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Allelic Variants of the Thiopurine S-Methyltranferase Deficiency in Patients With Ulcerative Colitis and in Healthy Controls

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OBJECTIVE: Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the inactivation of mercaptopurine, azathioprine, and thioguanine. The genetic polymorphisms in the TPMT gene that regulate TPMT activity are inherited as an autosomal recessive trait and patients with genetically determined low levels of TPMT activity develop severe myelosupression when treated with standard doses of the above-mentioned drugs. We have analyzed the frequencies of the allelic variants of the TPMT gene in a white European population of healthy blood donors from Spain and The Netherlands, and in a group of patients suffering from ulcerative colitis (UC) with a similar genetic background.

METHODS: Two hundred and thirteen unrelated healthy individuals (HC) and 146 UC patients were typed for the polymorphic sites at positions 460 (G → A) and 719 (A → G) of the TPMT gene using specific polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) methods.

RESULTS: There were no significant differences between the allele frequencies observed in the group of UC patients and those of the control group (10% of cases were heterozygous carriers of a TPMT mutant allele). The most frequent mutant allele in both UC and HC groups was TPMT3A ($A^{460} \rightarrow G^{719}$) (60% of carriers). TPMT3B ($A^{460} \rightarrow A^{719}$) and TPMT3C ($G^{460} \rightarrow G^{719}$) alleles were more often found in our study than in previously reported studies, reflecting the different genetic backgrounds of the European populations analyzed.

CONCLUSIONS: Genotyping methods provide a simple and reliable screening to identify patients with a high risk of developing severe bone marrow toxicity if treated with thiopurine drugs. In UC patients, TPMT genotype should be determined before the initiation of azathioprine therapy. (Am J Gastroenterol 2000;95:2313–2317. © 2000 by Am. Coll. of Gastroenterology)

INTRODUCTION

Thiopurine S-methyltransferase (TPMT, E.C.2.1.1.67) is an enzyme involved in the intracellular metabolism of mercaptopurine (1). The immunosupressant azathioprine (AZA) is converted to 6-mercaptopurine (6-MP), which may be metabolized by thiopurine S-methyltransferase (TPMT), oxydized by xanthine oxydase to thiouric acid, or catabolized to 6-thioguanine nucleotides (TGN) by hypoxanthine phosphoribosyl transferase (Fig. 1).

TPMT activity is usually measured in erythrocyte lysates, as the level of TPMT activity in human liver, kidney, and leukemic lymphoblasts correlates with that in red blood cells (2, 3). Clinical studies with 6-MP and AZA have established an inverse correlation between erythrocyte TPMT activity and accumulation of 6-MP metabolites in erythrocytes (4, 5). Patients with low TPMT activity accumulate higher levels of 6-MP metabolites and are at a higher risk of potential bone marrow toxicity after receiving standard doses of 6-MP and AZA than those with high enzyme activity (5, 6). In monitoring drug toxicity, the use of erytrhocyte 6-MP metabolites levels (TGN and 6-MMP levels) has proved clinically useful because it allows the optimization of AZA therapy based on inherent differences in drug metabolism.

Azathioprine or 6-MP have been shown to be useful treatments for a wide range of diseases, such as rheumatoid arthritis (7–11), transplants (6), acute lymphoblastic leukemia (5), and dermatological processes (12, 13). The immunomodulatory therapy with AZA and 6-MP has also become a treatment of choice to maintain remission in patients with refractory forms of inflammatory bowel disease (14, 15). Despite this widespread use, there is some controversy regarding safety, response, and side effects appearing during the treatment, mainly bone marrow toxicity (16–18).

The autosomal inheritance of the TPMT gene polymorphisms that regulate TPMT activity was demonstrated by Weinshilboum *et al.* in 1980 (19). They found that in a

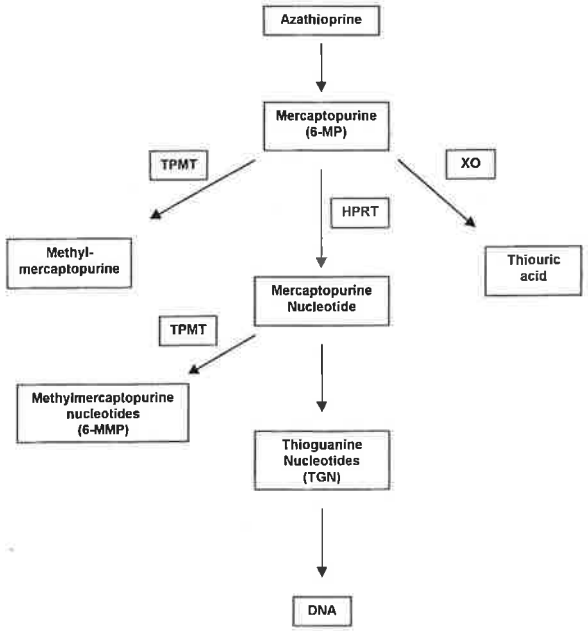

Figure 1. Pathways in thiopurine metabolism. The intracellular metabolic routes are catalyzed by TPMT (thiopurine methyl transferase), XO (xanthine oxidase), and HPRT (hypoxanthine phosphoribosyl transferase).

random population of 298 subjects, 86.6% of them had high TPMT activity, 11.1% intermediate, and 0.3% low enzymatic activity. The TPMT gene is located on chromosome 6p22.3, spans approximately 34 kb of genomic DNA, and contains 10 exons. Recent studies carried out to elucidate the molecular basis of the TPMT deficiency have led to the identification of different alleles that correlate with low enzymatic activity (20–23). Two polymorphic sites at positions 460 (G → A) and 719 (A → G) of the TPMT gene have been described; these nucleotide transitions are responsible for amino acid changes at codons 154 (Ala → Thr) in exon 7 and 240 (Tyr → Cys) in exon 10, respectively (20). The combination of these two polymorphisms results in four different alleles that are schematically shown in Table 1.

The study reported here was designed to examine, in a white European population from Spain and The Netherlands, the frequencies of the allelic variants of the human TPMT gene and to evaluate their clinical importance in a group of patients suffering from ulcerative colitis (UC) with a similar genetic background.

MATERIALS AND METHODS

Subjects

One hundred and forty-six patients suffering from UC (64 from Barcelona and 82 from Amsterdam) and 213 ethnically matched healthy individuals (105 from Barcelona and 108 from Amsterdam) were included in this study. All were unrelated white subjects from the Department of Gastroenterology in Sant Pau Hospital, Barcelona (Spain) and from the Department of Gastroenterology of the Vrije Universiteit, Amsterdam (The Netherlands). The diagnosis of UC was determined on the basis of conventional radiological, endoscopical, and histological findings according to the Truelove and Witts criteria (24).

Methods

Genomic DNA was extracted from peripheral blood nucleated cells according to Miller *et al.* (25).

TPMT MwoI RESTRICTION FRAGMENT-LENGTH POLYMORPHISM (RFLP). The region containing the MwoI polymorphic site was amplified by polymerase chain reaction (PCR) using a sense primer to intron 6 (5′-ATAA CAGAGTGGGGAGGCTGC-3′) and an antisense primer to exon 7 (5′-ACCTGGATTAATGGCAAC-3′) (20). Amplification was performed using a thermal cycler (Perkin-Elmer 9600; Perkin-Elmer, Norwalk, CN). A so-called "touch down" PCR reaction was performed with a progressive decrease in annealing temperature (from 70°C to 60°C), followed by 27 cycles of denaturation at 94°C for 40 s and annealing-extension at 57°C for 30 s, a final elongation at 65°C for 7 min, and then cooling to 4°C. The products of the reaction were digested with MwoI and analyzed by electrophoresis using acrylamide gels (GeneGel Excel 12.5, Pharmacia Biotech). MwoI digestion of the 284-bp PCR product resulted in fragments that either remained intact (mutant allele A^{460}) or were cut in two segments of 267 bp and 17 bp (wild-type allele G^{460}) (Fig. 2).

TPMT AccI RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP). The region containing the AccI polymorphic site was amplified by PCR under similar conditions to those discussed above, using the oligonucleotides 5′-TGTTGGGATTACAGGTGTGAGCCAC-3′ and 5′-CAGGCTTTAGCATAATTTTCAATTCCTC-3′ as primers (20, 21). Because the AccI restriction site was present

Table 1. Allelic Variants of the Human Thiopurine S-Methyltransferase

Allele Designation	Nucleotide at Position 460	Nucleotide at Position 719	Catalytic Activity*
TPMT 1 (Wild Type)	G^{460}	A^{719}	Normal
TPMT-3A	$G^{460} \rightarrow A$	$A^{719} \rightarrow G$	Complete loss
TPMT-3B	$G^{460} \rightarrow A$	A^{719}	9-fold reduction
TPMT-3C	G^{460}	$A^{719} \rightarrow G$	1.4-fold reduction

The $G^{460} \rightarrow A$ mutation leads to the aa substitution $Ala^{154} \rightarrow Thr$.
The $A^{719} \rightarrow G$ mutation leads to the aa substitution $Tyr^{240} \rightarrow Cys$.
* Data from reference 20.

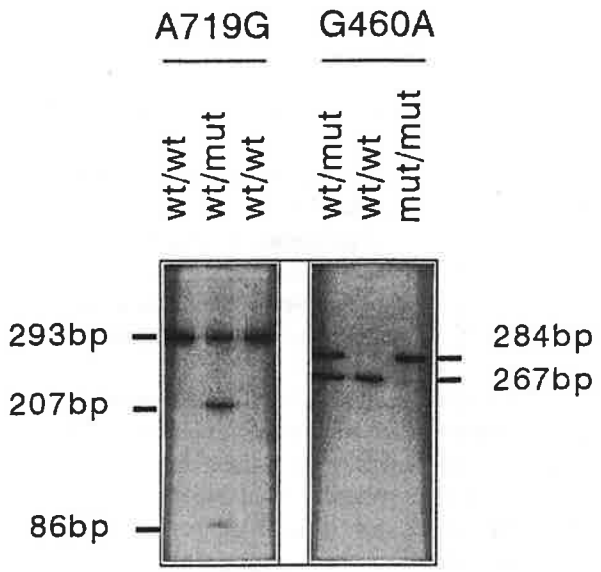

Figure 2. Silver-stained acrylamide gels showing the PCR-RFLP analysis. Lanes labelled A719G are PCR products digested by AccI to yield 207-bp and 86-bp fragments when the mutation is present. Lanes labelled G460A are PCR products digested by MwoI to yield a 284-bp fragment when the mutation is present. wt = wild type; mut = mutant.

when the G^{719} mutation existed, the PCR products containing this mutation were cleaved (207 bp and 86 bp fragments) when digested with AccI, whereas the wild-type fragments (A^{719}) were not digested (Fig. 2).

Statistics

The results are presented as genotype frequencies (95% confidence interval). χ^2 tests were performed on genotype frequencies. A two-sided p value <0.05 was considered statistically significant.

RESULTS

To evaluate the genetic polymorphism of the TPMT gene, which underlies the pharmacogenetic variation of this drug-metabolizing enzyme, we analyzed a large sample of European patients with UC from two different geographic areas (Spain and The Netherlands), together with a control group of healthy individuals with a similar ethnic background. Table 2 shows the genotypes of the TPMT gene observed in this study.

In the control group, which included 105 individuals from Spain and 108 from The Netherlands, we found 19 heterozygous carriers of a nonfunctional allele (8.9%). The most prevalent of the mutant alleles was TPMT3A, found in 12 of 19 heterozygous individuals. Three of 19 are carriers of allele TPMT3B and TPMT3C was detected only in Spanish control individuals.

In the UC group of patients, which included 64 patients from Barcelona and 82 from Amsterdam, 15 patients were carriers of a mutant allele (10.2%). As in the control group, the TPMT3A allele was the mutant allele most frequently observed (eight of 15).

Nonstatistically significant differences in genotype frequencies were found among the different groups of samples (Spanish controls *vs* Dutch controls; Spanish controls *vs* Spanish UC; Dutch controls *vs* Dutch UC; all controls *vs* all UC) (Table 2).

DISCUSSION

The role of TPMT in the human purine metabolic pathway is well known. Patients with low TPMT levels accumulate higher levels of thiopurine nucleotides when treated with standard doses of purine-related drugs, such as AZA or 6-MP. This accumulation of nucleotides usually leads to severe hematopoietic toxicity and the measurement of TPMT activity has been recommended before introducing these drugs. Measurement of TPMT activity is usually performed in erythrocyte lysates (26). This method, however, has a number of drawbacks: it is only available in specialized laboratories; a sample of 10–20 ml of fresh whole blood is required; samples can not be kept for later analysis; and the results can be masked by recent blood transfusions.

The characterization of the TPMT cDNA (27) allowed the study of a common genetic polymorphism that controls TPMT activity; the wild-type allele for high enzymatic activity has been designated as TPMT1 and a series of different alleles for reduced activity have been identified. Genotyping of white individuals with intermediate and low TPMT activity has shown that allele TPMT3A is the most prevalent mutant allele associated with the TPMT defi-

Table 2. Genotype for the Mutations in the TPMT Gene

	Controls						Ulcerative Colitis					
	Spanish			Dutch			Spanish			Dutch		
Genotypes	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
TPMT 1/TPMT 1	95	90.5	83.2–95.3	99	91.7	84.8–96.1	54	84.4	73.1–92.2	77	93.9	86.3–98.0
TPMT 1/TPMT 3A	5	4.76	1.57–10.8	7	6.48	2.64–12.9	5	7.81	2.58–17.3	3	3.66	0.76–10.3
TPMT 1/TPMT 3B	1	0.95	0.018–5.3	2	1.85	0.22–6.5	3	4.69	0.98–13.1	0	0.0	0.00–4.4
TPMT 1/TMPT 3C	4	3.81	1.04–9.5	0	0	0.00–3.4	2	3.12	0.38–10.1	2	2.24	0.29–8.5
Total	105			108			64			82		

Spanish controls *vs* Dutch controls ($p = 0.100$); Spanish controls *vs* Spanish UC ($p = 0.375$); Dutch controls *vs* Dutch UC ($p = 0.095$); all controls *vs* all UC ($p = 0.915$).
TPMT = thiopurine S-methyltransferase; CI = confidence interval.

ciency in whites. We have determined the fequencies of TPMT alleles in two large groups of healthy blood donors: one from Northern Europe (The Netherlands) and the other from Southern Europe (Spain). Moreover, the study sought to assess the usefulness of the genotypic screening of the TPMT gene in patients with UC in whom thiopurine drugs are currently used.

The major mutant alleles at the human TPMT locus can be reliably detected by PCR-based methods, which show an excellent concordance between genotype and phenotype (21). PCR is performed with a sample of few micrograms of genomic DNA extracted from peripheral blood leukocytes; samples can be stored as long as required, the results (when obtained) are already conclusive, the technique is relatively inexpensive, and it can be done in a short time.

Our results (Table 2) show that there are no significant differences in the frequencies of the TPMT mutant alleles found in the UC group of patients compared with those of the control group. In the group of UC patients included in this study, clinical data did not report any episode of hematopoietic toxicity in those patients treated with AZA. This finding agrees perfectly with the homozygous wild-type TPMT genotype identified in these treated patients.

Yates *et al.* (21) found TPMT3A to be the most prevalent deficient allele (26 of 33 mutant TPMT alleles). TPMT3B was not present in their group of patients and TPMT3C was relatively rare (six of 33 mutant alleles). Spire-Vayron de la Moureyre *et al.* (22) also reported allele TPMT3A in 68% of the mutant alleles (13 of 19) and TPMT3C in 10% (two of 19) of the TPMT nonfunctional alleles.

In this study, among 146 UC patients, 15 had a heterozygous genotype. TPMT3A was found in eight of 15 nonfunctional alleles (53%). TPMT3B allele was present in three of 15 (20%) heterozygous patients, and TPMT3C was noted in four of 15 (27%). TPMT3A was the most frequent allele, accounting for approximately half of the mutant alleles. TPMT3B and TPMT3C alleles occurred in these samples with a frequency higher than that reported previously, which reflects the genetic background of the two European populations analyzed.

Recent studies (21) have shown that genotyping the TPMT locus is a method with a sensitivity of 95.2% and can therefore be used as a diagnostic test for intermediate activity phenotypes. Such patients, if treated with thiopurine drugs, accumulate about 50% more thioguanine nucleotides than do patients who have high TPMT activity (TPMT wild-type homozygous) and thus they present an intermediate risk of toxicity. Fifteen UC patients from our study group are in this situation. Should the use of AZA be necessary in some patients of this group, the drug could be used without risk of toxicity provided that the dose were adjusted properly. The measurement of 6-MP metabolites in patients heterozygous for mutant alleles receiving AZA therapy will provide a useful tool to monitor drug toxicity in patients with decreased TMPT activity.

TMPT genotyping can be of interest in patients considered for high-dose AZA administration. In a recent study, Sandborn *et al.* (28) demonstrated that a loading dose of intravenous AZA is safe—but not effective—in decreasing the time to response in patients with steroid-treated Crohn's disease beginning AZA treatment. Patients included in this study represent a low-risk population because they were eligible for the study only if they had normal erythrocyte TPMT activity. In further controlled trials using high-dose intravenous or oral AZA at the start of oral therapy, TMPT genotyping will identify patients with intermediate or low TPMT activity, allowing accurate evaluation of efficacy and toxicity.

The TPMT polymorphism has a highly significant clinical impact, namely in the therapeutic efficiency of thiopurine drugs, which are widely used in the treatment of inflammatory bowel disease patients. Available diagnostic methods, such as the TPMT activity measurement, although reproducible and sensitive, are laborious and available only in specialized laboratories, require a sample of fresh blood, and the results can be masked by recent blood transfusions. TPMT genotyping by PCR-based methods performed on a sample of genomic DNA that can be stored for a long time is a simple and reliable test to identify patients at risk of severe bone marrow toxicity due to deficient TPMT activity if treated with AZA or 6-MP. In patients suffering from UC, TMPT genotype should be determined before the initiation of AZA therapy.

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