British Journal of Pharmacology (1998) 125, 879–887
http://www.stockton-press.co.uk/bjp

Genotypic and phenotypic analysis of the polymorphic thiopurine S-methyltransferase gene (*TPMT*) in a European population

[1]Catherine Spire-Vayron de la Moureyre, [1]Hervé Debuysere, [1]Bruno Mastain, [1]Elizabeth Vinner, [1]Delphine Marez, [1]Jean-Marc Lo Guidice, [1]Dany Chevalier, [2]Serge Brique, [2]Kokou Motte, [2]Jean-Frédéric Colombel, [2]Dominique Turck, [2]Christian Noel, [2]René-Marc Flipo, [2]Annie Pol, [1]Michel Lhermitte, [3]Jean-Jacques Lafitte, [2]Christian Libersa & [1,4]Franck Broly

[1]Laboratoire de Biochimie et Biologie Moléculaire, Hôpital Calmette, [2]Centre d'Investigation Clinique, Hôpital Cardiologique, and [3]Clinique de Pneumophtisiologie, Hôpital Calmette, Centre Hospitalier Régional et Universitaire de Lille, Bd. J. Leclercq, 59037 Lille, France

1 Characterization of allelic variants of the TPMT gene (*TPMT*) responsible for changes in TPMT activity, and elucidation of the mechanism by which these alleles act, are required because of the clinical importance of this polymorphism for patients receiving thiopurine drugs.

2 We defined the mutational and allelic spectrum of *TPMT* in a group of 191 Europeans. Using PCR–SSCP, we screened for mutation the entire coding sequence, the exon-intron boundaries, the promoter region and the 3′-flanking region of the gene. Six mutations were detected throughout the ten exons and seven *TPMT* alleles were characterized. Four of them, *TPMT*2*, **3A*, **3C* and **7*, harbouring the known mutations, G238C, G460A, A719G or T681G, were nonfunctional and accounted for 0.5, 5.7, 0.8 and 0.3% of the allele totality, respectively.

3 Within the promoter region, six alleles corresponding to a variable number of tandem repeats (VNTR), were identified. *VNTR*V4* and **V5a* which harbour four or five repeats of a 17–18 bp unit, were the most frequent (55% and 34%, respectively). The other *VNTR* alleles, having from five to eight repeats, were rarer.

4 The TPMT phenotype was correctly predicted by genotyping for 87% of individuals. A clear negative correlation between the total number of repeats from both alleles and the TPMT activity level was observed, indicating that VNTRs contribute to interindividual variations of TPMT activity. Therefore, additional analysis of the promoter region of TPMT can improve the phenotype prediction rate by genotyping.

Keywords: *TPMT* polymorphism; pharmacogenetics; PCR–SSCP analysis; genotype and phenotype correlation

Introduction

The thiopurine *S*-methyltransferase (TPMT; EC 2.1.1.67) is a cytosolic enzyme whose precise physiological role is unknown. It catalyzes the *S*-methylation of widely used immunosuppressive or cytotoxic thiopurine drugs such as 6-thioguanine, 6-mercaptopurine and azathioprine (Weinshilboum & Sladek, 1980; Woodson *et al*., 1982). The *in vivo* activity of this cytosolic enzyme is characterized by interindividual and interethnic variability caused by the genetic polymorphism of the TPMT gene, which was discovered by the existence of three major phenotypes, high (HM), intermediate (IM) and deficient (DM) methylators (Weinshilboum & Sladek, 1980). As a consequence, individuals greatly differ in the detoxication of thiopurine drugs to 6-methylmercaptopurine and thus, in the occurrence of side effects or therapeutic efficacy. In this regard, the TPMT polymorphism has been associated with severe and potentially fatal myelosuppression occurring in DMs treated by azathioprine that was not inactivated by the TPMT enzyme (Evans *et al*., 1991; Lennard *et al*., 1987, 1989, 1993; McLeod *et al*., 1993; Schütz *et al*., 1993). TPMT polymorphism has been also associated with the rejection of transplanted organ in HM recipient patients over-treated by immunosuppressive thiopurine medications (Lennard *et al*., 1990; Schütz *et al*., 1995). Therefore, in order to avoid 6-methylmercaptopurine concentration dependent side-effects, phenotyping/genotyping tests prior to the instigation of drug treatment might identify the patient at risk of treatment failure.

Evaluation of the phenotypic status of patients by the measurement of RBC TPMT activity has become a clinical test performed routinely before the initiation of thiopurine therapy and in the monitoring of treatment (Lennard *et al*., 1987; Szumlanski *et al*., 1992; Weinshilboum *et al*., 1978). However, the protocols for testing, and problems with the correct evaluation of the constitutive TPMT activity if patients have received transfusion of red blood cells, limit the use of the phenotyping procedures (Krynetski *et al*., 1995; Tai *et al*., 1996). The large interindividual variability in enzyme activity has been mainly explained by genetic factors and genotyping tests have been recently proposed to avoid these limitations. The gene (*TPMT*, MIM 187680) which codes for TPMT has been localized to chromosome 6 (6p22.3) and its sequence has been partially determined (Szumlanski *et al*., 1996). Presently, various *TPMT* alleles, carrying a point mutation or a combination of mutations in some of the ten exons or nine introns, as well as in the 5′-flanking promoter region, have been associated with deficient, intermediate or high TPMT activity (Krynetski *et al*., 1995; Otterness *et al*., 1996, 1997, 1998; Szumlanski *et al*., 1996; Tai *et al*., 1996; Yates *et al*., 1997). The three point mutations, G238C, G460A, and A719G represent the most common genetic defects identified in DMs (Krynetski *et al*., 1995; Szumlanski *et al*., 1996; Tai *et al*., 1996) and

[4]Author for correspondence at: Laboratoire de Biochimie et Biologie moléculaire, Hôpital Calmette, Centre Hospitalier Régional et Universitaire de Lille, Bd. J. Leclercq, 59037 Lille, France.

account for about 12, 81 and 88% of the detrimental mutations, respectively (Yates *et al*., 1997). A polymorphism in the 5′-flanking promoter region has also been identified due to a variable number of tandem repeats (VNTR), ranging from four to eight repeats (Krynetski *et al*., 1997; Spire *et al*., 1998a). Each repeat consists in 17 or 18 bp unit and contains a potential binding site for the transcription factor Sp1. The most frequent alleles *VNTR*V4* and **V5* harbor four or five tandem repeats and a heterozygosity rate of 0.44. This new polymorphism is suspected to contribute to the regulation of gene transcription (Spire *et al*., 1998a). Now, DNA-tests, based on PCR (polymerase chain reaction) amplification, are available to genotype individuals by identifying the major genetic defects of coding sequences on both chromosomes (Otterness *et al*., 1997; Yates *et al*., 1997).

The aim of this study was to determine, in a European population, (i) the overall mutational spectrum of the TPMT gene, (ii) the frequency of each mutation and their effect on TPMT activity, and (iii) the potential of using the genotype to predict individual TPMT activity. We have thus undertaken a complete analysis of the ten exons including the entire coding sequence of the TPMT gene and of the 5′- and 3′-untranslated regions of 191 unrelated individuals, using a previously described PCR–SSCP strategy (Spire *et al*., 1998a,b). As already demonstrated, this technique enables the detection of known *TPMT* mutations and is an effective method to screen for new mutations (Spire *et al*., 1998a,b). The present study improves our knowledge on the mutational spectrum of the TPMT gene, and assesses the accuracy of the genotyping method for predicting TPMT phenotype in a large population to determine patients at risk for thiopurine toxicity.

Methods

Subjects of investigations and DNA samples

A group of 191 unrelated subjects of European origin have been involved in the study after local ethic committee approval and informed consent were obtained. Among the 191 subjects, 146 were healthy volunteers and 45 were patients evaluated for TPMT activity before initiation of thiopurine therapy. Total genomic DNA was isolated from peripheral leukocytes by chloroform/phenol extraction and by isopropanol/sodium acetate precipitation, as described previously (Maniatis *et al*., 1989).

TPMT PCR–SSCP analysis

Each of the ten exons including their splice-site junctions and the 5′-flanking promoter region of *TPMT* were amplified by PCR for SSCP (single strand conformation polymorphism) analysis, as described previously (Spire *et al*., 1998a,b). Mutated sites analysed by PCR–SSCP in the present study are listed together with the corresponding references in Table 1. Additionally, the polyadenylation site, previously identified within the 3′-untranslated region of the exon 10 (Szumlanski *et al*., 1996), was also amplified by PCR for SSCP analysis. A 303 bp fragment was generated using a forward primer *TPMT-3′*F (5′-CAGTAGGGTT-CAAGG-TGGTC-3′; from nucleotide 1449) and a reverse primer *TPMT-3′*R (5′-TATAAGACCTGATGATTTAAA-CAG-3′; to nucleotide 1752). Primers were designed to avoid the unwanted amplification of fragments from the highly homologous *TPMT*processed pseudogene (Lee *et al*., 1995), and thereby to exclude false positive detection of mutations. The amplification reaction was carried out in a total volume of 15 μl in the presence of 100 ng of DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer and 0.25 U *Taq* DNA polymerase (Boehringer Mannheim GmbH, Germany). In addition, 0.6 μCi of [α-^{32}P]-dCTP (3000 Ci mmole^{-1}, Amersham, Buckinghamshire, U.K.) were added to label the amplified fragments. After an initial denaturation step at 94°C for 2 min, 35 cycles of 90 s at 94°C, 90 s at 58°C, 90 s at 72°C were performed, and a final extension period of 7 min was carried out at 72°C. For the SSCP electrophoresis, 3 μl of this PCR product were mixed with 3 μl of loading dye (10 mM NaOH, 20 mM EDTA, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 92°C for 2 min and chilled on ice. Three μl of the mixture were then loaded on a 10% glycerol non-denaturing MDE™ gel, prepared according to manufacturer's instructions (FMC Bioproducts, Rockland ME, U.S.A.). The electrophoresis was performed at room temperature in a 0.6× TBE buffer (53.4 mM Tris, 53.4 mM Boric acid, pH 8.3, and 1.2 mM EDTA) using an IBI™ sequencing gel apparatus (Apelex, Massy, France) at 6 W for 15 h. After electrophoresis, gels were transferred to filter paper, dried and subjected to autoradiography at −70°C for 72 h.

The population was tested using known wild-type and mutated sequences as controls (Spire *et al*., 1998a,b). In order to avoid erroneous shift of electrophoretic mobility due to mutation resulting from possible replication errors of the DNA polymerase, the PCR–SSCP procedure was repeated twice when a fragment displayed a SSCP pattern different from the controls.

TPMT sequencing

Samples with an abnormal SSCP pattern were sequenced using an automated DNA sequencer (Model 373A, Applied Biosystems Inc., Foster City, U.S.A.) with the ABIPRISM™ Dye Terminator Cycle Sequencing Ready Reaction FS kit (Applied Biosystems, ABI, Foster City, U.S.A.) according to manufacturer's instructions. Sequencing primers were the primers used in the SSCP analysis. The sequence of both strands was analysed from at least two independent PCR amplifications to ensure that all identified mutations were not PCR-induced artifact.

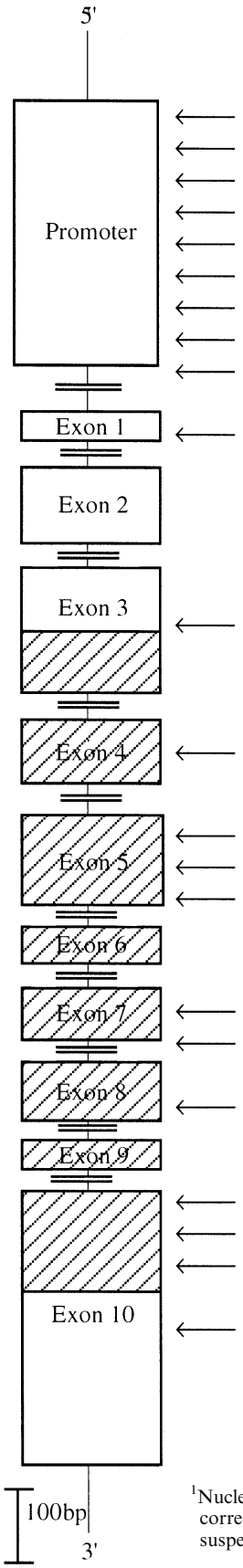
Characterization of TPMT alleles and frequency calculations

To characterize both *TPMT* alleles from each individual, we used the nomenclature to assess all previously known *TPMT* variants (Otterness *et al*., 1997). The mutations G460A and A719G are presumed to be combined on the same allele in Caucasians (Otterness *et al*., 1997; Yates *et al*., 1997). Genotypes were therefore defined assuming that G460A and A719G are present on the same allele *TPMT*3A*, when both mutations were detected in a particular DNA sample. Individuals were classified as DM, IM or HM according to the genotype determined using SSCP results. Homozygous subjects for the functional *TPMT* allele **1* were predicted as HMs. Heterozygous individuals for one nonfunctional *TPMT* allele **2*, **3A*, **3B*, **3C*, **3D*, **4*, **5*, **6* or **7* were predicted as IMs, while homozygous or compound heterozygous carriers of nonfunctional *TPMT* alleles were predicted as DMs.

TPMT phenotype determinations

TPMT activity was measured on erythrocyte lysates using a radiochemical enzymatic assay according to the modified procedure (Szumlanski *et al*., 1992) of the method of Weinshilboum *et al*. (1978). This assay is based on the methylation of 6-mercaptopurine with [^{14}C-methyl]-*S*-adenosyl-L-methionine as methyl donor. Erythrocyte lysates were

Table 1 Local distribution and nature of the mutations identified on TPMT gene and analysed by PCR–SSCP in this study



Position, nature[1]	*Consequences*	*References*
−168 T→G	None	Krynetski *et al*., 1997; Spire *et al*., 1998a
−91 A→G	None	Krynetski *et al*., 1997; Spire *et al*., 1998a
VNTR 3	–	Szumlanski *et al*., 1996
VNTR 4	–	Spire *et al*., 1998a
VNTR 5a	–	Krynetski *et al*., 1997; Spire *et al*., 1998a
VNTR 5b	–	Spire *et al*., 1998
VNTR 6	–	Spire *et al*., 1998a
VNTR 7	–	Spire *et al*., 1998a
VNTR 8	–	Spire *et al*., 1998a
−178 C→T	None	Spire *et al*., 1998b
−30 T→A	None	Otterness *et al*., 1997
146 T→C	49 Leu→Ser[3]	Otterness *et al*., 1997
238 G→C	80 Ala→Pro[2]	Krynetski *et al*., 1995
292 G→T	98 Glu→Stop[2]	Otterness *et al*., 1997
339 C→T	Silent	Otterness *et al*., 1997
460 G→A	154 Ala→Thr[2]	Tai *et al*., 1996; Szumlanski *et al*., 1996
474 T→C	Silent	Szumlanski *et al*., 1996
539 A→T	180 Tyr→Phe[3]	Otterness *et al*., 1997
AG→AA	Splicing defect[2]	Otterness *et al*., 1996
681 T→G	227 His→Glu[3]	Spire *et al*., 1998b
719 A→G	240 Tyr→Cys[2]	Tai *et al*., 1996; Szumlanski *et al*., 1996
AATAAA	Polyadenylation site	Szumlanski *et al*., 1996

[1]Nucleotides 5′-upstream from cDNA translation initiation codon and intronic nucleotides 5′-upstream from the corresponding exon are numbered negatively. [2]Mutations responsible of loss of TPMT activity. [3]Mutations suspected to be responsible of loss of TPMT activity. Dashed boxes represent ORF (open reading frame).

prepared according to the procedure from whole blood collected in heparinized tubes by experimented nurses from the Center of Clinical Investigation. The phenotype was assigned on the basis of TPMT activity (Otterness *et al*., 1997). The high methylator phenotype (HM) was defined as a TPMT activity higher than 13.7 U ml^{-1} of red blood cells (RBC). Individuals having TPMT activity from 5 to 13.7 U ml^{-1} RBC were defined as intermediate methylators (IMs). Deficient methylators (DMs) were individuals with values lower than 5 U ml^{-1} RBC.

Statistical analysis

Statistical analysis was performed with the Statview™ v1.0 program (Abacus Concepts, Inc., Berkeley CA, U.S.A.). Significant differences between the TPMT activity means was tested (a) between carriers of one and two functional alleles and (b) between carriers of eight to 12 total VNTR, by the use of one-way analysis of variance test.

Results

Detection of TPMT gene mutations by PCR–SSCP

One hundred and ninety-one unrelated individuals were analysed by PCR–SSCP for mutations in the ten exons of the TPMT gene and its 5′- and 3′-flanking regions. Patterns obtained for each amplified exon were compared with those obtained for control DNAs. Differences in SSCP migration patterns were observed for exons 1, 5, 7, 10 and for the 5′-flanking region. All mobility shifts observed in the different patterns corresponded to a mutation already characterized in controls (Spire *et al*., 1998a,b), except for two patterns observed in the 5′-flanking region. Further sequencing of samples with the two novel patterns allowed us to identify (i) a new VNTR allele termed **V5b*, which differ from **V5a* (or **V5*) by the nucleotide sequence of one repeated motif (Figure 1), and (ii) an individual homozygous for a VNTR containing seven repeats (allele **V7*). The nucleotide sequence of the allele *VNTR *V5b* reported in this paper have been submitted to the Genbank with accession number AF060074.

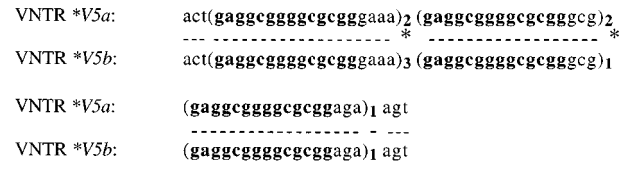


Figure 1 Nucleotide sequence of the two VNTR alleles containing five repeat units characterized in the 5′-flanking region of the TPMT gene from nucleotides −132 to −40 upstream from the exon 1. The 17 bp and 18 bp repeat units (written between brackets) differed from each other by three to four bases and have a consensus 14 bp nucleotide sequence (written in bold). The number of repeated units is indexed above the brackets. Differences in repeat numbers between the two sequences are noted with (*).

When the sequences of fragments were compared to the previously reported *TPMT*1* wild-type sequence (Szumlanski *et al*., 1996), a total of 14 mutations were detected in the population tested. Eight were characterized in the 5′-flanking region, including two point mutations found in all of the individuals. The six remaining sequence variations observed in the 5′-flanking region were the VNTRs (Spire *et al*., 1998a), with repeats varying from four to eight. Four missense mutations were detected in some of the ten exons, including the three deleterious mutations G238C, G460A and A719G and the T681G mutation suspected to be associated with a decrease of the TPMT activity. Additionally, two silent mutations were detected in exon 1 (C-178T) and exon 7 (T474C). The frequency of the inactivating point mutations and of the VNTRs in all the 191 individuals is given in Tables 2 and 3, respectively. The most prevalent inactivating mutations were A719G (89%) and G460A (79%). The rare inactivating mutations G238C and T681G represented 8% and 4%, respectively. These four mutations displayed a cumulated frequency of 12.7%. VNTR containing four repeats was the most frequent (55%), followed by the VNTRs with five repeats (34%). The number of VNTR sequences containing from six to eight repeats decreased progressively from 7% to 0.8% (Table 3).

TPMT allelic variants and their frequencies

Based on the *TPMT* allele nomenclature (Otterness *et al*., 1997), we characterized a total of seven *TPMT* alleles (Table 2). Two alleles, carrying mutations that did not alter encoded

Table 2 Allelic variants of TPMT gene and their frequency among 191 European individuals ($n = 382$ alleles)

	Alleles with single base changes[1] *at nucleotide number*								*Frequency*	
TPMT alleles	*146*	*238*	*292*	*460*	*539*	*(−1) exon 10*	*681*	*719*	n	*%*
*1[2]	T	G	G	G	A	G	T	A	354	92.7
*2	–	C	–	–	–	–	–	–	2	0.5
*3A	–	–	–	A	–	–	–	G	22	5.7
*3B	–	–	–	A	–	–	–	–	0	0.0
*3C	–	–	–	–	–	–	–	G	3	0.8
*3D	–	–	T	A	–	–	–	G	0	0.0
*4	–	–	–	–	–	A	–	–	0	0.0
*5	C	–	–	–	–	–	–	–	0	0.0
*6	–	–	–	–	T	–	–	–	0	0.0
*7	–	–	–	–	–	–	G	–	1	0.3
	Frequency of point mutations									
Data	*146*	*238*	*292*	*460*	*539*	*(−1) exon 10*	*681*	*719*		
n	0	2	0	22	0	0	1	25		
%	0	0.5	0	5.7	0	0	0.3	6.5		

[1]Inactivating single-base changes. [2]Wild-type allele (Wt).

amino acids (mutation T474C) or that was not located within the coding exons (mutation C-178T in exon 1), were considered as a functional allele *TPMT*1* which accounted for about 93% of all allelic variants in the total population. The three most common defective alleles **3A*, **3C* and **2* were detected. *TPMT*3A* was the most prevalent mutant allele (22 out of 28); *TPMT*3C* and **2* were rarer (three and two out of 28, respectively). The mutant allele **7* (with the mutation T681G in exon 10), suspected to be nonfunctional, was detected in one individual. None of the other alleles *TPMT*3B*, **3D* and **4* associated with a deficient TPMT activity, as well as alleles *TPMT*5* and **6* suspected to be defective alleles, were identified in our population.

The linkage analysis of the association between VNTR and allelic variants of *TPMT* was estimated for 96% of the population (Table 3). Six individuals for whom heterozygous SSCP patterns were observed for the 5′-flanking region and exons 7 and/or 10, have been therefore excluded for the calculation of allele frequency. Each VNTR, from **V4* to **V8*, was associated with the functional allele *TPMT*1* (Table 3). Each nonfunctional allele was associated at least once with **V4*. Additionally, the nonfunctional allele *TPMT*3A* can be also associated with **V5* or **V6*.

Phenotype determination in Europeans

Erythrocyte TPMT enzyme activity was measured in each of the 191 individuals participating in the study. The mean value was 20.85 U ml^{-1} RBC and TPMT activity values varied widely from 0.3–50 U ml^{-1} RBC in our population. The frequency distribution histogram differed from that reported for American subjects and did not include a clearly defined subgroup with intermediate activity (data not shown), probably due to the relatively small number of subjects tested. Out of the 191 individuals phenotyped, one was deficient, 32 were intermediate methylators and 158 were high methylators.

Phenotype/genotype correlation

On the one hand, individuals were classified according to the observed *TPMT* genotypes into groups of high (HMs), intermediate (IMs) or deficient methylators (DMs). In the population studied, 164 subjects carrying two functional alleles (genotype **1/*1*) were predicted as HMs (86%), 26 subjects heterozygous for one nonfunctional allele [**1/*2* ($n=2$), **1/*3A* ($n=20$), **1/*3C* ($n=3$), and **1/*7* ($n=1$)] were predicted as IMs (13.5%), and one subject homozygous for the nonfunctional allele **3A* was predicted as a DM (0.5%). On the other hand, erythrocyte activity was measured in a single blind analysis for the 191 genotyped individuals. The phenotype determined conventionally was then compared to that predicted by PCR–SSCP analysis. As shown in Figure 2, the subject predicted as a DM showed, as expected, a low TPMT activity (<5 U ml^{-1} RBC). Subjects predicted as IMs exhibited a TPMT activity range from 5–20 U ml^{-1} RBC and subjects predicted as HMs exhibited a TPMT activity range from 10–50 U ml^{-1} RBC. The median TPMT activity was significantly higher in predicted HMs than in predicted IMs (22.4 U ml^{-1} RBC versus 11.7 U ml^{-1} RBC; $P<0.0001$). Phenotypes were in good accordance with genotypes for 87% of individuals. However, nine out of the 26 heterozygotes for one nonfunctional allele presented a TPMT activity between the cutoff value of 13.7 U ml^{-1} RBC to 20 U ml^{-1} RBC. Likewise, 15 out of the 164 homozygotes for the functional allele **1* presented a TPMT activity from 10 to 13.7 U ml^{-1} RBC. Sequencing the ten exons and the 5′- and 3′-flanking regions of *TPMT* from these 15 DNA samples confirmed the genotypes as determined by our PCR–SSCP analysis. Therefore, there was an overlap between genotyped IM and HM groups in the functional range of 10–20 U ml^{-1} RBC and the genotype is in agreement with the phenotype for the DM, 53% of the IMs and 94% of the Hms.

Table 3 Frequency of *TPMT* alleles combined with the VNTR markers among 191 Europeans

	VNTR						
TPMT alleles	**V4*	**V5a*	**V5b*	**V6*	**V7*	**V8*	*Total*
*1	191	120	4	19	11	3	348
*2	2	–	–	–	–	–	2
*3A	11	5	–	3	–	–	19
*3C	1	–	–	–	–	–	1
*1, *3A, *3C or *7[1]	4	3	–	5	–	–	12

[1]Mutation-linkage pattern could not be defined for six individuals who were heterozygous for both VNTR and mutant *TPMT* alleles.

VNTR influence on TPMT activity

The influences of the genetic polymorphism of the 5′-flanking region of the TPMT gene on the basal TPMT activity and on the genotype/phenotype discrepancies were assessed. The *in vivo* functional consequence of the VNTRs on TPMT activity was first explored for individuals genotyped as HMs. For this purpose, the 164 HM subjects were classified according to their total number of repeats corresponding to the sum of both alleles. Seven groups with eight to 14 total VNTR repeats were constituted. Mean TPMT activity and its 95% confidence limits were calculated for groups containing more than one individual (five groups with eight to 12 total VNTR repeats). As shown in Figure 3a, a statistically-significant correlation between the TPMT activity and the total number of repeats was found, and an inverse relationship between the two variables was demonstrated. Secondly, a similar analysis was performed to estimate the influence of VNTR repeats on

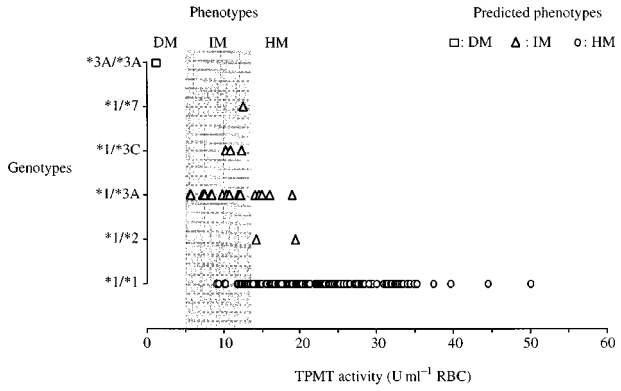


Figure 2 Distribution of thiopurine *S*-methyltransferase (TPMT) activity among 191 European individuals in relation to their TPMT genotypes determined by PCR–SSCP according to the previously described procedure (Spire *et al*., 1998b). The grey shaded area depicts the range of TPMT activity in erythrocytes that defines TPMT intermediate activity (5–13.7 U ml^{-1} of packed red blood cells) and which separates TPMT deficiency activity from TPMT high activity. Individuals genotyped homozygous for the functional allele *TPMT*1* were predicted as HMs, individuals genotyped heterozygous for the nonfunctional alleles **2*, **3A*, **3C* and **7* were predicted as IMS and individual genotyped homozygous for the nonfunctional allele **3A* was predicted as DM.

TPMT activity for individuals genotyped as IMs. Due to the small number of individuals ($n=26$), the mean TPMT activity and its 95% confidence limits were calculated for only three groups of IMs (groups with eight, ten and 11 total VNTR repeats). As shown in Figure 3b, no statistically-significant correlation between the TPMT activity and the total number of VNTR repeats was found although the data trend is clearly in the direction of an inverse relationship between the two variables.

Consequently, this polymorphism can be considered responsible for shifts to lower or higher TPMT activities observed among discordant individuals. Seven out of the nine phenotyped HMs but genotyped IMs were carrier of a total of eight VNTR repeats. This low number of repeat can account for the switch to high TPMT activities for these samples. Likewise, the 13 out of the 15 IMs, erroneously predicted as HMs by genotyping, were carriers of 9–11 VNTR repeats. The augmented number of repeats can be involved for the switch to low TPMT activity for these samples.

Discussion

Genetic polymorphism of TPMT activity is an important factor responsible for large individual differences in thiopurine toxicity and therapeutic efficacy. Therefore, it is essential to fully characterize the extent of allelic variation at the TPMT gene locus, to understand the functional consequences of each variant, and to develop accurate diagnostic tests for discriminating them in population. With this aim, we defined the mutational and allelic spectrum of *TPMT* in a large group of European individuals using an efficient and sensitive method of PCR–SSCP for *TPMT* mutation detection. The relationship between the TPMT activity and genotype of the 191 individuals participating in the study was further examined.

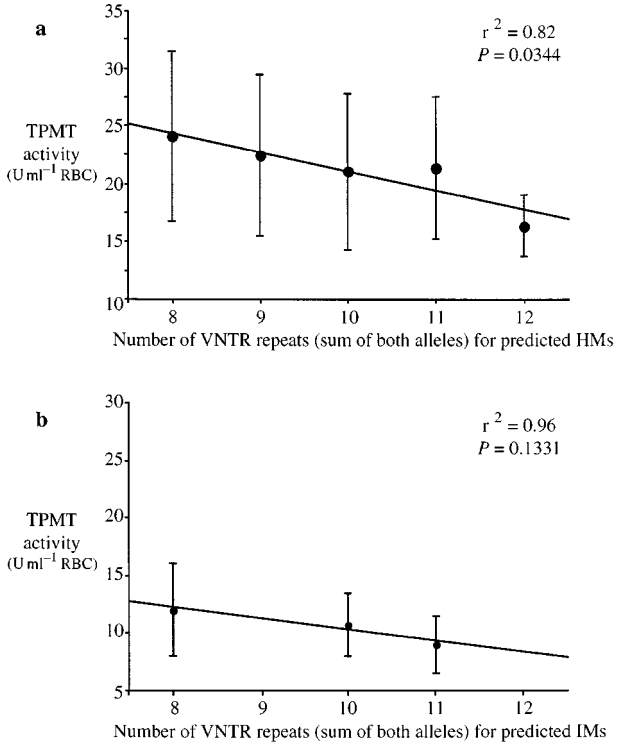


Figure 3 Correlation between TPMT activity and the total number of VNTR repeats carried by both chromosomes. (a) analysis of the genotyped HM population; (b) analysis of the genotyped IM population. Means of TPMT activity and their 95% confidence limits were calculated for groups consisting of more than one individual. Statistical analysis was performed using the Fisher *F*-test and by the use of one-way analysis of variance test (ANOVA). Significant differences were, for the HM group as follows: 8 versus 12 and 9 versus 12 with $P<0.05$.

Up to date, 20 mutations distributed on seven exons and on the 5′-flanking promoter region of the TPMT gene have been reported (Table 1). Five have been demonstrated as being responsible for a deficient TPMT phenotype and three others are suspected to be deleterious. Genotyping procedures based on PCR have been developed for the identification of the three mutations characterizing the alleles *TPMT *2*, **3A*, **3B* and **3C* (Yates *et al*., 1997). These genotyping tests are very simple, rapid and efficient, but have the disadvantage to be only focused on known variant nucleotides, allowing the prediction of only about 95% of the DM individuals (Otterness *et al*., 1997). Therefore, we designed a PCR–SSCP strategy for the identification of the 20 known mutations of the TPMT gene as well as for the detection of new mutations. This PCR–SSCP strategy was performed on the ten exons and the 5′- and 3′-flanking regions of *TPMT* and was applied to DNA samples of 191 Europeans. Fourteen mutations have been detected of which one is novel. Only four were deleterious mutations characterizing allelic variants *TPMT *2*, **3A*, **3C*, and **7*. Six were mutations characterizing different VNTRs in the promoter region, of which one corresponded to a new *VNTR* sequence termed **V5b*. The four remaining mutations were silent mutations or were out of the open reading frame (ORF). Mutation and allele frequencies of our population are in agreement with previous studies (Otterness *et al*., 1997; Weinshilboum & Sladek, 1980; Yates *et al*., 1997), and the allele **3A* was confirmed to be the most common detrimental mutations present in about 89% of the null alleles. Other mutations corresponding to the nonfunctional *TPMT* alleles **3D*, **4*, **5* and **6* have not been characterized in our population. Although our PCR–SSCP method would allow their detection, the absence of these mutations is not surprising since they are very rare and their frequency distribution can be different from a population to another (Otterness *et al*., 1997). However, no controls for those rare mutations could be included in the PCR–SSCP strategy and despite many precautions, it could be possible that they were not detected because of the chosen electrophoretic conditions. Nevertheless, the nucleotide sequence analysis performed on the discordant DNA samples with a HM genotype but corresponding to IM individuals did not allow the identification of those rare inactivating mutations and thus confirmed the genotype as determined by SSCP.

An additional PCR-based assay has to be developed for each new inactivating mutation identified. The SSCP analysis is therefore more efficient compared with conventional genotyping procedures since it allows simultaneously the detection of known and new mutations of the TPMT gene. However, as observed with conventional genotyping tests, it appears that the SSCP strategy does not distinguish the IM genotype **1/*3A* from the DM genotype **3B/*3C*, which slightly reduces the phenotype prediction capacity of genotyping.

In the present study, a relatively large population of 191 European individuals was genotyped and phenotyped for *TPMT* by using a PCR–SSCP method and a conventional radiochemical enzymatic assay, respectively. The distribution of RBC TPMT in the European population was different from a trimodal distribution as reported previously in the American population (Weinshilboum & Sladek, 1980) but was similar to a Gaussian distribution as previously reported in the French

population (Jacqz-Aigrain *et al*., 1994) or in the Korean population (Park-Hah *et al*., 1996). TPMT activity measured in our population was substantially higher than those previously reported for American subjects (Otterness *et al*., 1997) but range values for RBC TPMT activity varied from a population to another (Jacqz-Aigrain *et al*., 1994; Jang *et al*., 1996; Jones *et al*., 1993; McLeod *et al*., 1994; Park-Hah *et al*., 1996; Szumlanski *et al*., 1996). One individual was found to be DM by both genotyping and phenotyping tests. The genotyping method has allowed the correct assignation of HM and IM for 87% of the individuals and discrepancies between phenotype and genotype appeared for individuals with a TPMT activity close to the antimode value.

The reasons why phenotype/genotype discrepancies exist are multiple. The most likely explanation could result from mutations that our PCR–SSCP strategy failed to detect. However, in all cases, subsequent DNA sequencing of the *TPMT* exons and their flanking regions confirmed the genotype as determined by PCR–SSCP. Methodological problems with the TPMT assay could be also incriminated. Procedural variability of the TPMT phenotyping tests (Szumlanski *et al*., 1992), determination of the cut-off value used to separate IMs from HMs (Otterness *et al*., 1997), extrapolation of RBC lysate volumes to standard hematocrit (Klemetsdal *et al*., 1995), are different factors influencing the reproducibility of the phenotyping method from a laboratory to another. Moreover, phenotyping determination cannot be applied to individuals receiving blood transfusions as the test will then not be performed on their own red blood cells (Tai *et al*., 1996). Thus, some phenotype/genotype discrepancies may be explained by a lack of reliability of TPMT activity values.

Additionally, genetic as well as epigenetic factors are also known to influence TPMT activity (Krynetski *et al*., 1996;Vuchetich *et al*., 1995). Interindividual variability of the TPMT activity can be partially explained by VNTRs identified in the promoter region and highly suspected to be implicated in the regulation of *TPMT* expression (Spire *et al*., 1998a). Each repeat consists of a 17 or 18 bp unit which contains a potential binding site for the transcription factor Sp1. Analysis of the effect of each VNTR allele on promoter activity of a reporter gene, performed in various cell lines by transient transfection assay, revealed that alleles having six or seven tandem copies had a notable lower promoter activity than other alleles with less tandem copies (Spire *et al*., 1998a). Moreover, the inverse correlation between the total number of VNTR repeats present on both TPMT alleles (from eight to 12) and the TPMT activity reported in this study, supports this hypothesis. About 77% of the discordant genotyped IMs with a TPMT activity slightly higher than 13.7 U ml^{-1} RBC were carrier of only eight VNTR repeats. At the opposite, 87% of the discordant genotyped HMs with a TPMT activity slightly lower than the antimode value were carrier of more than eight VNTR repeats. Thus, the mechanism by which the VNTR decreases the expression of TPMT in a copy number-dependent manner has to be clarified, but it can explain the switch from a group to another for some individuals. Epigenetic factors can also influence the enzyme activity (Krynetski *et al*., 1996). Patients age (McLeod *et al*., 1995; Pacifici *et al*., 1991), renal function including chronic renal failure (Pazmino *et al*., 1980), as well as drug interactions with thiopurine therapy (for example with sulphasalazine or thiazide diuretics) (Bergan *et al*., 1997; Lysaa *et al*., 1996; Szumlanski & Weinshilboum, 1995) are known to influence *in vitro* and *in vivo* TPMT activity. The measurement of TPMT activity in patients on thiopurine therapy can also bias the phenotype determination, because of enzymatic induction phenomena (Chocair *et al*., 1992; Lennard *et al*., 1990; Mircheva *et al*., 1995). Discrepancies were not related to the nature of the included subjects as they were observed as well as for healthy volonteers than for patients. However, although none of the patients included in this study was on thiopurine therapy, physiological or pathological status of some individuals can be involved to explain some discrepancies. Results of *TPMT* phenotyping tests have to be interpreted with caution in light of these informations.

TPMT phenotype determination appears more complex than originally thought as both genotyping and phenotyping methods have their advantages and limits. The application of a phenotyping or a genotyping method to determine TPMT activity will depend on laboratory skills and experience, on the pathological status of patients, and on the aim of phenotype assignment. The elucidation of the molecular basis for TPMT polymorphism suggests that genotyping use to diagnose DMs and IMs offers advantages over RBC phenotype measurement, (i) for patients with chronic diseases or under multiple drug treatments, (ii) for patients receiving blood transfusions and (iii) for laboratory without experience in TPMT phenotyping. The development of a panel of PCR-based tests, or the use of PCR–SSCP analysis, for the detection of major deleterious *TPMT* mutations, would allow the identification of DMs with a predictive efficacy close to 100%. Guidelines for dose adjustments for such patients are now described (Andersen *et al*., 1998). In large-scale epidemiological studies, PCR–SSCP analysis strategy offers the advantage of its ability to detect simultaneously known and new mutations. We demonstrated that our TPMT genotyping test enables the determination of the metabolic capacity for 87% of individuals. However, it appears that genotyping is a poor predictor of TPMT metabolic capacity in HMs for whom the enzymatic activity varies over 4 fold. Identification of HMs with very high TPMT activity can only be performed by phenotyping and is useful to prevent them from under treatment and rejection episodes (Bergan *et al*., 1997). Additional analysis of the *TPMT* promoter using PCR-based tests for direct identification of the *VNTR* alleles might improve the phenotype predictive value of genotyping.

In conclusion, allelic variations at the *TPMT* locus result in large individual differences in the activity of the enzyme TPMT that the gene encodes and are responsible for large differences in toxicity and efficacy of thiopurine drugs. Therefore, it is important to fully characterize the extent of allelic variation at the TPMT gene locus in populations, to understand the functional consequences of each variant, and to develop accurate diagnostic tests for discriminating them. To our knowledge, the present study is the first extensive study of the mutational spectrum of the TPMT gene in a European population and of its consequence on TPMT activity. Data from this study improve our understanding of the molecular mechanisms of the polymorphism which affects TPMT activity. They also provide a rational basis for the choice of the methodology to apply, in clinic or in research, to explore the TPMT polymorphism.

This study was supported by the Centre Hospitalier Régional et Universitaire de Lille and cosupported by grants from the 'Agence Française du Médicament' and 'MSD[INSERM]'.

References

ANDERSEN, J.B., SZUMLANSKI, C., WEINSHILBOUM, R.M. & SCHMIEGELOW, K. (1998). Pharmacokinetics, dose adjustments, and 6-mercaptopurine/methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. *Acta Paediatr.,* **87,** 108 – 111.

BERGAN, S., RUGSTAD, H.E., KLEMETSDAL, B., GIVERHAUG, T., BENTDAL, Ø., SØDAL, G., HARTMANN, A., AARBAKKE, J. & STOKKE, O. (1997). Possibilities for therapeutic drug monitoring of azathioprine: 6-thioguanine nucleotide concentrations and thiopurine methyltransferase activity in red blood cells. *Ther. Drug. Monit.,* **19,** 318 – 326.

CHOCAIR, P.R., DULEY, J.A., SIMMONDS, H.A. & CAMERON, J.S. (1992). The importance of thiopurine methyltransferase activity for the use of azathioprine in transplant recipients. *Transplantation,* **53,** 1051 – 1056.

EVANS, W.E., HORNER, M., CHU, Y.Q., KALWINSKY, D. & ROBERTS, W.M. (1991). Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. *J. Pediat.,* **119,** 985 – 989.

JACQZ-AIGRAIN, E., BESSA, E., MEDARD, Y., MIRCHEVA, Y. & VILMER, E. (1994). Thiopurine methyltransferase activity in a French population: h.p.l.c. assay conditions and effects of drugs and inhibitors. *Br. J. Clin. Pharmac.,* **38,** 1 – 8.

JANG, I.J., SHIN, S.G., LEE, K.H., YIM, D.S., LEE, M.S., KOO, H.H., KIM, H.K. & SOHN, D.R. (1996). Erythrocyte thiopurine methyltransferase activity in Korean population. *Br. J. Pharmacol.,* **42,** 638 – 641.

JONES, C.D., SMART, C., TITUS, A., BLYDEN, G., DORVIL, M. & NWADIKE, N. (1993). Thiopurine methyltransferase activity in a sample population of black subjects in Florida. *Clin. Pharmacol. Ther.,* **53,** 348 – 353.

KLEMETSDAL, B., STRAUME, B., LYSAA, R., GIVERHAUG, T. & AARBAKKE, J. (1995). Erythrocyte fraction affects red blood cell thiopurine methyltransferase activity. *Eur. J. Clin. Pharmacol.,* **48,** 495 – 499.

KRYNETSKI E., FESSING M., YATES C., SUN D., SCHUETZ J. & EVANS W.E. (1997). Promoter and intronic sequences of the human thiopurine S-methyltransferase (TPMT) gene isolated from a human Pac1 genomic library. *Pharm. Res.,* **14,** 1672 – 1678.

KRYNETSKI, E., SCHUETZ, J., GALPIN, A., PUI, C.H., RELLING, M. & EVANS, W.E. (1995). A single point mutation leading to loss of catalytic activity in human thiopurine S-methyltransferase. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 949 – 953.

KRYNETSKI E., TAI H., YATES C., FESSING M., LOENNECHEN T., SCHUETZ J., RELLING M. & EVANS W.E. (1996). Genetic polymorphism of thiopurine s-methyltransferase: clinical importance and molecular mechanisms. *Pharmacogenetics,* **6,** 279 – 290.

LEE D., SZUMLANSKI C., HOUTMAN J., HONCHEL R., ROJAS K., OVERHAUSER J., WIEBEN E. & WEINSHILBOUM R.M. (1995). Thiopurine methyltransferase pharmacogenetics. Cloning of human liver cDNA and a processed pseudogene on human chromosome 18q21.1. *Drug. Metab. Dispos.,* **23,** 398 – 405.

LENNARD L., GIBSON B.E., NICOLE T. & LILLEYMAN J.S. (1993). Congenital thiopurine methyltransferase deficiency and 6-mercaptopurine toxicity during treatment for acute lymphoblastic leukaemia. *Arch. Dis. Child.,* **69,** 577 – 579.

LENNARD L., LILLEYMAN J.S., VAN LOON J. & WEINSHILBOUM R.M. (1990). Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. *Lancet,* **336,** 225 – 229.

LENNARD L., VAN LOON J.A., LILLEYMAN J.S. & WEINSHILBOUM R.M. (1987). Thiopurine pharmacogenetics in leukemia: correlation of erythrocyte thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations. *Clin. Pharmacol. Ther.,* **41,** 18 – 25.

LENNARD L., VAN LOON J.A. & WEINSHILBOUM R.M. (1989). Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism. *Clin. Pharmacol. Ther.,* **46,** 149 – 154.

LYSAA R., GIVERHAUG T., WOLD H. & AARBAKKE J. (1996). Inhibition of human thiopurine methyltransferase by furosemide, bendroflumethiazide and trichlormethiazide. *Eur. J. Clin. Pharmacol.,* **49,** 393 – 396.

MANIATIS T., SAMBROOK J. & FRITSH E.F. (1989). Commonly used techniques in molecular cloning. In *Molecular cloning, a laboratory manual*, eds. Ford, N., Nolan, C., Ferguson, M. & Ockler, M. pp E1 – E8. Cold Spring Harbor Laboratory Press.

MCLEOD H.L., KRYNETSKI E., WILIMAS J. & EVANS W.E. (1995). Higher activity of polymorphic thiopurine S-methyl transferase in erythrocytes from neonates compared to adults. *Pharmacogenetics,* **5,** 281 – 286.

MCLEOD H.L., LIN J.S., SCOTT E.P., PUI C.H. & EVANS W.E. (1994). Thiopurine methyltransferase activity in American white subjects and black subjects. *Clin. Pharmacol. Ther.,* **55,** 15 – 20.

MCLEOD H.L., MILLER D.R. & EVANS W.E. (1993). Azathioprine-induced myelosuppression in thiopurine methyltransferase deficient heart transplant recipient. *Lancet,* **341,** 1151.

MIRCHEVA J., LEGENDRE C., SORIA-ROYER C., THERVET E., BEAUNE P. & KREIS H. (1995). Monitoring of azathioprine-induced immunosuppression with thiopurine methyltransferase activity in kidney transplant recipients. *Transplantation,* **60,** 639 – 642.

OTTERNESS D., SZUMLANSKI C., LENNARD L., KLEMETSDAL B., AARBAKKE J., PARK-HAH J.O., IVEN H., SCHMIEGELOW K., BRANUM E., O'BRIEN J. & WEINSHILBOUM R.M. (1997). Human thiopurine methyl transferase pharmacogenetics: Gene sequence polymorphisms. *Clin. Pharmacol. Ther.,* **62,** 60 – 73.

OTTERNESS D., SZUMLANSKI C. & WEINSHILBOUM R.M. (1996). Human thiopurine methyltransferase pharmacogenetics: identification of a novel variant allele. *J. Invest. Med.,* **44,** 248A.

OTTERNESS D., SZUMLANSKI C., WOOD T. & WEINSHILBOUM R.M. (1998). Human thiopurine methyltransferase pharmacogenetics: kindred with a terminal exon splice junction mutation that results in loss of activity. *J. Clin. Invest.,* **101,** 1036 – 1044.

PACIFICI G., ROMOTI P., GUILIANI L. & RANE A. (1991). Thiopurine methyl transferase in humans: development and tissue distribution. *Develop. Pharmacol. Ther.,* **17,** 16 – 23.

PARK-HAH J.O., KLEMETSDAL B., LYSAA R., CHOI K.H. & AARBAKKE J. (1996). Thiopurine methyltransferase activity in a Korean population sample of children. *Clin. Pharmacol. Ther.,* **60,** 68 – 74.

PAZMINO P., SLADEK S. & WEINSHILBOUM R.M. (1980). Thiol S-methylation in uremia: Erythrocyte enzyme activities and plasma inhibitors. *Clin. Pharmacol. Ther.,* **28,** 356 – 367.

SCHÜTZ E., GUMMERT J., MOHR F., ARMSTRONG V. & OELLERICH M. (1995). Azathioprine myelotoxicity related to elevated 6-thioguanine nucleotides in heart transplantation. *Transplant. Proc.,* **27,** 1298 – 1300.

SCHÜTZ E., GUMMERT J., MOHR F. & OELLERICH M. (1993). Azathioprine-induced myelosuppression in thiopurine methyltransferase deficient heart transplant recipient. *Lancet,* **341,** 436.

SPIRE-VAYRON DE LA MOUREYRE C., DEBUYSÈRE H., FAZIO F., SERGENT E., BERNARD C., SABBAGH N., MAREZ D., LO GUIDICE J.M., D'HALLUIN J.C. & BROLY F. (1998a). Characterization of a Variable Number Tandem Repeat region (VNT) in the thiopurine S-methyltransferase gene promoter. *Pharmacogenetics,* In press.

SPIRE-VAYRON DE LA MOUREYRE C., DEBUYSÈRE H., SABBAGH N., MAREZ D., VINNER E., ALCAÏDÉ E., LO GUIDICE J.M. & BROLY F. (1998b). Detection of known and new mutations in the thiopurine S-methyltransferase gene by Single Strand Conformation Polymorphism analysis. *Human Mut.,* **12,** 177 – 185.

SZUMLANSKI C., HONCHEL R., SCOTT M. & WEINSHILBOUM R.M. (1992). Human liver thiopurine methyltransferase pharmacogenetics: biochemical properties, liver-erythrocyte correlation and presence of isozymes. *Pharmacogenetics,* **2,** 148 – 159.

SZUMLANSKI C., OTTERNESS D., HER C., LEE D., BRANDRIFF B., KELSELL D., SPURR N., LENNARD L., WIEBEN E. & WEINSHILBOUM R.M. (1996). Thiopurine methyltransferase pharmacogenetics: human gene cloning and characterization of a common polymorphism. *DNA Cell. Biol.,* **15,** 17 – 30.

SZUMLANSKI C. & WEINSHILBOUM R.M. (1995). Sulphasalazine inhibition of thiopurine methyltransferase: possible mechanism for interaction with 6-mercaptopurine and azathioprine. *Br. J. Clin. Pharmacol.,* **39,** 456 – 459.

TAI H.L., KRYNETSKI E.Y., YATES C.R., LOENNECHEN T., FESSING M.Y., KRYNETSKAIA N.F. & EVANS W.E. (1996). Thiopurine S-methyltransferase deficiency: two nucleotide transitions define the most prevalent mutant allele associated with loss of catalytic activity in Caucasians. *Am. J. Hum. Genet.,* **58,** 694–702.

VUCHETICH J.P., WEINSHILBOUM R.M. & PRICE R.A. (1995). Segregation analysis of human red blood cell (RBC) thiopurine methyltransferase (TPMT) activity. *Genet. Epidemiol.,* **12,** 1–11.

WEINSHILBOUM R.M., RAYMOND F.A. & PAZMINO P.A. (1978). Human erythrocyte thiopurine methyltransferase: radiochemical microassay and biochemical properties. *Clin. Chim. Acta.,* **85,** 323–333.

WEINSHILBOUM R.M. & SLADEK S.L. (1980). Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am. J. Hum. Genet.,* **32,** 651–662.

WOODSON L.C., DUNNETTE J. & WEINSHILBOUM R.M. (1982). Charmacogenetics of human thiopurine methyltransferase: kidney-erythrocyte correlation and immunotitration studies. *J. Pharmacol. Exp. Ther.,* **222,** 174–181.

YATES C.R., KRYNETSKI E.Y., LOENNECHEN T., FESSING M.Y., TAI H.L., PUI C.H., RELLING M.V. & EVANS W.E. (1997). Molecular diagnosis of thiopurine s-methyltransferase deficiency: Genetic basis for Azathioprine and Mercaptopurine intolerance. *Ann. Intern. Med.,* **126,** 608–614.

(Received May 26, 1998
Revised July 31, 1998
Accepted August 5, 1998)