GENETIC PREDICTORS OF AZATHIOPRINE TOXICITY AND CLINICAL RESPONSE IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

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ABSTRACT

Background
Thiopurines (Azathioprine (AZA) and 6-Mercaptopurine (6-MP) are considered a well-established therapy for patients with Inflammatory Bowel Disease (IBD) including ulcerative colitis (UC) and Crohn's Disease (CD). However, nearly 20% of patients discontinue thiopurines due to adverse events. Functional polymorphisms of several enzymes involved in the metabolism of thiopurines have been linked with toxicity. The clinical value of variant carriers such as TPMT, ITPA and GSTs in predicting toxicity and adverse events for IBD patients treated with thiopurines remains to be clarified.

Objectives
To determine if variation in TPMT, ITPA and GST genotypes can predict adverse effects such as neutropenia, pancreatitis, liver enzyme elevation, as well as clinical response for patients with IBD treated with thiopurines.

Methods
Patients known to have IBD and treated with AZA or 6MP were enrolled. Adverse effects were calculated and their correlation with TPMT, ITPA and GST genotypes was evaluated. Further, the correlation between clinical response and TPMT, ITPA and GST genotypes were assessed.

Results
A total of 53 patients were enrolled. 16/53 patients (28.6%) responded to AZA therapy. 17 patients experienced adverse events with 10 having to discontinue treatment. Three patients (5.4%) developed severe myelosuppression (WBC< 2.0 or neutrophils <1.0). Loss of function TPMT genotype was associated with adverse events (OR 3.64, 95% CI 0.55 - 24.23, p=0.0313). ITPA and GST polymorphisms were not associated with toxicity. GSTM1 deletion was associated with poor clinical response to therapy (OR 3.75, 95% CI 0.940 - 14.97, p=0.1028), however, neither TPMT*3A nor ITPA polymorphisms were associated with clinical response.

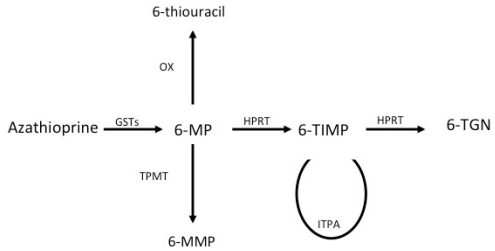
Conclusion
In addition to TPMT for adverse events, genotyping for GSTM1 appears to predict clinical response in IBD patients treated with thiopurines.

Key Words: *Inflammatory bowel disease, azathioprine, TPMT, GSTM1, ITPA*

Initially used as a chemotherapeutic agent in the 1950s for chronic lymphoblastic leukemia[1], azathioprine (AZA) has since become an important immunosuppressant in the management of the moderate to severe Inflammatory Bowel Diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD).[2-5] AZA is a pro-drug of 6-mercaptopurine (6-MP), which gives rise to active thioguanine nucleotide (TGN) metabolites by a multi-step process that includes enzymes hypoxanthine phosphoribosyl transferase (HPRT) and inosine triphosphate pyrophosphatase (ITPA).

Glutathione S-transferase (GST) has been implicated in the formation of 6-MP, which is then inactivated in the liver by thiopurine S-methyltransferase (TPMT), as well as by xanthine oxidase (XO), an enzyme not expressed in white blood cells (Figure-1).[6,7]

Figure 1. Azathioprine Metabolism



The Phase 2 drug-metabolizing enzyme TPMT is polymorphic, and the reduced function alleles TPMT*2, *3, and *4 result in distinct variability in enzymatic activity. The majority of the population (90%) is homozygous for the wild-type allele and possess normal TPMT activity (extensive metabolizer, EM).[8] Dependent on ethnic population, ranging from 4% to 11%, heterozygous carriers of a variant TPMT allele exhibit intermediate enzymatic activity (intermediate metabolizer, IM); whereas, approximately 0.3% of humans who are homozygous variant carriers are considered TPMPT deficient (poor metabolizer, PM).[8,9]

Patients with low or absent TPMT enzyme activity are reported to be at higher risk for bone-marrow toxicity with a standard weight-based dosing regimen for AZA.[10] However, it is unclear if other clinically relevant adverse events (AE) such as hepatotoxicity and diarrhea also relate to TPMT activity or if other mechanisms or enzymes are involved. A few studies have demonstrated a correlation between hepatotoxicity and high 6-MMP levels.[11] Moreover, bone marrow suppression is often reported in patients with normal or intermediate TPMT activity, suggesting that known TPMT genotype does not fully account for observed myelotoxicity. Recent studies suggest that polymorphisms in other genes involved in the bio-activation of AZA, particularly ITPA and GST, may play a role in the development of AZA toxicity in IBD patients.[12,13]

Furthermore, there is conflicting data regarding a potential role of TPMT genotype in clinical response to AZA. TPMT activity or phenotype has been previously associated with clinical response to AZA.[14-16] However, a prospective study failed to show that TPMT activity or serum 6-TGN metabolite levels were predictive of treatment outcome.[15-17] Some evidence suggests that the deletion of the GSTM1 gene may play a role; for example, carriers of the GSTM1 deletion showed a higher AZA dose requirement, lower 6-TGN levels, and reduced response to therapy.[18] Likewise, ITPA polymorphisms were associated with clinical response for IBD patients treated with AZA.[19]

To date, the importance of pharmacogenetic information on AZA/6-MP-related toxicity and treatment outcomes remains controversial and data are sparse.[13,20-22] Our objective was to study the relationship between genetic polymorphisms in

TPMT, ITPA and GST and AZA/6-MP-AEs and clinical response in IBD patients.

PATIENTS AND METHODS

Patient Population

All adult patients (age≥18 years) previously treated with AZA or 6MP or with the indication for treatment at the IBD clinic at the London Health Science Centre (LHSC), London, Ontario between 2005 and 2010 were identified and eligible for this study. All patients included in this study provided written consent for genetic testing. The study was approved by the Human Subjects Research Ethics Board at the University of Western Ontario.

Study Design

In this observational study, baseline clinical and demographic data were collected for each patient, and included the following: age, gender, ethnicity, personal history of smoking, concomitant medications and previous surgery. Hospital records were used to retrospectively obtain information for patients already on thiopurines at the time of recruitment. For patients initiated on thiopurines at the time of enrolment in the study, clinical data was collected prospectively. The starting dose of AZA was 50 mg once daily for one week, and in the absence of toxicity, increased to 2-2.5 mg/kg/day. 6-MP was typically prescribed at half the dose of AZA. All patients were followed for 3 months or until treatment was stopped due to toxicity or lack of response.

Clinical Assessment of Response to Therapy

Clinical response was determined after a three month treatment period using a modified Mayo score for UC and the Harvey-Bradshaw Index (HBI) for CD.[23,24] According to the here utilized Mayo scoring system, a score from 0 to 3 was assigned based on the frequency of bowel movements, the amount of blood in stool, and the physician's assessment of disease severity, with a total score generated by adding each individual score.[23,24] For UC, a complete response was defined as the achievement of clinical remission (modified Mayo score<3) after three months of AZA/6MP therapy without being on steroids at the time of the initial assessment. Treatment failure (non-response) was defined as the presence of a modified Mayo score of greater than 3 or the requirement of high dose prednisone (>20mg) during three months of AZA/6MP therapy. Partial responders were those who demonstrated a partial improvement in their modified Mayo score, but did not achieve clinical remission or required low doses of steroids (<10mg) during three months of AZA/6MP therapy. To assess CD activity, HBI takes into account four clinical parameters, general well-being, abdominal pain, number of liquid stools per day, and abdominal mass. In CD, complete response was defined as achieving clinical remission (HBI<5) after three months of AZA/6MP therapy without being on steroids. Treatment failure (non-response) was defined as the presence of high HBI (>5) or the requirement of high dose prednisone (>20mg) despite AZA/6MP therapy. Partial response was defined as an improvement in HBI without achieving clinical remission.

Clinical Assessment of Adverse Events

Adverse events (AEs) were recorded among all recruited patients and included myelosuppression (severe, defined as white cell counts $<2.0x10^9$/L or neutrophils < 1.0/L; mild, white cell counts 2-$4x10^9$/L), pancreatitis (defined as abdominal pain and a 3-fold increase in serum amylase and/or lipase), and hepatotoxicity (defined as a 2- fold increase in serum ALT and/or AST). Other AEs included skin rash, diarrhea, nausea, vomiting, dyspepsia, flu-like symptoms and arthralgia.

Genetic Analysis

Genotyping of previously described polymorphisms of potential relevance to AZA toxicity and response[12,13,25,26] was performed by TaqMan Real-time PCR for TPMT 238G>C (*2 allele; Assay ID C12091552_30), TPMT 460G>A (*3 allele; C30634116_20), TPMT 719A>G (*3 allele; C19567_20), TPMT 626-1G>A (*4 allele; C12091550_20), ITPA 94C>A (C27465000_10), and GSTP1 313A>G (C3237198_20). GSTT1 and GSTM1 deletion was assessed by PCR-based RFLP (polymerase-chain reaction restriction fragment length polymorphism analysis) as previously described[27,28]; the assay did not

discriminate between wild-type and heterozygous carriers (gene present) that were compared to those carriers completely lacking the gene (deletion). Variant carriers were confirmed with direct sequencing.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 5) or SPSS (IBM SPSS statistics, version 21). All statistical tests were two-sided and differences were considered significant when $p<0.05$. Fisher's exact test or univariate logistic regression was used to examine the association between TMPT, ITPA or GST polymorphism and toxicity or clinical response. Due to small numbers, patients with no response and partial response were combined as patients with reduced response and analyzed in comparison to normal response by binary logistic regression. Odds ratios (OR) and 95% confidence intervals (CI) were estimated. Multivariate logistic regression analysis was performed and the model included genotype, age, gender, smoking, disease and corticosteroid use (only for response) as covariates.

RESULTS

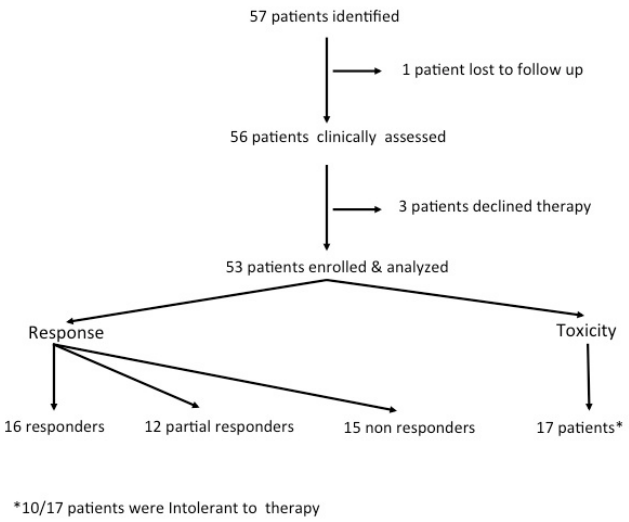
Subject Characteristics

The predominantly Caucasian study population analyzed consisted of 53 patients with a mean age of 41 years (min, max, 19, 80 years) (Table 1, Figure 2). Among those, 70% of patients (N=37) were enrolled in the study at the time of therapy initiation, and follow-up was prospective, whereas 30% of patients (N=16) had been previously on AZA, and were assessed retrospectively. Therapy in the latter patient group was discontinued either due to toxicity or lack of efficacy. More than half of the patients (60%) were males and 66% suffered from CD. Only one patient was treated with 6-MP. Co-medications prescribed were 5-ASA (31%) and corticosteroids (28.3%; Table 1). Eight patients were smokers (14.2%) and 19 (35.8%) patients had previous resection. Seventeen patients developed toxicity. However, only 10 patients were off therapy due to AEs. Genotype frequencies for the assessed genes are comparable to those reported in the literature in populations of Caucasian origin (13, 28, 29). TPMT*2 and *4 deficient alleles were not detected in this study population.

TABLE 1 Patient Characteristics

Sex (M/F), n (%)		
	Male	32 (60.4%)
	Female	21 (39.6%)
Age in years, median (range) Number of patients on treatment - Prospective follow-up, n (%) Number of patients on treatment – Retrospective follow-up, n (%)		41 (19-80) 37 (69.8%) 16 (30.2%)
Smoking, n (%)		8 (14.2%)
Ethnicity, n (%)		
	Caucasian	48 (90.6%)
	Other	5 (9.4%)
Diagnosis, n (%)		
	Ulcerative colitis	18 (34%)
	Crohn's disease	35 (66%)
Previous surgery, n (%)		18 (34%)
Medication, n (%)		
	5-ASA	16 (30.8%)
	Corticosteroids	15 (28.3%)
Azathioprine, mean (SD)	Dose, mg/kg	1.5 (1.0)
Genotype frequency, n (%)	TPMT*1/*3A	5/53 (9.4%)
	ITPA 94 C/A	7/53 (13.2%)
	GSTM1 deletion	27/52 (51.9%)
	GSTP1 313 A/G and G/G	27/53 (50.9%)
	GSTT1 deletion	11/52 (21.2%)

Figure 2. Patients Flow



Genetic Predictors of Adverse Events

Over the study period, a total of 17 patients reported AEs (Table 2), and AZA/6MP was either withdrawn, or in some cases, continued at a lower dosage (n =7). The most common adverse events were nausea and vomiting (18.9%), followed by mild myelotoxicity (7.5%). Severe myelotoxicity developed in three patients (Table 2). No patient developed hepatotoxicity.

The associations between TMPT, ITPA and GST genotypes and thiopurine-related toxicity are reported in Table 3. There was a higher incidence of AZA-induced AEs in heterozygous carriers of TPMT*3A compared to wild-type (OR 3.64, 95% CI 0.55 - 24.23, p = 0.0313). Five of 53 patients were heterozygous carriers for TMPT*3A, and 3 of these 5 patients (60%) developed adverse events including myelotoxicity (n=2) and diarrhea (n=1). Among those, one patient presented with febrile neutropenia and was admitted to the hospital for a week where AZA was discontinued upon admission and intravenous antibiotics were administered. No patient was homozygous for TMPT*3A. An association of ITPA, GSTP1, GSTT1 or GSTM1 genotypes with thiopurine toxicity were not observed in univariate analysis (Table 3) or multivariate analysis adjusted for age, gender, smoking and disease (data not shown).

Genetic Predictors of Reduced Response to Therapy

Univariate analysis suggested a potential role of GSTM1 deletion to reduced response to AZA therapy (OR 3.75, 95% CI 0.940 - 14.97, p=0.1028) but not TPMT*3A (Table 4). After adjustment for potential covariates including age, gender, smoking, disease and concomitant corticosteroids (Table 5), GSTM1 deletion was significantly associated with reduced treatment response with a 9-fold higher probability compared to normal (full) response (p=0.042). TPMT *3A heterozygous carrier status was not found to have a significant effect; however, the data may suggest a trend toward underrepresentation of this diplo-type in patients with reduced response. Male sex appears to be associated with a 6-fold increased probability of reduced AZA response compared to female sex (borderline significance; Table 5). Univariate analysis suggested smokers are more likely to respond poorly to therapy (OR 12.69, 95% CI 0.6726-239.5, p=0.0325). The inclusion of smoking into the multivariate model improved its predictive value; however, an OR could not be calculated due to the fact that 100% of patients (n=16) with complete response were non-smokers. Other clinical variables did not reach statistical significance.

TABLE 2 Adverse Events

Adverse Effects	N (%)
Myelotoxicity	7 (13.2%)
Mild (WBC 2.0-4.0)	4 (7.5%)
Severe (WBC<2.0 or PMN <1.0)	3 (5.6%)
Flu symptoms	2 (3.8%)
Diarrhea	3 (5.7%)
Dyspepsia	4 (7.5%)
Nausea/Vomiting	10 (18.9%)
Total	17 (32.1%)

TABLE 3 Genotype-toxicity associations among IBD patients treated with azathioprine

Genes	Patients with AE (n = 17)	Patients without AE (n = 36)*	Odds ratio	(95% CI)	*p*-value
TPMT *3A					
*1/*3A	3 (17.6%)	2 (5.6%)			
*1/*1	14 (82.4%)	34 (94.4%)			
			3.64	(0.55 - 24.23)	0.3127
ITPA 94C>A					
C/A	3 (17.6%)	4 (11.1%)			
C/C	14 (82.4%)	32 (88.9%)			
			1.714	(0.34 - 8.7)	0.6674
GSTM1					
Deletion	9 (52.9%)	18 (51.4%)			
Present	8 (47.1%)	17 (48.6%)			
			1.063	(0.33 - 3.39)	1
GSTP1 313A>G					
A/G and G/G	8 (47.1%)	19 (52.8%)			
A/A	9 (52.9%)	17 (47.2%)			
			0.80	(0.25 - 2.53)	0.7734
GSTT1					
Deletion	5 (29.4%)	6 (17.1%)			
Present	12 (70.6%)	29 (82.9%)			
			2.01	(0.52 - 7.88)	0.4701

AE, adverse event; TPMT, thiopurine S-methyltransferase; ITPA, inosine triphosphate pyrophosphatase; GST, glutathione S-transferase; *GSTM1 and GSTT1 genotypes only available for 35 patients

TABLE 4 Genotype-response associations among IBD patients treated with azathioprine

Genes	Reduced Response (n = 26)	Normal Response (n = 16)*	Odds ratio	(95% CI)	*p*-value
TPMT *3A					
*1/*3A	2 (7.7%)	2 (12.5%)			
*1/*1	24 (92.3%)	14 (87.5%)			
			0.583	(0.074 - 4.615)	0.628
ITPA 94C>A					
C/A	3 (11.5%)	2 (12.5%)			
C/C	23 (88.5%)	14 (87.5%)			
			0.913	(0.135 - 6.160)	1
GSTM1					
Deletion	15 (57.7%)	4 (26.7%)			
Present	11 (42.3%)	11 (73.3%)			
			3.75	(0.940 - 14.97)	0.1028
GSTP1 313A>G					
A/G and G/G	12 (46.2%)	8 (50.0%)			
A/A	14 (53.8%)	8 (50.0%)			
			0.857	(0.246 - 2.984)	1
GSTT1					
Deletion	6 (23.1%)	4 (26.7%)			
Present	20 (76.9%)	11 (73.3%)			
			0.825	(0.191 - 3.570)	1

AE, adverse event; TPMT, thiopurine S-methyltransferase; ITPA, inosine triphosphate pyrophosphatase; GST, glutathione S-transferase;* GSTM1 and GSTT1 genotypes only available for 15 patients

TABLE 5 Prediction of reduced response to azathioprine by clinical and genetic factors using multivariate logistic regression

Variable	Odds ratio	95% Confidence intervals	*P*-value
Age (yrs)	1.01	0.949-1.078	0.715
Gender, male	**6.167**	**0.967-39.36**	**0.054**
Smoking*	n.d.	n.d.	n.d.
Disease, UC	0.204	0.026-1.602	0.131
Corticosteroids	3.84	0.262-56.26	0.326
GSTM1 deletion	**9.22**	**1.081-78.62**	**0.042**
TPMT *1/*3A	0.403	0.027-6.013	0.510

*Smoking has been included into the model as a significant predictor of reduced response as suggested by univariate analysis (Odds ratio 12.69, 95% CI 0.6726-239.5, *p*-value 0.0325). However, an Odds ratio could not be calculated by multivariate logistic regression due to the fact that 100% of patients (n = 16) with complete response were non-smokers; n.d not determined

DISCUSSION

Thiopurines are widely used in the management of IBD patients and there is increasing evidence supporting the efficacy of combination therapy (AZA and infliximab) among CD patients (30-32). However 15-30% of patients treated with thiopurines develop AEs, which often leads to dose modification or withdrawal from treatment.[8,33] Haematological toxicity is the most common side effect of thiopurines with an incidence rate of myelotoxicity ranging between 2%-10.5%.[34,35] In a meta-analysis of 8302 IBD patients who were treated with AZA, the incidence rate of myelotoxicity was 3% per year, and 0.9% for severe myelotoxicity, defined by absolute neutrophil counts below 1 x 10^9 /L.[36] Likewise, the incidence rate of severe myelotoxicity in our study was (5.6%).

Genetic polymorphisms of TMPT has been associated with AEs among IBD patients treated with AZA/6MP and it is strongly linked to high 6-TGN blood levels and low TPMT activity.[11,31,37-39] In our study, we also found an association between TPMT*3 polymorphism and AEs (OR 3.64, 95% CI 0.55-24.23, p=0.0313). Zabala-Fernández et al reported similar results in a clinical study performed in 232 IBD patients who were treated with AZA and the incidence rate of AEs was 33.3% among TPMT variants (p=0.0304).[19] However, a systematic review performed by Booth et al to assess the sensitivity and the specificity of TPMT genotyping for TPMT enzymatic activity among IBD patients treated with AZA where 54 observational studies and one randomized controlled trial were included and there was insufficient evidence to address the effectiveness of TPMT testing for IBD.[22] In our study, the incidence rate of gastrointestinal (GI) intolerance was (10.7%), which is comparable with other reported studies.[16,35,40,41] Two out of six patients developed worsening diarrhea after starting AZA, and 40% of the TPMT heterozygous patients developed nausea, vomiting and diarrhea. In another study by Ansari et al, 272 IBD patients underwent genotyping for TMPT and the incidence rate of gastrointestinal intolerance were observed in 37% of TMPT heterozygous subjects. Likewise, Marinaki et al reported a significant association between TMPT heterozygous group and GI intolerance compared to the wild-type group (OR 5.5; 95% CI, 1.4-21.3, p=0.02).[12] The role of ITPA is not clearly understood. However, in normal cells ITP are produced by kinase from IMP and ITPase converts ITP back to IMP, to limit the accumulation of potentially harmful nucleotides, and ITPase hydrolyses 6-thioinosine triphosphate (6-TITP) into 6-thioinosine monophosphate (6-TIMP). Accordingly, ITPase deficiency leads to cellular accumulation of TITP.[12,42,43] In our study, there was not a significant association between ITPA polymorphism and clinical response or AEs. However, a significant association was found between the ITPA 94C>A variant and early therapy withdrawal in 71 CD patients who were treated with AZA (OR 7.8;95% CI, 2.1-29.1; p=0.002) (16, 44). Likewise, in a study by Zabala-Fernández et al, 30% of the ITPA variants allele reported AEs among 232 patients who underwent genotyping for TMPT and ITPA polymorphisms.[19] GSTs are cytosolic enzymes and they are responsible for the conjugation of a number of xenobiotics.[31] GSTs catalyze the release of 6-MP from its pro-drug AZA and high hepatic GST activity may lead to AZA toxicity.[45] Stocco et al, found a correlation between GST-M1 and adverse effect (OR 0.18; 95% CI, 0.037-0.72; p=0.007) among 70 IBD patients who were genotyped for GST-M1, GST-P1 and GST-T1.[13] However, our study did not support the role of GST polymorphisms in the development of adverse reactions in patients with IBD taking AZA. On the other hand, GST-M1 deletion was associated with clinical response in a multivariate analysis (OR 9.22; 1.08-78.62, p=0.042). Therefore, GST-M1 polymorphisms might be useful on predicating clinical response among patients who were treated with AZA.

There are several limitations to our study. The main limitations include a relatively small sample size, being a single center study, and that part of the study was retrospective. Accordingly, the findings need to be replicated in a larger prospective study. Notwithstanding these limitations, we note this is the second report of an association between the ITPA variant and efficacy of AZA treatment in IBD patients.

In conclusion, the mechanism that leads to thiopurine-mediated AEs appears to be multifactorial. Therefore, additional predictive biomarkers need to be determined and integrated into future pharmacogenomic testing panel for IBD patients who may benefit from AZA/6-MP therapy.

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