, 0.13%), and TPMT*1/*2 (*2 allelic frequency, 0.06%). All nine individuals with normal enzymatic activity were wild-type TPMT*1/*1. Thirteen individuals with intermediate activity were either TPMT*1/*3A (n = 12) or TPMT*1/*2 (n = 1). Eight individuals with low enzymatic activity were TPMT*3A/*3A (n = 4), TPMT*3A/*3C (n = 2), or TPMT*1/*3A (n = 2).

Conclusion: Next to wild type, the most frequent alleles in Sweden are TPMT*3A and TPMT*3C. A previously established phenotypic cutoff for distinguishing normal from intermediate metabolizers was confirmed. To identify the majority of cases (90%) with low or intermediate TPMT activity, it was sufficient to analyze individuals for only 3 of the 10 SNPs investigated. Nevertheless, this investigation indicates that other mutations might be of relevance for decreased enzymatic activity.

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The thiopurines 6-mercaptopurine (Purinethol®), azathioprine (Imurel®), and 6-thioguanine (Lanvis®) are used in the treatment of leukemia and inflammatory bowel disease (IBD)4 and to prevent organ transplant rejection (1–3). Approximately 30–40% of IBD patients fails to benefit from this treatment. In general, drug response is affected by several factors, such as sex, age, environment, drug–drug interactions, and drug–enzyme interactions (4). Another important factor for explaining interindividual differences in therapeutic efficacy and adverse reactions is genetic variation in the enzymes in the metabolic pathways for these drugs (1, 3, 5–10).

Thiopurines are subject to extensive metabolism by both anabolic and catabolic pathways (11). Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of heterocyclic and aromatic sulfhydryl compounds, such as thiopurines (11). It has been demonstrated that therapeutic efficiency and ad-
verse reactions are dependent on TPMT enzymatic activity (12–14). A causal relationship between interindividual variation in TPMT activity and certain genetic variants of TPMT has been established (1, 2, 5, 8, 9, 15–17).

The gene encoding TPMT is located on chromosome 6 (6p22) and consists of 9 introns and 10 exons (2). At least nine single-nucleotide polymorphisms (SNPs) that lead to decreased TPMT activity have been identified in the coding region of TPMT (1, 9, 15, 16). In addition, another SNP that leads to decreased TPMT enzymatic activity has been identified in the intron IX/exon X splice junction (Table 1) (8). Approximately 89% of the Caucasian population have normal TPMT activity and are therefore considered homozygous for a wild-type allele, whereas 11% have intermediate activity (heterozygous for a non-functional allele), and 1 in 300 has very low or undetectable TPMT activity (1, 18, 19). Decreased TPMT activity is inherited as an autosomal codominant trait, with a high risk of developing fatal adverse reactions if treated with standard doses of thiopurines (5, 20).

Most individuals with low TPMT enzymatic activity carry the TPMT*3A allele. This is the most frequent nonfunctional TPMT allele among Caucasians in Europe and America, with ~10% of the population carrying a nonfunctional allele (3, 21–24). In Asia, the overall frequency of nonfunctional TPMT alleles is comparatively lower. In western Asia, 2% of the population carry nonfunctional alleles, predominantly TPMT*3A (24). The corresponding value for eastern Asia is 4.7%, but here the TPMT*3C is predominant (24). In Africa, 14.8% of the population carry a nonfunctional TPMT allele (23, 25), mainly TPMT*3C.

Previously only quantitative assays for screening of TPMT activity were available. In addition to being labor-intensive, these assays do not always evaluate the inherent TPMT activity correctly. When applied to patients transfused with red blood cells, these assays may underestimate TPMT activity (9, 15). More recently, several genotyping techniques have been developed (5, 6, 26–29). However, each of these methods covers, to various degrees, only a subset of known TPMT SNPs that encode for nonfunctional alleles.

The aims of this work were (a) to establish a method based on pyrosequencing for genotyping of 10 TPMT polymorphisms, (b) to examine a general Swedish population (n = 800) for the most frequently reported nonfunctional genetic variants (TPMT*3A, TPMT*3B, TPMT*3C, and TPMT*2), and (c) to screen for 10 SNPs in a group of patients with IBD (n = 24) and in healthy volunteers (n = 6), selected on the basis of their TPMT activity.

**Materials and Methods**

**GENETIC VARIATION IN TPMT ACTIVITY AND CERTAIN GENETIC VARIANTS OF TPMT has been established (1, 2, 5, 8, 9, 15–17).**

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**Materials and Methods**

**GENERAL POPULATION, PATIENTS WITH IBD, AND HEALTHY VOLUNTEERS**

DNA samples (n = 800) were obtained from a regional DNA bank (Prof. Peter So¨derkvist, Department of Biomedicine and Surgery, Linköping University, Linköping, Sweden). The regional DNA bank consists of genomic DNA from 800 individuals living in the southeast region of Sweden: 400 males 24–80 (mean, 57) years of age and 400 females 25–80 (mean, 58) years of age. Individuals were selected randomly from the Swedish population register and anonymously included in the DNA bank after informed consent.

To evaluate the relationship between genotype and phenotype, we analyzed a group of patients with IBD (n = 24) and a group of healthy volunteers (n = 6). A total of 17 males 12–61 (mean, 44) years of age and 13 females 12–61 (mean, 31) years of age were included. These individuals were not a randomly selected population, but instead were selected on the basis of their previously (for clinical purposes) determined TPMT enzymatic activity. A description of these individuals and their respective therapies is given in the Supplemental Data Table that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue2/.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Exon*</th>
<th>SNP</th>
<th>Consequence</th>
<th>References</th>
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<td>V</td>
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<td>Ala80Pro</td>
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<td>VII</td>
<td>G460A</td>
<td>Ala154Thr</td>
<td>(15)</td>
</tr>
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<td></td>
<td>X</td>
<td>A719G</td>
<td>Tyr240Cys</td>
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</tr>
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<td>VII</td>
<td>G460A</td>
<td>Ala154Thr</td>
<td>(15)</td>
</tr>
<tr>
<td>TPMT*3C</td>
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<td>A719G</td>
<td>Tyr240Cys</td>
<td>(15)</td>
</tr>
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<td>Ala154Thr</td>
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<td>TPMT*4</td>
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<td>Disrupted splice junction</td>
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<td>Leu49Ser</td>
<td>(1)</td>
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<td>A539T</td>
<td>Tyr180Phe</td>
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<td>T681G</td>
<td>His227Gln</td>
<td>(5)</td>
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<td>TPMT*11</td>
<td>VI</td>
<td>G395A</td>
<td>Cys132Tyr</td>
<td>(17)</td>
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</table>

* Numbering based on Alves et al. (6).
Whole blood (4.5 mL) was collected in EDTA tubes. TPMT activity was assayed as described below, and genomic DNA was isolated from the same blood sample with a QIAamp DNA Blood Minikit (Qiagen). Genotyping was performed after informed consent, with approval from the local ethics committee.

**TPMT Activity Assay**

TPMT activity was estimated as described previously by Pettersson et al. (30). Briefly, TPMT activity was determined by measuring the formation of 6-methylmercaptopurine from 6-mercaptopurine with radiolabeled S-adenosyl-L-methionine used as the methyl donor. Product formation was measured by a liquid scintillation counter. One unit of enzyme activity represents the formation of 1 nmol of 6-methylmercaptopurine per milliliter of packed red blood cells (pRBCs) per hour of incubation. We used cutoffs of 9.0 U/mL of pRBCs to distinguish intermediate from low TPMT enzymatic activity and 5.0 U/mL of pRBCs to distinguish intermediate from low TPMT enzymatic activity (30).

**PCR**

The sequences of the PCR primers for amplification of exons IV and V were as described by Alves et al. (6). The TPMT gene was identified by use of the UCSC genome server (http://genome.ucsc.edu), and PCR primers for amplification of exons VII, VIII, and X were designed with PRIMER3, available through the Biology WorkBench (http://workbench.sdsc.edu) (31). Primers were checked for specificity by use of the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/blast/). All PCR primers were located in intronic sequences, with the exception of the exon X reverse primer. For the purification of single-stranded DNA, one primer in each pair was biotinylated in its 5’ end. All primers (Table 2) were obtained from Life Technologies.

For all reactions the same PCR conditions were used with either a PTC200 thermocycler (MJ Research) or a Mastercycler gradient (Eppendorf). PCRs were performed in a total volume of 25 µL with HotStarTaq master mixture (Qiagen) and with the MgCl₂ concentration adjusted to 2 mM. Each PCR primer was used at a concentration of 0.4 µM except for the forward biotinylated PCR primer for amplification of exon V (G292T), which was used at 0.1 µM. We used 25 ng of human genomic DNA per reaction as template. Amplification was performed with the following thermal profile: 1 cycle of 95 °C for 15 min; 50 cycles of 95 °C for 15 s, 55 °C for 8 s, and 72 °C for 30 s; followed by 1 cycle of 72 °C for 5 min.

**Pyrosequencing**

All pyrosequencing biochemicals were obtained from Sigma Aldrich, unless otherwise indicated.

The Pyrosequencing PSQ96MA system (Pyrosequencing AB) was used. Sequence-specific primers (Table 3) were designed with the software supplied by Pyrosequencing AB (http://www.pyrosequencing.com) and used for pyrosequencing. Pyrosequencing was carried out according to the manufacturer’s instructions. Briefly, for each genotype determination, single-stranded DNA was purified from the 25-µL PCR reaction with Dynabeads M-280 (Dynal AS) dissolved in BW buffer (10 mmol/L Tris-HCl, 2 mol/L NaCl, 1 mmol/L EDTA, 1 mL/L Tween 20, pH 7.6). The Dynabeads with captured DNA were then transferred into 0.5 mol/L NaOH and finally washed in annealing buffer (20 mol/L Tris-acetate, 5 mmol/L magnesium acetate, pH 7.6). The different genotypes were determined in separate reaction vessels by annealing 10 pmol of the corresponding sequence-specific primer to the single-stranded DNA (Table 3). Annealing was conducted in the annealing buffer before sequencing by heating the sample to 80 °C and then allowing it to cool to room temperature (25 °C).

The general population was genotyped for the most frequently reported genetic variants of TPMT (TPMT*3A, TPMT*3B, TPMT*3C, and TPMT*2; Table 1) with the sequence-specific primers for pyrosequencing (Table 3, primers 2, 4, and 6).

The population of patients diagnosed for IBD (n = 24) and healthy volunteers (n = 6) were genotyped for nine SNPs known to lead to decreased TPMT enzymatic activity (Table 1) with the sequence-specific primers for pyrosequencing (Table 3, primers 1–9) and for the G430C SNP (TPMT*10), for which the phenotypic significance is unknown at present, with sequence-specific primer 10 (Table 3).

<table>
<thead>
<tr>
<th>Target exon</th>
<th>Forward primer, 5’–3’</th>
<th>Reverse primer, 5’–3’</th>
<th>Product, bp</th>
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<td>bi*TACCACTGACTGGGTTGCTGTA</td>
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<td>V</td>
<td>bi*CGGGGCTGTTCTTTGAAACCTATG</td>
<td>TAAATAGGAACCATCCGACAC</td>
<td>379</td>
</tr>
<tr>
<td>VII</td>
<td>TGTTGAAGTACCAGCATGC</td>
<td>TAATCTCTCTCTCTCTCTCTC</td>
<td>180</td>
</tr>
<tr>
<td>VIII</td>
<td>CTCTCTCTCTCTCTCTCTCT</td>
<td>bi*AATCTCTCTCTCTCTCTCT</td>
<td>179</td>
</tr>
<tr>
<td>X</td>
<td>bi*CACCCAGCCTTTTGTGA</td>
<td>ACAGGTAAACATCTGGTGG</td>
<td>495</td>
</tr>
<tr>
<td>X</td>
<td>CACCCAGCCTTTTGTGA</td>
<td>bi*ACAGGTAAACATCTGGTGG</td>
<td>495</td>
</tr>
</tbody>
</table>

Numbering based on Alves et al. (6).

bio, biotinylated.
Statistical analysis was performed with Statistical Package for Social Sciences 11.0 for Windows (SPSS, Inc). For group comparisons, the Mann–Whitney U-test was used. A P value <0.05 was considered statistically significant.

Results

PCR PYROSEQUENCING

PCR amplification of the individual exons (IV, V, VII, VIII, and X) led to the formation of specific products, as judged from agarose gel electrophoresis (data not shown) and pyrograms after pyrosequencing (Fig. 1).

Table 3. Sequence-specific primers for pyrosequencing.

<table>
<thead>
<tr>
<th>No.</th>
<th>Exon*</th>
<th>Mutation</th>
<th>Sequencing direction</th>
<th>Primer sequence, 5’–3’</th>
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</thead>
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<tr>
<td>1</td>
<td>IV</td>
<td>T146C</td>
<td>Reverse</td>
<td>GAAAGATCTAAATGCTTCT</td>
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<tr>
<td>2</td>
<td>V</td>
<td>G238C</td>
<td>Reverse</td>
<td>TGTCGGGGCTGTG</td>
</tr>
<tr>
<td>3</td>
<td>V</td>
<td>G292T</td>
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<td>AGATCTCTGCTCTTGAAAAA</td>
</tr>
<tr>
<td>4</td>
<td>VII</td>
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</tr>
<tr>
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<td>9</td>
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</tr>
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<td>10</td>
<td>VII</td>
<td>G430C</td>
<td>Forward</td>
<td>TGATTGGGATAGAGAGG</td>
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</table>

*Numbering based on Alves et al. (6).

Allele frequencies in a general Swedish population

The frequencies of the three SNPs (G238C, G460A, and A719G) defining four of the nonfunctional TPMT alleles (*2, *3A, *3B, and *3C) were investigated in individual samples from a DNA bank (n = 800). Of the 800 samples, 730 (91%) had none of these SNPs and were therefore considered to be wild type (*1/*1) at the TPMT locus, whereas 70 (9%) were heterozygous for a nonfunctional TPMT allele. None of the samples contained two nonfunctional alleles for TPMT. In the population, TPMT*1/*3A was the most frequently occurring heterozygous genotype, with a genotype frequency of 7.5%, followed by TPMT*1/*3C (0.88%), TPMT*1/*3B (0.25%), and TPMT*1/*2 (0.13%; Table 4). Accordingly, the allelic frequencies of *3A, *3C, *3B, and *2 were 3.75%, 0.44%, 0.13%, and 0.06%, respectively.

Among males, 10% were carriers of a nonfunctional allele; among females, 7% were carriers of a nonfunctional allele. There was no significant difference in the presence of nonfunctional TPMT alleles between males and females.

TPMT genotype–phenotype relationship

Of the 30 individuals selected on the basis of their TPMT activity (Fig. 2; also see Supplemental Data Table) and analyzed for all 10 SNPs (Table 3), 8 (see Supplemental Data Table, individuals 1–8; all diagnosed with IBD) had low TPMT activity (0.3–0.8 U/mL of pRBCs). Four were genotyped as TPMT*3A/*3A and two as TPMT*3A/*3C. Two of these individuals with low activity (see Supple-
mental Data Table, individuals 1 and 3) were, however, genotyped in this assay as TPMT*1/*3A. This heterozygous genotype is in conflict with the low-activity phenotype obtained for these individuals. These two individuals were also wild type (GG) for the G430C SNP, for which the functional significance of TPMT activity is not known at present (32).

Thirteen individuals (all but 1 diagnosed with IBD) had intermediate TPMT activity (6.5–8.9 U/mL pRBCs; Fig. 2; also see Supplemental Data Table, individuals 9–21). These individuals were genotyped as either TPMT*1/*3A (n = 12) or TPMT*1/*2 (n = 1). Nine individuals (five healthy volunteers and four with IBD) had normal TPMT activity (>9.0U/mL of pRBCs; a previously established cutoff point distinguishing normal from intermediate TPMT activity; see Supplemental Data Table, individuals 22–30). None of these individuals carried any of the investigated SNPs, and they all were therefore considered to be wild type at the TPMT locus.

Discussion

General Population
Several genetic variants of the TPMT gene lead to decreased TPMT enzymatic activity (1, 2, 8, 9, 15). Both the TPMT genotype and phenotype have been associated with the occurrence of adverse reactions during treatment with thiopurine drugs (1, 3, 5–10); it therefore might be of value to determine a person’s TPMT genotype before initiating treatment with thiopurine drugs. For this to become a routine practice, simple and reliable genotyping assays must be available and the relevant genotype frequencies must be known. Accordingly, we developed a PCR-pyrosequencing-based method for TPMT genotyping and for the first time determined the frequency of currently known nonfunctional TPMT alleles in a general Swedish population.

The data for TPMT*3A and *3C (allelic frequencies, 3.75% and 0.44%, respectively) are in agreement with previous investigations of Caucasian populations in other European and Nordic countries. These previous investigations identified TPMT*3A as the most frequently occurring nonfunctional allele, followed by TPMT*3C, with allelic frequencies of 3.4–4.5% and 0.25–3.3%, respectively (21, 24, 33). However, for TPMT*2 we obtained an eightfold lower allele frequency (0.06% vs 0.5%) compared with a previous estimate (24). Furthermore, a few individuals (allele frequency, 0.13%) were identified as heterozygous for the TPMT*3B allele. This genotype has not, to our knowledge, been identified previously in a Nordic country. However, it was possible to obtain information only on the country of birth of the individuals constituting the DNA bank in the present study, which might explain the latter discrepancies, because earlier studies were performed mainly on homogeneous ethnic populations. None of the 800 individuals constituting the DNA bank was identified as a carrier of two nonfunctional TPMT alleles, although previous estimates indicated an occurrence of 1 in 300 individuals (19).

Because the DNA bank was not evaluated for the SNP G292T it is possible that some of the individuals genotyped as TPMT*1/*3A in fact are TPMT*1/*3D (A719G, G460A, and G292T). A previous study indicated that TPMT*3D is a rare allele, with an allele frequency of 0.18% in North America (1). Thus, provided that the *3D allele frequency is the same in our study population, we would have to expect that 3 of the 60 *3A individuals are in fact heterozygous for the *3D allele. The allele frequency of *3A would decrease accordingly, from 3.8% to 3.6%.

From the genotyping data we would predict that ~91% of the investigated population would exhibit normal TPMT enzymatic activity and that the remaining 9% would express intermediate or low enzymatic activity. A majority of these (99%) can be identified by use of only two genotyping reactions (for G460A and A719G). Nevertheless, genotyping for the *2 allele and other currently unknown mutations may be useful (Fig. 2 and Supplemental Data Table).

Clinical Material
Patients with IBD and healthy volunteers were genotyped for the nine known nonfunctional TPMT alleles and one SNP for which the phenotypic significance is not yet known; the data were then compared with the corresponding phenotypes. We conclude that the current assay correctly identified all individuals with normal TPMT activity (9 of 9) or intermediate activity (13 of 13), whereas 6 of 8 (75%) of those with a low-activity phenotype were identified by genotyping. Furthermore, the above-mentioned results would have been the same if we had investigated for only 3 (G238C, G460A, and A719G) of the 10 mutations. Other investigations have indicated a genotype–phenotype concordance of 83–100% (1, 22, 33, 34) compared with our 93%.

For the present study we used locally established cutoffs based on the frequency distribution of erythrocyte TPMT activity in a Swedish population to define the different phenotypic groups (30). The genotyping data in
the present study thus verify the previously defined cutoff (9.0 U/mL of pRBCs) in our laboratory for distinguishing normal from intermediate TPMT activity. As in previous studies, we also noted a high degree of variability in TPMT activity within the homozygous wild type (9.8–19.6 U/mL of pRBCs), indicating that factors other than TPMT nucleotide polymorphisms in the protein-encoding region also regulate the catalytic activity.

In this study two IBD patients were genotyped as TPMT*1/*3A despite their low TPMT enzymatic activities (0.3 and 0.4 U/mL of pRBCs, respectively; Supplemental Data Table, individuals 1 and 3). These individuals were not treated with thiopurines; one, however, was treated with an aminosaliclylate. There have been studies that indicate a modest reduction in TPMT activity in some patients during treatment with aminosaliclylates compared with TPMT activity at baseline (35–39). For an individual with a heterozygous genotype to present a low-activity phenotype, the aminosaliclylate treatment would have to cause a reduction in TPMT activity up to 15-fold. Considering the following, it seems unlikely that concomitant aminosaliclylate treatment would have produced a low-activity phenotype in a heterozygous individual. Four of the individuals with normal TPMT activity were treated with aminosaliclylates. These individuals were, as expected, genotyped as carriers of two functional alleles. In addition, seven of the individuals with an intermediate phenotype were on concomitant aminosalicylate treatment. As expected, all of these individuals were genotyped as heterozygous.

The individuals with low TPMT activity who were genotyped as TPMT*1/*3A were also wild type (GG) for the G430C SNP, for which the functional significance of TPMT activity is unknown at present (32). Instead, the SNPs identified in these two individuals may be interpreted as a TPMT*3B/*3C genotype. This genotype would be in agreement with the obtained phenotype, but based on established allele frequencies, this is considered an improbable interpretation. However, the TPMT*3B/*3C genotype can be excluded only by haplotyping. This is a limitation with pyrosequencing as well as many other SNP detection techniques. Recently, a novel SNP (G395A; TPMT*11) that leads to reduced TPMT activity has been identified in a patient with acute lymphoblastic leukemia (17). Our population was not investigated for this SNP.

Taken together, the results obtained support pyrosequencing as a method for TPMT genotyping and indicate that the two TPMT*1/*3A individuals with low TPMT activity actually are compound heterozygous, either TPMT*3B/*3C or TPMT*3A/*11 (or a hitherto unknown TPMT mutation).

**METHODOLOGY**

Pyrosequencing represents a flexible and convenient method for SNP analysis (40–42). The throughput of the pyrosequencing instrument was 96 genotypes (96-well microplate) in 15 min, with a total turnaround time for the system (including the prepyrosequencing PCR step) of 3.5 h. The total reagent cost (including disposable material, DNA extraction, PCR using a biotinylated primer, PCR purification, and pyrosequencing) was approximately US $10.00 per genotype (pyrosequencing reagent, including disposables, US $4.70). The cost of the PSQ96MA pyrosequencing system was approximately US $84,000. However, the pyrosequencing system has, since the study was performed, been updated, reducing reagent costs and increasing throughput.

On the basis of our experiences, pyrosequencing is a specific and reliable method for SNP analysis. However, for TPMT genotyping, the method is not sufficient by itself for haplotype determination, which would distinguish the intermediate TPMT activity genotype TPMT*1/*3A from the low TPMT activity genotype TPMT*3B/*3C. Of these two genotypes, *1/*3A is by far the most common. Other SNP detection techniques, for example, single-strand conformation polymorphism (SSCP) analysis, PCR with restriction fragment polymorphism analysis, horizontal conformation-sensitive gel electrophoresis, and denaturing HPLC (DHPLC), are also unable to differentiate between the TPMT*1/*3A and TPMT*3B/*3C genotypes. Recently, long-range PCR combined with intramolecular ligation has been described as a method for TPMT haplotyping (43). Although this would allow for the identification of the rare TPMT*3B/*3C genotype, it would, however, be a significantly more labor-intensive method for routine diagnostics.

Previously, PCR with restriction fragment polymorphism analysis (26), SSCP analysis (5), DHPLC (28), horizontal conformation-sensitive gel electrophoresis (6), TaqMan technology (44), and LightCycler with hybridization probes (29) have been described for genotyping of TPMT. However, only the LightCycler assay described by Schütz et al. (29) account for most (eight) of the known TPMT SNPs that lead to nonfunctional alleles. Furthermore, most of these methods cannot be considered practical for routine analysis in a clinical laboratory. Both PCR with restriction fragment polymorphism analysis and SSCP analysis are time-consuming and labor-intensive methods. SSCP analysis, DHPLC, and horizontal conformation-sensitive gel electrophoresis require both a high skill level and extensive training in running the assays and interpreting the genotype from the various band patterns and elution profiles that are generated, and SSCP analysis is especially sensitive to the assay conditions used. Indeed, of the above-mentioned methods, only the TaqMan and the LightCycler assays can be considered easy to use because they are highly automated and do not require post-PCR manipulation. However, because these methods do not generate sequence data, their reliability depends on the greater likelihood that a known mutation will be present compared with an unknown mutation in the probe area. Thus, compared with these methods, pyrosequencing generates sequence information, easily interpretable results, and avoids the use of expensive
fluorescently labeled hybridization probes. In addition, because of the 96-well format, pyrosequencing is amenable to a high degree of automation, in common with the TaqMan assay, DHPLC, and in principle, the LightCycler assay. The LightCycler assay developed by Schütz et al. (29), however, needs to be transferred to a real-time PCR instrument based on the 96-well format.

In conclusion, TPMT*3A and TPMT*3C were the most frequently occurring nonfunctional TPMT alleles in our Swedish population, and analysis for 3 of the 10 SNPs investigated (G238C, G460A, and A719G) was sufficient to identify the majority (90%) of the cases with low or intermediate enzymatic activity. The data, however, indicate that hitherto uncharacterized mutations may be of relevance in the Swedish population. In addition, the previously established phenotypic cutoff (9.0 U/mL of pRBCs) for distinguishing normal from intermediate metabolizers was confirmed. Finally, we conclude that pyrosequencing is a flexible method for parallel SNP identification that can be used as a complementary tool for assessing a person’s inherent capacity for metabolizing thiopurine drugs.

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