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Thiopurine S-Methyltransferase Pharmacogenetics: Genotype to Phenotype Correlation in the Slovenian Population

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Key Words
Genetic polymorphism · Slovenian population · Thiopurine S-methyltransferase · Pharmacogenetics

Abstract
The toxicity of thiopurine drugs has been correlated to the activity of thiopurine S-methyltransferase (TPMT), whose interindividual variation is a consequence of genetic polymorphisms. We have herein investigated the relevance of some genetic markers for the prediction of thiopurine-related toxicities and to determine the genotype to phenotype correlation in the Slovenian population. The most prevalent mutant allele in the Slovenian population is TPMT*3A (4.1%), followed by TPMT*3C (0.5) and TPMT*3B (0.3), while the TPMT*2 allele was not found in any of the examined samples. TPMT enzyme activity distribution in the subgroup sample was bimodal and as such correlated with genetic data. Using a cutoff value of 9.82 pmol/10^7 RBC per h, the genetic data correctly predicted TPMT enzyme activity in 91.6% of the examined individuals. Pharmacogenetic TPMT analyses have therefore proved to have significant clinical implications for prediction of individuals' responses to treatment with thiopurine drugs in order to avoid possible life-threatening therapy-related toxicities.

Introduction

Therapy-related toxicity is a serious disadvantage of thiopurine drugs, e.g. 6-mercaptopurine (6-MP), 6-thioguanine and azathioprine (AZA), which are commonly used in the treatment of acute lymphoblastic leukemia (ALL), autoimmune disorders, and as immunosuppressants in solid organ transplantations [1]. They are metabolized along three pathways [2]. In the case of 6-MP the S-methylation by thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) leads to the detoxification of the drug by the conversion to 6-methylmercaptopurine (6-MMP) [3]. It represents the only inactivation pathway, since the activity of xanthine oxidase in the anabolic pathway is negligible in hematopoietic tissues [4]. The multienzyme catabolic pathway leads to the formation of 6-thioguanine nucleotides (6-TGN) responsible for the cytotoxic effects of 6-MP [5]. Decreased TPMT activity leads to a higher concentration of 6-TGN and, consequently, to greater cytotoxicity [6]. The incorporation of 6-TGNs into DNA and RNA is the most commonly accepted mechanism of cytotoxic effect [4]. However, it has also been shown that the inhibition of de novo purine synthesis by TPMT-methylated intermediates of the catabolic pathway contributes largely to the antiproliferative effects of 6-MP [7]. Furthermore, a recent study identified an interesting role of AZA and its metabolite 6-MP in the

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Table 1. Allelic frequencies of polymorphisms TPMT*2, *3A, *3B and *3C in different populations

Population	Number[a]	Mutant alleles, %	TPMT*2 %	TPMT*3A %	TPMT*3B %	TPMT*3C %	Reference
African-American	23	4.6	0.4	0.8	N/A	2.4	34
American Caucasian	21	3.8	0.2	3.2	N/A	0.2	34
Argentinian	147	4.1	0.7	3.1	N/A	N/A	35
Brazilian[b]	306	5.3	0.8	2.0	N/A	2.5	36
Brazilian[c]	204	5.2	2.2	1.5	0.5	1.0	37
British Caucasian	199	5.3	0.5	4.5	N/A	0.3	21
Chinese	192	2.3	N/A	N/A	N/A	2.3	17
Chinese[d]	160	1.9	0	0.3	0	1.6	18
Chinese[e]	213	0.2	0	0	0	0.2	19
Colombian	140	4.0	0.4	3.6	N/A	N/A	38
Egyptian	200	0.016	0	0.003	0	0.013	39
French Caucasian	304	4.1	0.7	3.0	N/A	0.4	40
German Caucasian	1,214	5.0	0.2	4.4	N/A	0.4	12
Ghanaian	217	7.6	N/A	N/A	N/A	7.6	41
Indian	200	1.3	N/A	0.5	N/A	0.8	20
Italian	103	5.4	0.5	3.9	N/A	1.0	42
Japanese	192	0.8	N/A	N/A	N/A	0.8	29
Kazakh	327	1.2	N/A	0.3	N/A	0.9	43
Kenyan	101	5.4	N/A	N/A	N/A	5.4	21
Malayan	200	2.3	N/A	N/A	N/A	2.3	20
Norwegian Caucasian	66	3.7	0	3.4	N/A	0.3	25
Northern Portuguese	310	2.4	N/A	2.4	N/A	N/A	44
Polish	358	3.2	0.4	2.7	N/A	0.1	45
Saami[f]	194	3.3	0	0	N/A	3.3	25
South-west Asian	99	1.0	N/A	1.0	N/A	N/A	17
Swedish	800	4.4	0.4	3.8	0.1	0.1	46
Unknown[c]	31,742	4.8	0.3	3.6	0.0063	0.9	7
Slovenian [this study]	194	4.9	0	4.1	0.3	0.5	

[a] Number of individuals studied; [b] European origin; [c] diverse population; [d] Uygur, Caucasian; [e] Han, Yao, non-Caucasian; [f] Northern Norwegian, non-Caucasian.

control of T cell apoptosis, clarifying AZA's immunosuppressive mechanisms of action as well as contributing to the already complex cytotoxic pathways of thiopurines [8].

The genetic polymorphism of TPMT is a cause of interindividual variation in enzyme activity and thus serves as a predictive measure of thiopurine-related toxicities [6, 9]. The TPMT gene is sublocalized to the chromosome band 6p22.3 and spans a 27-kb region consisting of 10 exons and 9 introns [10]. Interpatient differences in toxicity and therapeutic efficacy of thiopurine drugs exist due to genetic polymorphism in the TPMT gene [11]. While 20 variants of TPMT have been described, the molecular basis of clinically significant decrease in TPMT activity is primarily associated with 3 mutant alleles, i.e. TPMT*2 (mutation G238C), TPMT*3A (G460A and A719G) and TPMT*3C (A719G) [12]. On the proteome level, nonsynonymous mutations result in loss of enzyme activity due to greater susceptibility to protein degradation [13] by the ubiquitin-proteasome pathway and in a higher association with chaperone proteins such as hsp90 [14–16]. Recently, four novel and potentially clinically significant nonsynonymous mutations were identified, which contribute to an excellent TPMT genotype-to-phenotype concordance rate (98.4%) of the study [12].

There is notable variation in the frequency of mutant alleles between different ethnicities as shown in table 1. The frequency distribution of TPMT enzyme activity in the Caucasian population is trimodal, such that wild-type homozygotes (TPMT*1) have high enzyme activity (89.5%), while heterozygous and homozygous individuals

for TPMT*2, TPMT*3A and TPMT*3C alleles show intermediate and low enzyme activity (9.9 and 0.6%, respectively) [12]. Such a distribution has not been found in non-Caucasian populations [17–21]. Individuals with intermediate or deficient TPMT activity thus have a higher risk for toxic events such as life-threatening myelosuppression and/or secondary tumor development after receiving standard doses of thiopurine drugs [22]. Therefore, a reduction to 6–14 and 50–100% of the standard dosage of thiopurine drugs is required for homozygous and heterozygous patients, respectively [23–25]. On the other hand, patients with a very high TPMT activity may be undertreated and/or have a greater risk of early leukemia relapse [26, 27]. Therefore, pharmacogenetic analyses of the TPMT locus are potent clinical markers for thiopurine therapy-related toxicity.

The objective of this study was to set the basis for pharmacogenetic testing in the Slovenian population. We thus aimed to determine the frequency of clinically significant, low-activity TPMT alleles, i.e. TPMT*2 (mutation G238C), TPMT*3A (G460A and A719G), TPMT*3B (G460A) and TPMT*3C (A719G) by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) or Taqman® genotyping methods. The TPMT enzyme activity was determined by high-performance liquid chromatography (HPLC) assay. The genotype-to-phenotype concordance rate in the presented study was 91.6%.

This study contributes to a better understanding of the pharmacogenetics of purine metabolism and provides potential implemented clinical applications for appropriate dose adjustments in individuals with partial TPMT deficiency undergoing thiopurine therapy.

Materials and Methods

Study Participants

Venous blood (3–5 ml) was obtained from 194 unrelated healthy Caucasian volunteers and used for TPMT genotyping. A subgroup of 95 individuals was randomly chosen for TPMT activity measurements. The study was approved by the Slovenian national regulatory authority. Written informed consent was obtained from all participating subjects. The experiments comply with the current laws of the Republic of Slovenia.

DNA Extraction

DNA was extracted from the samples by the modified salting out procedure [28]. Briefly, 2 ml of blood was centrifuged, the pellet was suspended in CLB solution (11% saccharose, 1% Triton, 5 mmol/l of $MgCl_2$, 10 mol/l of Tris; pH = 8.2), centrifuged and resuspended in SLR solution (14 mmol/l of Tris, 7 mmol/l of $MgCl_2$, 10 mmol/l of NaCl), followed by further centrifugation and resuspension in 1.5 ml of ice-cold NLB solution (10 mmol/l of Tris, pH = 8.2, 0.4 mmol/l of NaCl, 20 mmol/l of EDTA), 100 μl of 10% SDS solution and 250 μl of proteinase K (1 mg/ml). After overnight incubation at 42°C, aliquots of saturated NaCl solution were added to the samples. Following centrifugation, ice-cold absolute ethanol was used for DNA precipitation.

TPMT Genotyping

PCR-RFLP-based assays were used for the analyses of G460A (TPMT*3B) and A719G (TPMT*3C) point mutations as described previously [29]. Briefly the allele-specific primers P460Fb (5′-AAG CAG CTA GGG AAA AAG AAA GGT G-3′) and P460Rb (5′-CAA GCC TTA TAG CCT TAC ACC CAG G-3′), both from Promega GmbH, Mannheim, Germany, were used for the amplification of the genomic DNA segment containing TPMT*3B mutation. The PCR product was digested with *Mwo*I (New England Biolabs, Beverly, Mass., USA) overnight at 60°C. The wild-type but not the mutant TPMT*3B allele contains a *Mwo*I restriction site, which results in digestion of 694-bp PCR product, yielding fragments of 443 and 251 bp.

The primers P719Fb (5′-GAG ACA GAG TTT CAC CAT CTT GG-3′) and P719Rb (5′-CAG GCT TTA GCA TAA TTT TCA ATT CCT C-3′), both from Promega, were used to obtain a 373-bp DNA fragment spanning the TPMT*3C mutation. The PCR product was further digested with *Acc*I (New England Biolabs) for 16 h at 37°C. The mutant TPMT*3C allele but not the wild-type allele contains an *Acc*I restriction site, which results in a digestion of 373-bp PCR product, producing 283 and 90 bp fragments.

An excess amount of both enzymes (1 U) was used for the RFLP analyses to achieve complete digestion of the PCR products, ensuring specificity and validity of the described method.

A custom Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, Calif., USA) was used for the determination of the TPMT*2 polymorphism (G238C). Differently labeled fluorescent wild-type (5′-FAM-TTT ATG CAG GTT TGC AGA C-3′) and mutant-specific (5′-VIC-ATT TTA TGC AGG TTT CCA GAC-3′) probes were used for allelic discrimination upon amplification of the fragment defined by the forward (5′-ACT CTA ATA TAA CCC TCT ATT TAG TCA TTT GAA AAC ATA ATT T-3′) and reverse (5′-CTG ATT TCC ACA CCA ACT ACA CTG T-3′) oligonucleotide primers. The reporter dyes used were 6-carboxy-fluorescein (FAM) and VIC®, while 6-carboxy-tetramethylrhodamine (TAMRA) was used as the quencher dye. The PCR was performed using a 96-well optical tray with caps at a final reaction volume of 20 μl. The samples contained 10 μl of Taqman Universal PCR Master Mix, No. AmpErase® uracil-*N*-glycosylase, 2 μl of genomic DNA, an additional 2.5 U of AmpliTaq Gold (Perkin Elmer, Norwalk, Conn., USA), 2.5 μmol/l of each of the primers and 150 nmol/l of each of the fluorescently labeled probes. Initial denaturation at 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase was followed by 40 cycles of denaturation at 92°C for 15 s, and annealing and extension at 60°C for 1 min. The fluorescence intensities were recorded at excitation maxima for FAM and VIC reporter dyes at 518 and 548 nm, respectively (ABI Prism 7000 Sequence Detection System).

The heterozygous TPMT*2 samples (genotype TPMT*1/*2), which served to validate the method, were kindly provided by Dr. Matthias Schwab [12].

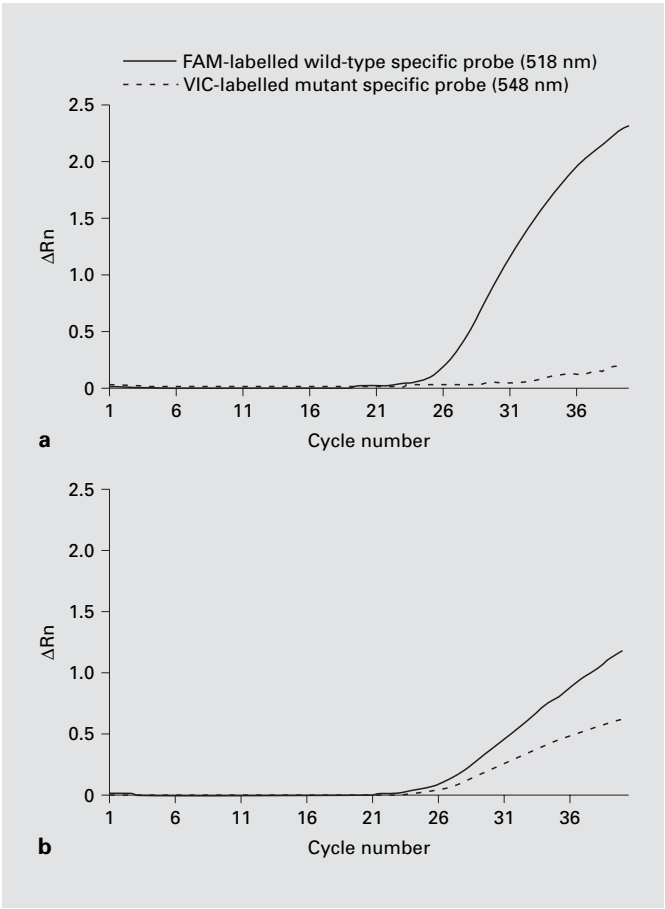

Fig. 1. Allelic discrimination of wild-type and TPMT*2 alleles. Fluorescent wild-type (FAM) and mutant (VIC) specific probes were used for the determination of individual's genotype. **a** For a homozygous wild-type individual (TPMT*1/*1) only the wild-type-specific fluorescent signal was elevated from the baseline fluorescence. **b** In the case of heterozygous TPMT*1/*2 positive control the wild-type-specific probe was annealed to the wild-type allele and the mutant-specific probe to the TPMT*2 allele. Upon amplification, both fluorescent signals were elevated. Relative fluorescence of VIC and FAM (ΔRn) is plotted as a function of the cycle number. Fluorescence was measured at 518 (FAM) and 548 nm (VIC).

TPMT Enzyme Activity Assay

A group of 95 randomly chosen blood samples was used for the isolation of red blood cells (RBC). The sample preparation and HPLC analysis was adopted from a previously described procedure [30]. Briefly, RBC were isolated from heparinized blood, lysed, and supernatants were collected and stored at –80°C until further usage.

The RBC lysates were first chelated by 100 μl of Chelex 100 resin (Bio-Rad), followed by incubation with 90 μmol/l of S-adenosyl-*L*-methionine, 450 μmol/l of dithiothreitol, 36 μmol/l of allopurinol and 4.3 mmol/l of 6-MP in potassium phosphate buffer (all the products were from Sigma Co., St. Louis, Mo., USA). The reaction was stopped by the addition of $HClO_4$, the supernatant neutralized with K_2HPO_4 and used for HPLC analyses.

An automated Agilent Technologies 1100 HPLC system and Chemstation for LC 3D Systems software were used for the HPLC analyses (Agilent Technologies Inc., Palo Alto, Calif., USA). The samples (100 μl of supernatant) were eluted with 30% methanol (pH was adjusted to 3.00 with acetic acid) with an operating pressure of approximately 290 bar and a flow rate of 1.5 ml min^{-1}. The detection wavelength on the diode array detector was 290 nm. TPMT activity was calculated as the amount (pmol) of 6-MMP formed per 10^7 RBC/h (pmol/10^7 RBC/h).

Statistical Analysis

Significant differences among experimental and expected TPMT genotype frequencies in the Slovenian population were analyzed using the χ^2 test, with a significant confidence of $p = 0.05$. The expected genotype frequencies and numbers were calculated from experimentally obtained allelic frequencies and according to the Hardy-Weinberg law. Median and mean values of TPMT activity were assessed using nonparametric statistics and t tests, respectively. $p < 0.05$ was considered to be statistically significant. The TPMT activity cutoff value between intermediate- and high-activity groups was determined by receiver operating characteristics analysis using JMP Statistical Discovery software version 6.0.0 (SAS Institute Inc., Cary, N.C., USA). The χ^2 test was used to compare the experimental and expected frequencies of TPMT phenotypes with respect to the determined genotypes.

Results

Inherited differences in TPMT activity have been defined at the molecular level by the identification of activity modulating polymorphisms. Since allelic distribution is population-specific, 194 Slovenian subjects were genotyped for the most common mutant alleles in the Caucasian population, namely TPMT *2, *3A, *3B and *3C.

TPMT*2 allele contains a G-to-C transversion at position 238 leading to the substitution of a rigid proline for a more flexible alanine residue (P80A), resulting in protein instability and decreased activity [13, 15]. Allelic discrimination was based on a newly established fluorogenic probe 5′ nuclease PCR assay. Two differently labeled internal MGB Taqman probes were designed; VIC-labeled mutated sequence for the detection of the TPMT*2 allele and an FAM-labeled probe that binds to the wild-type sequence. The amount of fluorescent dye released during PCR is measured by a real-time PCR system and is directly proportional to the amount of PCR product generated. Figure 1 shows the fluorescence signals of wild-type and mutant-specific reporter dyes as a function of the number of PCR cycles. Thus in the case of the wild-type allele only the wild-type specific FAM fluorescence was elevated, while the mutant-specific probe did not anneal and its signal remained constant (fig. 1a). Converse-

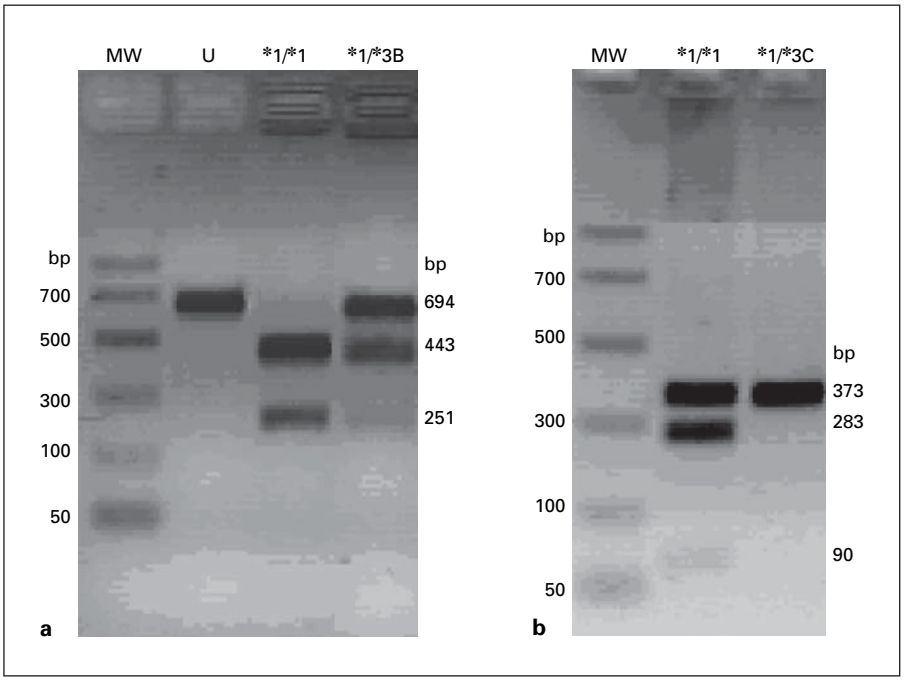

Fig. 2. Identification of wild-type, TPMT*3B and TPMT*3C alleles. Allelic discrimination is based on electrophoresis patterns for TPMT alleles on 2% agarose gels after RFLP analyses. **a** Distinction between wild-type and TPMT*3B alleles. PCR product (694 bp) was digested by the restriction enzyme *Mwo*I. In the case of the wild-type TPMT*1/*1 genotype only 2 fragments are obtained (443 and 225 bp), while in the case of TPMT*1/*3B the uncut fragment (694 bp) can also be observed. **b** Distinction between wild-type and TPMT*3C allele. PCR product (373 bp) was digested by the restriction enzyme *Acc*I. In the case of the wild-type TPMT*1/*1 genotype only 1 PCR product is obtained, while in the case of a TPMT*1/*3C individual 2 smaller fragments are observed as well (283 and 90 bp). MW = molecular weight ladder; U = uncut PCR product; *1/*1 = homozygous wild-type; *1/*3B = heterozygous TPMT*3B mutant; *1/*3C = heterozygous TPMT*3C mutant.

ly, when the heterozygous positive control was used as a template, both fluorescent signals of FAM and VIC were generated, since both probes annealed to the specific allele (fig. 1b).

TPMT*3 variant alleles also contain transition polymorphisms causing amino acid changes resulting in rapid degradation of the enzyme and lower enzymatic activity [13, 15]. The TPMT*3B allele contains G460A polymorphism in exon 7, and the TPMT*3C allele is a result of A719G mutation in exon 10, while the TPMT*3A allele contains both polymorphisms (G460A and A719G). The allelic discrimination among wild-type, TPMT*3B and TPMT*3C alleles was based on RFLP. TPMT*3B allele was amplified as a 694-bp fragment, while its wild-type variant allele contains a *Mwo*I restriction site, resulting in two restriction fragments (443 and 251 bp). Figure 2a shows the electrophoretic separation of the uncut PCR product, the wild-type allele and the mutant heterozygote TPMT*1/*3B. Similarly RFLP analysis was employed for the distinction between the wild-type and TPMT*3C alleles. Single nucleotide base substitution (A719G) creates a new restriction site for *Acc*I endonuclease. The TPMT*3C allele is thus represented by two digested fragments (283 and 90 bp), the wild-type allele by one 373-bp fragment (fig. 2b). Homozygote patterns for genotypes TPMT*3B/*3B and TPMT*3C/*3C were not observed in any of the samples analyzed. The presence of both mutant alleles TPMT*3B and TPMT*3C in the individual's genomic DNA is denoted as the TPMT*3A allele.

Table 2. Allelic and genotype frequencies of TPMT variants in a sample of 194 Slovenian individuals

Allele	Number of alleles	Allelic frequency, %	Genotype frequency, %
Number of alleles	388		
Wild-type TPMT*1	369	95.1	90.2
TPMT*2	0	0	0
TPMT*3A	16	4.1	8.2
TPMT*3B	1	0.3	0.5
TPMT*3C	2	0.5	1.0
Total mutant alleles	19	4.9	9.8

In the majority of the examined sample population (90.2%) the homozygous wild-type allele TPMT*1 was present. Mutant alleles were found in 9.8% of the sample population, with TPMT*3A found in 16 individuals (8.2%), while TPMT*3B and TPMT*3C were present in 1 (0.5%) and 2 (1.0%) individuals, respectively (table 2). All the mutants determined were heterozygotes for a given allele. No major difference between experimental and expected genotypic frequencies was observed (χ^2 lower than $\chi^2_{theoretical} = 3.841$; $p > 0.05$; degree of freedom 1). Therefore we concluded that our sample is in the Hardy-

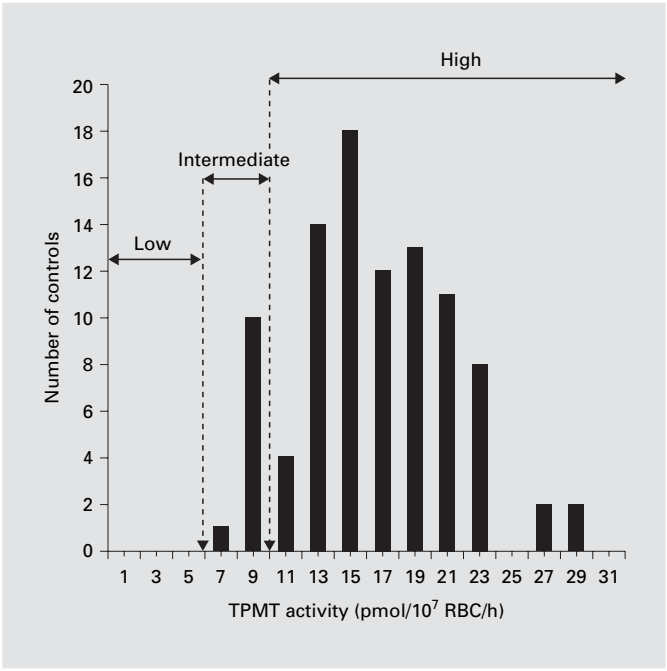

Fig. 3. Frequency distribution histogram of TPMT activity. TPMT activity was determined in 95 randomly selected Slovenian subjects. The statistically determined cutoff value between high and intermediate TPMT activity is 9.82 pmol/10^7 RBC per h. No subjects with low activity (<5.8 pmol/10^7 RBC per h) were observed in our study.

Weinberg equilibrium and that the result can be applied to the whole population. The identification of TPMT*3A as the prevalent allele in the Slovenian population is in accord with previous studies of Caucasian populations, as are the allelic frequencies (tables 1, 2). According to the Hardy-Weinberg law the frequency of any homozygous TPMT*3/*3 genotype for low TPMT activity in the Slovenian population is expected to be 0.24%.

For HPLC analyses of TPMT enzymatic activity, 95 blood samples were used. Among these, 87 individuals were wild-type homozygous (TPMT*1/*1) and predicted to have high TPMT activity, while 8 heterozygote individuals were predicted to have intermediate activity (6 were TPMT*1/*3A, 1 was TPMT*1/*3B and 1 was TPMT*1/*3C heterozygote).

The frequency histogram revealed a bimodal distribution of erythrocyte TPMT activities (fig. 3), and their correlation with previously determined TPMT genotypes demonstrated that the wild-type homozygous group had a higher median and mean TPMT activity than mutant heterozygous samples ($p < 0.05$).

The TPMT activity cutoff value between wild-type and mutant samples was determined by statistical receiver operating characteristics analysis. Hence the calculated cutoff activity between high (wild-type) and intermediate (heterozygous) TPMT activity values was 9.82 pmol/10^7 RBC per h. This cutoff value is in accord with the previously published correlation study [30], where the cutoff value between the high (wild-type) and intermediate (heterozygous) activity groups was 10 pmol/10^7 RBC per h, while the cutoff value between the low- (mutant homozygous) and intermediate-activity groups was shown to be 5.8 pmol/10^7 RBC per h. Since no low-activity samples were found in our study, the latter value was assumed to be the cutoff between low and intermediate TPMT activity samples.

According to the cutoff value determined, the samples are distributed in two groups of high and intermediate TPMT activity (fig. 3). Five samples from the heterozygous group TPMT*1/*3A showed an intermediate TPMT activity of 8.60 ± 0.22 pmol/10^7 RBC per h, although 1 sample from this group showed a higher activity (13.5 ± 0.81 pmol/10^7 RBC per h). The TPMT*1/*3B heterozygous mutant showed an intermediate TPMT activity of 7.87 ± 0.46 pmol/10^7 RBC per h, while, unexpectedly, the TPMT*1/*3C heterozygous mutant showed a high TPMT activity of 15.0 ± 0.45 pmol/10^7 RBC per h (fig. 4). The average TPMT activity of the 87 samples from the wild-type homozygous group was 16.3 ± 4.5 pmol/10^7 RBC per h, although 6 (6.9%) of them showed an average intermediate activity of 8.36 ± 1.26 pmol/10^7 RBC per h. Therefore, the phenotype based on genetic data was predicted correctly in 91.6% of samples, as also seen in previous studies [31–33]. No significant differences were observed between experimental and expected frequencies of TPMT intermediate- and high-activity phenotypes (χ^2 lower than $\chi^2_{theoretical}$ = 3.841; $p > 0.05$; d.f. 1).

Discussion

Based on the statistical method and the Hardy-Weinberg law, the heterozygous TPMT*1/*3 and homozygous TPMT*3/*3 genotype frequency in the Slovenian population are predicted to be 9.31 and 0.24%, respectively. Although no homozygous mutants were found in our population sample, we can expect that approximately 1 in every 400 Slovenians has a homozygous TPMT*3/*3 genotype with a very low or even immeasurable TPMT activity. In previous studies of Caucasian populations

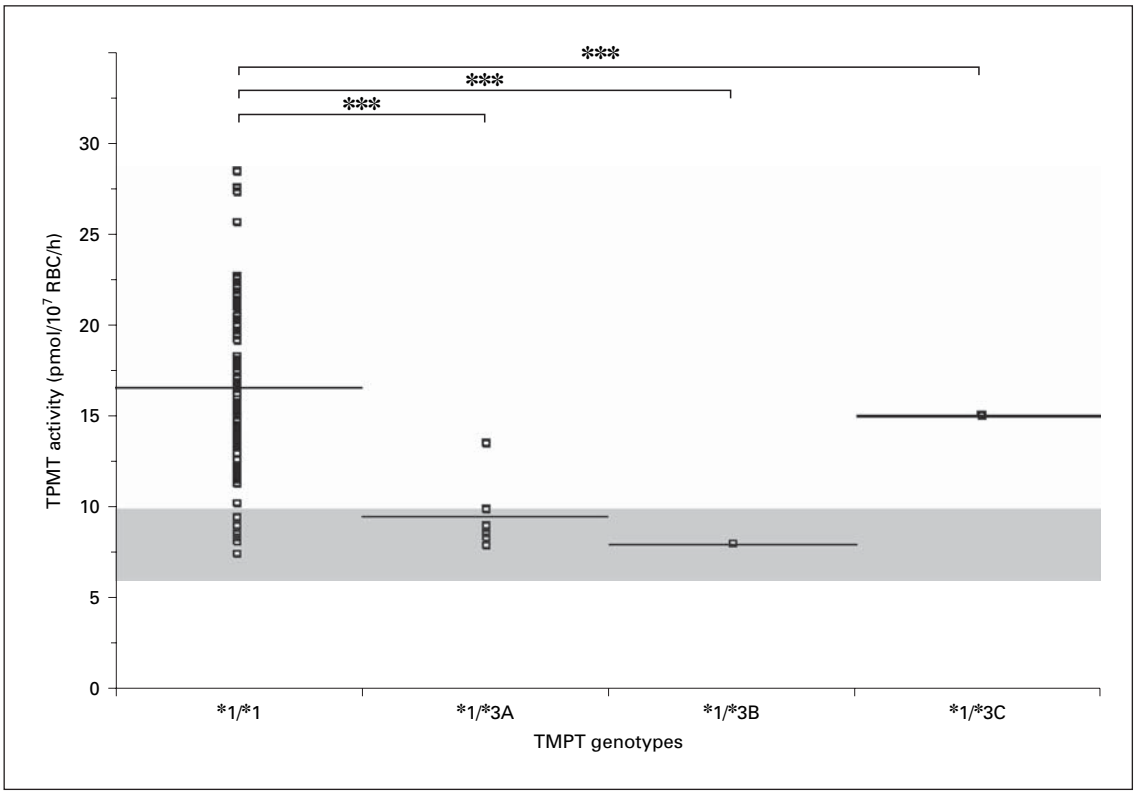

Fig. 4. Distribution of TPMT activity among 95 individuals in relation to their TPMT genotypes. The grey shaded area represents the intermediate TPMT activity range as defined by 2 cutoff values. The wild-type individuals (TPMT*1/*1) were predicted to have high TPMT activity, while individuals with a mutant heterozygous genotype (TPMT*1/*3A, TPMT*1/*3B or TPMT*1/*3C) were expected to have intermediate TPMT activity. Some overlapping of activity values between intermediate and high TPMT activity groups are evident, as not all activities correspond to the observed genotype. The prediction of TPMT phenotype based on TPMT genotyping data was successful in 91.6% of the samples analyzed. *** $p < 0.05$.

(originally reported by Weinshilboum and Sladek [9]) the frequency of mutant homozygotes has been shown to be only slightly higher (0.3%).

The most prevalent mutant TPMT allele in the predominantly Caucasian Slovenian population (TPMT*3A, 4.1%) has a very similar allelic frequency among all the investigated TPMT mutant alleles in Caucasians. It has been determined as the most prevalent mutant allele in Argentinian (3.1%), Colombian (3.6), Italian (3.9), American Caucasian (3.2), Norwegian (3.4), French (3.0), German (4.4), British (4.5), Swedish (3.8) and Polish Caucasian populations (2.7), and it is the only TPMT mutant allele found in the Northern Portuguese population sample (2.4%; for all refs see table 1). TPMT*3C allele is most frequent in individuals of black or Asian ethnicity but is rarely found in Caucasians, having an allelic frequency of 0.1% in Swedish, 0.1 in Polish, 0.2 in American Caucasian, 0.3 in British and Norwegian, and 0.4 in French and German populations, which are most similar to the Slovenian one (0.5%). The frequency of TPMT*3B allele is relatively high (0.3%) in the Slovenian population. This allele has only been found in Brazilian (0.5), Swedish (0.1) and a large US-based diverse population (0.0063). The latter two values were obtained on larger population samples, hence the numerical overrepresentation of the TPMT*3B allele in the 194 Slovenian individuals analyzed is most likely due to the sample size.

The TPMT*2 allele is the predominant nonfunctional allele in the Brazilian population (2.2%) and has been found in Argentinian (0.7), Polish (0.4), Colombian (0.4), British Caucasian (0.5), Italian (0.5), Swedish (0.4), American Caucasian (0.2) and even in African-American (0.4) populations. In our study no carriers of the TPMT*2 allele were identified. Again this is most probably due to

the relatively low allelic frequency of TPMT*2 in Caucasian populations and the size of the population studied. To validate the custom Taqman genotyping method for the determination of TPMT*2 polymorphism we used positive control samples with a known genotype (TPMT*1/*2). The described method requires very low amounts of DNA and is as such highly sensitive. Its simplicity, very short processing times and automation capabilities are the main benefits for the implementation of this method into clinical practice.

High genotype-to-phenotype correlation corresponds to trimodal activity distribution in Caucasians, such that wild-type allele homozygotes have a high enzymatic activity, while heterozygous and mutant homozygous individuals have an intermediate and low enzymatic activity, respectively [9, 12]. The distribution of TPMT enzymatic activity in the population sample studied was bimodal, due to the lack of individuals with a mutant homozygous genotype. The statistically determined cutoff value between the two groups with high and intermediate activity was 9.82 pmol/10^7 RBC per h.

TPMT activity above the cutoff was consequently found in 90.8% (81/87) of the wild-type samples, while the remaining 9.2% (6/87) fell below the cutoff. The intermediate activity was determined in 75% (6/8) of the heterozygous individuals (5 were TPMT*1/*3A, 1 was TPMT*1/*3B), while the remaining 25% (2/8) of the heterozygous samples (1 TPMT*1/*3A and 1 TPMT*1/*3C) exhibited moderately high activity (fig. 4). According to this data, the concordance rate between TPMT enzyme activity and TPMT genotype in our study was 91.6%. This result is in accord with previously published results on small populations [31–33] and slightly lower than the high concordance rate (98.4%) from a recent large-scale study [12].

Further analysis of other TPMT polymorphisms, such as variable number of tandems repeated in the 5′ region of the TPMT gene, may reveal the basis for 2 mutant samples with unexpectedly high TPMT activity, while the undetermined and/or recently identified TPMT*16, *17, *18 and *19 low-activity alleles could underlie the discrepancies in 6 wild-type samples with lower than expected TPMT activity. Accordingly, the genotype to phenotype correlations could be improved in future studies, allowing an even better prediction of thiopurine-related toxicity and fundamentals for individual dose adjustments.

In conclusion, we have identified TPMT*3A as the prevalent mutant allele in the Slovenian population, but also the exceptional presence of TPMT*3B and the absence of TPMT*2 alleles. A genotype to phenotype correlation was established in 91.6% of the examined individuals. Since only limited known TPMT polymorphisms were determined, further genotyping assays could establish an even better prediction of the phenotype. The research presented herein has already been implemented in clinical practice. Upon determination of the heterozygous phenotype, the dose was adjusted in individuals treated with thiopurine drugs. The identification of the individual's genetic TPMT status could facilitate the development of rapid DNA-based assays for patients at high risk or those showing inadequate responses to thiopurine therapy. Routine TPMT genotyping prior to treatment with 6-MP is required for increasing the efficacy and/or decreasing treatment toxicity. The results of genetic and biochemical analyses may be useful for the determination of genotype to phenotype correlation even before the beginning of individualized therapy. Thus pharmacogenetic assessment of patients undergoing thiopurine therapy may give rise to significant improvements in the therapeutic outcome and consequent cost benefits. The ultimate outcome of interest relates to improved therapy, reduced adverse events and optimized dosage of thiopurine drugs.

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