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Characterisation of novel defective thiopurine S-methyltransferase allelic variants

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ABSTRACT

Human thiopurine S-methyltransferase (TPMT, EC 2.1.1.67) is a key enzyme in the detoxification of thiopurine drugs widely used in the treatment of various diseases, such as inflammatory bowel diseases, acute lymphoblastic leukaemia and rheumatic diseases. The *TPMT* gene is genetically polymorphic and the inverse relationship between TPMT activity and the risk of developing severe hematopoietic toxicity is well known. In this study, the entire coding sequence of *TPMT*, together with its 5′-flanking promoter region, was analysed in patients with an intermediate phenotype for thiopurine drug methylation. Four polymorphisms were identified, two previously described, c.356A>C (p.Lys^{119}Thr, *TPMT**9) and c.205C>G (p.Leu^{69}Val, *TPMT**21), and two novel missense mutations, c.537G>T (p.Gln^{179}His, *TPMT**24) and c.634T>C (p.Cys^{212}Arg, *TPMT**25). Structural investigations, using molecular modeling, were undertaken in an attempt to explain the potential impact of the amino acid substitutions on the structure and activity of the variant proteins. Additionally, in order to determine kinetic parameters (K_m and V_{max}) of 6-thioguanine (6-TG) methylation, the four variants were expressed in a recombinant yeast expression system. Assays were performed by HPLC and the results were compared with those of wild-type TPMT. The p.Leu^{69}Val and the p.Cys^{212}Arg substitutions encode recombinant enzymes with a significantly decreased intrinsic clearance compared to that of the wild-type protein, and, consequently, characterise non-functional alleles of *TPMT*. The p.Lys^{119}Thr and the p.Gln^{179}His substitutions do not affect significantly the catalytic activity of the corresponding variant proteins, which prevents to unambiguously describe these latter alleles as defective *TPMT* variants.

1. Introduction

Thiopurine S-methyltransferase (TPMT, EC 2.1.1.67) is a cytosolic enzyme that catalyses the S-methylation of the thiopurine drugs, 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and azathioprine (AZA). 6-MP and 6-TG are used in the treatment of acute leukaemia, whereas AZA is widely used as an immunosuppressant for the treatment of inflammatory

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bowel diseases (IBDs) and certain auto-immune diseases, as well as in the prevention of organ transplant rejection [1]. In order to exert their cytotoxic effects, thiopurines must be metabolised to 6-thioguanine nucleotides (6-TGNs) by the metabolic pathway of Hypoxanthine Guanine Phosphoribosyl Transferase (HGPRT). Two alternative metabolic pathways catalysed by Xanthine Oxidase (XO) and TPMT lead to the formation of inactive metabolites [2]. Numerous studies have shown that patients with defective TPMT activity have elevated 6-TGN concentrations that can result in severe or even fatal hematopoietic toxicity when they are treated with standard doses of thiopurine drugs [3–5].

The human *TPMT* gene exhibits genetic polymorphism and 90% of Caucasians inherit high TPMT activity, 10% intermediate TPMT activity and 0.3% low TPMT activity [6]. Three major variants, *TPMT*2* (c.238G>C), *TPMT*3A* (c.460G>A, c.719A>G) and *TPMT*3C* (c.719A>G), represent the most prevalent mutant alleles in Caucasian and Afro-American populations and account for 80–95% of intermediate and deficient methylator phenotypes [7]. Additional rare allelic variants are continuously being identified in intermediate or deficient methylators and, to date, a total of 29 different alleles of *TPMT* have been characterised. It is important to note that for a few rare allelic variants, their implication in TPMT deficiency has not yet been proven and further functional analysis is necessary to assess their deleterious effect on TPMT activity or expression.

In the present study, we investigated the entire coding sequence and the 5′-flanking promoter region of *TPMT* in DNA samples from five patients who were previously classified as intermediate methylators during a routine phenotyping assay, but were genotyped as homozygous wild-type for the c.238G>C, c.460G>A and c.719A>G mutations, corresponding to the *TPMT*2*, **3A*, **3B*, **3C* and **3D* alleles. A total of four missense mutations were identified in a heterozygous state, two of which, p.Lys^{119}Thr and p.Leu^{69}Val, had been previously reported and characterised as *TPMT*9* and *TPMT*21* alleles, respectively [8,9]. The two other mutations are novel polymorphisms located in exon 8 (c.537G>T) and in exon 10 (c.634T>C) and lead to the amino acid substitutions p.Gln^{179}His and p.Cys^{212}Arg, respectively. The c.634T>C substitution was found in a heterozygous state in two DNA samples. The distribution of these four single nucleotide polymorphisms (SNPs) was analysed in genomic DNA samples from 230 unrelated Caucasian subjects comprising 100 healthy volunteers and 130 patients treated with thiopurine drugs. Additionally, their functional consequences on the catalytic activity of TPMT were studied using heterologous expression in yeast cells. Structural investigations using molecular modeling were also performed to predict the potential impact of the amino acid substitutions in the variant proteins.

2. Materials and methods

2.1. TPMT phenotype determination

TPMT activity was measured in erythrocyte lysates, using a radiochemical enzymatic assay, according to the procedure described by Spire-Vayron de la Moureyre C et al. [10], based on the methylation of 6-mercaptopurine with [^{14}C-methyl]-S-adenosyl-L-methionine as the methyl donor. TPMT activity was expressed as nanomoles of 6-methylmercaptopurine formed per mL of red blood cells per hour (nmol/(h mL) RBC = Units/mL RBC = U/mL RBC). The phenotypes are defined as deficient (DM), intermediate (IM) and high methylator (HM) for a TPMT activity lower than 5 U/mL RBC, ranging from 5 to 13.7 U/mL RBC and greater than 13.7 U/mL of RBC, respectively [10–13].

2.2. Genomic DNA samples

Genomic DNA of five Caucasian patients with an intermediate TPMT phenotype was extracted from peripheral blood leukocytes using the Nucleon BACC3 kit (GE Healthcare, Saclay, France) according to the manufacturer's instructions. Additionally, 100 unrelated healthy volunteers and 130 patients treated by thiopurines, all of Caucasian origin, were included in the study. For all participants, blood samples were collected after ethical committee approval and written informed consents had been obtained.

2.3. TPMT sequence analysis

For the five patients with intermediate TPMT phenotype, the entire coding sequence of *TPMT*, including the splice-site junctions, were amplified by PCR, as described previously [10]. For nucleotide sequence analysis, amplicons were purified with Qiaquick spin columns (Qiagen, Courtaboeuf, France) and then sequenced on both strands, using the CEQ^{TM}DTCS-Quick Start Kit (Beckman Coulter, Fullerton, USA). Analysis of DNA sequence variations was performed with the CEQ^{TM}8000 Genetic Analysis System Software (Beckman Coulter).

Additionally, the polymorphic variable number of tandem repeat (VNTR) within the 5′-flanking region of *TPMT* was studied using a PCR-based assay, as described previously [14].

2.4. Genotyping of the c.205C>G, c.356A>C, c.537G>T and c.634T>C mutations

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based assays were developed in order to allow rapid genotyping of the c.205C>G, c.356A>C, c.537G>T and c.634T>C mutations. In a first step, four fragments were generated by PCR with couples of primers listed in Table 1. These primers were designed to avoid co-amplification of the highly similar processed pseudogene *TPMTP1* [15]. PCRs were performed in a total volume of 25 μL of 20 mM Tris–HCl buffer (pH 8.4) containing 50 mM KCl, 200 ng of genomic DNA, 0.4 μM of each primer and 0.5 U of Taq DNA Polymerase (Invitrogen, Cergy-Pontoise, France). The $MgCl_2$ concentration was optimised for each primer set (Table 1). After an initial denaturation of 3 min at 94 °C, amplicons were generated for 35 cycles of 1 min at 94 °C, 1 min at an optimised annealing temperature (Table 1), and 1 min 30 s at 72 °C, followed by a 10 min final extension step at 72 °C. Size and specificity of PCR fragments were evaluated on ethidium bromide-stained agarose gels (Invitrogen).

In a second step, PCR products were digested with endonucleases, as summarised in Table 1. For the c.205C>G

Table 1 – Conditions for PCR-RFLP analysis

Mutations	Primers[a]	Primer sequence (5′ → 3′)[b]	PCR			Digestion conditions		Restriction fragments (bp)	
			Size (bp)[c]	T_m (°C)[d]	$MgCl_2$ (mM)[e]	Endo-nuclease	Time, T °C	Wild-type allele	Variant allele
c.205C>G Leu[69]Val	TPMT4F	TAAAACCTTTTTTTTTTTCCTCTTC	146	62	2	*Mnl*I	2 h/37 °C	47, 40, 30, 29	77, 40, 29
	TPMT4R	TTCTGTTAATGTTTATCTGCTCAT							
c.356A>C Lys[119]Thr	TPMT5F	TAAGTGTAAATGTATGATTTTATGC	187	60	2	*Taa*I	16 h/65 °C	140, 47	104, 47, 36
	TPMT5R	CAATTATTTACCCAAATCAAAACAA							
c.537G>T Gln[179]His	TPMT8F	TCCCTCCTGGGAAAGAAGTCTC	97	62	4	*Dde*I	3 h/37 °C 20 min/65 °C	75, 22	97
	TPMT8R	GAAAAAAAAAAAACCCAACAACTTT							
c.634T>C Cys[212]Arg	TPMT10F	TTAACATGTTACTCTTTCTTGTTTC	166	58	2	*Hpy*CH4V	3 h/37 °C 20 min/65 °C	129, 37	166
	TPMT10R	GTGATTTTATTTTATCTATGTCTCAT							

[a]F, forward; R, reverse; [b]primers were designed according to the GenBank sequence NC_000006; mismatch introduced to create a *Dde*I site is underlined; [c]size of amplified fragments; [d]optimised annealing temperature for each set of primers; [e]optimised $MgCl_2$ concentration for the PCR.

mutation, amplicons encompassing the mutation were digested with *Mnl*I (New England Biolabs, Beverly, MA), which cleaves the wild-type allele in four fragments (47, 40, 30 and 29 bp) and the mutant allele in three fragments (77, 40 and 29 bp). For the genotyping of the c.356A>C substitution, PCR products encompassing the mutation location were digested with *Taa*I (New England Biolabs) which cleaves the wild-type allele in two fragments (140 and 47 bp) and the mutant allele in three fragments (104, 47 and 36 bp). For the c.537G>T mutation, amplicons were digested with *Dde*I (Fermentas, St. Leon-Rot, Germany), which cleaves the wild-type allele in two fragments (75 and 22 bp), but leaves uncut the mutant allele. For the c.634T>C mutation, PCR products were digested with *Hpy*CH4V (Fermentas), which cleaves the wild-type allele in two fragments (129 and 37 bp), but leaves uncut the mutant allele.

The digested amplicons were analysed on a 12% acrylamide ethidium bromide-stained gel (BioRad, Marnes-la-Coquette, France). One DNA sample homozygous for the wild-type allele of *TPMT* (reference sample) and four DNAs heterozygous for the c.205C>G, c.356A>C, c.537G>T or c.634T>C SNP were respectively used as controls (Fig. 1).

2.5. Molecular modeling simulations

Structural analysis of variant proteins was studied from the crystal structure at 1.89 Å resolution (PDB ID: 2h11.pdb) of the human TPMT complexed with the S-adenosyl-L-homocystein (SAH) [16]. A specific docking simulation was performed with the software package GOLD [17] in order to predict the relative orientation of the cofactor S-adenosyl-L-methionine (SAM) in the wild-type TPMT and in the Leu[69]Val mutant. The substitution and the conformational rearrangement of Val69 side chain were refined using the Tripos molecular forcefield [18] on the platform Sybyl software (Sybyl 6.9.2 ed., Tripos Associates Inc., St. Louis, MO) running on HP Linux workstations (Fig. 2).

2.6. Site-directed mutagenesis

The yeast expression vector pYeDP60 containing the wild-type *TPMT* cDNA [19] was used as a template for site-directed mutagenesis using the mutagenic primers listed in Table 2 and the QuickChange™ Site-Directed Mutagenesis kit (Stratagene, Amsterdam, Netherlands), according to the manufacturer's instructions. Sequencing of each construct (Val69, Thr119,

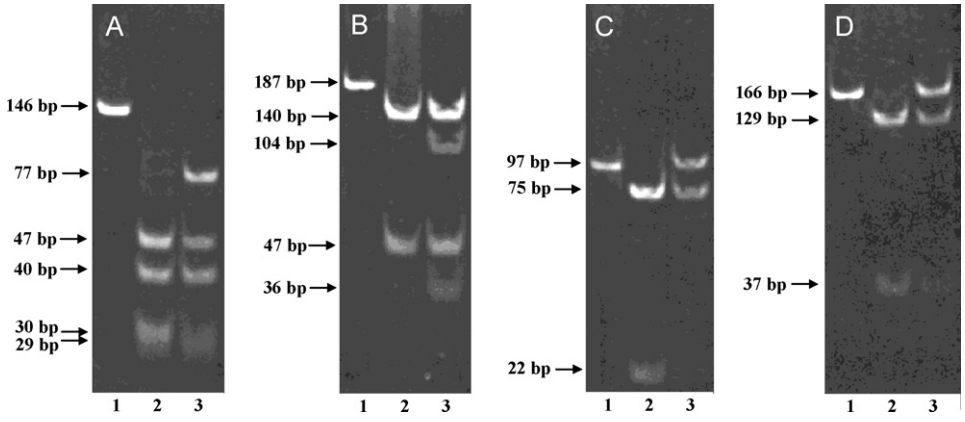

Fig. 1 – PCR-RFLP analysis for TPMT mutations c.205C>G in exon 4 (A), c.356A>C in exon 5 (B), c.537G>T in exon 8 (C) and c.634T>C in exon 10 (D). PCR fragments of exons 4, 5, 8 and 10 were digested with restriction enzymes *Mnl*I, *Taa*I, *Dde*I and *Hpy*CH4V, respectively. Lanes 1: non-digested PCR fragments; lanes 2: restriction fragments of wild-type individuals; lanes 3: restriction fragments of heterozygous individuals.

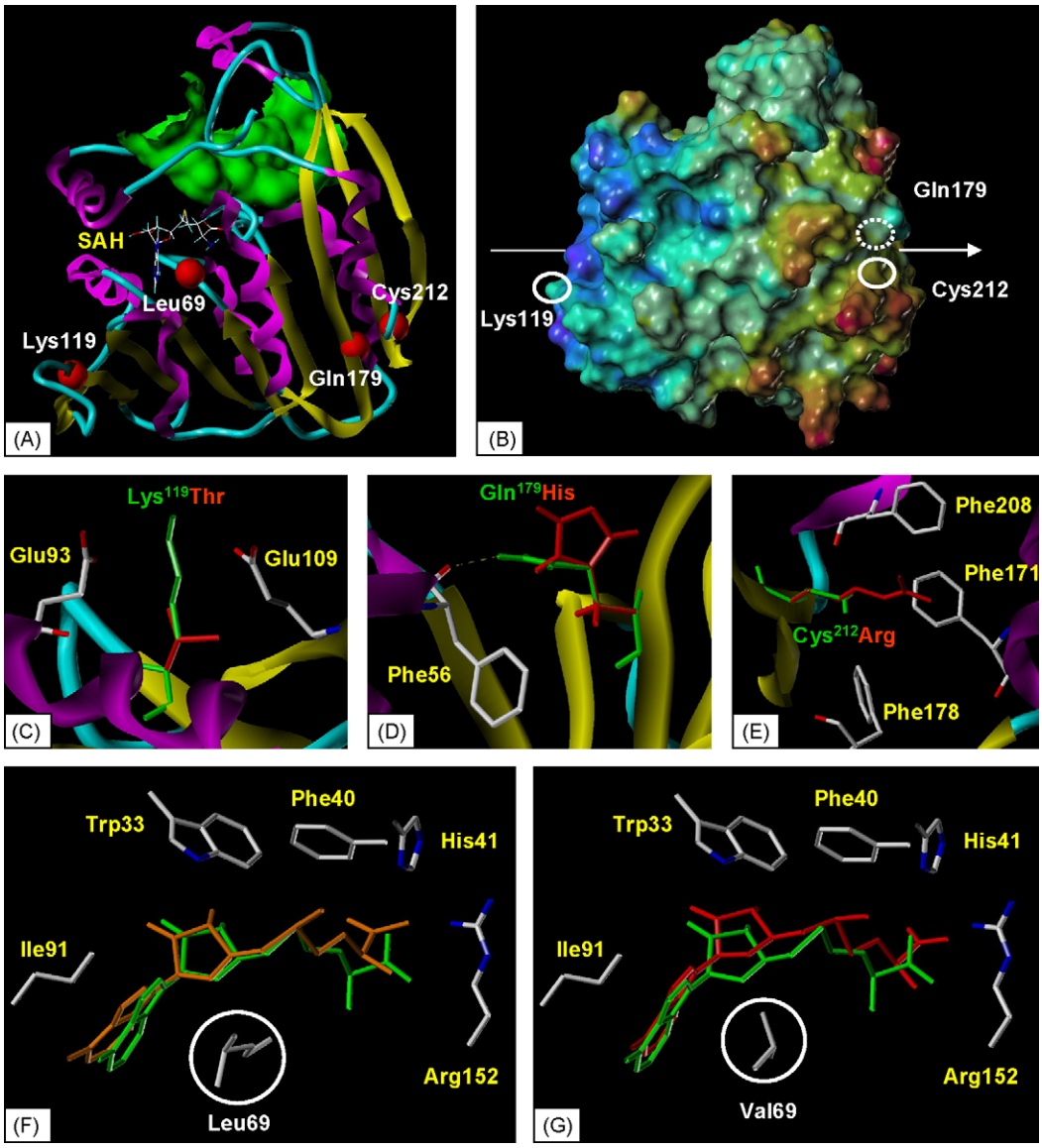

Fig. 2 – Structural analysis of human TPMT. (A) Crystal structure of the human TPMT (PDB Id: 2h11.pdb). Protein structure is represented as ribbons (α-helix in magenta) and tubes (β-sheet in yellow); point mutations are marked as red spheres. The cavity for the binding of the substrate is illustrated by the green-shaded molecular surface, and the SAH by the sticks coloured by atom type. (B) Electrostatic nature of the TPMT molecular surface. The shaded molecular surface of the protein displays the electrostatic amino acids from blue (electronegative amino acids) to red (electropositive amino acids) and orients the dipolar moment (arrow). Solvent-exposed mutations are encircled as bold (front surface) and dashed lines (back surfaces). (C–E) Structural environment of Lys119Thr (C), Gln179His (D) and Cys212Arg (E) substitutions in TPMT; native residues are coloured in green, mutant residues in red. Yellow dashed line in figure D illustrates the H-bond interaction. (F and G) Docking simulation of the SAM cofactor in the wild-type TPMT (F, SAM in orange) and in Val69 TPMT variant (G, SAM in red) and superposition with the SAH cofactor from the crystal structure (in green). Only heavy atoms of amino acids are represented as sticks coloured by atom type.

His179, Arg212 and wild-type construct) was carried out on both strands to assess their strict identity with the expected mutated sequence.

2.7. *Heterologous expression of TPMT cDNA in yeast cells*

Saccharomyces cerevisiae W(R) strain (generous gift from Dr. Pompon) was transformed with wild-type or mutant cDNAs, by the lithium acetate method published by Gietz et al. [20]. As a negative control, an insertless vector was used to transform yeast cells. Cytosolic fractions of transformed cells were prepared as described previously [19].

Protein concentrations of cytosolic fractions were determined by the method of Lowry et al. [21] with the Uptima BC Assay Protein Quantification Kit (Interchim, Montluçon, France).

Table 2 – Primers used for site-directed mutagenesis

Primers[a]	Primer sequences (5′ → 3′)[b]	Mutated codon	Amino acid substitution
L69VF	CTGAGGGTATTTTTTCCTGTTTGCGGAAAAGCGG	CTT → GTT	$Leu^{69}Val$
L69VR	CCGCTTTTCCGCAAACAGGAAAAAATACCCTCAG		
K119TF	CCGAAATTCCTGGAACCACAGTATTTAAGAGTTCTTCG	AAA → ACA	$Lys^{119}Thr$
K119TR	CGAAGAACTCTTAAATACTGTGGTTCCAGGAATTTCGG		
Q179HF	CTCCTGGGAAAGAAGTTTCATTATCTCCTGTGTGTTC	CAG → CAT	$Gln^{179}His$
Q179HR	GAACACACAGGAGATAATGAAACTTCTTTCCCAGGAG		
C212RF	GGTTGTTTGGTAAAATACGCAATATACGTTGTCTTGAGAAGG	TGC → CGC	$Cys^{212}Arg$
C212RR	CCTTCTCAAGACAACGTATATTGCGTATTTTACCAAACAACC		

[a] F, forward; R, reverse.
[b] Underlined nucleotides correspond to mutated nucleotides.

Cytosolic fractions (5 μg of total proteins per lane) were electrophoresed using a 10% SDS-containing polyacrylamide gel (Biorad), transferred to a 0.45 μm nitrocellulose membrane (Biorad) and reacted with a polyclonal rabbit antiserum against human TPMT diluted 1 to 10,000 (generous gift from Drs. Krynetski and Evans). The nitrocellulose membrane was then incubated with a goat anti-rabbit horseradish peroxidase conjugate diluted 1 to 2000 (ECL Plus™ Western Blotting Reagent Pack, GE Healthcare, Orsay, France) and antibody complexes were detected using the ECL Plus Western Blotting Detection System (GE Healthcare) following the manufacturer's recommendations. The intensity of the bands was measured by the Chemidoc XRS system (BioRad) and analysed with the Quantity One®1D Analysis Software (BioRad) (Fig. 3).

2.8. 6-Thioguanine S-methylation assay

TPMT activity of yeast cytosolic fractions (100 μg of total proteins) was assayed in a total volume of 1 mL of 10 mM phosphate buffer pH 7.4 (Sigma–Aldrich, Saint-Quentin Fallavier, France), 1 mM of SAM (Sigma–Aldrich), 250 μmol of dithiothreitol (DTT, Sigma–Aldrich), 20 μmol of allopurinol (Sigma–Aldrich), and 0–350 μM of 6-TG (Sigma–Aldrich) as a probe-substrate. For the wild-type and the Val69 constructs, additional assays were performed using a constant concentration of 6-TG (500 μM) and 0–400 μM of SAM. Incubations were performed at 37 °C for 10 min and stopped by addition of 100 μL of 1 M HCl (Sigma–Aldrich). Mixtures were then centrifuged at 10,000 × *g* for 10 min. Supernatants were stored at −80 °C until use. In order to quantify the 6-methylthioguanine metabolite (6-MeTG) produced by cytosolic fractions, 2.75 μg of an external standard, metoclopramide (Sanofi-Aventis, Paris, France) were added to 200 μL of each supernatant. Fifty microlitres of mixtures were directly injected onto a C18 column (4.6 mm × 150 mm) (Waters, Saint-Quentin en Yvelines, France) for reverse phase HPLC (600 multisolvent pump, Waters). Elution was monitored at 310 nm and performed at a flow rate of 1 mL/min in an isocratic elution mode with a mobile phase comprising 0.05 M NaH_2PO_4 pH 3.6 (90%) (Sigma–Aldrich) and acetonitrile (10%) (Sigma–Aldrich). Kinetic parameters (K_m and V_{max}) were estimated from the Eadie-Hoffstee equation ($V_i = f\ (V_i/[S]) = -K_m \times V_i/[S] + V_{max}$) using the Grafit software (Version 3.0, Erithacus Software, Surrey, UK). The intrinsic clearance was evaluated as the ratios of V_{max} to K_m. Assays were repeated in three independent experiments, each experiment comprising three replicates.

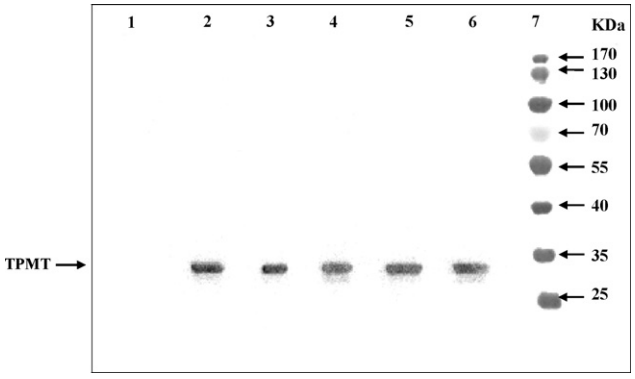

Fig. 3 – Immunoblot analysis of recombinant human TPMT proteins. Cytosolic fractions (5 μg per lane) of yeast cells expressing the Arg212, His179, Thr119, Val69 TPMT variants and the wild-type TPMT protein, were loaded in lanes 2, 3, 4, 5 and 6, respectively. The cytosolic fraction of yeast cells transformed with an insertless vector was used as negative control (lane 1). A molecular mass marker was applied line 7.

2.9. Statistical analysis

Kinetics parameters were statistically analysed using the software R version 2.4.1 (http://www.cran.r-project.org). Significance was evaluated using the Wilcoxon Rank–Sum test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Identification of TPMT mutations and promoter VNTRs

During routine TPMT phenotyping, prior to the instigation of thiopurine therapy, we identified five patients with intermediate methylating activity who were not carriers of the defective *TPMT*2* or **3A-D* alleles (Table 3). Consequently, to elucidate the genetic basis of the intermediate phenotype in these patients, the entire *TPMT* coding sequence, including exon–intron boundaries, was sequenced. Four heterozygous

Table 3 – Genotypic and phenotypic TPMT status of the five studied patients

Patients	Genotype		Phenotype
	Promoter VNTR	Coding sequence	Enzyme activity (U/mL RBC)
A	*V4/*V5	*TPMT**1/TPMT*21*	5.7
B	*V4/*V5	*TPMT**1/TPMT*9*	6.3
C	*V4/*V5	*TPMT**1/TPMT*24*	13.3
D	*V4/*V4	*TPMT**1/TPMT*25*	5.8
E	*V4/*V4	*TPMT**1/TPMT*25*	10.8

single nucleotide substitutions were detected in exons 4, 5, 8, and 10, respectively. The c.205C>G and c.356A>C substitutions have already been described in previous studies. They encode the p.Leu[69]Val and p.Lys[119]Thr substitutions and are characterised as *TPMT*21* and *TPMT*9* alleles, respectively [8,9]. The c.537G>T and c.634T>C polymorphisms are novel missense *TPMT* mutations and result in p.Gln[179]His and p.Cys[212]Arg exchanges, respectively. According to the *TPMT* allele nomenclature, these sequence variations were named *TPMT*24* and *TPMT*25*. Additionally, the 5′-flanking promoter region of *TPMT* was analysed using a PCR-based assay to determine the VNTR genotype of the five patients. Two different VNTR genotypes, that correspond to the two most frequent ones in Caucasians [14,22] were identified. The genotypic and phenotypic features of the five studied patients are summarised in Table 3.

3.2. *Frequency analysis of c.205C>G, c.356A>C, c.537G>T and c.634T>C mutations in Caucasians*

In order to estimate the frequency of the four detected SNPs, we developed specific PCR-RFLP assays and applied them to 230 unrelated Caucasian subjects comprising 100 healthy volunteers and 130 patients undergoing thiopurine therapy. Of the 230 DNA samples studied, electrophoretic patterns did not differ from those of the reference sample and their genotypes were thus established as homozygous wild-type for the investigated mutations. Consequently, the c.205C>G, c.356A>C, c.537G>T and c.634T>C mutations are rare polymorphisms, with a frequency of less than 0.1% in the studied population.

3.3. *Structural analysis of TPMT variants*

At the structural level, using molecular modeling (Fig. 2), it is possible to rationalise the impact of the identified *TPMT* mutations on protein functionality. They can be grouped into two categories: those exposed to the solvent and located on the protein surface away from the catalytic site (p.Lys[119]Thr, p.Gln[179]His and p.Cys[212]Arg), and the one directly involved in cofactor binding (p.Leu[69]Val).

3.3.1. *Structural analysis of solvent-exposed mutations*

Even though the mutations studied could be tolerated since the polar nature of the substituted amino acids is conserved, the relevant surface regions could be destabilised by electrostatic perturbations. The p.Lys[119]Thr substitution is characterised by a loss of a positive charge that seems to destabilise the local electrostatic balance in the native protein. The Lys119 residue establishes salt bridges with two acidic residues (Fig. 2C) and, disrupting charge–charge interactions, the mutation can significantly alter the local stability of the protein [23].

Although the two other mutations, p.Gln[179]His and p.Cys[212]Arg, are also exposed to solvent, their side chains tend to orientate themselves toward the core of the protein. As illustrated in Fig. 2D, the Gln179 in the native structure binds with Phe56 of the ''near'' α-helix via a hydrogen bond. In contrast, the His179 mutated residue cannot establish this type of interaction in this native state of TPMT. His179 could bind to Phe56 residue if there was a local structural rearrangement between the β-sheet carrying the His179 amino acid and the ''near'' α-helix. In this case, this might modify the local stability of the protein.

The p.Cys[212]Arg substitution (Fig. 2E) induces an increase of side chain volume. However, even though this residue is oriented toward the protein core, there is an internal cavity that is able to accommodate the side chain of Arg212. In addition, the positive charge of this residue could be well tolerated by the surrounding electronic environment conferred by aromatic residues Phe171, Phe178 and Phe208. Nevertheless, these usually called cavity-filling mutations of residues lining these cavities can be detrimental to stability. For instance, mutations that do not allow the presence of internal water molecules and H-bond networks can be destabilising [24].

3.3.2. *Structural analysis of the p.Leu[69]Val mutation*

This substitution would appear to represent the most unambiguous explanation for the observed deficiency in TPMT activity since Leu69, a strictly conserved amino acid in orthologous enzymes (Fig. 4), is directly involved in SAH binding site. This deeply buried substitution is located in a turn and may modify the Van der Waals packing surrounding the cofactor binding site. In order to predict the potential orientation of the cofactor relatively to the substrate-binding pocket, a docking experiment of the methyl donor (SAM) was conducted with the native form of the TPMT and the Val69 mutant. As seen in Fig. 2F–G, docking of the SAM is very close to the SAH position in the crystal structure. Even if the methionyl moiety part of the molecule is not well superposed, it remains in a region which allows electrostatic binding between the carboxylate group of the cofactor and two basic residues, His41 and Arg152. Besides, the binding of the adenosyl group is consistent with the crystal structure with a hydrophobic stabilising cluster (Trp33, Phe40 and Ile91). Moreover, the sulfur atoms which are involved in the methyl transfer catalysis are also well superposed. In contrast, the docking of SAM in the Val69 mutant shows a spatial shift in the superposition with the native structure but retaining the same hydrophobic and electrostatic interactions. This placement is

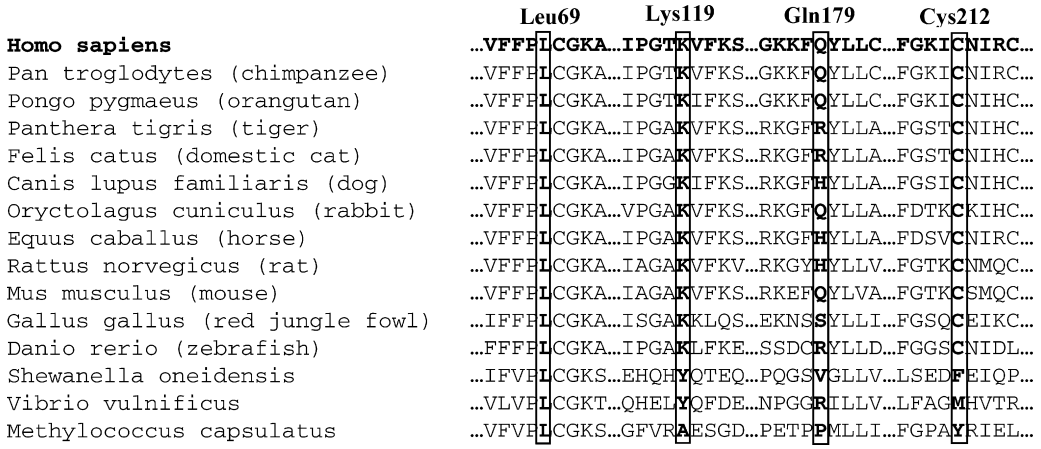

Fig. 4 – Alignment of the wild-type human TPMT amino acid sequence with those of vertebrate and prokaryote orthologs. The comparison of amino acid sequences is restricted to regions surrounding the residues Leu69, Lys119, Gln179 and Cys212. This alignment was performed with the software ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

due to the valine side chain whose refined conformation introduces a steric bulk surrounding the adenosyl group and induces a shift of the sulfur atom as well as its methyl group. However, in our structural studies with this mutant, we could not provide an accurate value of the shift in distance of the transferable methyl group since this distance is smaller than the resolution (1.89 Å) provided by the X-ray diffraction of the TPMT crystal structure.

3.4. *Functional analysis of the four TPMT variants*

To examine the functional impact of the p.Leu^{69}Val, p.Lys^{119}Thr, p.Gln^{179}His and p.Cys^{212}Arg missense mutations on TPMT activity, wild-type and mutant *TPMT* cDNAs were expressed in *S. cerevisae* cells. Western immunoblot analysis confirmed the presence of recombinant TPMT in cytosolic fractions prepared from transfected cells (Fig. 3). No immunodetectable protein was observed in cytosolic preparation from yeast cells transfected with an insertless vector. Compared to the wild-type construct, the relative expression levels of TPMT protein were 0.95, 0.87, 0.94 and 1.12 for the Val69, Thr119, His179 and Arg212 variants, respectively.

Following incubation of cytosolic fractions with 6-TG, monophasic Michaelis–Menten kinetics were observed with each recombinant TPMT enzyme (data not shown). For each variant, the kinetic parameters (K_m and V_{max}), as well as the in vitro intrinsic clearance (V_{max}/K_m) value of 6 Me-TG production were determined and then compared with the values obtained with the wild-type protein. V_{max} values are expressed as nmol of 6-MeTG/min/mg total cytosolic protein, after normalising the TPMT protein content of the mutant lysates relative to that of the wild-type. As expected, cytosolic fractions from cells transfected with the insertless vector did not convert 6-TG to a metabolite. The Thr119 and His179 variants significantly increased the apparent K_m of TPMT relative to wild-type ($p < 0.05$), whereas the Val69 and Arg212 substitutions did not significantly affect the K_m (Table 4).

The Val69 variant encoded a protein exhibiting a significant decrease of V_{max}, whereas the His179 variant displayed an increased V_{max} relative to wild-type. With the two other variants, Thr119 and Arg212, the V_{max} was similar to that of the wild-type.

When comparing intrinsic clearances, the V_{max}/K_m ratio significantly differed for two variants, relative to wild-type. Accordingly, the Val69 and the Arg212 variants encoded proteins with reduced catalytic efficiency, retaining only 24% and 68% of the V_{max}/K_m value of the wild-type protein, respectively.

Table 4 – Kinetic parameters of 6-thioguanine *S*-methylation by recombinant TPMT variants expressed in yeast cells

Recombinant protein	K_m (μM)	V_{max} (nmol/(min mg prot))	V_{max}/K_m (mL/(min mg prot))
Wild-type	62.70 ± 13.35	14.15 ± 0.45	0.25 ± 0.05
Val69 Variant	56.20 ± 7.35	3.35 ± 0.20**	0.06 ± 0.01** (24%)[a]
Thr119 Variant	79.85 ± 6.35*	14.15 ± 0.35	0.19 ± 0.01 (76%)
His179 Variant	83.05 ± 12.95*	21.00 ± 2.80**	0.26 ± 0.07 (104%)
Arg212 Variant	72.00 ± 16.45	12.15 ± 1.90	0.17 ± 0.02* (68%)

Cytosolic fractions were incubated with 0–350 μM of 6-TG and constant concentration of SAM (1 mM).
All values are mean ± standard deviation from three independent experiments.
[a] Values in parentheses represent relative rates of V_{max}/K_m as a percentage of the wild-type value, calculated with raw data.
* $p < 0.05$.
** $p < 0.01$.

Table 5 – Kinetic parameters of 6-thioguanine S-methylation by recombinant TPMT variants expressed in yeast cells

Recombinant protein	K_m (μM)	V_{max} (nmol/(min mg prot))	V_{max}/K_m (mL/(min mg prot))
Wild-type	34.35 ± 2.90	11.00 ± 1.15	0.32 ± 0.03
Val69 Variant	13.70 ± 2.30**	2.40 ± 0.20**	0.18 ± 0.03**(55.5%)[a]

Cytosolic fractions were incubated with 0–400 μM of SAM and constant concentration of 6-TG (500 μM).
All values are mean ± standard deviation from three independent experiments.
[a] Value in parentheses represent relative rates of V_{max}/K_m as a percentage of the wild-type value, calculated with raw data.
** $p < 0.01$.

As the p.Leu[69]Val substitution is directly involved in the cofactor binding site, additional assays with a constant concentration of 6-TG, but increasing concentrations of SAM, were performed with the Val69 construct (Table 5). A significant decrease of K_m, V_{max} and V_{max}/K_m values (55%) compared to those of the wild-type protein was observed.

4. Discussion

It is now well known that genetic polymorphisms that modify the enzymatic activity of TPMT can contribute to interindividual differences in susceptibility to thiopurine therapy [3,5]. Individuals who inherited one or two defective *TPMT* alleles can develop severe haematopoietic toxicity when treated with standard doses of 6-MP or AZA because of the accumulation of cytotoxic metabolites. It is now recognised that TPMT genotyping or phenotyping should be performed prior to thiopurine administration in order to predict the risk for patients to develop severe hematotoxicity. However, both genotyping and phenotyping procedures have their limitations [8]. The determination of TPMT activity in RBC can be misleading if the patient has received a recent blood transfusion and it is well known that several currently used drugs can alter TPMT activity, giving rise to misinterpretation by phenocopy. Genotyping assays have been proposed to circumvent these limitations. However, conventional TPMT genotyping assays are based on the identification of the most frequent polymorphisms and do not allow the detection of rare SNPs unless the whole gene is sequenced. The characterisation of additional rare inactivating mutations will then improve the reliability of genotyping tests to identify TPMT deficient and intermediate patients.

In the present study, we analysed the entire coding sequence, including exon–intron boundaries of the *TPMT* gene in DNA samples from five patients who had been previously phenotyped as intermediate methylators prior to thiopurine therapy, and genotyped as wild-type homozygotes for the three most frequent inactivating mutations of the gene. A total of four different allelic variants, each carrying a single missense mutation, were characterised. As a polymorphic VNTR in the promoter region of *TPMT* (*V3 to *V9) has been described to modulate RBC TPMT activity [14,22], with, in particular, moderately decreased activity correlating with the >*V5 alleles, the VNTR genotype of the five patients were also determined. We could exclude a major influence of this polymorphic promoter region on the apparent IM phenotype of our patients, as none of them carried an allele with more than five repeats.

We identified two previously reported mutations in exons 4 (c.205C>G; p.Leu[69]Val) [9] and in exon 5 (c.356A>C; p.Lys[119]Thr) [8], corresponding to the *TPMT**21 and *TPMT**9 alleles, respectively. The two other polymorphisms we identified are novel and located in exons 8 (c.537G>T) and 10 (c.634T>C). They result in p.Gln[179]His and p.Cys[212]Arg amino acid substitutions. The corresponding alleles were respectively designated *TPMT**24 and *TPMT**25, according to the *TPMT* allele nomenclature.

The distribution of *TPMT**9, *TPMT**21, *TPMT**24 and *TPMT**25 alleles was investigated in a Caucasian population using PCR-RFLP-based assays. None of these variants was detected in DNA samples of the 230 tested individuals. *TPMT**9 and *TPMT**21 have already been described as extremely rare alleles in Caucasian populations. Schaeffeler et al. [9] failed to identify the *TPMT**21 allele in a control population of 1048 individuals and only two patients carrying the *TPMT**9 allele were found in a population of 1214 Caucasians [8]. In a similar manner, the *TPMT**24 and *TPMT**25 alleles are not expected to frequently occur in Caucasian populations. In support of this comment, the p.Gln[179]His substitution (*TPMT**24) is described in the NCBI SNP database as rs6921269 with a frequency of 0% in the European tested-population. However, because of inter-ethnic variations in the distribution of *TPMT* variant alleles [25], the frequency of the novel alleles may be different in other ethnic groups, as it seems the case for the rs6921269 which appears less rare in Africans with an allelic frequency of 2.6%.

In order to characterise the functional impact of the four missense mutations, wild-type and mutant *TPMT* cDNAs, generated by site-directed mutagenesis, were expressed in *S. cerevisiae*. Cytosolic fractions of yeast cells transfected with these different cDNAs expressed recombinant proteins, as confirmed by immunoblot analyses. The fact that TPMT protein levels were relatively similar between the wild-type and the four mutant constructs suggests that the variants tested in the present study exhibit negligible impact on translation or protein stability in yeast cells, contrary to other alleles, such as *TPMT**2 and *TPMT**3A, which are associated with a 20–400-fold decrease in TPMT expression in yeast cells [26], as a result of enhanced proteolysis [27]. After incubation of cytosolic fractions with 6-TG, the kinetic parameters (K_m and V_{max}), as well as the in vitro intrinsic clearance (V_{max}/K_m), of each TPMT variant were compared with the values obtained for the wild-type protein.

The p.Gln[179]His substitution affects an evolutionarily unconserved residue in vertebrates. A His residue at position 179 is also found in several mammalian species (*Canis lupus familiaris*, *Equus caballus* and *Rattus norvegicus*). Amino acid substitutions at evolutionarily conserved residues are thought to be more deleterious than those at unconserved positions. As a consequence, the His179 variant might not have any influence on TPMT catalytic activity. Our in vitro studies are in favour of this hypothesis, since we did not observe any significant difference of V_{max}/K_m ratio between this variant and the wild-type protein.

The three other detected substitutions (p.Leu^{69}Val, p.Lys^{119}Thr and p.Cys^{212}Arg) affect highly conserved amino acid residues in vertebrates, suggesting a possible alteration of enzymatic activity for the corresponding variant proteins. The availability of the human TPMT crystal structure has allowed us to perform the structural mapping and analysis of these mutations.

The p.Lys^{119}Thr and p.Cys^{212}Arg mutations (*TPMT*9* and *TPMT*25*) are located on the protein surface away from the catalytic site. These solvent-exposed mutations are

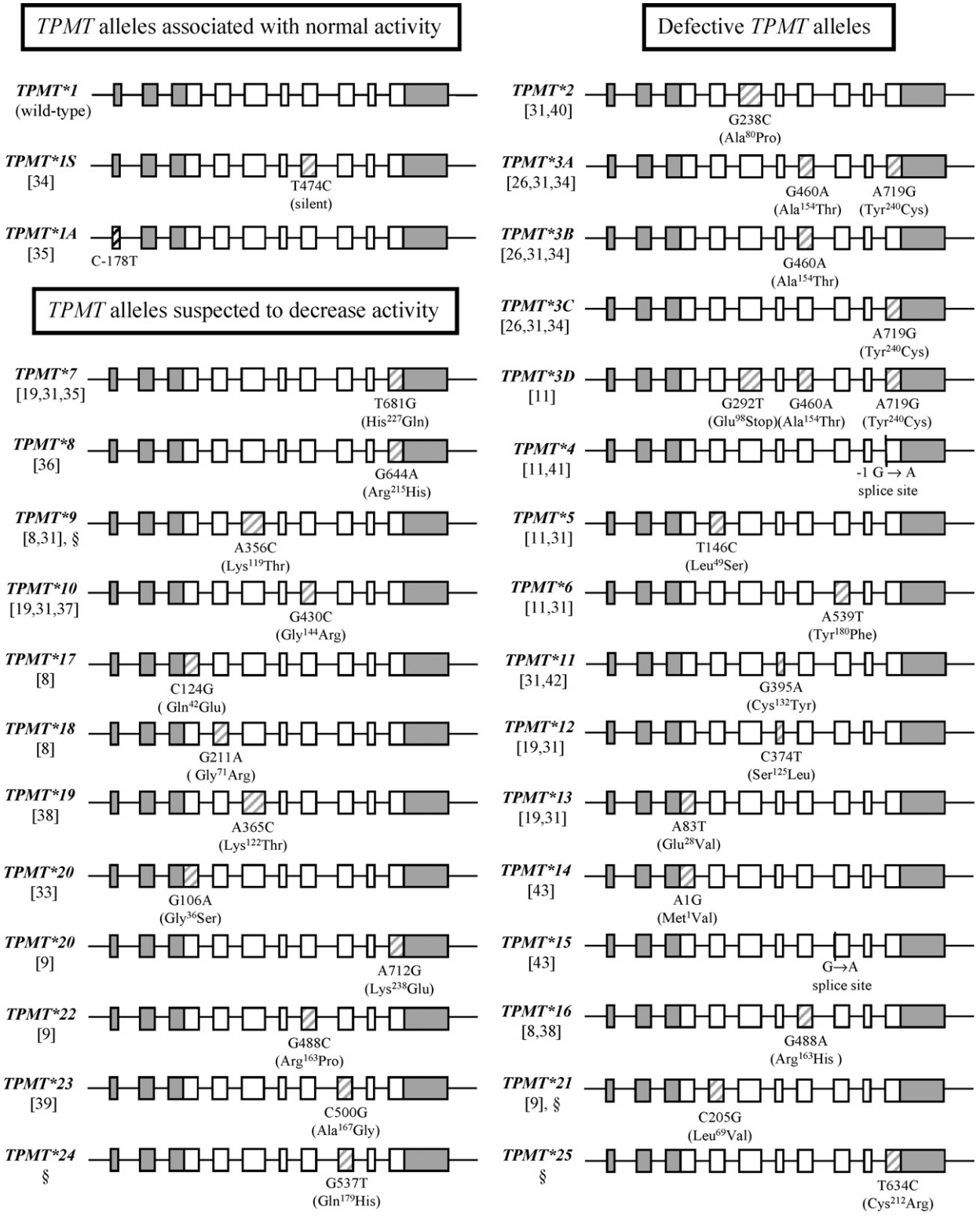

Fig. 5 – Allelic variants of the human *TPMT* gene. Hatched boxes, exons containing at least one mutation; white boxes, exons with normal sequence. Solid gray boxes, non-coding exonic sequence. Exons sizes are proportional to their relative lengths, but introns not. §: present study.

characterised by modifications of ionic charge that could destabilise the local electrostatic balance in the native protein. Even though these mutations occur far away from the catalytic and substrate binding sites, it is well known that, in some cases, structural shifts can propagate away from the site of the mutation, sometimes, up to 15–20 Å [28–30]. Independently, an additional study of the molecular surface of TPMT (Fig. 2B) highlights a particular electrostatic propensity characterised by a dipolar moment through the protein. Since the p.Lys^{119}Thr and p.Cys^{212}Arg mutations introduce a loss or gain of positive charges and thus a variation of electrostatic potential in these crucial exposed regions, they might modify that dipolar moment and cause electronic perturbations through the catalytic and binding sites, as well as structural shifts. On the basis of our in vitro data, the intrinsic clearance of the Thr119 variant (*TPMT**9) was reduced by less than 25% but was not statistically different compared with the wild-type protein, even if close to significance ($p = 0.052$). Furthermore, kinetic parameters of this variant expressed in COS-1 cells were shown not to be significantly different from those of the wild-type protein [31]. The *TPMT**9 allele, that we identified in a heterozygous state in a patient with an IM TPMT phenotype, had been previously detected in an individual with an IM phenotype, but also in a patient with a normal TPMT activity [8]. As a consequence, the definite functional status of the *TPMT**9 allele remains difficult to determine. Concerning the p.Cys^{212}Arg variant encoded by *TPMT**25, the V_{max}/K_m value was reduced by more than 30% and was statistically different compared with the wild-type enzyme ($p = 0.041$). This mutation was found in two patients displaying an intermediate status during routine phenotyping (Table 3).

The p.Leu^{69}Val mutation is located close to the cofactor binding site. Previous in silico analysis had predicted that the *TPMT**21 allele carrying this mutation could encode a potentially inactive protein [9]. At a structural level, the critical position of the relevant p.Leu^{69}Val substitution in the tightly packed cofactor binding site allowed us to show, by docking simulation, a certain displacement of SAM in the mutant protein. Even if this displacement seems minor, we hypothesise that it might be important enough to alter the methyl transfer from the SAM toward the guanine substrate and explain the dramatic decrease of TPMT activity that we observed in vitro for the Val69 variant, retaining only 24% of that of the wild-type enzyme.

In total, according to structural and functional analyses, the *TPMT**21 and *TPMT**25 alleles encode variant proteins with a significantly reduced catalytic activity toward 6-TG methylation compared to the wild-type enzyme. Consequently, these allelic variants can be regarded as non-functional alleles of *TPMT*. The definite functional status of the two other alleles we studied, *TPMT**9 and *TPMT**24, remains difficult to ascertain and further in vitro analyses would be needed to demonstrate their impact on TPMT expression and/or stability. It was shown that certain *TPMT* variant alleles, that carry non-synonymous cSNPs, encode proteins that are rapidly degraded via enhanced proteasomal degradation, leading to reduced levels of TPMT enzyme [27,32]. We cannot exclude that the four TPMT variants we describe here undergo such an enhanced proteolysis and the use of a more appropriate expression system, such as mammalian cells, could be helpful to test this mechanism [31,32].

In conclusion, we report the identification and functional characterisation of four *TPMT* allelic variants, identified in five patients phenotyped as intermediate methylators, two previously described alleles, *TPMT**9 and *TPMT**21, as well as two novel alleles, *TPMT**24 and *TPMT**25. Thus, to date, a total of 28 different allelic variants, in addition to the wild-type *TPMT**1 allele, were described (Fig. 5) [8,9,11,19,26,31,33–43]. We should notice that the *TPMT**20 allele designation has been used to describe two different nucleotide variations [9,33]. From various in vivo and in vitro studies, 16 allelic variants are now well recognised as being defective *TPMT* alleles, leading to significantly decreased TPMT activity (Fig. 5). The consequence of the 12 remaining allelic variants on TPMT expression and activity, and, consequently, on the in vivo TPMT phenotype, is less clearly established. This difficulty to ascertain the functional status of these alleles comes from either a lack of in vitro functional analysis, or the absence of or discrepancies between in vitro and in vivo data. Even though they correspond to very rare allelic variants of *TPMT* in most of the studied populations and their contribution to the overall deficiency of TPMT activity can be considered as minor, additional analyses would be necessary to definitely classify the alleles as defective *TPMT* alleles or not.

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