Genetic polymorphisms of thiopurine S-methyltransferase in a cohort of patients with systemic autoimmune diseases

C. Tani[1], M. Mosca[1], R. Colucci[2], G. Gori[2], A. d'Ascanio[1], N. Ghisu[2], M. Fornai[2], A. Di Paolo[2], C. Blandizzi[2], M. Del Tacca[2], S. Bombardieri[1]

[1]Rheumatology Unit and [2]Division of Pharmacology and Chemotherapy, Department of Internal Medicine, University of Pisa, Pisa, Italy.

Chiara Tani, MD
Marta Mosca, MD
Rocchina Colucci, PhD
Giovanni Gori, MD
Anna d'Ascanio, MD
Narcisa Ghisu, PhD
Matteo Fornai, PhD
Antonello Di Paolo, MD
Corrado Blandizzi, MD
Mario Del Tacca, MD
Stefano Bombardieri, Professor

Please address correspondence to:
Marta Mosca, MD,
Rheumatology Unit,
Department of Internal Medicine,
University of Pisa,
Via Roma 67, 56126 Pisa, Italy.
E-mail: marta.mosca@int.med.unipi.it

Received on February 25, 2008; accepted in revised form on July 24, 2008.

Key words: Pharmacogenomics, azathioprine, TPMT polymorphism, systemic autoimmune diseases, drug toxicity.

Competing interests: none declared.

ABSTRACT

Objectives. *Azathioprine (AZA) is a purine antimetabolite, prodrug widely used as a disease modifying drug in several rheumatic conditions. The aim of the present study was to evaluate the prevalence of TPMT genetic polymorphisms in a cohort of Italian Caucasian patients affected by rheumatic diseases and treated with AZA, and to establish correlations with the tolerability of AZA treatment.*

Results. *Seventy-eight Caucasian patients, 16 males and 62 females, median age 41 years (min-max: 24-76) were enrolled. At the time of evaluation, the median duration of treatment with AZA was 8 months (min-max: 2-150 months); the median dose of AZA per kg of body weight was 1.42 mg (min-max: 0.5-2). Among the 78 patients evaluated, 76 presented a wild type genotype (TPMT *1), while polymorphic alleles were identified in 2 patients (2.6%). Twenty-five patients (32%) experienced different types of adverse events (AE) under AZA treatment. Eighteen patients (23.1%) discontinued AZA because of AE. No correlation was observed between polymorphic TPMT alleles and the development of AE.*

Conclusions. *Our analysis supports the view that TPMT genotyping alone is not sufficient to adequately personalize the AZA dosage in rheumatic patients. Further studies based on phenotypic analysis of TPMT enzyme and assay of AZA metabolite appear to be required.*

Introduction

Response to pharmacotherapy can be influenced by several factors including disease-specific variables (severity and complications), comorbidity, concomitant therapies, environmental factors and genetic factors, and it is estimated that gene polymorphisms can account for 20-95% of variability in drug effects (1). Pharmacogenomics studies the association between variability in drug response and/or drug toxicity and gene polymorphisms in order to adapt pharmacological therapy to a patient's specific genetic background, to obtain greater efficacy and safety (2-4).

Azathioprine (AZA) is a purine antimetabolite, a prodrug widely used as a disease modifying drug in several rheumatic conditions, such as systemic lupus erythematosus (SLE), systemic vasculitis, rheumatoid arthritis (RA) and systemic sclerosis (SSc) (5-7). After oral intake, AZA is non-enzymatically converted into 6-mercaptopurine (6MP), which undergoes a series of subsequent enzymatic reactions to form 6-thioguanine nucleotides (6-TGNs), the active metabolites which can be incorporated into DNA and produce antiproliferative therapeutic effects. Alternatively, 6MP can be inactivated by xanthine oxidase or by thiopurine S-methyltransferase (TPMT).

TPMT pharmacogenetics has been well characterized in large population studies and there is consistent evidence to suggest that polymorphisms of TPMT gene can affect the inactivation rate of 6-thiopurine derivatives. To date, a total of 21 TPMT genetic polymorphisms have been identified. TPMT*1 is the wild type allele, and the most common variant alleles are TPMT*3A (G460A and A719G) and TMPT*3C (A719G alone) in Caucasians (5%) and Asians (2%), respectively. These two variant alleles, together with the less common TPMT*2 (G238C), account for over 95% of cases of inherited TPMT deficiency in Caucasian subjects (8). The aim of the present study was to evaluate the prevalence of TPMT genetic polymorphisms in a cohort of Italian Caucasian patients affected by rheumatic diseases and subjected to therapy with AZA, and to establish correlations with the tolerability of AZA treatment.

Materials and methods

Patients followed at the Rheumatology Unit of the University Hospital of Pisa between 2005 and 2006 were included in the present study. All patients were under treatment with AZA at the time of evaluation. Epidemiological and clinical characteristics were collected both prospectively and retrospectively from patients'clinical charts. The appearance of adverse events (AE) during AZA treatment was evaluated in accordance with the *Common Terminology Criteria for Adverse Events* (CTCAE v.3.0) (9). Briefly, haematological (leukopenia,

anemia, thrombocytopenia) and liver toxicity, the occurrence of gastrointestinal symptoms, fever, cutaneous lesions or severe infections not attributable to any underlying disease, other therapies or concomitant independent events were taken into account. The severity of AE was categorized according to CTCAE (grade 1-2: mild to moderate; grade 3-4: severe-very severe), and AE were classified as dose-dependent or dose-independent, based on their belonging to first category, related mainly to intracellular concentrations of 6-TGN (*e.g.* myelosuppression, opportunistic infections and hepatotoxicity), or to second category, related to allergic sensitization (*e.g.* skin rash, fever, malaise, gastrointestinal symptoms, colestatic hepatitis) (10). All patients provided an informed written consent and ethical approval was granted by the local ethics committee.

Analysis of allele-specific TPMT polymorphisms

Venous blood samples were collected, using EDTA as an anticoagulant and stored at -20°C. At the time of analysis, blood was defrosted to 37°C and total genomic DNA was isolated using a commercial kit (Nucleo Spin, Macherey-Nagel, Germany). TPMT polymorphisms *2 (G238C), *3A (G460A and A719G), *3B (G460A) and *3C (A719G) were detected by means of polymerase chain reaction (PCR) assays. Briefly, the G238C mutation was detected by means of allele specific primers. Mutations G460A and A719G were detected by means of primers flanking the polymorphic nucleotides and subsequent digestion of PCR products with the restriction enzymes MwoI at 60°C and AccI at 37°C, respectively, for 100 minutes. Primer sequences are shown in Table I. The presence of TPMT*2 and the length of digestion products for the detection of TPMT*3A, *3B or *3C were determined by electrophoresis on 2% agarose gel using a DNA marker and detection by UV rays.

Statistical analysis

The odds ratio (OR) with 95% confidence intervals (CI) for adverse reactions in individuals with and without aberrant genotypes was calculated.

Table I. Primer sequences used for PCR analysis of TPMT polymorphisms.

P460-F	AGG CAG CTA GGG AAA AAG AAA GGT G
P460-R	CAA GCC TTA TAG CCT TAC ACC CAG G
P719-F	AAT CCC TGA TGT CAT TCT TCA TAG TAT TT
P719-R	CAC ATC ATA ATC TCC TCT CC
P238-WT	GTA TGA TTT TAT GCA GGT TTG
P238-MUT	GTA TGA TTT TAT GCA GGT TTC
P238-C	TAA ATA GGA ACC ATC GGA CAC

Numerical data were non-parametric and represented by median and interquartile ranges. Comparison of medians was performed using the Mann-Whitney test.

Results

Seventy-eight Caucasian patients, 16 males and 62 females, median age 41 years (min-max: 24-76) were enrolled. Only one patient presented a mild chronic renal failure. At the time of evaluation, the median duration of treatment with AZA was 8 months (min-max: 2-150 months); the median dose of AZA per kg of body weight was 1.42 mg (min-max: 0.5-2). All patients were under concomitant treatment with drugs other than AZA (Table II). In particular, 74 (95%) patients were chronically subjected to treatment with steroids, 17 (22%) were taking hydroxy-cloroquine, 2 (2.6%) sulphasalazine and 3 (4%) infliximab.

Genotyping

Among the 78 patients evaluated, 76 presented a wild type genotype (TPMT *1), while polymorphic alleles were identified in 2 patients (2.6%). One mutated patient was homozygote and the other one was heterozygote with the TPMT*3A allele (G460A and A719G). Therefore, the cumulative prevalence of the variant alleles in our cohort resulted 1.92%.

Adverse events

Twenty-five patients (32%) experienced different types of AE under AZA treatment. Eighteen patients (23.1%) discontinued AZA because of AE. In the group with AE, the median treatment duration until the event was 1 month (min-max: 0.1-60) and the median AZA dose per kg was 1.4 mg (min-max: 0.7-2).

With regard to AZA treatment duration, no statistical difference was observed between patients who experienced AE and those who did not. Similar results were obtained by comparison of the length of AZA treatment in patients with dose-dependent and dose-independent AE. Likewise, no difference was detected when comparing patients who underwent AZA toxicity and those who did not experience AE.

Sixteen dose-dependent AEs and 15 dose-independent AEs were recorded. In 5 additional cases a hepato-biliary injury occurred, and it was not possible to clearly classify this AE as dose-dependent or independent in nature. Twenty-nine AEs were mild–moderate (grade 1-2 CTCAE), while 7 were severe-very severe (grade 3-4). Eight patients presented haematological toxicity: leukopenia (<3000/mmc) was observed in 7 cases, and megaloblastic anemia with mild thrombocytopenia (<100000/mmc) in 1 case. Six of these patients discontinued AZA and achieved a spontaneous remission within one month

Table II. Concomitant drug therapies in patients under treatment with AZA.

Drug	Number of patients (%)
Glucocorticoids	74 (94.8)
Hydroxychloroquine	17 (21.8)
Sulfasalazine	2 (2.6)
Anti-TNF	3 (3.8)
Protonic pump inhibitors	39 (50)
Calcium and Vit D	30 (3.8)
Antiaggregants	23 (29.5)
ACE- inhibitors	16 (20.5)
Anticoagulants	11 (14.1)
Calcium antagonists	9 (11.5)
Bisphosphonates	10 (12.8)
Gabapentin	5 (6.4)
Folinic acid	4 (5)
Insulin	4 (5)
NSAID	4 (5)
Thyroxine	3 (4)
Gardenal Fenobarbital	2 (3)
Colchicine	3 (3)
Furosemide	4 (5)

after stopping the treatment. Twelve patients developed hepato-biliary injury with elevation of biochemical indexes, but only two patients experienced accompanying symptoms such as nausea, malaise and fever. Five patients discontinued AZA because of gastrointestinal complications (nausea, pyrosis, vomiting) without any laboratory change and/or because of inexplicable fever. In all these cases, symptoms disappeared within two weeks after stopping AZA administration. Two patients presented generalized pruriginous cutaneous lesions one day and one month from the start of AZA therapy, respectively. In both cases the lesions disappeared after interruption of AZA followed by few days of glucocorticoid and antihistaminic medications. During the study period, severe infections occurred in four patients: in three cases a diagnosis of *Herpes zoster* infection was made (12, 15 and 16 months after the beginning of AZA therapy), and in one patient an interstitial pneumonia (probably due to *Cytomegalovirus* infection) was detected after four months of AZA therapy. Of note, three of these 4 patients experienced also other AEs in different periods of the study: one patient developed leukopenia and the others developed hepato-biliary damage. One patient definitively discontinued AZA because of opportunistic infection. In two cases, after a temporary suspension because of active infection, the treatment was restarted, but subsequently the patients developed leukopenia or hepatotoxicity. Only one patient restarted AZA treatment without further AE. One of the two patients with polymorphic TPMT alleles developed an AE characterised by diffuse, pruriginous, erythematous papular cutaneous rash in concomitance with nausea and vomiting one month after the beginning of therapy. The second one was treated with AZA for more than 120 months at the dose of 1.66 mg/kg, obtaining a good clinical response without any AE.

Discussion

AZA is a purine antimetabolite widely used in rheumatology as steroid sparing agent. Pharmacogenetic research on AZA metabolic pathways has led to the identification of a total of 21 TPMT polymorphisms, three of which have been associated with decreased levels of TPMT enzyme activity. It has been proposed the existence of a relationship between TPMT activity and TPMT variant alleles, and the distribution of these variant alleles differs significantly in different ethnic populations (8, 11). Among Caucasians, 0.03% of the population is homozygous for the non-functional variant alleles, and consequently they have low to absent enzyme activity; 6 to 11% of Caucasian subjects are heterozygous, having a partial reduction in enzyme activity; subjects with a wild type genotype exhibit normal to high levels of TPMT activity when treated with standard doses of thiopurines. Homozygous patients for the variant alleles are at high risk of developing severe or life-threatening myelotoxicity. On the other hand, a marked myelosuppression can expose the patients to the risk of severe opportunistic infections (12-14). In this study, we analyzed the TPMT genotype in a cohort of 78 rheumatic patients treated with AZA and detected variant alleles in two cases. No correlations were observed between TPMT genotype and appearance of AE to AZA, and 96% of patients who developed AE presented a normal TPMT genotype. These data suggest that, at least in the present cohort of patients, the analysis of TPMT genotype did not provide information on the possible risk of AE during AZA treatment.

In several studies on patients with inflammatory bowel diseases, up to 100% of severe myelotoxicity has been reported in heterozygous or homozygous patients with TPMT variant alleles treated with standard doses of thiopurine (10, 12, 15, 16). Based on these results, some authors have suggested reducing AZA dosage by at least 50% in heterozygous patients and to avoid the use of thiopurine derivatives in homozygous patients (10, 16). Furthermore, pharmacoeconomic studies have emphasized the cost-effectiveness of genetic screening in order to avoid severe drug-induced toxicities (15, 17).

There are few pharmacogenetic studies of TPMT in rheumatic patients and results are somewhat conflicting (14, 15, 17-22). Black *et al*. evaluated 67 patients treated with AZA for different rheumatic diseases. In this study, 6 patients (9%) exhibited a heterozygous TPMT status and 5 withdrew from AZA treatment within one month because of a low white cell count (14), showing a strong correlation between the analysis of TPMT genotype and the development of AZA-induced mielotoxicity. Different data were reported by Naughton *et al.,* who analyzed the genetic TPMT status of 120 SLE patients, 78 of whom were under AZA treatment. They found 7 subjects with variant polymorphic alleles; 4 of these were taking AZA, but only one patient (the homozygous one) developed severe myelotoxicity. On the other hand, these authors reported a total of 10 cases of non-disease-related neutropenia in patients with a normal TPMT genotype (18). The analysis carried out by Jun *et al*. reached similar conclusions. They analyzed the TPMT genotype of 342 Korean SLE patients, 94 of whom under AZA therapy, and found variant alleles in 5% (TPMT *3C and TPMT *6) without any evident relationship between genotype and drug-mediated toxicity (19). Finally, Okada *et al*. studied TPMT genotype and enzyme activity in a group of 68 Japanese SLE patients, obtaining a good correlation between the two parameters and the ability of genotyping to predict some, but not all cases of AZA-related toxicity. Indeed, two of four patients with variant alleles were taking AZA and one of these developed severe myelotoxicity (21). Overall, it is likely that the results obtained in different studies could be influenced by variables other than TPMT polymorphisms, such as other genetic determinants in the metabolic pathway of thiopurine derivatives or phenotypic variations, comorbidities and drug interferences.

In our study, the prevalence of AE was similar to that reported in previous investigations (32% *vs*. 15-30%). Differences with studies on inflammatory bowel diseases could be explained partly by the different dose regimens adopted in gastroenterology with respect to rheumatology. Indeed, previous data from patients with inflammatory bowel

disease reported an AZA mean dosage significantly higher (2-3 mg/kg) than that currently used in rheumatic diseases (10, 22). It could be hypothesized that the lower clinical impact of TPMT genotyping in rheumatic patients depends on the fact that these patients were not treated with such high AZA doses to expose them to drug-related risks in the presence of genetically determined susceptibility. The same reason could explain the high frequency of AZA withdrawal because of inefficacy. A point of weakness in this study is the wide heterogeneity of patient cohort in terms of diagnosis, which did not allow us to evaluate the efficacy of AZA treatment. Nevertheless, our analysis supports the view that TPMT genotyping alone is not sufficient to adequately personalize the AZA dosage in rheumatic patients. Therefore, further studies on large homogeneous populations, based on phenotypic analysis of TPMT enzyme and assay of AZA metabolite, are required.

References

1. EVANS WE, MCLEOD HL: Pharmacogenomics-drug disposition, drug targets, and side effects. *New Engl J Med* 2003; 348: 538-49.
2. DANESI R, MOSCA M, BOGGI U, MOSCA F, DEL TACCA M: Genetics of drug response to immunosuppressive treatment and prospects for personalized therapy. *Mol Med Today* 2000; 6: 475-82.
3. RUBINI M, PADOVAN M, BARICORDI O, FOTINIDI M, GOVONI M, TROTTA F: The c.1298A>C polymorphism in the methylenetetrahydrofolate reductase gene is associated with rheumatoid arthritis susceptibility in Italian patients. *Clin Exp Rheumatol* 2008; 26: 163.
4. PHILLIP R, LUQMANI R: Mortality in systemic vasculitis: a systematic review. *Clin Exp Rheumatol* 2008; 26 (Suppl. 51): 594-S104.
5. PAONE C, CHIAROLANZA I, CUOMO G *et al.*: Twelve-month azathioprine as maintenance therapy in early diffuse systemic sclerosis patients treated for 1-year with low dose cyclophosphamide pulse therapy. *Clin Exp Rheumatol* 2007; 25: 613-6.
6. IPPOLITO A, PETRI M: An update on mortality in systemic lupus erythematosus. *Clin Exp Rheumatol 2008*; 26 (Suppl. 51): S72-S79.
7. JAYNE D: Part 3: Newer therapies for ANCA-associated vasculitis. *Clin Exp Rheumatol* 2007; 25 (Suppl. 44): S77-9.
8. WANG L, WEINSHILBOUM R: Thiopurine S-methyltransferase pharmacogenetics: insight, challenges and future directions. *Oncogene* 2006; 25: 1629-38.
9. TROTTI A, COLEVAS AD, SETSER A *et al.*: CTCAE v 3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin Radiat Oncol* 2003; 13: 176-81.
10. AL HADITHY AF, DE BOER NHK, DERIJKS LJJ, ESCHER JC, MULDER CJ, BROUWERS JR: Thiopurines in inflammatory bowel disease: pharmacogenetics, therapeutic drug monitoring and clinical recommendation. *Dig Liv Dis* 2005; 37: 282-297.
11. ROSSI AM, BIANCHI M, GUARNIERI C, BARALE R, PACIFICI GM: Genotype-phenotype correlation for thiopurine S-methyltransferase in healthy Italian subjects. *Eur J Clin Pharmacol* 2001; 57: 51-4.
12. COLOMBEL JF, FERRARI N, DEBUYSERE H *et al.*: Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* 2000; 118: 1025-30.
13. GEARRAY RB, BARCLAY ML: Azathioprine and 6-mercaptopurine pharmacogenetics and metabolite monitoring in inflammatory bowel disease. *J Gastroenterol Hepatol* 2005; 20: 1149-57.
14. BLACK AJ, MC LEOD HL, CAPELL HA *et al.*: Thiopurine methyltransferase genotype predicts therapy-limiting severe toxicity from azathioprine. *Ann Intern Med* 1998; 129: 716-8.
15. OH KT, ANIS AH, BAE SC: Pharmacoeconomic analysis of thiopurine methyltransferase polymorphism screening by polymerase chain reaction for treatment with azathioprine in Korea. *Rheumatology* 2004; 43: 156-63.
16. PIERIK M, RUTGEERTS P, VLIETINCK R, VARMEIRE S: Pharmacogenetics in inflammatory bowel disease. *World J Gastroenterol* 2006; 12: 3657-67.
17. MARRA CA, ESDAILE JM, ANIS AH: Practical pharmacogenetics: the cost effectiveness of screening for thiopurine S-methyltransferase polymorphisms in patients with rheumatological conditions treated with azathioprine. *J Rheumatol* 2002; 29: 2507-12.
18. NAUGHTON MA, BATTAGLIA E, O'BRIEN S, WALPORT MJ, BOTTO M: Identification of thiopurine methyltransferase (TPMT) polymorphisms cannot predict myelosuppression in systemic lupus erythematosus patients taking azathioprine. *Rheumatology* 1999; 38: 640-4.
19. JUN JB, CHO DY, KANG C, BAE SC: Thiopurine S-methyltransferase polymorphisms and the relationship between the mutant alleles and the adverse effects in systemic lupus erythematosus patients taking azathioprine. *Clin Exp Rheumatol* 2005; 23: 873-6.
20. CLUNIE GP, LENNARD L: Relevance of thiopurine methyltransferase status in rheumatology patients receiving azathioprine. *Rheumatology* 2004; 43: 13-8.
21. OKADAY, NAKAMURA K, KODAMA T *et al.*: Thiopurine methyltransferase genotype and phenotype status in Japanese patients with systemic lupus erythematosus. *Biol Pharm Bull* 2005; 28: 2117-9.
22. COROMINAS H, DOMÈNECH M, LAÌZ A *et al.*: Is thiopurine methyltransferase genetic polymorphism a major factor for withdrawal of azathioprine in rheumatoid arthritis patients? *Rheumatology* 2003; 42: 40-5.