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Thiopurine S-methyltransferase polymorphisms: efficient screening method for patients considering taking thiopurine drugs

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Abstract Objective: More than 11% of the Caucasian population are heterozygous or homozygous carriers of thiopurine S-methyltransferase (TPMT) mutants and are at risk for toxic side effects when treated with thiopurine drugs. Therefore, screening for TPMT polymorphisms in a patient prior to prescribing these agents is recommended. The goal of this study was to determine a cut-off concentration of the TPMT activity assay beyond which genotyping of the TPMT gene should be performed.

Methods: The TPMT activity of 240 unrelated Caucasian subjects was measured using high-performance liquid chromatography. Genotyping for the most frequent allelic variants, TPMT*2, *3A, *3B, *3C and *7 was performed by LightCycler technology and sequencing.

Results: The inter-individual TPMT activity showed a range from 23 nmol MTG/g*Hb*h⁻¹ to 97 nmol MTG/g*Hb*h⁻¹ with a median of 56 nmol MTG/g*Hb*h⁻¹. Using a cut-off concentration of 45.5 nmol MTG/g*Hb*h⁻¹, a test sensitivity of 100% and a specificity of 89% were reached for heterozygous carriers of a TPMT mutation. We identified 1 carrier of TPMT*2, 14 carriers of TPMT*3A and 3 carriers of TPMT*3C, resulting in a TPMT heterozygosity prevalence of 7.5%.

Conclusions: This study defines the cut-off value for the TPMT phenotyping assay at 45.5 nmol/g*Hb*h⁻¹, beyond which additional genotyping elucidates the individual risk for drug therapy. Using this cut-off concentration, the number of genotyping assays could be reduced by about 60%.

Keywords Thiopurine methyltransferase · Thiopurine drugs · Single nucleotide polymorphisms

Introduction

Thiopurine S-methyltransferase (TPMT; EC 2.2.2.67) is a cytosolic enzyme, which catalyzes the S-methylation of widely used immunosuppressive but also cytotoxic thiopurine drugs, such as 6-thioguanine, 6-mercaptopurine and azathioprine [1]. The in vivo activity of this enzyme is characterised by inter-individual and inter-ethnic variability caused by genetic polymorphisms in the TPMT gene. The frequency distribution of TPMT activity in Caucasian population studies is trimodal: 1 in 300 subjects has undetectable activity, 11% have intermediate activity and 89% inherit high enzyme activity [2]. The gene that codes for TPMT has been localised to chromosome 6 (6p22.3). Various TPMT alleles, carrying a point mutation or a combination of mutations in the ten exons and nine introns, as well as in the 5'-flanking promoter region, have been associated with deficient, intermediate or high activity [3, 4, 5, 6, 7]. As a consequence, individuals greatly differ in the detoxification of thiopurine drugs to 6-methylmercaptopurine and, thus, in the occurrence of side effects or reduced therapeutic efficacy. TPMT polymorphisms have been linked with severe and potentially fatal myelosuppression in deficient metabolisers and rejection of transplanted organs in high metabolisers [8, 9, 10]. Therefore, phenotyping and/or genotyping tests for TPMT prior to thiopurine therapy should become routine practice to avoid severe haematotoxicity in TPMT-deficient patients and to lower the incidence of haematological side effects in heterozygous individuals. But it has to be considered that not every risk can be eliminated by the analysis of the TPMT gene. It is well known that during an established thiopurine drug therapy in some wild-type patients different side effects

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can occur, which are independent of TPMT polymorphisms, such as pancreatitis, hepatotoxicity, pancytopenia, nausea, vomiting or abdominal pain [11, 12, 13]. Nevertheless, congenital patients who are at risk can be detected, and drug-induced toxicity can be prevented from the beginning of the therapy.

TPMT molecular pharmacogenetic studies resulted in the discovery of a series of variant alleles (single nucleotide polymorphisms) associated with significantly decreased levels of TPMT activity. The most common of these variant alleles in Caucasian populations, TPMT*3A, is characterised by the combined appearance of two single nucleotide polymorphisms (SNPs) in exon 7 (G460A) and exon 10 (A719G), which result in Ala → Thr and Tyr → Cys substitutions, respectively. TPMT*3B (G460A) and the TPMT*3C (A719G) alleles are caused by the sole presence of the exon 7 and exon 10 mutations, respectively [3, 5, 14]. For TPMT*2, a G238C mutation in exon 5 causes an Ala → Pro substitution [7]. Further, a polymorphic tandem repeat was identified within a GC-rich area in the 5'-flanking region of the TPMT gene. The most common alleles were those with four and five 17-bp or 18-bp repeats. An inverse relationship was found: the larger the number of repeats, the lower the activity. As a conclusion, a modulation of TPMT activity by the variable number of tandem repeats was discussed [15]. Various detection possibilities of TPMT genotyping assays have been published, e.g. polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) [16], denaturing high-performance liquid chromatography (HPLC) [17], sequencing [6] and melting-curve analysis [18].

Phenotyping represents a possibility to test the metabolic capacity of TPMT. At the beginning, the enzyme activity was measured by a radiochemical assay [19]; more recently, HPLC methods have been published [20, 21, 22]. All these methods are based on the *in vitro* conversion of 6-mercaptopurine to 6-methylmercaptopurine or 6-thioguanine to 6-methylthioguanine, using S-adenosyl-L-methionine as the methyl donor. The product of the enzymatic reaction is extracted by liquid-liquid or solid-phase extraction and is measured by HPLC with ultraviolet (UV) or fluorescence detection.

The general advantage of phenotyping is a reliable detection of an individual's enzyme activity at less costs per analysis compared with genotyping analysis, which includes laborious DNA extraction, time-consuming thermocycling programs and interpretation of agarose gels or chromatograms. Overall, for the implementation, the infrastructure for the phenotyping assay, such as HPLC-UV systems, is more often available in laboratories than, for example, realtime thermocycling systems, such as LightCycler technology. We report a phenotype-genotype comparison of the TPMT enzyme and develop a new screening strategy for patients prior to taking thiopurine drugs.

Materials and methods

Reagents and chemicals

Ammonium formate and sodium 1-hexansulfonate monohydrate were supplied by Fluka (Buchs, Switzerland). Methanol and dichloromethane were obtained from Scharlau (Barcelona, Spain), acetonitrile from Biosolve (Valkenswaard, Netherlands) and potassium dihydrogenphosphate, 2-propanol and acetic acid from Merck (Darmstadt, Germany). Acetonitrile and methanol were of HPLC grade; all other chemicals were of analytical grade. 6-Methylthioguanine, 6-thioguanine and S-adenosyl-L-methionine were purchased from Sigma-Aldrich (Steinheim, Germany).

Oligonucleotides

Primers for sequencing TPMT *3C and *7 (exon 10) were designed with the Oligo 4.0 software, purchased from Microsynth (Balgach, Switzerland). The LightCycler primers and probes for TPMT *2 and TPMT *3B were synthesised by TibMolbiol (Berlin, Germany). The hybridisation probes were labelled with LCRed640, Cy5.5 or FL (fluorescein) with a phosphate molecule at the 3' end.

Subjects

Venous blood from 102 unrelated healthy volunteers and 138 patients with inflammatory bowel disease from the University Hospital Zurich was collected in blood collection tubes containing ethylenediaminetetraacetate. The local ethics committee approved this study, and an informed consent was obtained from all blood donors. DNA was isolated with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Phenotyping

The TPMT activity assay was performed according to Kroplin [22] with minor modifications. The haemoglobin content (Hb) of the red blood cell lysate was measured by a Microcellcounter F500 (Sysmex, Hamburg, Germany). The sample volume of each patient consisted of 50 µl red blood cell lysate and was equal to the published method, whereas the extraction of the product was made with dichloromethane/2-propanol (90/10; v/v) instead of chloroform/2-propanol (90/10; v/v). The HPLC system consisted of a 9010 pump, a 9100 autosampler and a 9050 UV-VIS detector (Varian, Sunnyvale, CA, USA). The detector was set at 290 nm and the injection volume was 100 µl. 6-Thioguanine and 6-methylthioguanine were separated using a reversed-phase C18 column Phenomenex Aqua (250×4.6 mm, 5 µm, 125A, Torrance, California, USA). Eluent A consisted of ammonium acetate buffer 5 mM/pH 3, methanol, acetonitrile (95/2.5/2.5; v/v/v) with 0.2% hexanesulfonic acid; eluent B consisted of ammonium acetate buffer 5 mM/pH 3, methanol, acetonitrile (5/75/20; v/v/v). The mobile phase was mixed in a gradient system starting with 100% A, after 5 min changing to 50% A, 50% B during 5 min, which was then maintained for 10 min. The flow rate was 1.0 ml/min.

Genotyping

The genotyping of TPMT*3C (A719G) was carried out using PCR, followed by Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. For TPMT *3C, the primers were L1 5'ATCTTTACATCATAAATCTCCTCTCCAA and U1 5' AATCCCTGATGTCATTCTTCATAGTATT, according to Otterness et al. [6] with minor modifications. The PCR

amplifications were performed in a thermal cycler (Perkin-Elmer, Gene Amp PCR System 9600). The 50 µl reaction volume included 5 µl genomic DNA (ca 50 ng/µl) as template, 1.0 µl of each primer *3C L1 and *3C U1 (10 mM), 1.0 µl of dNTP mixture (10 mM), 5.0 µl 10× buffer II, 8.0 µl of magnesium chloride (25 mM), 0.4 µl Gold *Taq* (5 U/µl) and 28.6 µl of water. The following cycling conditions were used: 10 min at 94 C, 40 cycles of 94 C for 30 s, 63 C for 30 s and 72 C for 60 s and a final extension of 7 min at 72 C. The 10 µl sequencing reaction volume contained 4.0 µl of Big Dye Reaction Mix, 1.0 µl *3CU1 (10 mM) and 5.0 µl of purified amplification product. The sequencing reaction conditions involved 45 s at 94 C, 25 cycles of 10 s at 96 C, 5 s at 55 C and 90 s of 60 C. Sequencing analysis was carried out on an ABI Prism Model 310 DNA sequencer (Perkin Elmer, Applied Biosystems).

The LightCycler PCR assays for TPMT* 2 and TPMT* 3B were applied from Schutz et al. [18]. The primers and probes for TPMT*2 (exon 5) were cctgcatgttctttgaaaccctatgaa (intron4for), gcttgagtacagagagcctttgacctc (exon5rev), LCR-ggctgcaaacctgataa-PHO (tpmt *2) and CY-atttccacaccaactacactgtc-FL (anchor) and for TPMT*3B (exon 7) ctcccacccaggtccacacatt (intron6for), gta-tagtatactaaaaaattgaacagcctaacc (exon7rev), ggcaactaatgctctcatcatcc-FL (tpmt *3b) and CY-atcatgtcaaatgtccaatattgtcctaccag-PHO (anchor). The assay conditions were identical to Schutz et al. [18].

Statistical analysis

Descriptive statistics and multiple correlations were calculated using Excel 97 (Microsoft, USA) and StatView (SAS, Cary, NC, USA). Median and mean values of TPMT activity were compared using nonparametric statistics and *t*-tests, respectively. A *P* value of less than 0.05 was considered to be statistically significant. Receiver-operating-characteristics (ROC analysis) were applied for the calculation of the cut-off concentration for the TPMT phenotyping assay. The chi-square test was used to compare the frequencies of TPMT phenotypes between subgroups. For diagnostic significance analysis, calculations were made as follows: test sensitivity = $RP/(RP + FN)$, test specificity = $RN/(RN + FP)$, test efficiency = $(RP + RN)/(RP + FN + FP + RN)$ and positive predictive value = $RP/(RP + FP)$ (abbreviations: *RP* right positive, *FP* false positive, *RN* right negative, *FN* false negative).

Results

Genotyping was performed for the five most frequent SNPs: TPMT *2, *3A, *3B, *3C and *7. The total frequency of heterozygous subjects in our collective was 7.5%. The frequencies of the TPMT *2, *3A and *3C alleles were 0.4%, 5.8% and 1.3%, respectively (Table 1).

A typical chromatogram of the TPMT phenotyping assay is shown in Fig. 1. The retention time for 6-methylthioguanine was about 13.7 min.

Minor modifications of the phenotyping assay conditions, such as the extraction step and the UV detection instead of the fluorescence detection resulted in an increase of the median TPMT activity of 44% (56 nmol versus 38.8 nmol MTG/g*Hb*h⁻¹) compared with Kroplin et al. [22]. The inter-individual TPMT enzyme activity ranged from 23 nmol MTG/g*Hb*h⁻¹ to 97 nmol MTG/g*Hb*h⁻¹.

Based on the genotyping results, mean values of TPMT activity were calculated for wild-type and heterozygous subjects, respectively (Table 2). Significant TPMT activity differences between the two groups resulted in a *P* value < 0.0001. The normal distribution of TPMT activity among wild-type and heterozygous carriers is depicted in Fig. 2. The analysis of the healthy controls and the group taking thiopurine drugs demonstrated no significant differences in the mean of TPMT activity between the two collectives; consequently, no enzyme-induction phenomena could be observed. Additionally, gender differences could not be detected.

The correlation between phenotype and genotype according to the SNPs is demonstrated in Fig. 3. As expected, samples with one mutated allele had a lower TPMT activity than samples with two wild-type alleles, but a well-defined cut-off concentration could not be set.

The optimal cut-off activity for the TPMT phenotype assay to predict a heterozygous carrier was chosen by ROC analysis (Fig. 4), with the aim to include all heterozygous samples. The calculated cut-off activity was 45.5 nmol MTG/g*Hb*h⁻¹ with a test sensitivity of 100% and a specificity of 89%. The test efficiency was 90% and the positive predictive value of a TPMT activity below the cut-off amounted to 42%.

Discussion

The genetic TPMT polymorphism represents a striking example of the potential clinical importance of pharmacogenetic variation of a drug-metabolising enzyme [23]. The incidence of TPMT mutations for the Caucasian population is reported to be 0.3% for homozygous, 11% for heterozygous and 89% for wild-type carriers [2]. Homozygous mutated individuals are at increased risk for potentially life-threatening toxicity when exposed to standard doses of thiopurine drugs, while

Table 1 Frequencies of thiopurine *S*-methyltransferase (TPMT) mutations analysed in a Swiss population sample

TPMT genotype	Exon	Nucleotide substitution	Amino acid exchange	No. of subjects	Observed frequency [%]
TPMT *1/*1		Wild type		222	92.5
TPMT *1/*2	5	G238C	Ala80Pro	1	0.4
TPMT *1/*3A	7/10	G460A/A719G	Ala154Thr/Tyr240Cys	14	5.8
TPMT *1/*3B	7	G460A	Ala154Thr	0	0.0
TPMT *1/*3C	10	A719G	Tyr240Cys	3	1.3
TPMT *1/*7	10	T681G	His227Glu	0	0.0
Total heterozygous				18	7.5

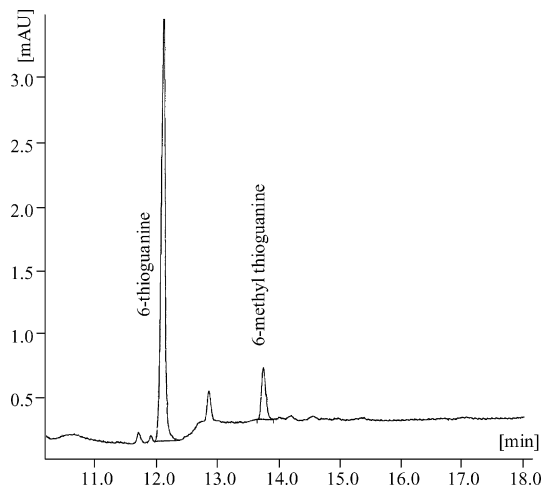


Fig. 1 Chromatogram of the thiopurine *S*-methyltransferase phenotyping assay: the educt 6-thioguanine is detected at $R_t = 12.1$ min and the product 6-methylthioguanine at $R_t = 13.7$ min

heterozygous carriers have to start the immunosuppressive therapy with 50–60% of the recommended dose [24].

The molecular basis of TPMT deficiency is well understood; TPMT *2, *3A, *3B, *3C and *7 are the most common variants and account for 80% of all possible mutations [4]. Rare variants, such as *3D, *4, *5 and *6 were omitted in this genotyping study. The incidence of these mutations was 0% in a collective of 191 subjects [25]. Furthermore, promoter polymorphisms were ignored because of their minor effect on the modulation of TPMT activity compared with SNPs in the open reading frame [15].

As the consequences of individualised thiopurine drug therapy would be of potential benefit for a single patient, we compared the TPMT activity, as determined by a non-radiochemical assay, with the TPMT genotype, assessed using a realtime PCR-based technique and sequencing.

In our cohort, TPMT*2, *3A, *3B, *3C and *7 mutations had similar prevalences (7.5%), as seen in reference populations [2, 4, 6, 25, 26]. The heterozygosity rate varies in these studies between 7.4% and 13.5%. The higher prevalence of mutated subjects in these clinical trials can be attributed to the preselection of the

subjects, such as leukaemia patients with toxicity under thiopurine drugs [27].

In order to determine the distribution of TPMT activity, blood samples from randomly collected healthy donors were obtained and compared with the levels measured in samples from patients undergoing a thiopurine drug therapy. Apart from the mean differences between wild-type and heterozygous carriers, no other influencing factors could be observed. The trimodal enzyme activity distribution could not be shown thus far, due to the absence of a homozygous-mutated individual, who is deficient in TPMT activity. The prevalence of 0.3% lets assume that our collective of 240 individuals has to be enlarged for this purpose. The TPMT activity distribution between heterozygous carriers and wild-type subjects led to the definition of a cut-off concentration ($45.5 \text{ nmol/g*Hb*h}^{-1}$) beyond which genotyping has to be performed. The cut-off of the phenotyping assay was set at an activity with a probability of 100% to detect a heterozygous subject. The sensitivity and specificity analysis was optimised to detect all heterozygous samples so that the test can be used as a screening assay for genotyping. The specificity accounts for 89%, which can be accepted, as the number of genotyping assays is reduced to a reasonable number. Phenotype/genotype discrepancies, such as false positives that result in TPMT activity measurements (i.e. low TPMT activity) despite wild-type genotype, were explained thus far by missed SNPs in the open reading frame, the variable number of tandem repeats in the promoter region [16] or patients' age [28] and renal function, as well as drug interactions [29]. The analysis of our patients with wild-type genotype and an activity beyond the defined cut-off concentration resulted in subjects of all age, who ranged from 17 years to 78 years. Further, the phenomenon was observed in healthy controls ($n=8$), as well in patients under thiopurine drugs ($n=17$). No correlation was found between renal function, measured by creatinine clearance, and TPMT activity. Our observation showed that patients who had a TPMT activity below the cut-off concentration, but without a mutation on the TPMT gene, tolerated a thiopurine drug therapy with standard doses.

The aim of the study was not to detect each possible mutation on the TPMT gene, but to extract those

Table 2 Thiopurine *S*-methyltransferase (TPMT) genotype compared to TPMT activity means according to sex and collective

Genotype	Collective	No. of subjects	Sex	No. of subjects	Mean \pm SD (nmol MTG/g*Hb*h ⁻¹)
Wild type	Healthy	95	Male	59	58.8 \pm 9.8
			Female	36	55.5 \pm 10.5
	Under thiopurine drug therapy	127	Male	61	59.3 \pm 12.4
			Female	66	57.8 \pm 12.1
			222	58.1 \pm 11.4*	
Heterozygous	Healthy	7	Male	5	31.1 \pm 5.1
			Female	2	32.1; 39.8
	Under thiopurine drug therapy	11	Male	4	39.8 \pm 7.0
			Female	7	34.9 \pm 5.6
			18	35.4 \pm 6.1*	

* $P < 0.0001$

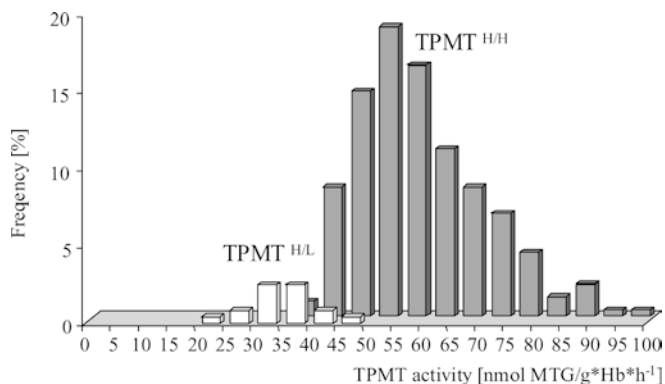


Fig. 2 Frequency of thiopurine S-methyltransferase (TPMT) activity distribution in 240 subjects according to wild-type and heterozygous carriers of TPMT mutations

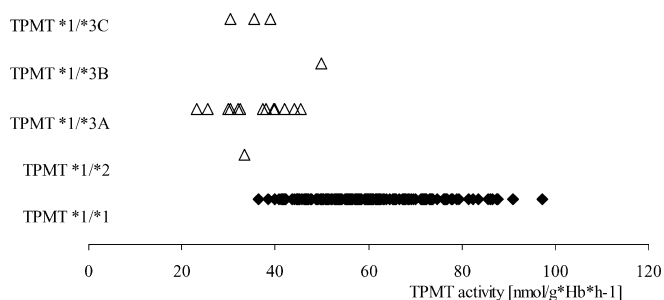


Fig. 3 Distribution of thiopurine S-methyltransferase (TPMT) activity in relation to their TPMT genotypes. Individuals genotyped homozygous for the functional allele TPMT *1/*1 were predicted as high metabolisers (*closed diamond*), and individuals genotyped heterozygous for the non-functional alleles *2, *3A, *3B, *3C were predicted as intermediate metabolisers (*open triangle*)

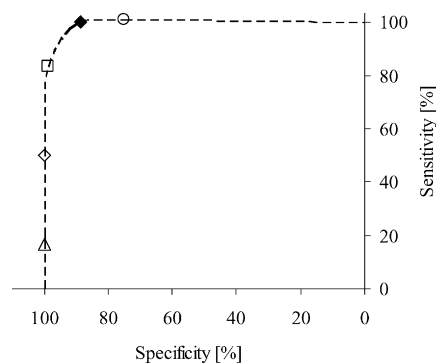


Fig. 4 Receiver-operating-characteristics analysis: the cut-off concentration of 30 nmol MTG/g*Hb*h⁻¹ is represented by an *open triangle*, 35 nmol by an *open diamond*, 40 nmol by an *open square*, 45.5 nmol by a *closed diamond* and 50 nmol by an *open circle*

patients supposed to be at risk for toxicity during intake of thiopurine drugs. The number of genotyping assays could be reduced to 40% of the samples, which is cost effective and timesaving. By applying our recommended phenotyping method, possible savings amounted to between 40% and 50% of the overall costs, depending on

the standard rates of each hospital. In addition, it allowed an earlier start of therapy for most of the patients, as they are wild type and have a low risk for toxicity problems under thiopurine drugs.

In conclusion, we report a screening procedure for patients prior or subsequent to initiation of thiopurine drug treatment. With this new combined concept, patients can be tested rapidly for TPMT mutations, and drug therapy can be started at individually chosen dosages.

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