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Identification and functional analysis of two rare allelic variants of the thiopurine *S*-methyltransferase gene, *TPMT*16* and *TPMT*19*

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Abstract

Human thiopurine *S*-methyltransferase (TPMT) catalyses the *S*-methylation of thiopurine drugs. TPMT is genetically polymorphic and is associated with large interindividual variations in thiopurine drug toxicity and therapeutic efficacy. During routine genotyping of patients with Crohn's disease, one novel missense mutation, 365A > C (*TPMT*19*, Lys[122]Thr), and a recently described missense mutation, 488G > A (*TPMT*16*, Arg[163]His), were identified in a Caucasian and a Moroccan patient, respectively. Using a heterologous yeast expression system, kinetic parameters (K_m and V_{max}) of the two variants with respect to 6-thioguanine *S*-methylation were determined and compared with those obtained with the wild-type enzyme. The Lys[122]Thr exchange did not significantly decrease the intrinsic clearance value (V_{max}/K_m) of the variant enzyme. In contrast, the Arg[163]His substitution significantly decreased the intrinsic clearance value by three-fold. The Arg[163] is located in a highly conserved region of the human TPMT protein and, as such, the Arg[163]His substitution is expected to result in a marked reduction of enzyme activity, as confirmed by the in vitro data. Phenotyping by measurement of red blood cell TPMT activity indicated that the patient heterozygous for the Lys[122]Thr mutation had normal TPMT activity, whereas the patient heterozygous for the Arg[163]His mutation was an intermediate methylator, which demonstrated a positive correlation between TPMT phenotyping and the in vitro data. The identification of a novel non-functional allele of the TPMT gene improves our knowledge of the genetic basis of interindividual variability in TPMT activity. These data further enhance the efficiency of genotyping methods to predict patients at risk of an inadequate response to thiopurine therapy.

Keywords: TPMT; Genetic polymorphism; Azathioprine; 6-Thioguanine; Deficient methylator; Heterologous expression

1. Introduction

The thiopurines 6-mercaptopurine, azathioprine and 6-thioguanine (6-TG) are used in the treatment of leukaemia and inflammatory bowel disease, and in the prevention of organ transplant rejection [1]. Thiopurines are metabolised extensively by both anabolic and catabolic pathways. Human thiopurine *S*-methyltransferase (TPMT; EC2.1.1.67) is a cytosolic enzyme that catalyses the *S*-methylation of heterocyclic and aromatic compounds, including thiopurines. TPMT exhibits a genetic polymorphism with 89% of Caucasians and Afro-Americans exhibiting a high methylator (HM) phenotype, 11% an intermediate activity (intermediate methylator, IM) and 0.33% a TPMT deficiency (deficient methylator, DM) [2–4].

Several clinical studies have found that high methylators may be under-treated with conventional doses of thiopurine drugs, whereas intermediate and deficient methylators are recognized to be at risk of moderate to profound haematopoietic toxicity when treated with standard doses of these medications [5–10]. These observations are explained by an inverse relationship between TPMT activity and the production of active thiopurine metabolites, designated as thioguanine nucleotides (TGN) [5–7].

Abbreviations: RBC, red blood cell; 6-TG, 6-thioguanine; TPMT, thiopurine *S*-methyltransferase

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The gene encoding TPMT is located on chromosome 6 (6p22) and consists of 9 introns and 10 exons. To date, at least 16 variant alleles causing TPMT deficiency have been defined [11–18]. In Caucasian and Afro-American populations, *TPMT*2*, *TPMT*3A* and *TPMT*3C* represent the most prevalent mutant alleles, and account for 80–95% of intermediate and deficient methylator phenotypes [14,19]. It is obvious that the prediction of TPMT phenotype from genotyping methods will increase as more rare alleles affecting enzyme function are discovered [18].

During routine genotyping, two additional novel *TPMT* allelic variants, which are characterized by a single missense mutation, have been identified in patients with Crohn's disease. In order to characterize and assess the functional impact of these mutations on TPMT enzyme activity, an in vitro analysis, using a yeast heterologous expression system, and subsequent phenotyping of the patients were carried out.

2. Materials and methods

2.1. Patients

In order to determine, by genotyping, the TPMT status of Crohn's disease patients ($n = 550$) prior to azathioprine or 6-mercaptopurine treatment, venous blood (10 ml) was collected in EDTA treated tubes, after written informed consent had been obtained. Two patients, one of North-African origin and a Caucasian were found to carry novel mutations in the open reading frame of the TPMT gene.

2.2. TPMT genotyping protocol

Total RNA was isolated from venous blood using Trizol reagent (Life Technologies), and retro-transcribed by Superscript™ RNase H-reverse transcriptase (Life Technologies) according to the manufacturers' instructions. Wild-type *TPMT* cDNA was amplified by PCR using *Ampli Taq* DNA polymerase (Applied Biosystems) and a pair of primers that anneal to sequences within exon 4 and exon 10 of the published sequence of the *TPMT* cDNA (NCBI: accession number BC009596), as follows: sense primer, 5′-GCATTTAGATACTTTCCTTAAAGGCA-3′; anti-sense primer, 5′-AAAAACATGTCAGTGATTTTATTTT-3′. Primers were designed to avoid co-amplification of the highly similar processed pseudogene [20]. The anticipated size of the PCR product was 625 bp. The PCR protocol consisted of an initial denaturation step at 95 °C for 2 min, followed by 32 PCR cycles (94 °C for 50 s, 56 °C for 40 s and 72 °C for 2 min) and a final extension at 72 °C for 10 min.

cDNA sequencing was performed on both strands with the Big Dye® terminator cycle sequencing kit (Applied Biosystems), using an automated ABI 3700 capillary sequencer (Applied Biosystems). Sequences were compared with the wild-type sequence using the SeqScap version 2.0 software (Applied Biosystems), which identifies variant and sequence matches from an allele library.

2.3. Site-directed mutagenesis

The yeast expression vector (pYeDP60) [16] containing the wild-type *TPMT* cDNA was used as the template for site-directed mutagenesis using the mutagenic primers listed in Table 1 and the QuickChange™ Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. Sequencing of each of the mutated cDNA constructs was carried out on both strands to assess their strict identity with the expected mutated sequence.

2.4. Heterologous expression and Western blot analysis

Saccharomyces cerevisiae W(R) strain (generous gift from Dr. Pompon) was transformed by the lithium acetate method with wild-type or mutant cDNA [21]. Cultures of yeast cells expressing each *TPMT* cDNA and the preparation of cytosolic fractions were performed as described previously [16]. The total protein content of cytosolic fractions was determined with the BCA protein assay kit (Interchim). Rabbit antibodies raised against human TPMT (generous gift from Drs. Krynetski and Evans) were used for Western blot analysis as described previously [16]. The intensity of bands revealed by the anti-TPMT serum was measured by a digital camera (DC120, Kodak Digital Science) equipped with the ID Image Analysis Software (Kodak Digital Science).

2.5. 6-Thioguanine S-methylation assay

Enzymatic activity of the yeast cytosolic fractions was assessed using 6-thioguanine as a substrate and metabolite formation assayed according to a published HPLC method [16]. Apparent K_m and V_{max} values for *S*-methylation of 6-TG, as well as V_{max}/K_m ratios (in vitro intrinsic clearance),

Table 1
Primers used for site-directed mutagenesis

Primers	Sequence (5′ → 3′)[a]	Mutated codon	Amino acid substitution
K122TF	CCTGGAACCAAAGTATTTACGAGTTCTGGGAAC	AAG → ACG	$Lys^{122}Thr$
K122TR	GTTCCCCGAAGAACTCGTAAATACTTTGGTTCCAGG		
R163HF	CCATTCCAGATCACAAATGCTATGCAGATA	CGC → CAC	$Arg^{163}His$
R163HR	GTATCTGCATATTTGTGATCACCTGGATTAATG		

[a] Underlined nucleotides correspond to mutated nucleotides.

Table 2
Kinetic parameters of 6-thioguanine *S*-methylation by recombinant TPMT variants expressed in yeast

TPMT variant	K_m (μM)	V_{max} (nmol/min/mg prot)	V_{max}/K_m (ml/min/mg prot)
Wild-type (*TPMT*1*)	15.2 ± 0.3	19.2 ± 1.0	1.25 ± 0.09
$Lys^{122}Thr$ (*TPMT*19*)	18.8 ± 1.7	16.8 ± 3.3	0.87 ± 0.11 (69%)[a]
$Arg^{163}His$ (*TPMT*16*)	44.8 ± 7.2*	18.0 ± 0.3	0.40 ± 0.05* (32%)[a]

All values are mean ± S.D. from three independent experiments.
[a] Values in parentheses represent relative rates of V_{max}/K_m as a percentage of the wild-type value.
* $p < 0.05$.

were determined for each variant form of TPMT and compared with the values obtained for the wild-type enzyme (Table 2).

Kinetic parameters were treated statistically with ANOVA and the Tuckey post hoc test for multiple parameters. A value of $p < 0.05$ was considered as statistically significant.

2.6. TPMT phenotype determination

TPMT activity was assessed using erythrocyte lysates of the two patients carrying novel *TPMT* variants, according to a previously published protocol [22]. The HM phenotype was defined as a TPMT activity greater than 13.7 U ml^{-1} of red blood cell (RBC). Individuals having a TPMT activity ranging from 5 to 13.7 U ml^{-1} RBC were defined as IMs. DMs exhibited values lower than 5 U ml^{-1} RBC.

3. Results and discussion

Individuals who inherit the autosomal recessive TPMT deficiency trait can develop severe haematopoietic toxicity when treated with standard dosages of mercaptopurine or azathioprine, due to accumulation of the cytotoxic metabolites, 6-thioguanine nucleotides, in haematopoietic tissues. Prospective measurement of erythrocyte TPMT activity is a routine safety measure prior to thiopurine therapy in order to prevent toxic side effects. However, there are a number of limitations with respect to the determination of constitutive TPMT enzyme activity. For example, the reliability of the phenotyping assay can be questioned because this measurement may not reflect constitutive TPMT activity of patients who received donor erythrocyte transfusions within the previous two to three months or who received medications that can induce or inhibit TPMT activity. Therefore, the possibility of performing a genetic diagnosis of TPMT deficiency or heterozygosity before the initiation of thiopurine treatment offers an alternative approach to assess the inherent capacity of an individual to metabolise thiopurine drugs.

Genotyping two Crohn's disease patients by *TPMT* cDNA sequencing revealed the presence of one novel and a recently identified [25] missense mutations, corresponding to 365A > C ($Lys^{122}Thr$) and 488G > A ($Arg^{163}His$, *TPMT*16*) substitutions in exons 5 and 7, respectively. The location of mutations referred to the ATG translation start site with A at position +1. According to the *TPMT* allele nomenclature, the new variant was designated *TPMT*19*. Additionally, DNA sequence analysis of the 10 exons of *TPMT* and their 5′- and 3′-consensus splice sites was performed as described to confirm the identification of the variants, and to exclude the presence of other unidentified mutations [23]. However, no other sequence alteration was found. *TPMT*16* and *TPMT*19* can be considered as very rare variants of *TPMT* with a frequency <0.1% as each was identified in a heterozygous state in one out of a total of 550 patients with Crohn's disease, all being genotyped by cDNA sequencing (data not shown). However, due to the existence of inter-ethnic variations in the distribution of *TPMT* mutant alleles [10], the possibility arises that the frequency of these new alleles may be different in certain ethnic groups. Particularly, the frequency of *TPMT*16* allele identified in the Morrocan patient could be greater in North-African populations.

In order to characterize the functional impact of the novel missense mutations, mutant cDNAs generated by site-directed mutagenesis, were expressed heterologously in yeast cells. Expression of wild-type and mutant *TPMT* cDNAs in *S. cerevisiae* enabled us to prepare cytosolic fractions containing the recombinant proteins and to analyse their level of expression and catalytic activity. Expression of mutant forms of TPMT in yeast has already been successfully used to functionally characterize various naturally occurring mutations of *TPMT* [11,13,16,24].

When expressed in yeast cells, the apparent K_m of the $Arg^{163}His$ variant (*TPMT*16*) for the 6-thioguanine *S*-methylation, showed a significant three-fold increase compared to the wild-type enzyme (44.8 μM versus 15.2 μM, respectively, $p < 0.05$), whereas the $Lys^{122}Thr$ substitution in the *TPMT*19* variant did not affect the K_m value (18.8 μM versus 15.2 μM). V_{max} values for each mutant form did not differ significantly from that of the wild-type (Table 2).

The *TPMT*16* allele identified in a Morrocan patient encodes a mutant enzyme which significantly reduced the intrinsic clearance value (V_{max}/K_m) with respect to 6-TG methylation by approximately three-fold compared with the wild-type allele. It is of note that both the *TPMT*12* ($Ser^{125}Leu$) and *TPMT*10* ($Gly^{144}Arg$) defective alleles, which were previously assessed with the same method [16], decreased the intrinsic clearance value to the same

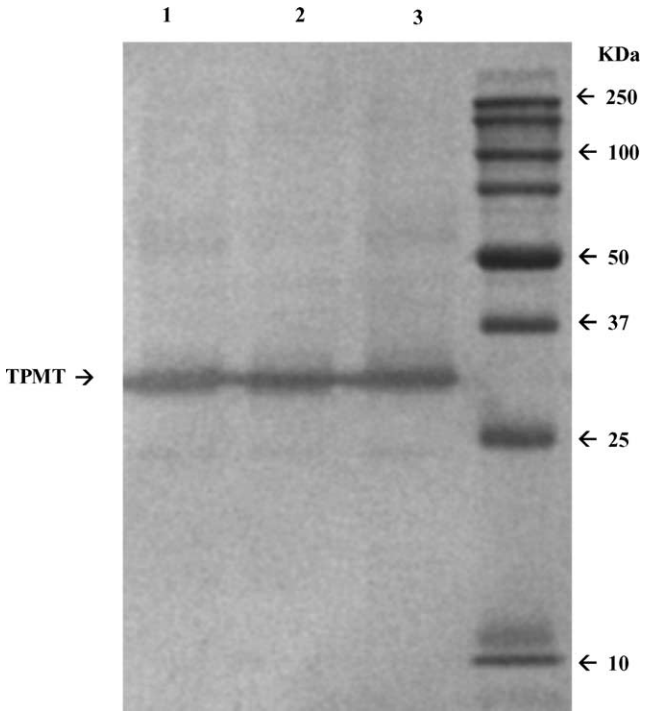

Fig. 1. Immunoblot analysis of recombinant human TPMT proteins. Cytosolic proteins (5 μg per lane) of yeast cells expressing the wild-type, Lys^{122}Thr and Arg^{163}His forms of TPMT were loaded in lanes 1, 2 and 3, respectively. The proteins were probed with a rabbit antiserum against human TPMT diluted 1–2000. A molecular mass marker was applied to lane 4.

extent [~3.5 folds]. Furthermore, it appears that Arg^{163}His exchange associated with *TPMT*16* did not alter the level of protein expression in the yeast expression system when compared to wild-type protein (relative intensity compared to that of the wild-type: 0.9) (Fig. 1). Consistent with our in vitro data, an intermediate TPMT activity (12.8 U ml^{-1} RBC) was found in the patient genotyped as *TPMT*1/*16*. This result is also corroborated by the recent observation of another subject heterozygous for Arg^{163}His, who also presented with an intermediate TPMT activity [25]. Taking these findings together, it can be assumed that *TPMT*16* encodes in vivo a protein with a severely impaired catalytic efficiency and could then be considered as a non-functional allele of *TPMT* in the context of phenotype prediction via genotyping tests.

Regarding the *TPMT*19* allele (Lys^{122}Thr exchange) identified in the Caucasian patient, the V_{max}/K_m value did not significantly decrease compared to that of the wild-type TPMT. Furthermore, the patient had an apparent normal TPMT activity (36.7 U ml^{-1} RBC), which is in accordance with our in vitro data. Like with the *TPMT*16* allele, the TPMT protein level in the cytosol preparation of *TPMT*19* was similar to that of the wild-type preparation (relative intensity to that of the wild-type: 1.1) (Fig. 1). Consequently, *TPMT*19* can be considered as a functional *TPMT* allele.

The molecular/structural basis for the detrimental influence of the *TPMT*16* amino acid substitution on the enzymatic activity remains to be elucidated. According to multiple alignments of TPMT protein sequences from different species, Arg^{163}His substitution is located in a highly conserved region of the protein. Moreover, based on the three-dimensional homology model of human TPMT constructed by Lysaa et al. [26], Arg^{163} is situated in an α-helix, in proximity of residues surrounding the substrate/inhibitor binding cleft, that have been suggested to influence ligand orientation and affinity. In agreement with our in vitro data, Arg^{163}His might, therefore, alter the substrate affinity of TPMT.

In conclusion, we report the identification and the functional analysis of two rare allelic variants of the human TPMT gene, *TPMT*16* and *TPMT*19*. According to the in vitro data and TPMT phenotyping results, it can be postulated that *TPMT*16* corresponds to a novel loss-of-function allele of *TPMT*, whereas *TPMT*19* appears to have no major influence on expression and activity of TPMT. The present study further indicates that rare mutations of *TPMT* might be of relevance with regard to decreased enzymatic activity and that their identification by specific-genotyping assays are of clinical relevance in order to improve the TPMT phenotype prediction rates from genotyping data.

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