A Pharmacogenetics Study of TPMT and ITPA Genes Detects a Relationship with Side Effects and Clinical Response in Patients with Inflammatory Bowel Disease Receiving Azathioprine

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Abstract

Background&Aims: Pharmacogenetic studies in inflammatory bowel diseases (IBD) are mainly focused on genes involved in the metabolism of Azathioprine (AZA). Use of AZA is limited by its toxicity, which occurs in 20-30% of patients. Variants in the Thiopurine S-methyltransferase (TPMT) and Inosine triphosphate pyrophosphatase (ITPA) genes have been associated with AZA toxicity, but also can contribute to the lack of response. The aims of this study were to determine the contribution of TPMT and ITPA variants in the development of AZA-related toxicity and response. **Methods**: Variants associated with the decrease of enzyme activity in TPMT and ITPA genes were genotyped with the Snapshot system in 232 IBD patients treated with AZA, and correlated with the clinical response and development of adverse drug reactions in a retrospective case-control study. **Results**: Genotypic analysis showed that there is a statistical significance between c.94C>A variant on ITPA gene with non response to AZA treatment (p=0.005) and arthralgia (OR 8.2353; 95%CI 1.752-38.87, p=0.0041), as well as between mutant TPMT alleles and myelosuppression (OR 7.5; 95%CI 1.4456-38.91, p=0.0304). **Conclusions**: There is a positive correlation between c.94C>A variant on ITPA with clinical response. Mutant alleles on TPMT and the variant c.94C>A on ITPA gene predict side effects induced by AZA in our population (myelosuppression and arthralgia).

Key words

Azathioprine – ITPA – pharmacogenetics – TPMT.

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Introduction

The thiopurines drugs: Azathioprine (AZA in Europe) and 6-mercaptopurine (6-MP in the USA) are widely used for the treatment of active Inflammatory Bowel Disease (IBD) and maintaining remission of both Crohn's disease (CD) and ulcerative colitis (UC) [1, 2]. After it is absorbed, AZA is transformed to 6-MP in the liver then it is metabolized by three major enzymatic pathways mediated by the Xanthine oxidase (XO), Thiopurine S-methyltransferase (TPMT) and Hypoxanthine phosphoribosyl transferase (HPRT) enzymes (Fig. 1) [3, 4].

The main focus of pharmacogenetics research in IBD has relied on the analysis of the TPMT gene (OMIM *187680). Approximately 90% of the population has normal enzyme activity, and are homozygous for the wild type allele. About 10% have an intermediate activity, being heterozygous, and may demand therapeutic dose adjustments or withdrawal of AZA/6-MP [5, 6]. Approximately 1 in 300 individuals have a low or null activity of TPMT enzyme, which means they are homozygous for the non-functional variant or mutant alleles [7]. Expressing lower or null TPMT activity means a significant increase in the risk for toxicity on a standard weight-based dosing regime [8]. However, the presence of a wild type allele is not sufficient to ensure non-occurrence of adverse effects during AZA/6-MP treatment.

In recent years, another enzymatic deficiency has been associated with AZA-6-MP toxicity [9, 10]. Inosine triphosphate pyrophosphatase (ITPA-OMIM *147520) catalyzes the pyrophosphohydrolysis of inosine triphosphate (6-TITP) to inosine monophosphate (6-TIMP), thereby preventing an abnormal accumulation of 6-TITP nucleotides in cells and their incorporation into RNA and DNA (Fig. 1). ITPA deficiency has been related with non-myelosuppression adverse effects such as pancreatitis, nausea, flu-like symptoms and skin rashes in patients treated with AZA/6-MP [10]. At least five variants have been identified on the

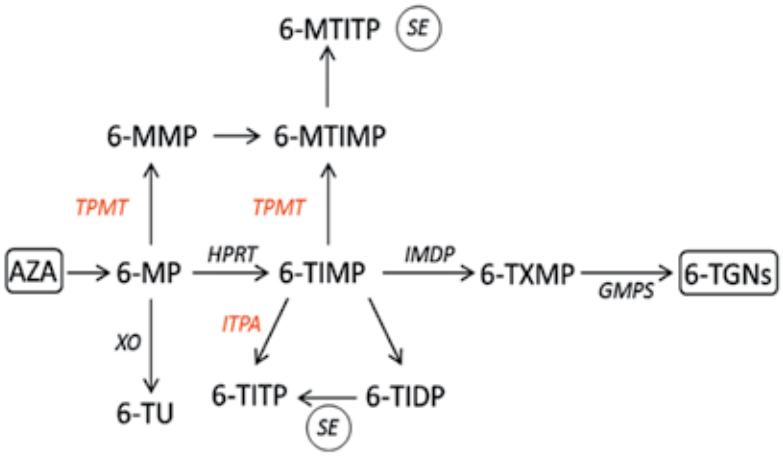

Fig 1. Azathioprine metabolism.

ITPA gene, and two of them, c.94C>A and IVS2+21A>C have been associated with decreased enzyme activity [11]. To date, the role of TPMT and ITPA polymorphisms in the AZA/6-MP-related adverse effects is still debatable [11, 13]. Therefore, in our study we aimed to investigate the relationship between the genetic polymorphisms of TPMT and ITPA gene and AZA/6-MP-adverse effects in IBD patients.

Material and methods

This retrospective study included all patients with IBD who had been taking AZA (Imurel®) for at least 6 months or who had experienced adverse effects. Patients that were receiving concomitant drugs such as infliximab or adalimumab were not included. Two hundred and thirty-two unrelated IBD patients were recruited from four hospitals of EIGA (Galician Inflammatory Bowel Disease Group). All the subjects were clinically evaluated and the IBD diagnosis was according to Lennard-Jones criteria [14]. Patients were classified as cases and controls based on the side effects developed after AZA treatment as well as the time exposure to AZA. In the case of myelosuppression and hepatotoxicity where the time of onset is usually longer, patients were selected as controls who matched the same exposure time to treatment with AZA, from 28 to 86 months in the case of myelosuppression and from 18 to 86 in hepatotoxicity. The study was performed with the approval of the local Ethics Committee. Written consent was obtained from each participant.

The choice of the dose of AZA was based, as usually recommended, on the weight of the patient (2-2.5 mg/kg/day), and not on the TPMT level. In keeping with previous reports [15, 16], treatment with AZA was considered successful (responders) if the drug achievement of a clinical remission (defined as CDAI<150 in CD and Mayo score <3 for UC) without steroids was for at least 12 months. Treatment failure (non responder) was assumed if the drug was discontinued for clinically judged non-response after an adequate period of treatment. Undetermined response was indicated in those patients in whom the drug exposure time was not sufficient to evaluate the clinical response.

The criteria for defining adverse effects were: myelosuppression as a reduction in the number of white cells (<3.0x10^9/L), including neutropenia (neutrophil count <1.5x10^9/L), and/or thrombocytopenia (platelets count <100x10^9/L); hepatotoxicity as an increase of the liver enzymes ALAT or GGT of at least 2-fold; pancreatitis was defined as severe abdominal pain and a 3-fold elevation of serum amylase and/or lipase; digestive intolerance by the presence of diarrhea, nausea or vomiting; skin reaction when significant redness of the skin developed; arthralgia when generalized joint ache or pain developed, and alopecia by hair loss [17]. All patients were closely monitored by blood testing in order to screen adverse events and clinical effects. Additional information was recovered from clinical records.

Genotyping was made at the Galician Public Foundation of Genomic Medicine in Santiago de Compostela, Spain. DNA samples were obtained from all patients using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) from peripheral blood. Variants associated with decrease of enzyme activity in TPMT and ITPA genes were genotyped using the Snapshot® Multiplex System, developed by Applied Biosystems Inc. (AB Inc., Carlsbad, CA, USA), and followed by capilary electrophoresis on an ABI 3730 genetic analyzer (AB). Alleles were determined automatically by creating a panel analysis with the Gene Mapper v0.37 software (AB) and checked manually. In order to verify the accuracy of the above genotyping, results were confirmed by bidirectional sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (AB).

We screened for gene polymorphisms that reduce TPMT and ITPA activities, selecting the variants: c.238G>C (rs1800462), c.460G>A (rs1800460) and c.719A>G (rs1142345) in the TPMT gene (which define the alleles *1, *2,*3A,*3B and *3C). The c.460G>A and c.719A>G are combined on the allele TPMT*3A, and c.460G>A, c.719A>G, and c.238G>C variants define the alleles TMPT

Table I. Summary of polymorphisms and design of primers used in the multiplex PCR

GENE/SNP		dbSNP	Aminoacidic Change	TM (ºC)	Length of Amplicons (pb)	Length of Primers (pb)	Sequence (5´-3´)
TPMT	c.238G>C	rs1800462	Ala80Pro	59.32	242	26	F-TCTGAGTAAGAAAGATTCTGCTCTGT
				60.01		21	R- TCTGCTTTCCTGCATGTTCTT
	c.460G>A	rs1800460	Ala154Thr	58.83	380	27	F-CAAAGCTAGTATTGGATTTAGGTTTT
				59.96		20	R- CCAGGTCCACACATTCCTCT
	c.719A>G	rs1142345	Tyr240Cys	59.41	174	23	F-CATCCATTACATTTTCAGGCTTT
				58.12		22	R-TCTTGAGAAGGTTGATGCTTTT
ITPA	c.94C>A	rS3177087	Pro32Thr	60.04	177	20	F- CTCATTGGTGGGGAAGAAGA
				60.25		20	R- CGAACTGCCTCCTGACATTT
	IVS2 + 21A>C	rs7270101	-	57.14	300	22	F-CACATGGAGAATCACTAGATGG
				60.30		20	R-AGAGCAAGTGTGGGACAAGG

Table II. Demographic distribution of the population.

	Patients with adverse effects (cases 75)	Patients without adverse effects (controls 157)	All patients (232)
Median age (years[range])	36.8 [14-70]	30.6 [8-65]	32.6 [8-70]
Sex F/M	37/38	80/77	117/115
IBD diagnosis (CD/UC)	56/19	100/57	156/76
Median AZA dose (mg/kg/day[range])	2.4 [1.5-2.5]	2.2 [1.5-3]	2.3 [1.5-3]
Median length of treatment (months[range])	4.7 [0.33-45]	35.4 [10-86]	25.5 [0.33-86]
Response to AZA (Responder/Non Responders/Indeterminate)	-	-	133/34/65

*3B, *3C, and *2. In the ITPA gene we chose the c.94C>A (rs3177087) and IVS2+21A>C (rs7270101) polymorphisms (Table I).

Statistical analysis was performed using G-Stat 2.0 software (http://www.e-biometria.com/g-stat/index.html). Allelic and genotypic frequencies were calculated. The association between adverse effects and TPMT and ITPA polymorphisms was tested using the two-sided Fisher's exact test and odds ratios and 95% confidence intervals were calculated. A P-value <0.05 was considered as statistically significant. Hardy Weinberg Equilibrium was also calculated for each polymorphism studied.

Results

Two hundred and thirty two IBD patients were included. One hundred and fifty six were diagnosed with CD (67.2%) and 76 with UC (32.7%); the median age was 32.6 years, without differences between genders (Table II). All patients received standard recommended doses with a median of 2.32 mg-kg-day. All patients were closely monitored through blood tests and regular medical visits (every 15 days during the first month and monthly during the first three months, later every three months) in order to evaluate the clinical response and presence of adverse events. According to toxicity, 75 patients presented side effects to AZA (Table III). AZA was withdrawn or reduced due to adverse effects in 72 patients (21.0%). Three cases of myelosuppression were resolved with dose reduction; all others adverse effects were resolved with drug withdrawal. The median of time of treatment was 25.4 months. In 133 patients the clinical response was positive, whereas 34 did not respond to treatment. In 65 cases the response was indeterminate because the time to exposure was insufficient to evaluate it as a consequence of the development of AZA side effects.

Table III. AZA-Side effects observed in the enrolled patients.

Type of adverse effect	n	%
Pancreatitis	19	25.3
Digestive intolerance	16	21.3
Myelosuppression	15	20.0
Skin reaction (Rash)	13	17.3
Hepatotoxicity	5	6.6
Arthralgia	6	8.0
Alopecia	1	1.3

The genotypic distribution for the TPMT and ITPA variants is shown in Table IV. All the polymorphisms were in the Hardy-Weinberg equilibrium and the pattern of distribution was similar to other population reports, especially those with Caucasian origin [18, 19]. Regarding the TPMT polymorphism 217 individuals carried wild-type alleles (*1/*1), no mutant alleles were found in homozygosis, and only 15 mutant alleles were found (11/*3A, 1/*3C and 3/*2) (Table IV). Of these, five developed side effects, three myelosuppression, one pancreatitis and the other,

Table IV. Genotypic frequencies distribution and statistical analysis of the TPMT and ITPA genes.

Gene/Variants	Genotype	All patients (232)		Cases (75)		Controls (157)		OR (CI 95%)	p-value
		n	*	n	*	n	*		
TPMT	*1/*1	217	0.93	70	0.93	147	0.93		
	*1/*3A	11	0.04	4	0.05	7	0.04	1.60	0.36
	*1/*3C	1	0.004	0	0.0	1	0.006	(0.41-6.20)	
	*1/*2	3	0.013	1	0.013	2	0.013		
ITPA c.94 C>A	CC	210	0.90	70	0.93	140	0.89	0.76	0.76
	CA	22	0.09	5	0.06	17	0.10	(0.24-2.45)	
	AA	0	0.0	0	0.0	0	0.0		
ITPA IVS2+21A>C	AA	158	0.68	49	0.65	109	0.69	1.18	0.35
	AC	70	0.30	24	0.32	46	0.29	(0.62-2.26)	
	CC	4	0.017	2	0.027	2	0.01		

* Frequencies

Table V. Distribution of the TPMT and ITPA genotypes and side effects with statistical estimates. Calculation of the OR (CI 95%), and p-value in relation to the presence of a mutated allele and the development of side effects (SE).

		Controls	Cases						
Gene	Genotype	No SE a/b/c*	Pancreatitis (19)	Digestive intolerance (16)	Skin reaction (13)	Arthralgia (6)	Alopecia (1)	Myelo-suppression (12)	Hepatotoxicity (5)
TPMT	*1/*1	147/90/119	18	15	13	6	1	9	5
	*1/*3A	7/4/5	1	1	0	0	0	2	0
	*1/*3C	1/0/0	0	0	0	0	0	0	0
	*1/*2	2/0/1	0	0	0	0	0	1	0
OR (CI95%)			0.81 (0.09-6.75)	0.98 (0.11-8.19)	3.13 (0.28-34.49)	0.33 (0.01-9.85)	0.34 (0.00-1077.26)	7.5 (1.44-38.91)	0.33 (0.00-23.61)
p-Value			0.726	0.667	0.441	1.0	1.0	0.030	1.0
ITPa 94 C>A	CC	140/86/114	19	15	12	3	1	12	5
	CA	17/8/11	0	1	1	3	0	0	0
	AA	0/0/0	0	0	0	0	0	0	0
OR (CI95%)			0.29 (0.05-1.42)	0.549 (0.06-4.42)	1.45 (0.17-11.91)	8.23 (1.75-38.87)	0.32 (0.00-182.12)	3.34 (0.34-32.20)	0.32 (0.01-7.94)
p-Value			1.0	0.841	0.588	0.004	1.0	0.369	1.0
ITPa IVS2+21A>C	AA	109/65/86	11	9	10	4	0	9	4
	AC	46/27/37	8	6	2	2	1	3	1
	CC	2/2/2	0	1	1	0	0	0	0
OR (CI95%)			1.65 (0.62-4.36)	1.76 (0.62-5.01)	1.46 (0.38-5.57)	1.13 (0.20-6.41)	25.14 (0.36-1740.47)	1.33 (0.33-5.30)	1.13 (0.20-6.41)
p-Value			0.221	0.210	0.414	0.597	0.310	0.481	0.597

* a. All patients controls (157); b. Controls to myelosuppression (94); according to time to exposure to AZA: between 28-86 months; c. Controls to hepatotoxicity (125); according to time to exposure to AZA: between 18-86 months.

digestive intolerance. Concerning the c.94C>A variant of the ITPA gene, 210 individuals were homozygous for the wild-type allele (C/C), 22 were heterozygous (A/C), and no homozygous for the mutant allele were found (A/A) (Table IV). Five patients developed side effects. For IVS2+21A>C, 158 individuals were homozygous for the wild-type (A/A), 70 were heterozygous (A/C) and 4 were homozygous for the mutated allele (C/C). Twenty two developed side effects; thirty five were responders and eight non responders. The allelic frequencies for these variants are 0.953 for c.94C>A, and 0.8327 for IVS2+21A>C.

Table V reflects the distribution of genotypes between cases and controls considering each side effect in a stratified way. Related to response, there was a statistically significant

Table VI. Relationship between TPMT and ITPA variants with response.

Gene	Genotype	Responders (133)	Non response (34)	Indeterminate (65)
TPMT	*1/*1	125	30	62
	*1/*3A	7	2	2
	*1/*3C	0	1	0
	*1/*2	1	1	1
OR (CI95%)			2.74 0.81-9.22	
p-Value			0.096	
ITPa 94 C>A	CC	123	26	61
	CA	10	8	4
	AA	0	0	0
OR (CI95%)			4.32 1.57-11.87	
p-Value			0.005	
ITPa IVS2+21A>C	AA	93	24	41
	AC	38	10	22
	CC	2	0	2
OR (CI95%)			0.83 0.36-1.88	
p-Value			0.736	

Responder Status was defined according to clinical criteria and time to exposure to treatment. OR, IC95% and p-value fisher two tailed test is indicated.

association between the variant c.94C>A in the ITPA gene and non drug response (p=0.005) (Table VI).

Discussion

Azathioprine/6-MP is a widely used drug in IBD treatment which is especially effective for the maintenance of remission in CD and UC, but around 15-30% of patients have adverse effects making a modification of the doses or withdrawal from treatment necessary [6, 21], other important groups of patients do not respond and there are unknown predictive factors. This is a large multicenter study evaluating the pharmacogenetic role of the two genes involved in AZA metabolism, TPMT and ITPA, which uses an adequate genotyping methodology with quality control that guarantees the accuracy of the results.

Several genetics variants in TPMT have been systematically related to the occurrence of toxicity to AZA/6-MP-treatment. It is accepted that patients carrying alleles associated with low or null TPMT enzymatic activity, especially in homozygosis, show a significant increase in the risk of myelosuppression as a result of excessive accumulation of 6-TGN [22, 23], so AZA/6-MP is not recommended for them.

In our study, we found an association between the TPMT variants and the AZA-side effect of myelosuppression (p=0.0304); this result is in agreement with previous reports where it is well recognized that TPMT deficient patients are at major risk for developing bone marrow suppression (24). However, the clinical significance of evaluation of TPMT polymorphism pre treatment is a matter of controversy. In this sense, several authors have found that only a small percentage of the IBD patients who developed AZA/6-MP-related adverse effects are in fact carriers of TPMT mutant alleles [25]. For example, Colombel et al reported that only 27% of 41 patients with CD and myelosuppression carried a low TPMT activity allele, so they concluded that this phenotype could be only partially attributable to the presence of these alleles [23].

Moreover, we did not find any association between the TPMT mutant alleles and other side effects different to myelosuppression. In 232 patients analyzed in our study, no homozygous alleles were found for variants of low TPMT enzymatic activity. Only 15 (6.5%) were heterozygous and among them, only 33.3% presented adverse effects. These results are comparable with the study reported by Stocco et al [26], which failed to reveal a significant association between AZA/6-MP-related toxicity and TPMT genotype (p=0.061).

How the reduction of ITPA activity can determine adverse effects is unknown. ITPase catalyzes the breakdown of ITP in a futile cycle related to the purine metabolic pathway. With the exposure to thiopurines, ITPase deficiency can lead to the cellular accumulation of 6 thio-inosine triphosphate (6-TITP), a substrate of ITPA potentially toxic. Von Ahsen et al [10] and Ansari et al [27] reported a significant association

between the c.94C>A variant and early therapy withdrawal due to adverse events probably due to the accumulation of 6-TITP. In our study, we found a positive correlation between this variant and non responder status to AZA treatment (p=0.005). To date, this is the first report of an association between the ITPA variant and efficacy of AZA treatment in IBD patients. On the other hand, Okada et al [28] have shown that patients with systemic lupus erythematous carrying the ITPA 94CA or 94AA genotypes exhibited a significantly better response to low doses of AZA than those carrying the ITPA 94CC genotype. With regard to this, Hawwa et al reported that carriers of the c.94C>A variant on ITPA had lower concentrations of TGNs; however, this result did not reach statistical significance [29]. Thus, the concentration of TGNs might be limited as a predictor of clinical outcome for the carriers of the c.94C>A variant allele. Stepchenkova et al [30] proposed that the P32T mutation exerts its effect in certain human tissues by cumulative effects of destabilization of transcripts, protein stability, and availability, so according to this, the presence of this variant might alter several metabolic pathways related with the physiological cell cycle or even some still unknown, and thus affects the cell response to the pharmacological effect of AZA. However, the real mechanism of this correlation remains unclear and it must be corroborated by more comprehensive studies.

Otherwise, we found an association between c.94C>A variant and the development of arthralgia. Of the 6 patients who developed it, 50% were carriers of one mutant allele. However, after assessing the patients with IBD not receiving AZA in treatment, about 10% will develop arthralgia in the course of the disease, which makes it difficult to conclude that the development of arthralgia is a side effect to treatment, triggered by drug toxicity or if its development is related to disease progression.

The present study has some limitations: it is a retrospective study and the value of activity index is limited, but we have only included patients in whom these values were present in their clinical history. Another limitation is that we have categorized patients as responders and non responders but we were not able to calculate specific values in the variation of CDAI or Mayo scores.

The development of adverse effects to AZA/6-MP is a multifactorial event, caused by the confluence of factors other than variants in the TPMT and ITPA genes. Further investigations must be instigated into finding new effective biomarkers to translate into clinical practice. It is therefore advisable to continue the search for other candidate genes that may be involved in the toxicity to AZA/6-MP using recent technical studies of whole genomes.

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Conflicts of interest

No conflict to declare.

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