Division of Pharmaceutical Care Sciences[1], Center for Social Pharmacy and Pharmaceutical Care Sciences, Faculty of Pharmacy; Division of Rheumatology[2], Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

Effect of genetic polymorphisms of azathioprine-metabolizing enzymes on response to rheumatoid arthritis treatment

A. Tsuchiya[1], T. Aomori[1,*], M. Sakamoto[1], A. Takeuchi[1], S. Suzuki[1], A. Jibiki[1], N. Otsuka[1], E. Ishioka[2], Y. Kaneko[2], T. Takeuchi[2], T. Nakamura[1]

Received August 15, 2016, accepted September 12, 2016

**Corresponding author: Tohru Aomori, Division of Pharmaceutical Care Sciences, Center for Social Pharmacy and Pharmaceutical Care Sciences, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8521, Japan*
aomori-th@pha.keio.ac.jp

Pharmazie 72: 22–28 (2017) *doi : 10.1691/ph.2017.6799*

Azathioprine (AZA) is increasingly being prescribed to rheumatoid arthritis (RA) patients. Following oral administration, AZA is converted into its active form. Inflammatory bowel disease (IBD) and systemic lupus erythematosus (SLE) patients with low thiopurine (S)-methyltransferase (TPMT) activity tend to respond well to AZA therapy. In a previous study of Japanese SLE patients under low-dose AZA therapy, the group with the 94C>A mutation in inosine triphosphatase (*ITPA*) showed greater improvement in their disease activity index. However, it is not yet clear how genotypes relate to responsiveness to RA treatment. The genotypes *ITPA* 94C>A, *TPMT*3C*, *NUDT15* 595C>T, *GST-M1*, *GST-T1* and *MRP4/ABCC4* 2269G>A of Japanese patients with RA were determined. The relationship between these genotypes and response to AZA therapy was evaluated using the Disease Activity Score 28 (DAS28) and various medical data. Of the 22 patients 15 had the *ITPA* 94C/C genotype, 7 had the *ITPA* 94C/A genotype, none had the *TPMT**3C mutation, 4 had the *NUDT15* 595C>T mutation, 8 had the GST-M1 and T1 null genotypes and 9 had the *MRP4/ABCC4* 2269G>A mutation. Changes in DAS28 at 6 months after baseline were similar in both *ITPA* genotype groups. However, the maintenance dose of AZA was significantly lower in the C/A group than in the C/C group (0.85±0.30 mg/kg/day vs. 1.2±0.46 mg/kg/day, respectively; $p = 0.043$). The *ITPA* 94C/A group showed the same response to RA treatment as the C/C group, but at a lower dose. This demonstrates that RA patients with the *ITPA* 94C>A mutation are more responsive to AZA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that causes destructive synovitis. RA affects not only joints but also various organs such as lungs, kidneys, and subcutaneous tissues. The primary goal of RA treatment is to improve the clinical condition which results in long-term good prognosis by inhibiting joint destruction, particularly in the prevention of physical function impairement. As joint destruction is irreversible and occurs in the early stage of the disease, it is recommended that treatment is started immediately on. RA is usually treated with disease-modifying antirheumatic drugs (DMARDs) including methotrexate (MTX) as an anchor drug, with non-steroidal anti-inflammatory drugs or low-dose steroids used adjunctively. If therapeutic efficacy is not sufficient within 3 months, it is standard to combine other DMARDs or to use biological drugs (Schuna 2011).

Azathioprine (AZA) is an immunosuppressive agent that is used widely in organ transplantation, acute lymphoblastic leukemia, inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), and RA (Gisbert and Scott 2009; Gaffney et al. 1998; Pearson et al. 1995; Sahasranaman et al. 2008). The first-choice drug for RA treatment is MTX, but for patients who cannot take this medicine because of a decline in liver or kidney function, AZA is administered as one of the second-line drugs. To avoid irreversible joint destruction, finding a marker to predict AZA response would be beneficial.

AZA is a prodrug that exerts its effect after metabolic activation. The metabolic pathways of AZA are described in the Figure. AZA is absorbed and then converted into 6-mercaptopurine (6-MP) by glutathione S-transferase (GST). After intracellular incorporation by nucleoside transporters, 6-MP is metabolized by three competitive enzymatic pathways, two of which are catabolic pathways: xanthine oxidase (XO) and thiopurine S-methyl-transferase (TPMT). XO generates 6-thiouric acid, which is an inactive metabolite and excreted in the urine. TPMT methylates 6-MP into 6-methylmercaptopurine, which is converted to 6-methylmercapto-8-hydroxypurine by aldehyde oxidase. In the anabolic pathway, 6-MP is converted into active metabolites, 6-thioguanine nucleotides (6-TGNs), by some enzymes (Chouchana et al. 2012). Intracellular 6-TGNs are incorporated into DNA instead of guanine nucleotides, which leads to inhibition of nucleic acid or protein synthesis by inhibiting the cell cycle and inducing apoptosis (Tieda et al. 2003). The nucleoside diphosphate-linked moiety X motif 15 (NUDT15) converts 6-thioguanine triphosphate (6-TGTP) into 6-thioguanine monophosphate (6-TGMP) (Moriyama et al. 2016). 6-TGMP is exported to the extracellular matrix by multidrug-resistance protein 4/ATP-binding cassette subfamily C member 4 (MRP4/ABCC4) (Chouchana et al. 2012).

Approximately 15–28% of patients treated with AZA need to be withdrawn from this therapy because of adverse drug reactions (ADRs) such as myelosuppression (Ansari et al. 2002; Lennard 2002; Marinaki et al. 2004a; Schwab et al. 2002). It was considered that these ADRs were mainly associated with genetic polymorphisms of *TPMT* (Ansali et al. 2008; Evans et al. 2001; Fabre et al. 2004; Relling et al. 1999; Zelinkova et al. 2006); however, in Japanese patients with low allele frequencies of *TPMT* polymorphisms, ADRs are not associated with *TPMT* polymorphisms (Ban et al. 2008; Takatsu et al. 2009).

Inosine triphosphate pyrophosphatase (ITPA) is an enzyme that hydrolyses 6-thioinosine triphosphate (6-TITP) into 6-thioinosine monophosphate and prevents the accumulation of 6-TITP, which is considered to be toxic. *ITPA* 94C>A (Pro 32 Thr: rs1127354) is

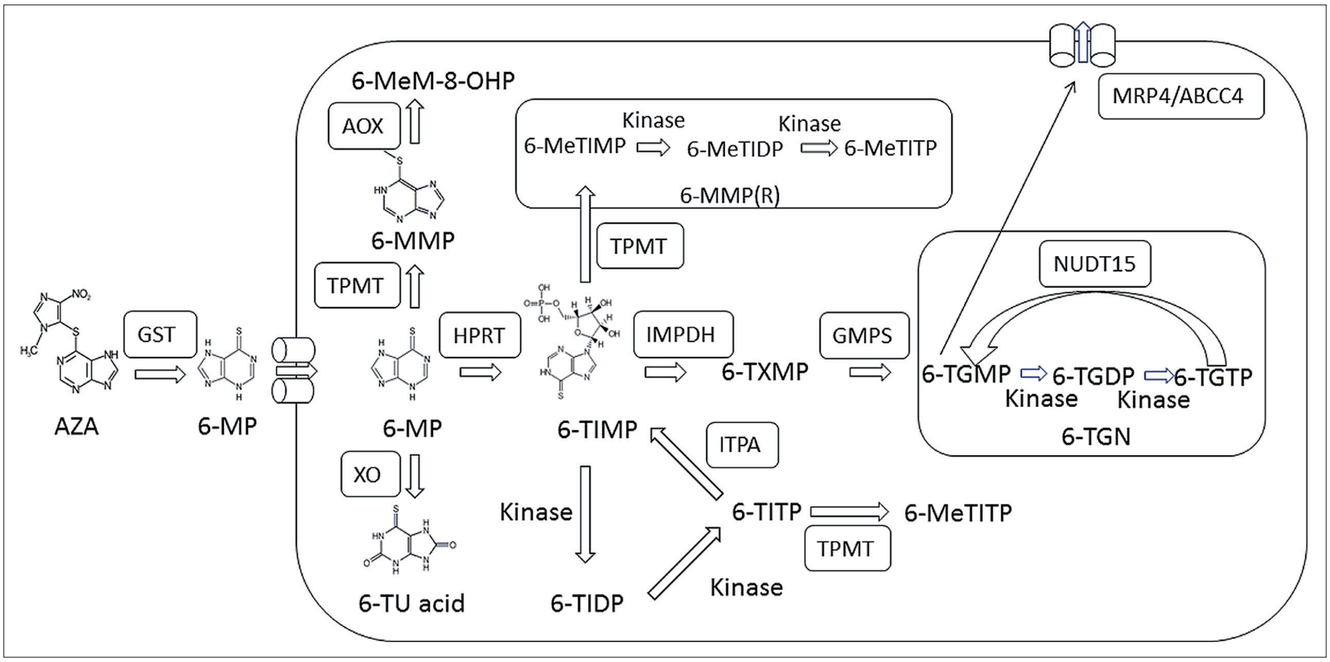

Fig.: Metabolic pathways of AZA.
1) Metabolites
AZA: azathioprine, 6-MP: 6-mercaptopurine, 6-MMP: 6-methylmercaptopurine, 6-MeM-8-OHP: 6-methylmercapto-8-hydroxypurine, 6-TU acid: 6-thiouric acid, 6-TXMP: 6-thioxanthosine monophosphate, 6-TGMP: 6-thioguanine monophosphate, 6-TGDP: 6-thioguanine diphosphate, 6-TGTP: 6-thioguanine triphosphate, 6-TGN: 6-thioguanine nucleotides, 6-MeTIMP: 6-methylthioinosine monophosphate, 6-MeTIDP: 6-methylthioinosine diphosphate, 6-MeTITP: 6-methylthioinosine triphosphate, 6-MMP(R): 6-methylmercaptopurine ribonucleotides, 6-TIMP: 6-thioinosine monophosphate, 6-TIDP: 6-thioinosine diphosphate, 6-TITP: 6-thioinosine triphosphate
2) Metabolic enzymes and drug transport carriers.
GST: glutathione S-transferase, TPMT: thiopurine S-methyl-transferase, XO: xanthine oxidase, AOX: aldehyde oxidase, HPRT: hypoxanthine-guanine phosphoribosyltransferase, IMPDH: inosine monophosphate dehydrogenase, GPMS: guanosine monophosphate synthetase, NUDT15: nucleoside diphosphate-linked moiety X motif 15, ITPA: inosine triphosphate pyrophosphatase, MRP4/ABCC4: multidrug resistance-associated protein-4/ATP-binding cassette sub-family C member 4

one of the genetic polymorphisms of *ITPA*. The enzyme activity of heterozygous mutant *ITPA* is 22.5% and homozygous mutant *ITPA* is 0% (Marinaki et al. 2004b; Sumi et al. 2002). In a previous report, patients with low ITPA activity had a high possibility of influenza-like symptoms, rash, and pancreatitis (Marinaki et al. 2004a), although some reports suggest that the *ITPA* 94C>A genetic polymorphism is not associated with ADRs (Van Dieren et al 2007). However, these studies were conducted with Caucasian patients, and allele frequencies are different between Asian and Caucasian populations. The allele frequency of the *ITPA* 94C>A polymorphism is higher in Asians (11–19%) than in Caucasians (1–7%) (Cao and Hegele 2002; Maeda et al. 2005; Marsh et al. 2004). As for *TPMT* polymorphisms, *TPMT*3A* (460G>A: rs1800460 and 719A>G: rs1142345) is the most prevalent allele in Caucasians, while *TPMT*3C* (719A>G: rs1142345) is the most prevalent allele in the Japanese population. The allele frequency of *TPMT*3C* has been reported to be 0.003–0.016 in the Japanese population (Kubota and Chiba 2001; Kumagai et al. 2001; Hiratsuka et al. 2000). *TPMT*3A* has negligible activity, while *TPMT*3C* has moderate activity compared with the wild-type allele (Tai et al. 1996).

In some previous reports, Japanese SLE patients with the *ITPA* 94C>A mutation receiving low-dose AZA showed a great improvement in the SLE Disease Activity Index (Okada et al. 2009; Nagamine et al. 2012). Similar to SLE patients, whether the effectiveness of AZA is greatly influenced by the *ITPA* 94C>A mutation in Japanese RA patients remains unclear.

Many enzymes, including ITPA and TPMT, are involved in AZA metabolism. Recent studies revealed that *NUDT15* 595C>T (Arg 139 Cys: rs116855232) is a genetic factor responsible for thiopurine-induced leukocytopenia and hair loss in East Asian population (Asada et al. 2016; Kakuta et al. 2016; Yang et al. 2014). The allele frequency of the mutation is approximately 10-20% in East Asian people (Asada et al. 2016; Kakuta et al. 2016; Yang et al. 2014). By contrast, it is much lower in Caucasians with an occurrence of 0.4% (Roberts and Barclay 2015). GST participates in the early stage of AZA metabolism, and has *GST-M1* and *GST-T1* isoforms. Approximately 50% of the Japanese population carry a null type of each gene (Cotton et al. 2000; Piacentini et al. 2011). Also, the allele frequency of *MRP4/ABCC4* 2269G>A (rs3765534), which affects MRP4/ABCC4 function, is 0.147 or over 0.187 in the Japanese population (Ban et al. 2010; Krishnamurthy et al. 2008). However, the association between these genetic polymorphisms and therapeutic efficacy and safety remains unclear.

The aim of this study was to investigate the genetic polymorphisms of AZA-metabolizing enzymes that are present at a high allele frequency in the Japanese population, and to evaluate the association between these genetic polymorphisms and their influence on RA treatment.

2. Investigations and results

2.1. Characteristics of the patients

The characteristics of the 22 patients are shown in Table 1. Three patients were male and 19 were female. The allele frequencies of *ITPA* 94C>A, *TPMT**3C, NUDT15 595C>T and *MRP4/ABCC4* 2269G>A, and the deletion frequencies of *GST-M1* and *GST-T1* are summarized in Table 2. The genotype frequencies of all polymorphisms did not deviate from Hardy-Weinberg's equilibrium.

There was no significant association of age, weight, sex, duration of RA and stage of disease with each genotype of *ITPA* 94C>A, *GST-M1*, and *GST-T1* (Table 3). Age and stage of disease were associated with the *NUDT15* 595C>T but weight, sex and duration of RA were not. The *MRP4/ABCC4* 2269G>A genotype were associated only with age and weight. The gene mutations were not related to each other.

The primary reason for starting AZA treatment was interstitial pneumonia due to MTX in 9 patients. The second reason was lymphoproliferative disease in 3 patients.

Table 1: Characteristics of patients

Number of Patients, n	22
Age. years, mean±SD	66±9.1
Weight, kg, mean±SD	54±12
Sex, n (%)	
Male	3 (14)
Female	19 (86)
Duration of RA, month, mean±SD	15±12
Stage of disease, n (%)§1	
I	2 (9.5)
II	7 (33)
III	1 (4.8)
IV	11 (52)
Maintenance dose of AZA, mg/kg/day, mean±SD	1.1±0.45
Biologic antirheumatic drugs, n (%)§2	
With	17 (81)
Without	4 (19)
Use of PSL, n (%)	
Yes	7 (32)
No	15 (68)
DAS28 score, mean±SD§3	
Baseline	3.6±2.1
After 6 months	1.9±0.63

§1,2: n=21, because one patient is unknown
§3: n=16, because 6 patients are unknown
RA: rheumatoid arthritis, AZA: azathioprine, PSL: prednisolone,

Table 2: Allele or deletion frequency

***ITPA* 94C>A mutation, n (%)**		
C/C	15	(68)
C/A	7	(32)
A/A	0	
A allele frequency	0.16	
***TPMT** 3C mutation, n (%)**		
*1/*1	22	(100)
*1/*3C	0	(0)
*3C allele frequency	0	
NUDT15 595C>T mutation, n (%)		
C/C	18	(82)
C/T	4	(18)
T/T	0	
A allele frequency	0.09	
***GST-M1* deletion, n (%)**		
Present	8	(36)
Null	14	(64)
***GST-T1* deletion, n (%)**		
Present	10	(45)
Null	12	*(55)*
***MRP4/ABCC4* 2269G>A mutation, n (%)**		
G/G	13	(59)
G/A	8	(36)
A/A	1	(5)
A allele frequency	0.23	

2.2. *Effects of genetic polymorphisms on AZA treatment*

In a comparison of the *ITPA* 94C>A genotype, the maintenance dose of AZA was significantly lower in the heterozygous mutant group (C/A group) than in the wild-type group (C/C group) (0.85±0.30 mg/kg/day vs. 1.2±0.46 mg/kg/day, respectively, $p = 0.043$; Table 4). The response of treatment was evaluated by using the Disease Activity Score 28 (DAS28) and EULAR response criteria. Changes in the DAS28 value at 6 months after baseline were not significant in both *ITPA* groups (-1.9±0.22 in C/A vs. -1.6±2.1 in C/C, $p = 0.39$; Table 4). There was also no significant difference in the good/moderate response rate of the EULAR criteria (40% in C/A vs. 55% in C/C, $p = 0.89$; Table 4).

No genotype of *NDT15* 595C>T, *GST-M1*, *GST-T1*, and *MRP4/ABCC4* 2269G>A was associated with the maintenance dose of AZA, changes in the DAS28 value, and response rate of the EULAR criteria (Table 4).

Two out of 14 patients were using prednisolone (PSL) in both or either present group with *GST-M1* and *T-1*, while 5 out of 8 patients were using PSL in both null groups. There was no significant difference in PSL use between the groups, but the use of PSL tended to be higher in both null groups ($p = 0.052$).

2.3. *Safety*

Of the 22 patients, 7 patients discontinued AZA treatment within 20 months. The reasons for this were as follow: hepatic disorder in 3 patients, nausea in 1, and the reason was unknown in 3. AZA was discontinued in 4 out of 15 patients (27%) in the *ITPA* 94C/C group, and in 3 out of 7 (43%) in the *ITPA* 94C/A group, but this was not significant. Similarly, the other genetic polymorphisms, including *NUDT15* 595C>T, were not associated with AZA discontinuance.

3. Discussion

This is the first report of an association between the *ITPA* 94C>A, *NUDT15* 595C>T, *GST-M1*, *GST-T1*, and *MRP4/ABCC4* 2269G>A genetic polymorphisms and the effect of RA treatment with AZA. In this study, the allele frequency of *ITPA* 94C>A was 0.16, *NUDT15* 595C>T was 0.09, and *MRP4/ABCC4* 2269G>A was 0.23. Previous studies of the East Asian population have reported allele frequencies of *ITPA* 94C>A as 0.135-0.155 (Marinaki et al. 2004b; Maeda et al. 2005), *TPMT*3C* as 0.003-0.042 (Kubota and Chiba 2001; Kumagai et al. 2001; Hiratsuka et al. 2000; Ishioka et al. 1999), *NUDT15* 595C>T as 0.102-0.233 (Asada et al. 2016; Kakuta et al. 2016; Yang et al. 2014) and *MRP4/ABCC4* 2269G>A as 0.147 or 0.187 and over (Ban et al. 2010; Krishnamurthy et al. 2008). These allele frequencies were close to the frequencies detected in our study. In the present study, the deletion frequencies of *GST-M1* and *GST-T1* were 64% and 55%, respectively. Similarly to the allele frequencies, these detection frequencies are comparable with previous analysis of the Japanese population (Cotton et al. 2000).

The maintenance dose of AZA was significantly lower in the heterozygous mutant group (C/A group) than in the wild-type group (C/C group) of *ITPA* 94C>A (0.85 ± 0.30 mg/kg/day vs. 1.2±0.46 mg/kg/day, respectively, $p = 0.043$; Table 4). There was no significant difference in changes in the DAS28 values at 6 months after baseline or in the response rate of the EULAR criteria between the C/C and C/A groups. On the basis of these findings, it is considered that the C/A group has the same response to RA treatment as the C/C group, but at a lower dose. In previous reports of Japanese patients with SLE who received low-dose AZA, those with the *ITPA* 94C>A mutation showed a great improvement in the SLE Disease Activity Index for 1 year (Okada et al. 2009; Nagamine et al. 2012). However, these studies did not use a different AZA dose between the C/C and C/A groups. These reports support our findings in terms of the good response to AZA of patients carrying the *ITPA* 94C/A mutation.

The *ITPA* 94C>A mutation induces the accumulation of 6-TITP. 6-TITP is considered a toxic compound (Marinaki et al. 2004a), but its relationship with toxicity is unclear (Van Dieren et al. 2007). In our study, the *ITPA* 94C>A genetic mutation was not associated with AZA discontinuation. For this reason, we considered the three following possibilities. First, the patients in this study were receiving AZA at a low dose; 2–3 mg/kg/day AZA is recommended in Caucasians (Andoh et al. 2008), whereas in the Japanese population, a lower dose (1 mg/kg/day) is usually given. As the concentrations of 6-TGNs in the erythrocytes of Japanese patients who received low-dose AZA were not different from those in Caucasians, low-dose AZA treatment was successful in Japanese

Table 3: Characteristics of RA patients according to each genotype

	ITPA 94C>A genotype			*NUDT15* 595C>T genotype			*GST-M1, T1* genotype			*MRP4* 2269G>A genotype		
	C/C	C/A	P value	C/C	C/T	P value	Both or either present	Null and Null	P value	G/G	G/A or A/A	P value
Number of Patients, n (%)	15 (68)	7 (32)		18 (82)	4 (18)		14 (64)	8 (36)		13 (59)	9 (41)	
Age, years, mean±SD	65±10	66±6.6	0.40	68±7.4	57±12	0.040	66±9.5	65±8.8	0.19	63±6.0	71±11	0.0036
Weight, kg, mean±SD	54±14	54±7.2	0.23	54±13	51±9.3	0.37	54±14	53±8.9	0.36	49±8.4	60±14	0.027
Sex, n (%)			1.00			1.00			0.53			0.54
Male	2 (9.1)	1 (4.5)		3 (14)	0 (0)		1 (4.5)	2 (9.1)		1 (4.5)	2 (9.1)	
Female	13 (59)	6 (27)		15 (68)	4 (18)		13 (59)	6 (27)		12 (55)	7 (32)	
ITPA 94C>A genotype, n (%)						1.00			0.34			0.65
C/C				12 (55)	3 (14)		11 (50)	4 (18)		8 (36)	7 (32)	
C/A				6 (27)	1 (4.5)		3 (14)	4 (18)		5 (23)	2 (9.1)	
GST-M1, T1 genotype, n (%)			0.34			0.60						0.074
Both or either present	11 (50)	3 (14)		12 (55)	2 (9)					6 (27)	8 (36)	
Null and Null	4 (18)	4 (18)		6 (27)	2 (9)					7 (32)	1 (4.5)	
MRP4 2269G>A genotype, n (%)			0.65			0.62			0.074			
G/G	8 (36)	5 (23)		10 (46)	3 (14)		6 (27)	7 (32)				
G/A or A/A	7 (32)	2 (9.1)		8 (36)	1 (4.5)		8 (36)	1 (4.5)				
NUDT15 595C>T genotype, n (%)			1.00						0.602			0.62
C/C	12 (54)	6 (27)					12 (55)	6 (27)		10 (45)	8 (36)	
C/T	3 (14)	1 (4.6)					2 (9.1)	2 (9.1)		3 (14)	1 (4.6)	
Duration of RA, month, mean±SD	16±12	15±12	0.37	16±11	14±19	0.18	15±10	16±15	0.40	14±11	17±13	0.35
Stage of disease, n (%)§1			0.97			0.039			0.97			0.97
I	1 (4.8)	1 (4.8)		0 (0)	2 (9)		1 (4.8)	1 (4.8)		1 (4.8)	1 (4.8)	
II	4 (18)	3 (14)		7 (32)	0 (0)		5 (24)	2 (9.5)		5 (24)	2 (9.5)	
III	1 (4.8)	0 (0)		1 (4.6)	0 (0)		1 (4.8)	0 (0)		1 (4.8)	0 (0)	
IV	8 (36)	3 (14)		9 (41)	2 (9)		6 (29)	5 (24)		6 (29)	5 (24)	

§1: n=21, because one patient is unknown, RA: rheumatoid arthritis

patients with inflammatory bowel disease and SLE (Okada et al. 2009; Hibi et al. 2003). When the maintenance dose of AZA was maintained at less than 1.5 mg/kg/day, a previous study could not find an association between the *ITPA* 94C>A mutation and ADRs (Yamamoto et al. 2010). In contrast, this association was found if the dose of AZA was maintained at more than 1.5 mg/kg/day. In our study, the maintenance dose of AZA did not exceed 1.5 mg/kg/day. Second, AZA dose adjustment was performed frequently by taking into account the symptoms of the patients. Third, all *ITPA* 94C>A mutant patients in our study were heterozygous for this mutation (C/A group). As the enzyme activity of the heterozygous mutant protein is reportedly 22.5% of that of the wild-type protein, and the activity of the homozygous mutant protein is 0% (Marinaki 2004b), individuals carrying the heterozygous mutant protein may retain some enzyme activity. Further investigation with inclusion of a homozygous mutant group (A/A group) is needed to investigate this hypothesis. These reasons provide an explanation for why we could not detect a difference in AZA discontinuation according to the *ITPA* 94C>A polymorphism.

In summary, in the *ITPA* 94C/A group, the maintenance dose of AZA was significantly lower without influencing its effects on RA, and the frequency of ADRs was not increased. These results indicate that the C/A group is more responsive to AZA treatment than the C/C group. With the low dose of AZA used in Japanese patients, it is considered that accumulation of 6-TITP by the *ITPA* genetic polymorphism affects the immunosuppression of AZA, without affecting the incidence of ADRs. The influence of the *ITPA* genetic polymorphism might be involved strongly in the Japanese population who have a high allele frequency of *ITPA* 94C>A.

The use of PSL tended to be higher in the both *GST-M1* and *GST-T1* null group compared with both or either present group (p = 0.052). GST is mostly expressed in the liver and converts AZA into 6-MP. GST deletion causes the disappearance of enzyme activity and a decrease in conversion to 6-MP (Chouchana et al. 2012). In spite of the same therapeutic efficacy in both groups, the reason why the use of PSL is higher in the both *GST-M1* and *GST-T1* null group is assumed to be that PSL complements the insufficient efficacy of AZA because of a decrease in conversion to active 6-TGNs.

The GST family consists of GST-M1, GST-T1, and GST-P1. Previous reports showed that patients with the *GST-M1* genotype have an increased probability of developing ADRs and a higher incidence of lymphopenia, so that the *GST-M1* genotype is a factor associated with the occurrence of AZA-induced ADRs (Stocco et al. 2007). However, in our study, there was no significant difference in AZA discontinuation between the *GST-M1* positive and null groups.

NUDT15 convert 6-TGTP into 6-TGMP. In patients with 595C>T mutation, higher levels of 6-TGTP were measured (Motiyama et al. 2016). Besides, IBD patients with this missense variant were shown to more likely to develop leukopenia and hair loss (Asada et al. 2016; Kakuta et al. 2016; Yang et al. 2014). Hence, in East Asian population, which has especially high incidence of this SNP, it is considered to be an important risk factor. However, in this study, these adverse event had not been detected among our patients, thus, we could not discuss about the impact of this SNP to AZA therapy.

MRP4/ABCC4 is an organic anion transporter that exports monophosphate nucleosides such as 6-MP and 6-TGN. It protects against hematologic toxicity by limiting the intracellular accumulation of the active metabolite 6-TGN. The *MRP4/ABCC4* 2269G>A mutation dramatically reduces MRP4/ABCC4 function by impairing it at the cell membrane (Krishnamurthy et al. 2008). It has been shown that 6-TGN levels were significantly higher and the blood cell count was significantly lower in patients with the

Table 4: Pharmacotherapy and response to treatment according to each genotype

	ITPA 94C>A genotype			*NUDT15* 595C>T genotype			*GST-M1, T1* genotype			*MRP4* 2269G>A genotype		
	C/C	C/A	P value	C/C	C/T	P value	Both or either present	Null and Null	P value	G/G	G/A or A/A	P value
Maintenance dose of AZA, mg/kg/day, mean±SD	1.2±0.46	0.85±0.30	0.043	1.1±0.50	1.1±0.14	0.43	1.2±0.51	0.95±0.27	0.12	1.1±0.49	1.7±0.42	0.36
Biologic antirheumatic drugs, n (%)§1			0.25			0.15			0.62			0.27
With	10 (48)	7 (33)		15 (71)	2 (9.5)		11 (52)	6 (29)		11 (52)	6 (29)	
Without	4 (19)	0 (0)		2 (9.5)	2 (9.5)		2 (9.5)	2 (9.5)		1 (4.8)	3 (14)	
Use of PSL, n (%)			0.14			1.00			0.052			1.00
PSL	3 (14)	4 (18)		6 (27)	1 (5)		2 (9.1)	5 (23)		4 (18)	3 (14)	
No PSL	12 (55)	3 (14)		12 (55)	3 (14)		12 (55)	3 (14)		9 (41)	6 (27)	
AZA dose adjustment, n (%)			0.90			0.86			0.90			0.39
Reduction	4 (18)	1 (4.5)		4	1		3 (14)	2 (9.1)		2 (9.1)	3 (14)	
No change	9 (41)	6 (27)		12	3		9 (41)	6 (27)		11 (50)	4 (18)	
Escalation	2 (9.1)	0 (0)		2	0		2 (9.1)	0 (0)		0 (0)	2 (9.1)	
AZA administration			0.79			1.00			1.00			0.65
Discontinuation	4	3		6	1		4	3		5	2	
Continuation	11	4		12	3		10	5		8	7	
Discontinuance rate (discontinuation/all, %)	4/15 (27)	3/7 (43)		6/18(33)	1/4 (25)		4/14 (29)	3/8 (38)		5/13 (39)	2/9 (22)	
DAS28 score, mean±SD												
Baseline	3.5±2.1	3.7±2.2	0.48	3.7±2.11	2.84	0.38	3.5±2.1	4.0±2.3	0.41	4.4±2.3	2.7±1.3	0.072
After 6 month	2.0±0.65	1.9±0.69	0.45	2.0±0.65	1.8±0.85	0.42	1.9±0.65	2.2±0.65	0.23	2.0±0.63	2.0±0.73	0.43
Changes between baseline evaluation and 6 month follow-up in DAS score, mean±SD	-1.6±2.1	-1.9±2.2	0.39	-1.7±2.15	-1.64	0.38	-1.6±2.0	-2.1±2.6	0.50	-2.5±2.4	-0.69±1.1	0.086
Response§2	5	2	0.89	6	1	0.19	5	2	0.76	5	2	0.70
Good response	1	0		1	0		1	0		1	0	
Moderate response	5	3		8	0		6	2		3	5	
No response Response rate (responder/all, %)	6/11 (55)	2/5 (40)		7/15(47)	1/1 (100)		6/12 (50)	2/4 (50)		6/9 (67)	2/7 (29)	

§ 1: n=21, because one patient is unknown, §2: n=16, because 6 patients are unknown, AZA: azathioprine, PSL: prednisolone, DAS: disease activity score

MRP4/ABCC4 2269G>A mutation (Ban et al. 2010). Accordingly, we thought that the *MRP4/ABCC4* 2269G>A mutation might influence RA therapeutic efficacy and safety, but such an influence was not shown in our study.

Many enzymes, such as XO and inosine monophosphate dehydrogenase (IMPDH), are involved in AZA metabolism. Genetic polymorphisms have been identified in these enzymes, such as *XO* 837C>T, *XO* 2211C>T, *XO* 3030C>T, and *IMPDH* 1575A>G (Kudo et al. 2008; Kudo et al. 2009). However, these genetic polymorphisms were not shown to have an effect on clinical efficacy in SLE patients (Nagamine et al. 2012). For this reason, these genetic polymorphisms were excluded from our study.

In conclusion, we found that *ITPA* 94C>A is associated with the maintenance dose of AZA in Japanese patients with RA. It was suggested that the C/A group showed the same response to RA therapy as the C/C group, but at a lower dose.

4. Experimental

4.1. Patients

We enrolled a total of 22 Japanese adult patients with RA who were treated with AZA for more than 6 months in the rheumatology unit of Keio University Hospital. Patients who had a hepatic disorder greater than moderate severity as assessed by the Child-Pugh score or were judged to be unsuitable for study enrollment by the doctor were excluded. This study was approved by the Institutional Review Board of Keio University Hospital (approval number: 150608-1). All patients provided written informed consent before participating in this study.

4.2. DNA isolation

Genomic DNA was extracted from peripheral blood using a QIAamp® DNA Blood Mini Kit (QIAGEN, Valencia, CA) according to the spin protocol of the product manual.

4.3. Genotyping

The following genetic polymorphisms related to AZA metabolism and transportation were analyzed: *ITPA* 94C>A, *TPMT*3C*, *NUDT15* 595C>T, *GST-M1*, *GST-T1*, and *MRP4/ABCC4* 2269G>A. *ITPA*, *TPMT*, *NUDT15* and *MRP4/ABCC4* genotypes were determined by using a TaqMan® genotyping assay obtained from Applied Biosystems (Foster City, CA, USA). *GST-M1* and *GST-T1* genotypes were determined by PCR as described previously (Abdel-Rahman et al 1996).

4.4. Statistical analysis

The comparison between the wild-type group and mutant group in each genotype was performed with respect to age (years), weight (kg), duration of RA (months), maintenance dose of AZA (mg/kg/day), DAS28 value at baseline, DAS28 value at the 6-month follow-up, and change in the DAS28 value using the Mann-Whitney U-test. Results were presented as mean ± SD.

Similarly, the comparison of sex, the other polymorphisms, stage of disease, use of PSL, use of biologic antirheumatic drugs, AZA dose adjustment, AZA administration, and response were analyzed using the Yates 2×2 chi square test, Fisher exact probability, or Yates m×n chi square test. P-values < 0.05 were considered to be statistically significant.

Acknowledgments: This work was supported by JSPS KAKENHI Grant Number 15K18933 and by The Research Foundation for Pharmaceutical Sciences, Japan.

Author contributions: A.T., T.A., S.S., A.J., N.O., and T.N. wrote the manuscript. T.A., T.T., and T.N. designed the research. A.T., T.A., M.S., E.I., and Y.K. performed the research. A.T., T.A., S.S., A.J., and N.O. analyzed the data.

Conflict of interest/disclosure: All authors declare no conflict of interest.

References

Abdel-Rahman SZ, el-Zein RA, Anwar WA, Au WW (1996) A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies. Cancer Lett 107: 229-233.

Andoh A, Tsujikawa T, Ban H, Hashimoto T, Bamba S, Ogawa A, Sasaki M, Saito Y, Fujiyama Y (2008) Monitoring 6-thioguanine nucleotide concentrations in Japanese patients with inflammatory bowel disease. J Gastroenterol Hepatol 23: 1373-1377.

Ansari A, Hassan C, Duley J, Marinaki A, Shobowale-Bakre EM, Seed P, Meenan J, Yim A, Sanderson J (2002) Thiopurine methyltransferase activity and the use of azathioprine in inflammatory bowel disease. Aliment Pharmacol Ther 16: 1743-1750.

Ansari A, Arenas M, Greenfield SM, Morris D, Lindsay J, Gilshenan K, Smith M, Lewis C, Marinaki A, Duley J, Sanderson J (2008) Prospective evaluation of the pharmacogenetics of azathioprine in the treatment of inflammatory bowel disease. Aliment. Pharmacol Ther 28: 973-983.

Asada A, Nishida A, Shioya M, Imaeda H, Inatomi O, Bamba S, Kito K, Sugimoto M, Andoh A (2015) NUDT15 R139C-related thiopurine leukocytopenia is mediated by 6-thioguanine nucleotide-independent mechanism in Japanese patients with inflammatory bowel disease. J Gastroenterol 51: 22-9.

Ban H, Andoh A, Tanaka A, Tsujikawa T, Sasaki M, Saito Y, Fujiyama Y (2008) Analysis of thiopurine S-methyltransferase genotypes in Japanese patients with inflammatory bowel disease. Intern Med 47: 1645-1648.

Ban H, Andoh A, Imaeda H, Kobori A, Bamba S, Tsujikawa T, Sasaki M, Saito Y, Fujiyama Y (2010) The multidrug-resistance protein 4 polymorphism is a new factor accounting for thiopurine sensitivity in Japanese patients with inflammatory bowel disease. J Gastroenterol 45: 1014-1021.

Cao H, Hegele RA (2002) DNA polymorphisms in ITPA including basis of inosine triphosphatase deficiency. J Hum Genet 47: 620-622.

Cotton SC, Sharp L, Little J, Brockton N (2000) Glutathione S-transferase polymorphisms and colorectal cancer: a HuGE review. Am J Epidemiol 151: 7-32.

Chouchana L, Narjoz C, Beaune P, Loriot MA, Roblin X (2012) Review article: the benefits of pharmacogenetics for improving thiopurine therapy in inflammatory bowel disease. Aliment Pharmacol Ther 35: 15-36.

Evans WE, Hon YY, Bomgaars L, Coutre S, Holdsworth M, Janco R, Kalwinsky D, Keller F, Khatib Z, Margolin J, Murray J, Quinn J, Ravindranath Y, Ritchey K, Roberts W, Rogers ZR, Schiff D, Steuber C, Tucci F, Kornegay N, Krynetski EY, Relling MV (2001) Preponderance of thiopurine S-methyltransferase deficiency and heterozygosity among patients intolerant to mercaptopurine or azathioprine. J Clin Oncol 19: 2293-2301.

Fabre MA, Jones DC, Bunce M, Morris PJ, Friend PJ, Welsh KI, Marshall SE (2004) The impact of thiopurine S-methyltransferase polymorphisms on azathioprine dose 1 year after renal transplantation. Transpl Int 17: 531-539.

Gaffney K, Scott DG (1998) Azathioprine and cyclophosphamide in the treatment of rheumatoid arthritis. Br J Rheumatol 37: 824-836.

Gisbert JP, Linares PM, McNicholl AG, Maté J, Gomollón F (2009) Meta-analysis: the efficacy of azathioprine and mercaptopurine in ulcerative colitis. Aliment Pharmacol Ther 30: 126-137.

Hibi T, Naganuma M, Kitahora T, Kinjyo F, Shimoyama T (2003) Low-dose azathioprine is effective and safe for maintenance of remission in patients with ulcerative colitis. J Gastroenterol 38: 740-746.

Hiratsuka M, Inoue T, Omori F, Agatsuma Y, Mizugaki M (2000) Genetic analysis of thiopurine methyltransferase polymorphism in a Japanese population. Mutat Res 448: 91-95.

Ishioka S, Hiyama K, Sato H, Yamanishi Y, McLeod HL, Kumagai K, Maeda H, Yamakido M (1999) Thiopurine methyltransferase genotype and the toxicity of azathioprine in Japanese. Intern Med 38: 944-947.

Kakuta Y, Naito T, Onodera M, Kuroha M, Kimura T, Shiga H, Endo K, Negoro K, Kinouchi Y, Shimosegawa T (2016) NUDT15 R139C causes thiopurine-induced early severe hair loss and leukopenia in Japanese patients with IBD. Pharmacogenomics J 16: 280-285.

Krishnamurthy P, Schwab M, Takenaka K, Nachagari D, Morgan J, Leslie M, Du W, Boyd K, Cheok M, Nakauchi H, Marzolini C, Kim RB, Poonkuzhali B,Schuetz E, Evans W, Relling M, Schuetz JD (2008) Transporter-mediated protection against thiopurine-induced hematopoietic toxicity. Cancer Res 68: 4983-4989.

Kubota T, Chiba K (2001) Frequencies of thiopurine S-methyltransferase mutant alleles (TPMT*2, *3A, *3B and *3C) in 151 healthy Japanese subjects and the inheritance of TPMT*3C in the family of a propositus. Br J Clin Pharmacol 51: 475-477.

Kudo M, Moteki T, Sasaki T, Konno Y, Ujiie S, Onose A, Mizugaki M, Ishikawa M, Hiratsuka M (2008) Functional characterization of human xanthine oxidase allelic variants. Pharmacogenet Genomics 18: 243-251.

Kudo M, Saito Y, Sasaki T, Akasaki H, Yamaguchi Y, Uehara M, Fujikawa K, Ishikawa M, Hirasawa N, Hiratsuka M (2009) Genetic variations in the HGPRT, ITPA, IMPDH1, IMPDH2, and GMPS genes in Japanese individuals. Drug Metab Pharmacokinet 24: 557-564.

Kumagai K, Hiyama K, Ishioka S, Sato H, Yamanishi Y, McLeod HL, Konishi F, Maeda H, Yamakido M (2001) Allelotype frequency of the thiopurine methyltransferase (TPMT) gene in Japanese. Pharmacogenetics 11: 275-278.

Lennard L (2002) TPMT in the treatment of Crohn's disease with azathioprine. Gut 51: 143-146.

Maeda T, Sumi S, Ueta A, Ohkubo Y, Ito T, Marinaki AM, Kurono Y, Hasegawa S, Togari H (2005) Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency in the Japanese population. Mol Genet Metab 85: 271-279.

Marinaki AM, Ansari A, Duley JA, Arenas M, Sumi S, Lewis CM, Shobowale-Bakre el-M, Escuredo E, Fairbanks LD, Sanderson JD (2004) Adverse drug reactions to azathioprine therapy are associated with polymorphism in the gene encoding inosine triphosphate pyrophosphatase (ITPase). Pharmacogenetics 14: 181-187.

Marinaki AM, Sumi S, Arenas M, Fairbanks L, Harihara S, Shimizu K, Ueta A, Duley JA (2004) Allele frequency of inosine triphosphate pyrophosphatase gene polymorphisms in a Japanese population. Nucleosides Nucleotides Nucleic Acids 23: 1399-1401.

Marsh S, King CR, Ahluwalia R, McLeod HL (2004) Distribution of ITPA P32T alleles in multiple world populations. J Hum Genet 49: 579-581.

Moriyama T, Nishii R, Perez-Andreu V, Yang W, Klussmann FA, Zhao X, Lin TN, Hoshitsuki K, Nersting J, Kihira K, Hofmann U, Komada Y, Kato M, McCorkle R, Li L, Koh K, Najera CR, Kham SK, Isobe T, Chen Z, Chiew EK, Bhojwani D, Jeffries C, Lu Y, Schwab M, Inaba H, Pui CH, Relling MV, Manabe A, Hori H, Schmiegelow K, Yeoh AE, Evans WE, Yang JJ (2016) NUDT15 polymorphisms alter thiopurine metabolism and hematopoietic toxicity. Nat Genet 48: 367-373.

Nagamine A, Takenaka M, Aomori T, Okada Y, Hiromura K, Nojima Y, Araki T, Nakamura T, Yamamoto K (2012) Effect of genetic polymorphisms on effectiveness of low-dose azathioprine in Japanese patients with systemic lupus erythematosus. Am J Health Syst Pharm 69: 2072-2078.

Okada Y, Nakamura K, Hiromura K, Nojima Y, Horiuchi R, Yamamoto K (2009) Pro32Thr polymorphism of inosine triphosphate pyrophosphatase gene predicts efficacy of low-dose azathioprine for patients with systemic lupus erythematosus. Clin Pharmacol Ther 85: 527-530.

Pearson DC, May GR, Fick GH, Sutherland LR (1995) Azathioprine and 6-mercaptopurine in Crohn disease. A meta-analysis. Ann Intern Med 123: 132-142.

Piacentini S, Polimanti R, Porreca F, Martínez-Labarga C, De Stefano GF, Fuciarelli M (2011) GSTT1 and GSTM1 gene polymorphisms in European and African populations. Mol Biol Rep 38: 1225-1230.
Sahasranaman S, Howard D, Roy S (2008) Clinical pharmacology and pharmacogenetics of thiopurines. Eur J Clin Pharmacol 64: 753-767.
Schuna AA (2011) Rheumatoid Arthritis. In: DiPiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, L. Posey LM (ed.) Pharmacotherapy: A pathophysiologic approach, 8th ed., McGraw Hill New York, p. 1583-1632.
Schwab M, Schäffeler E, Marx C, Fischer C, Lang T, Behrens C, Gregor M, Eichelbaum M, Zanger UM, Kaskas BA (2002) Azathioprine therapy and adverse drug reactions in patients with inflammatory bowel disease: impact of thiopurine S-methyltransferase polymorphism. Pharmacogenetics 12: 429-436.
Stocco G, Martelossi S, Barabino A, Decorti G, Bartoli F, Montico M, Gotti A, Ventura A (2007) Glutathione-S-transferase genotypes and the adverse effects of azathioprine in young patients with inflammatory bowel disease. Inflamm Bowel Dis 13: 57-64.
Sumi S, Marinaki AM, Arenas M, Fairbanks L, Shobowale-Bakre M, Rees DC, Thein SL, Ansari A, Sanderson J, De Abreu RA, Simmonds HA, Duley JA (2002) Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency. Hum Genet 111: 360-367.
Relling MV, Hancock ML, Rivera GK, Sandlund JT, Ribeiro RC, Krynetski EY, Pui CH, Evans WE. (1999) Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. J Natl Cancer Inst 91: 2001-2008.
Roberts RL, Barclay ML (2015) Update on thiopurine pharmacogenetics in inflammatory bowel disease. Pharmacogenomics 16: 891-903.
Takatsu N, Matsui T, Murakami Y, Ishihara H, Hisabe T, Nagahama T, Maki S, Beppu T, Takaki Y, Hirai F, Yao K (2009) Adverse reactions to azathioprine cannot be predicted by thiopurine S-methyltransferase genotype in Japanese patients with inflammatory bowel disease. J Gastroenterol Hepatol 24: 1258-1264.
Tai HL, Krynetski EY, Yates CR, Loennechen T, Fessing MY, Krynetskaia NF, Evans WE (1996) Thiopurine S-methyltransferase deficiency: two nucleotide transitions define the most prevalent mutant allele associated with loss of catalytic activity in Caucasians. Am J Hum Genet 58: 694-702.
Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R,Walczak H, Iven H, Galle PR, Ahmadian MR, Neurath MF (2003) CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. J Clin Invest 111: 1133-1145.
Van Dieren JM, Hansen BE, Kuipers EJ, Nieuwenhuis EE, Van der Woude CJ (2007) Meta-analysis: Inosine triphosphate pyrophosphatase polymorphisms and thiopurine toxicity in the treatment of inflammatory bowel disease. Aliment Pharmacol Ther 26: 643-652.
Yamamoto K, Okada Y, Nakamura K, Hiromura K, Nojima Y, Nakamura T (2010) Inosine triphosphate pyrophosphatase 94C>A polymorphism: clinical implications for patients with systemic lupus erythematosus treated with azathioprine. Expert Opin Drug Saf 9: 447-457.
Yang SK, Hong M, Baek J, Choi H, Zhao W, Jung Y, Haritunians T, Ye BD, Kim KJ, Park SH, Park SK, Yang DH, Dubinsky M, Lee I, McGovern DP, Liu J, Song K (2014) A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leukopenia. Nat Genet 46: 1017-1020.
Zelinkova Z, Derijks LJ, Stokkers PC, Vogels EW, van Kampen AH, Curvers WL, Cohn D, van Deventer SJ, Hommes DW (2006) Inosine triphosphate pyrophosphatase and thiopurine s-methyltransferase genotypes relationship to azathioprine-induced myelosuppression. Clin Gastroenterol Hepatol 4: 44-49.