Importance of thiopurine S-Methyltransferase gene polymorphisms for prediction of azathioprine toxicity

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Abstract

OBJECTIVES: Our study aims to find the relationship between metabolic enzyme thiopurine S-methyltransferase (TPMT) gene polymorphisms and clinical output of the therapy with azathioprine. We focused on patients who experienced leucopenia caused by high blood levels of active azathioprine metabolites.
DESIGN: Our group consists of 87 patients who have been treated by azathioprine. 21 individuals experienced leucopenia during treatment with standard dose of azathioprine. We have used PCR-REA and "real-time" PCR methods for genotype detection G238C, G460G and A719G substitutions in TPMT gene.
RESULTS: We have found statistical association between the presence of non-standard TPMT alleles and adverse event associated with azathioprine treatment – leucopenia (p=0.0033).
CONCLUSION: Our results confirm that TPMT genotyping prior to the treatment with azathioprine could predict patients with genetic predisposition for serious leucopenia and seems to be a useful genetic marker for individualisation of the therapy.

ORIGINAL ARTICLE

INTRODUCTION

The inflammatory bowel disease (IBD) is the result of defects of mucosal immunity and intestinal epithelial barrier function. The IBD includes two clinical subtypes: Crohn's disease (CD) and the ulcerative colitis (UC).These are chronic and lifelong diseases with onset in young adulthood. Although the etiology of these disorders is not fully understood, there is a strong evidence to suggest that they emerge from a combination of constitutional and environmental factors. Familiar accumulation of disease, monozygotic twins' concordance or ethnic differences give the evidence about the influence of genetic factors involved in the disease development (Yammoto-Furusho &

Abbreviations:

6-MP	– 6-mercaptopurine
6-TGN	– 6-thioguanine nucleotide
AZA	– azathioprine
CD	– crohn's disease
IBD	– Inflammatory bowel disease
ITPase	– inosine triphosphate pyrophosphatase
SNP	– single nucleotide polymorphism
TIMP	– 6-Thioinosic monophosphate
TNF-α	– tumor necrosis factor alpha
UC	– ulcerative colitis
TPMT	– thiopurine S-methyltransferase
WBC	– white blood cell

Podolsky, 2007; Braat *et al.* 2005). The environmental hypothesis emerges from an observation that the IBD is more common in urban than in rural areas. Prevalence of IBD has been rising for the last 40 years in the developed world, and its occurrence has significantly shifted from the South to the North. Incidence and prevalence of CD in the Czech Republic is 1.7-2 and 18-22/100 000 respectively.

Nowadays, there is no curative therapy and hence the pharmacological treatment focuses on inflammation control, elimination of the disease symptoms and improvement of the quality of life. IBD pharmacology utilises a wide scale of drugs like salicylates, glucocorticoids, immunosupressives (e.g. thioguanine derivates, methotrexate and cyclosporine). Many authors indicate that TNF-α as a pro-inflammatory cytokine has a crucial role in the pathogenesis of many diseases (Maes, 2008) include IBD (Tracey *et al.* 2008). The use of monoclonal antibodies which neutralize TNF-α seems to be a very effective method of treatment. IBD requires a long-term treatment. Patients are potentially exposed to side effects of many drugs. Some toxic effects could be caused by overdose induced by low metabolism and elimination of effective substances. However, mechanisms of metabolism and elimination are complex; there are some crucial enzymatic proteins whose activity affects metabolism on a therapeutically important level. Single nucleotide polymorphisms (SNPs) of these genes affect enzyme structure and activity and therefore are therapeutically significant.

Thioguanine derivates - azathioprine (AZA) and 6-mercaptopurine (6-MP) are immunosuppressives commonly used in variety of indications as a treatment of inflammatory conditions such as IBD, hematological malignancies and transplantations (Teml *et al.* 2007; Wang *et al.* 2008; Stanulla *et al.* 2006; Ponticelli *et al.* 1999). Azathioprine is non-enzymatically transformed to 6-mercaptopurine and converted to pharmacologically active substance (6-TGN). Partial metabolites (6-MP and TIMP) are methylated and inactivated by cytosolic enzyme thiopurine S-methyltransferase (TPMT) to methyl catabolites, which lose their activity. 6-TGNs act as purine antagonists, which incorporate into lymphocytes DNA and induce immunosuppression. Thioguanines are indicated for induction and maintenance of remission in steroid-dependent or steroid-refractory cases of IBD. The drugs are given to patients on corticosteroids with an aim to decrease and take off steroids (Lavy *et al.* 2003). TPMT activity shows interindividual variations. It was found out that 0.3 % of individuals have low enzyme activity, 11 % intermediate, and 89 % normal or high activity (Krynetski *et al.* 1996). Patients with low TPMT activity are commonly intolerant to AZA therapy due to high TGN levels and risk of toxic effects, such as dose-related bone marrow suppression (Lavy *et al.* 2003). Clinically relevant leucopenia means decreasing the WBCs counts under $<3.5x10^9$/l and forces clinicians to alter the azathioprine dose.

Studies have established that variations in the activity of TPMT reflect variations in TPMT gene itself (Krynetski *et al.* 1995). Gene coded TPMT located on 6p22.3 consist of 9 exons and 8 introns (Seki *et al.* 2000). Genome studies confirmed many single nucleotide polymorphisms (SNPs) in promoter region, as well as exons and introns sequence of TPMT gene. The presence of some non-standard alleles of TPMT gene leads to lower enzyme activity due to its increasing cellular degradation (Tai *et al.* 1997). Nomenclature of four clinically important non-standard TPMT alleles - respective SNPs are shown in ***Table 1***. These four non-standard alleles account for 80-95 % of low activity alleles in human populations (Wei *et al.* 2005). Individuals with nucleotide substitution on both alleles have little or undetectable TPMT activity, whereas individuals carrying one wild-type allele show intermediate TPMT activity (Seki *et al.* 2000). TPMT*3A is the most prevalent TPMT non-standard allele in the Caucasian population is (Schaeffeler *et al.* 2004), whereas TPMT*3C is predominant in African and Asian population (McLeod *et al.* 1999; Zhang *et al.* 2004).

MATERIAL AND METHODS

The test group consisted of 87 patients who have been treated with azathioprine. 21 individuals experienced leucopenia during the treatment. This serious adverse drug reaction was the reason to discontinue the azathioprine therapy. Other patients (n=66) were treated with standard doses of azathioprine without significant decrease of WBCs counts. We tested the presence of most important SNPs on TPMT gene (C238G, G460A, A719G) in these two groups of patients.

Basic characteristics (e.g. age, gender) of the tested groups of patients are shown in ***Table 2***. Diagnosis was made at the Internal/hepatogastroenterology Department in the University Hospital Brno (GAEK FN Brno) (Czech Republic) and in the Derer's Faculty Hospital with Polyclinic in Bratislava-Kramare (Slovak Republic) by means of standard examination methods. All participants signed informed consent. Study was approved by the Ethical Committee.

Table 1: Nomenclature of five most common TPMT alleles

TPMT alelle	SNP	Amino acid change	reference
TPMT*1	wt	---	
TPMT*2	G238C	Ala80Pro	Krynetski et al, 1995
TPMT*3A	G460A	Ala154Thr	Tai et al. 1997
	A719G	Tyr240Cys	
TPMT*3B	G460A	Ala154Thr	Sahasranaman et al, 2008
TPMT*3C	A719G	Tyr240Cys	Otterness et al., 1997

Table 2: Basic characteristics of patients

Patients with	Diagnosis		Male	Female	Average Age
	CD	UC			
- normal response	53	13	39	27	35.0
- intolerant response	18	3	12	9	34.7
Total	71	16	51	36	34.85

Table 3: Primers and restriction endonucleases used to detect G460A and A719G polymorphisms on TPMT gene

SNP	primer sequence	restriction endonuclease	Fragments (bp)		
			wt/wt	wt/mu	mu/mu
G460A					
P460F	5´ - ATA ACA GAG TGG GGA GGC TGC - 3´	MwoI	267, 98	365, 267 and 98	365
P460R	5´ - CTA GAA CCC AGA AAA AGT ATA G - 3				
A719G					
P719F	5´ - CAG GCT TTA GCA TAA TTT TCA ATT CCT C - 3´	AccI	293	293, 207 and 86	207, 86
P719R	5´ - TGT TGG GAT TAC AGG TGT GAG CCA C - 3´				

Table 4: Probes sequences used to "real time" PCR detection of C238G polymorphism of TPMT gene

SNP C238G	probe sequence
Probe VIC	5´ - CCAACTACACTGTGTCCCCGGTCTG**C**AAACCTGCATAAAATCATACATTTA - 3´
Probe FAM	5´ - CCAACTACACTGTGTCCCCGGTCTG**G**AAACCTGCATAAAATCATACATTTA - 3´

VIC dye is associated with 238C allele, FAM dye with 238G allele.

Genomic DNA isolation. Total genomic DNA was isolated from peripheral blood leucocytes by the Quick Gene DNA whole blood kit S (*FujiFilm*).

Genotyping study. Three SNPs (C238G, G460A and A719G) were determined using the modification of the previously described PCR-based method (Yates *et al.* 1997).

Detection of G460A polymorphism. PCR amplification was done for 35 cycles consisting of denaturation at 94°C for 15 seconds, annealing at 50°C for 20 seconds and extension at 72°C for 1 minute. Primers sequences show ***Table 2*** (Yates *et al.* 1997). PCR reaction was performed on 20 µl volume with HOTStar MasterMix (QIAGEN) using thermocycler PTC-200. Amplified fragment of DNA was subsequently digested by a suitable restriction endonuclease *MwoI* (New England Biolabs). Reaction conditions are shown in ***Table 3***. Only wild-type allele 460G has a restriction site and yields 267 and 98 bp DNA fragments. Uncleaved DNA fragments of 365 bp represent allele 460A.

Detection of A719G polymorphism. PCR amplification was done for 35 cycles under the above described conditions with exemption of annealing at 57°C for 40 seconds. Products of amplification were digested by *AccI* restiction endonuclease (New England Biolabs) as described in ***Table 3***. *AccI* restriction site is present only in 719G allele and yields 207 and 86 bp fragments. Wild-type allele 719A remains uncleaved (293bp).

The digestion products were separated by electrophoresis in 2% agarose gel containing ethidium bro-

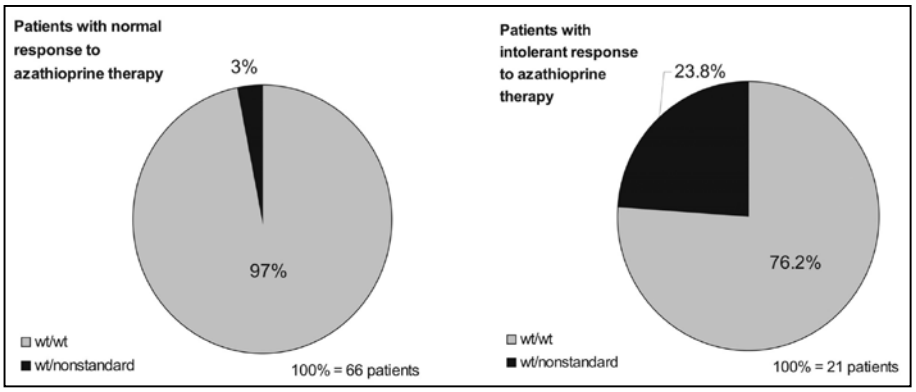

Figure 1 : Percentage occurrence of non-standard genotypes in both groups of patients (with normal and intolerant response)

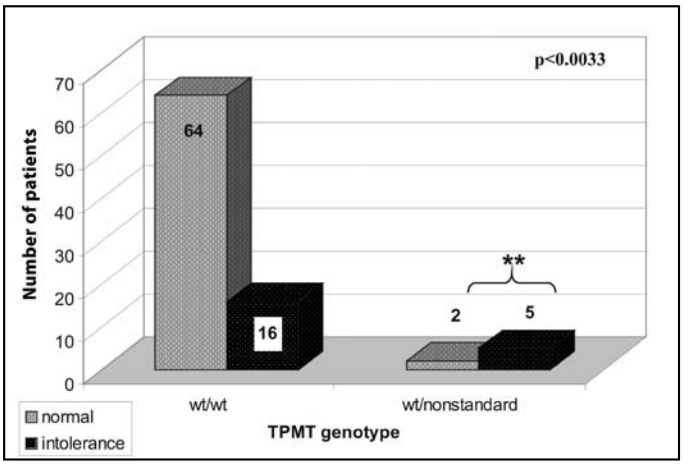

Figure 2: Presence of TPMT genotypes in patients on the basis of clinical effect during the azathioprine treatment. Normal = normal response to the azathioprine treatment; Intolerance = intolerant response to the azathioprine treatment = leucopenia

mide (0.15 mg/ml). Visualization was performed on the UV transilluminator using 312 nm light.

Detection of G238C polymorphism. Real time PCR method was used to detect one SNP (C238G) in TPMT gene. Reaction was prepared in 20µl volume in compliance with the manufacturer's directions. Sequences of allele specific probes (AppliedBiosystems) are shown in ***Table 4***.

Statistical analysis. We used chi-squared test performed on statistical software Unistat 5.1 for the statistical evaluation. Values of $p < 0.05$ were considered significant.

RESULTS

The TPMT genotypes were determined in 87 patients with IBD diagnosis and who were treated by azathioprine. No homozygote for TPMT non-standard alleles was detected. However, we found 6 TPMT*1/TPMT*3A and 1 TPMT*1/TPMT*3B genotypes among patients.

Patients were divided into two groups on the basis of adverse event presence – leucopenia (66 individuals without and 21 patients who experienced leucopenia). Individuals heterozygous for non-standard TPMT alleles were detected in both groups (2 and 5 individuals respectively). Presence of heterozygous genotype is significantly higher in patients with clinically relevant leucopenia (***Figures 1 and 2***).

Chi-squared test confirmed statistical association between the presence of non-standard TPMT alleles and the adverse effect of the azathioprine treatment – leucopenia (p=0.0033). Our results confirm that presence of non-standard allele of TPMT gene in genome is associated with the risk of leucopenia in patients treated with azathioprine.

DISCUSSION

Pharmacogenetics studies a role of inheritance in the individual variations in the drug response. The aim is to find genetic markers that are able to identify the appropriate drug and dose for each patient and make pharmacotherapy safer and more effective. Individual differences in drug response can be due to age, sex, disease or drug interactions (Weinshilboum, 2003). On the other hand, genetic factors also affect both the effi-

cacy and the likelihood of the adverse reaction (Weber, 2001). Many candidate genes and their polymorphisms (non-standard alleles) that influence drug response and metabolism are under investigation.

There are many SNPs in TPMT gene. The most important TMPT non-standard alleles, TPMT*2, TPMT*3A and TPMT*3C, are responsible for 80–95% of intermediate or low enzyme activity (Tai *et al.* 1997). These polymorphisms (C238G, G460A and A719G) account approximately for 95% cases of inherited TPMT deficiency (Sahasranaman *et al.* 2008). The most prevalent TPMT non-standard allele in the Caucasian population is TPMT*3A (Schaeffeler *et al.* 2004), whereas TPMT*3C is predominant in African and Asian population (McLeod *et al.* 1999; Zhang *et al.* 2004).

The presence of the SNPs causes amino acid changes on TPMT protein and leads to higher protein degradation and TPMT activity decrease (Tai *et al.* 1997). Accumulation of an active metabolite of azathioprine 6-TGN leads to suppression of bone marrow and decreasing count of WBCs – leucopenia (Sahasranaman *et al.* 2008). This is a serious adverse effect occurring during the IBD treatment with azathioprine. Population studies confirmed that about 0.3% of individuals show very low or undetectable activity and 11% intermediate TPMT activity. It became possible to detect TPMT inactivating mutations and phenotype of low enzyme activity with more than 95% concordance (Krynetskaia *et al.* 2000). Our data also confirmed that patients with leukopenia have significantly more often non-standard allele compared to patients who don't shown significantly decreased WBCs level during the azathioprine therapy. Patients with insufficient TPMT activity, but treated with normal doses of azathioprine, are in high risk of severe hematopoietic toxicity. These patients could avoid this complication if the dose is properly adjusted (Lennard *et al.* 1993). Both TPMT activity measurement and genotyping methods can be used to diagnose TPMT deficiency (Coulthard *et al.* 2000). TPMT genotyping prior to the treatment can help to identify patients who require reduction of thiopurine dose to avoid hematopoietic toxicity. On the other hand, there are other factors that affects metabolism of azathioprine and 6-TGN levels. Co-administration of azathioprine and aminosalicylates seems to induce higher risk of bone marrow depression (Lavy *et al.* 2003; Lowry *et al.* 2001). Another candidate gene, coded ITPase, also affects levels of TGN (Marinaki *et al.* 2004). Although DNA-based tests can be used to detect sequence variations, the results of such tests will not necessarily account for all possible phenotypic variations (Weinshilboum, 2003). Our results confirm that TPMT genotyping prior to the azathioprine treatment could predict patients with predisposition to leucopenia. Although we detected individuals heterozygous for non-standard TPMT alleles in both groups, the frequency of nonstandard TPMT alleles is significantly higher in the group of patients with leucopenia. Despite the fact that most drug are metabolized by several enzymes and many other drug-to-drug interactions are possible, patients with TPMT non-standard allele in their genome are in risk of dose-related bone marrow suppression and their WBC counts should be carefully monitored during the azathioprine treatment.

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