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Pharmacogenetics of thiopurines: can posology be guided by laboratory data?

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Background. *The purpose of this study was to investigate the relationships between the presence of mutations in the TPMT gene, the consequent reduced enzymatic activity, and the clinical toxicity of the treatment with thiopurine antimetabolite drugs.*
Materials and methods. *The study was performed on 44 patients with inflammatory bowel disease treated with AZA. DNA was extracted from blood samples collected from each patient, and genotyping was performed using specific polymerase chain reaction assays in order to detect the three more frequent mutations of the gene. Enzymatic activity was measured on red blood cell lysates by HPLC.*
Results. *Among the subjects, 4 (9.0%) were heterozygous for mutations in the TPMT gene; no subject was homozygous for mutations in the TPMT gene. A complete concordance between TPMT mutated genotype and reduced enzymatic activity could be determined. The incidence of toxicity in the subjects with a mutated genotype was not different from that observed in the patients with a normal TPMT gene.*
Conclusion. *Genotyping methods provide a simple and reliable DNA-based strategy to identify TPMT homozygotes that should avoid thiopurines administration. However, it seems that the most common, less dangerous forms of thiopurine toxicity could be caused by factors different from TPMT gene mutations examined.*

Key words: inflammatory bowel diseases – drug therapy; 6-mercaptopurine; azathioprine; genotype; phenotype

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Introduction

6-mercaptopurine (6MP) is an antimetabolite drug that oncologists have been using for more than 30 years to treat acute lymphoblastic leukemia in children. It is a prodrug that undergoes a complex metabolism[1]: it requires an intracellular activation to its thioguanine nucleotides (TGNs) in order to exert cytotoxic effects. Particularly relevant

for the cytotoxicity of 6MP, and thus of TGNs, is the interference with *de novo* purine biosynthesis, and the modification of DNA structure after incorporation of TGNs that produces an alteration of the function of DNA processing enzymes.[2,3]

In vivo biotransformation of thiopurines also leads to their metabolic inactivation, either by oxidation to thiouric acid catalyzed by xanthine oxidase (XO), or by methylation of the thiol moiety of the molecule by thiopurine-S-methyltransferase (TPMT).

The treatment of patients with thiopurines can cause various adverse effects that can be so severe (even life-threatening) to require the cessation of therapy. The most common adverse effects that have been described include nausea, bone marrow suppression, hepatitis and pancreatitis. The toxicity of the treatment with thiopurines, and the occurrence of bone marrow toxicity in particular, have been ascribed to a genetically determined deficiency of the enzyme TPMT which is held responsible for the metabolic deactivation of the drug.[4-6] TPMT exhibits genetic polymorphism in all large ethnic groups studied to date[7-10]; approximately one individual in 300 inherits two mutant TPMT alleles and is TPMT deficient, and about 10% are heterozygous at the TPMT gene locus and have intermediate enzyme activity. It has been reported that subjects who inherit a deficiency in TPMT exhibit intolerance to thiopurines medications, including 6MP and azathioprine (AZA). Unless TPMT-deficient patients are treated with 10- to 15- fold lower doses of these medications, they develop a profound haematopoietic toxicity that precludes the administration of thiopurines and also of other chemotherapeutic agents and that can be fatal.[4,5,10,11] Moreover, some reports indicate that, in ALL patients who are treated with 6MP carrying a mutant allele for TPMT and who receive intracranial irradiation, there is a greater risk of developing secondary fatal tumours.[12]

More than 10 non-functional mutant alleles for TPMT have been reported, and reliable polymerase chain reaction (PCR) based assays were developed to detect the three most prevalent mutant alleles: TPMT*2, TPMT*3A and TPMT*3C.[13] These variants result from the following point mutations in the TPMT open reading frame: G238C transversion for TPMT*2 alleles and the G460A and A719G transitions for TPMT*3 alleles. While variant alleles other than TPMT*2 and TPMT*3 may lead to a reduced enzyme activity, the frequency of these variant alleles is likely to be very low. Indeed, genotyping for TPMT*2 and TPMT*3 mutant alleles yielded 95% concordance between genotype and phenotype in different populations.[13-17]

Currently, there is a great debate about the opportunity of suggesting genetic testing for TPMT when prescribing 6MP aiming to identify, before treatment, the subjects with a higher risk of severe toxicity. The question of whether to add on an advisory gene testing to the 6MP package label is now before FDA; 6MP is the first drug to be evaluated as possibly requiring a gene test before use.[18]

It has to be noted that thiopurine medications are employed for some 'off label' uses, in particular to treat inflammatory disease like ulcerative colitis, Crohn's disease and rheumatoid arthritis.[19-21] There are indications that the number of prescriptions of thiopurines could be 10 times higher for these patients as compared to cancer patients, which constitutes the registered indication for the use of these agents.[18] Although thiopurines are used to treat inflammatory diseases at a dose lower than that employed for cancer therapy, the treatment is usually much more prolonged, providing a strong argument in favor of testing TPMT genotype, in order to prevent the appearance of serious adverse effects in both types of patients.

In the last two years, a collaborative study run by the Department of Biomedical Sciences of the University of Trieste and the Burlo Garofolo Children's Research Hospital

of Trieste is being implemented in order to examine the occurrence of thiopurine induced adverse effects and the presence of mutations in the TPMT genes. The samples are being obtained from the hemato-oncology and gastroenterology clinics that use thiopurines to treat leukaemia and inflammatory bowel disease (IBD), respectively. This paper reports the current results about the relationship between the outcome of the treatment with AZA used as an immunosuppressive drug to treat IBD, and TPMT genotype in children population or young adults.

Materials and methods

Patients

Between July 2002 and July 2003, 44 patients with IBD were enrolled. The average age at the time of analysis was 16.4 years (range 4-38); among these patients, 20 (45.4%) were female. They all received AZA at an average dose of 2 mg/kg/day (range 1-5 mg); the average length of the treatment with AZA was of 20.6 months (range 0.5-63).

Blood sample preparation

Blood samples of the patients were obtained in Vacutainer Tubes, using EDTA as an anticoagulant. Total genomic DNA was isolated using a commercial kit (Talent, Trieste Italy) according to the suppliers' instructions. Collected DNAs were dissolved in distilled water to a final concentration of 20 ng/μl, as determined by UV spectrophotometry; these solutions were used as template in the PCR reactions.

Erythrocyte lysates were prepared from the blood samples to measure TPMT activity according to the procedure already described.[22] Erythrocytes (RBC) were collected by centrifugation at 800xg for 10 minutes at 4°C, washed twice with two volumes of an isotonic sodium chloride solution (0.9% w/v), and lysed with distilled water added to the final volume of 10 ml.

TPMT enzyme assay

TPMT activity was measured with the HPLC assay previously described.[22] This assay is based on the *in vitro* conversion of 6MP to 6-methylmercaptopurine (6MMP), using S-adenosyl-L-methionine (SAM) as the methyl donor. Briefly, RBC lysates were purified from bivalent cations that could interfere with the assay by an incubation of 1 hour at 4°C with the chelating resin Chelex 100 (BIO-RAD, Richmond, CA, USA). The purified lysates were than incubated in the presence of 6-MP and SAM for one hour at 37°C. 6-MMP produced during the reaction was separated by an HPLC system Hewlett Packard HP Agilent 1100 including a G1311A Quaternary Pump, G1315A Diode Array Detector, G1313A Autosampler, G1322A Vacuum Degasser. The analytical column was a C18 reverse phase 250 mm long (VARIAN, Palo Alto, CA, U.S.A.); the mobile phase consisted of a solution of acetic acid 0.1% and 15% acetonitrile, with a flow rate of 1 ml/min. The detection wavelength was 290 nm, and the quantitative determination was performed comparing the area of the 6MMP peak with a standard curve of the compound dissolved in water. All chemicals were obtained from Sigma-Aldrich (Milan, Italy).

TPMT genotyping

The genotype of each individual at the TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C alleles was determined using previously described polymerase chain reaction (PCR)-based assays.[13,23,24] The mutations present in the variants of the alleles TPMT*3 (G460A and A719G) were assayed by restriction fragment length polymorphism (RFLP) analysis. TPMT exon 7 and exon 10 were amplified in two separate reactions by the use of primers that hybridized

with the sequences flanking each of these polymorphic nucleotides. The sequences of the primers employed to amplify exon 7 were P460F: AGG CAG CTA GGG AAA AAG AAA GGTG and P460R: CAA GCC TTA TAG CCT TAC ACC CAG G[23], for exon 10 were P719F: AAT CCC TGA TGT CAT TCT TCA TAG TAT TT and P719R: CAC ATC ATA ATC TCC TCT TCC.[24] The exon 7 amplicon was digested for 1 hour at 60°C with MwoI restriction enzyme (New England Biotechnologies, Beverly, MA, U.S.A). MwoI restriction site was present in the wild type allele, but not in the mutant allele. The exon 10 amplicon was digested for 1 hour at 37°C with Acc I (New England Biotechnologies, Beverly, MA, U.S.A). AccI restriction site was present in the mutant allele, but not in the wild-type allele. The unpurified products of the enzymatic reaction where recognized by electrophoresis in a 2-per cent agarose gel stained with ethidium bromide. An allele specific PCR was used for the analysis of the G238C mutation (TPMT*2). DNA was amplified in two specific reactions, one containing a forward primer wild-type specific (P2W: GTA TGA TTT TAT GCA GGT TTG) and one a mutant specific primer (P2M: GTA TGA TTT TAT GCA GGT TTC); the reverse primer (P2C: TAA ATA GGA ACC ATC GGA CAC) was the same in both reactions.[13] Unpurified PCR products were analyzed after electrophoresis in a 2-per cent agarose gel stained with ethidium bromide. A DNA fragment was amplified with P2M and P2C primers when C238 (mutant) was present, or with P2W and P2C primers when G238 (wild type) was present. The primers were purchased from Invitrogen (Milan, Italy).

Results

TPMT genotype distribution

The distribution of the TPMT genotype in 44 patients with IBD so far enrolled in the study is reported in Table 1. Among these subjects, 40 had a wild type TPMT genotype, while 4 subjects were heterozygous for a mutation in the TPMT gene. Three patients displayed a TPMT*3A mutated allele, and one patient displayed a TPMT*2 mutated allele; no patients were homozygous for TPMT variant alleles.

Table 1. TPMT genotype in 44 patients with IBD

Genotype	N
Normal (wild type/wild type)	40 (90.9 %)
Heterozygous (wild type/mutant)	4 (9.1%)
Homozygous (mutant/mutant)	0
Total	**44 (100.0 %)**

The considered mutations for the TPMT gene are G238C, G460A and A719G, identified as described in the experimental section.

Table 2. TPMT activity in patients with IBD according to TPMT genotype.

	TPMT activity (nmol h^{-1} ml^{-1} RBC)
Wild type (n=23)	11.2 ± 0.1
Heterozygous (n=4)	6.0 ± 0.4 *

TPMT activity is expressed as nmol of 6MMP produced during 1 hour of incubation by 1 ml of RBCs and is reported as mean ± SE. * Means significantly different, t student's test, $p<0.0001$.

TPMT activity

TPMT activity was measured in 27 out of these 44 subjects enrolled in the study, and the results obtained are reported in Table 2. The average activity among these patients was 10.4 nmol/hour/ml RBC; 23 of these subjects had a normal TPMT gene, whereas 4 had a mutated TPMT gene. The average TPMT activity in the subjects with a normal gene was 11.2 nmol/hour/ml RBC, whereas in those with a mutated gene was 6.0 nmol/hour/ml RBC ($p < 0.0001$ t student's test).

Clinical toxicity of AZA treatment and correlation with TPMT genotype

The toxicity of the treatment in the 44 subjects is reported in Table 3. During AZA ad-

ministration, 22 patients (50%) developed side effects. Because of this toxic event, the dosage was reduced in 8 patients and the treatment was suspended in 14 cases. In 13 subjects (29.5%), the bone marrow toxicity manifested as lymphopoenia or thrombocytopoenia. In 9 patients, other side effects were observed: pancreatitis, n = 2 (4.5%); hepatotoxicity, n = 2 (4.5%); infections, n = 3 (6.8%), neuropathy, n = 2 (4.5%). Among the 4 subjects with a mutated TPMT allele, 2 responded normally to therapy whereas 2 developed neuropathy.

Discussion

Thiopurine drugs play an important role in the treatment of leukemias and of some chronic inflammatory diseases. However, the use of these drugs is limited due to serious adverse effects, among which bone marrow suppression may require even the cessation of therapy. Individual differences in susceptibility to AZA/6MP have been observed, and they were attributed to variable intracellular concentrations of the cytotoxic metabolites of the drugs, TGNs. It is known that TPMT deficiency, caused by a frequent genetic polymorphism, might induce a profound bone marrow suppression in the patients receiving thiopurines because it causes a reduced methylation of 6MP and the consequent accumulation of TGNs toxic metabolites. About 10% of Caucasians inherit a mutant allele of the TPMT; they also have a reduced TPMT activity; on the other hand, the subjects homozygous for the variant alleles of TPMT are encountered at the much lower rate of about 1 per 300 Caucasians, and have no measurable TPMT activity. In the 44 subjects examined in the present study, no subject homozygous for TPMT mutated gene was identified, presumably because of the insufficient number of subjects genotyped; at the same time, 4 subjects (9.1%) were found to be heterozygous for a mutated allele of the TPMT gene. The overall frequency of defective alleles obtained in the present study is comparable with that reported in the Italian population[25] and also in other populations of Caucasian origin by other researchers.[13,24,26]

As far as phenotype is concerned, TPMT activity has been so far measured in 27 of the 44 subjects enrolled. The average value of the enzymatic activity measured in the patients with a mutated TPMT genotype was significantly lower than that determined in the remaining group not carrying the mutations considered. This finding is in agreement with the studies showing a strong correlation for TPMT between genotype and phenotype in patients with IBD.[13-17] This finding also confirms the view that genotyping for the identification of the considered TPMT mutations may allow an approach, effective in identifying the subjects with a reduced inherited TPMT activity.

Several studies have been recently published reporting the investigations about the relationship between TPMT reduced enzymatic activity, observed in IBD patients with inherited mutated alleles of TPMT gene, and bone marrow toxicity, which occurred after

Table 3. Correlation between TPMT genotype and the toxicity of AZA treatment in patients with IBD

		TPMT genotype		
	n	Wild type	Heterozygous	Homozygous
Without adverse effects	22 (50.0 %)	20 (45.5 %)	2 (4.5 %)	0
With adverse effects	22 (50.0 %)	20 (45.5 %)	2 (4.5 %)	0
Total	44	40 (90.9 %)	4 (9.1 %)	0

The adverse effects considered are bone marrow toxicity (lymphopoenia, thrombocytopoenia, hepatotoxicity, pancreatitis, infections and neuropathy).

thiopurine treatment.[27,28] The results reported in these studies show in some instances a significant increase in the occurrence of profound bone marrow toxicity in the subjects with a mutated TPMT gene; on the other hand, other side effects (such as hepatotoxicity, pancreatitis, mild lymphopenia and reduced platelet counts) appeared not to be related to TPMT mutations. In particular, in a group of 41 patients with Crohn's disease displaying significant myelotoxicity following the treatment with thiopurines, only 10% were homozygous for the considered TPMT mutations which lead to a severe enzymatic deficiency, and 17% were heterozygous for the same mutated alleles. These mutations, which are associated with the reduced TPMT activity, were overrepresented in this group as compared with a general population, although in most patients, the bone marrow toxicity was not associated with the genotype corresponding to low TPMT activity.[27] Similar results were published also in a further study, reporting the TPMT genotype of 50 patients with IBD treated with thiopurines who suffered from adverse effects that required to discontinue the drug administration: five patients (10%) were heterozygous for TPMT mutated alleles, and one (2%) was homozygous. Even if the single subject with the homozygous mutated genotype developed bone marrow suppression after AZA treatment, the treatment toxicity could not be related to a reduced TPMT activity in most of the patients.[28]

When the adverse effects of AZA in the IBD patients were examined in the group of 44 patients considered in the present investigation, the toxicity of the treatment was in general moderate (grade II or III). The most frequent toxicity encountered was bone marrow toxicity (13 subjects, 29.5%), and none of these patients had any of the considered mutated TPMT alleles. Four subjects were heterozygous for a TPMT mutation; among them, two responded normally to the therapy, whereas two developed an idiosyncratic form of myalgia and arthralgia, which required to discontinue the treatment. Myalgia and arthralgia are more associated with with Type I hypersensitivity reaction than direct drug toxicity, and are unlikely to be associated with TPMT mutated alleles.[29] These results thus appear to agree with those showing that the more common forms of myelosuppression during AZA administration to the IBD patients have causes different from genetic factors such as the considered mutations leading to a reduced TPMT activity.[27,28]

Drug toxicity is a multifactorial phenomenon involving multiple biological and environmental processes, including drug interactions. As far as AZA is concerned, almost all the subjects treated with this drug receive also other medications, which might be responsible for the interactions leading to adverse effects. Drugs like aminosalycilates are commonly used to treat IBD in combination with AZA; at the same time, they have been reported to reduce the metabolic inactivation of thiopurines, through TPMT inhibition.[30,31]

Moreover, genetic factors different from TPMT mutated alleles, such as the polymorphism of genes whose transcripts are involved in the detoxification of xenobiotics, could also influence the pharmacokinetics of thiopurines, and consequently, their clinical efficacy. In this connection, a study is being carried on by the authors aiming to examine the relationships between the clinical toxicity of the treatment with AZA and polymorphisms in the genes for P-glycoprotein[32], a transporter involved in the extrusion of xenobiotics from cells, and polymorphisms in the genes for glutathione-S-transferase, the enzyme responsible for the conjugation of drugs with glutathione.[33]

High throughput techniques are currently available for the investigation of the relationships between the response to drug treatment and the genetic characteristic of the patients. Microarray technology allows the study of the expression of thousands of genes in a single

sample, and has been employed to study the molecular basis of diseases, including various cancers [34], and the molecular mechanisms of drug action.[35] One of the authors (S.G.) spent a short period as visiting scholar at St.Jude Children's Hospital in Memphis (TN, USA) in order to learn the basis of microarray data analysis. These methods were applied to the study of thiopurine cytotoxicity and gene expression aiming to find new markers of drug action that could assist the clinician in determining the patients' individual sensitivity to the effectiveness and toxicity of a drug. In this study, the expression of genes involved in the small GTPase signaling pathways were determined in relation to the effectiveness of the drug, expressed as the magnitude of the decrease of white blood cell number in the 4 days following a single drug administration. Interestingly, the gene expression of Rac1 small guanosine triphosphatase seems to be related to the efficacy of thiopurines: indeed, 6MP is less effective in the subjects with a higher expression of this protein 24 hours after the treatment. This finding is in agreement with a recent observation, indicating a new possible mechanism for thiopurines toxicity, consisting of the inhibition of a Rac1, a critical regulator in mammalian T cells.[36]

These results allow to conclude that genotyping provide a simple and reliable DNA-based strategy to identify TPMT homozygotes who should avoid AZA/6MP administration at conventional dosages. However, it seems that the most frequent, even if less dangerous, forms of thiopurine toxicity in IBD patients could be attributable to factors different from a mutated TPMT genotype. Although TPMT genotyping may be useful to identify subjects with a risk of very severe toxicity, clinicians should still need to monitor carefully the patients treated with these toxic medications, in order to detect other common forms of toxicity. Further studies are needed to enlight the genetic characteristics of subjects experiencing toxic effects after thiopurine treatment, which may lead to the identification of additional genetic markers of toxicity that could assist the clinician in the treatment of patients with these toxic medications.

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