ORIGINAL ARTICLE

Thiopurine S-methyltransferase (TPMT) Mutation Prevalence and Myelosuppression Frequency in North Indian Patients with Autoimmune Disorders

Garg Ambar[1], Arya Vandana[1], Duggal Lalit[2], Kotwal Jyoti[1*]

Abstract

Background: For many years, azathioprine and its active metabolite 6-merceptopurine are used as immunosuppressants for treatment of autoimmune disorders. However, azathioprine has low therapeutic index with myelosuppression as its predominant toxicity which is linked with thiopurine S-methyltransferase (TPMT) enzyme activity, which is involved in drug metabolism. TPMT activity is controlled by variants in TPMT gene. We aimed to estimate prevalence of TPMT gene mutations in North Indian patients with autoimmune disorders and to assess myelosuppression in these patients.

Methods: We analysed 176 adult patients with autoimmune disorders coming to SGR hospital. TPMT mutation was analysed by PCR-RFLP and further validated by reverse dot blot. Patients with wild type TPMT genotype were followed for development of myelosuppression.

Results: Out of total 176 patients studied, TPMT mutation was present in 3 patients showing prevalence of 1.7%. Two patients (1.13%) were heterozygous for TPMT*1/*3C genotype and only one patient (0.56%) was heterozygous for TPMT*1/*3A. Other TPMT mutant alleles (TPMT*2 and *3B) were not identified. No homozygous TPMT mutants were identified. Allele frequencies of TPMT*3A; TPMT*3C were 0.28% and 0.56% respectively. Three patients positive by PCR-RFLP were also found to be positive by reverse dot blot analysis; depicting 100% concordance between two methods. Excluding three positive patients, follow up was available in 114/173 patients. Follow up ranged from 28 days to 1.5 years. Twenty (17.5%) patients developed myelosuppression with majority (60%) within 1-5 months of therapy. Leucopenia along with neutropenia was the most common presentation (11.4%) followed by anaemia (7.8%) and thrombocytopenia (6.1%).

Conclusion: TPMT mutation prevalence is low in North Indian adult patients with autoimmune disorders as compared to Western population. In our study patients even with wild type TPMT genotype (17.5%) developed myelosuppression underscoring the importance of TPMT mutation as the only factor contributing to myelosuppression.

Introduction

Azathioprine (AZA) and its active metabolite 6-merceptopurine (6MP) are immunosuppressants. These have been used for many years for treatment of conditions like systemic lupus erythematosus (SLE), acute lymphoblastic leukemia (ALL), rheumatoid arthritis (RA), uveitis and inflammatory bowel diseases (IBD). However, along with treatment of the underlying conditions, azathioprine also has adverse effects like gastro-intestinal toxicity and severe myelosuppression in few patients.[1] Severe myelotoxicity which may lead to drug intolerance and withdrawal of the drug is related to an anomaly in thiopurine metabolic pathway, resulting from deficient thiopurine S-methyltransferase (TPMT) activity.[2] AZA is a pro-drug which acts through conversion into 6MP. The TPMT enzyme is responsible, in part, for the methylation of 6-MP into the inactive metabolite 6-methylmercaptopurine and thus prevents 6-MP from further conversion into active, cytotoxic thioguanine nucleotide (TGN) metabolites which are responsible for causing myelosuppression.[3] Many studies done in West have shown increased myelosuppression in patients on thiopurine therapy, having low TPMT enzyme activity[4], which is largely influenced by genetic variants in the TPMT gene. More than 20 variants in the TPMT gene, associated with decreased TPMT activity, have been identified[5]. However, four major variant alleles, TPMT*2 (G238C), TPMT*3A (G460A, A719G), TPMT*3B (G460A) and TPMT*3C (A719G), account for more than 90% of intermediate or low activity cases.[6] At present, US Food and Drug Administration (FDA) recommends TPMT phenotype/genotype testing prior to starting treatment with thiopurines.[7] However, TPMT genotyping has not been universally adopted, and the cost effectiveness and optimal clinical circumstances in which to perform routine testing are not well-defined. Worldwide prevalence of TPMT mutations has been found to vary between 3 to 14%[7] with ethnic variations between different populations, ranging from 10.1% in Caucasians,[8] 9.2% in African Americans[9] to 2% in South West Asians.[8] Scanty data is available from India regarding prevalence of TPMT mutations. Prevalence variability has been found to vary from 2.7% in South Indian population[10] to 4.9% and 10% in two North Indian pediatric ALL patient populations.[11,12] Also,

[1]Department of Hematology, [2]Department of Rheumatology, Sir Ganga Ram Hospital, New Delhi; *Corresponding Author
Received: 14.07.2017; Accepted: 29.11.2017

the type of polymorphism causing decreased enzyme activity is different in different ethnic groups.[7] No data of the frequency of TPMT mutations, decreasing enzyme activity, is available from our region [Delhi NCR] in adult patients, with immunological diseases, for which azathioprine is commonly prescribed as an adjunct to steroids and other immunosuppressive therapy. The patients in whom TPMT mutations are present, either alternative second line expensive therapy has to be given or dose reductions and frequent monitoring is required from the beginning of the therapy, to save them from severe side effects of the drug. The question which arises is, whether the prevalence of TPMT polymorphism in Indian adult patients is same as in South India or higher as seen in studies done in North Indian pediatric ALL patients? And which is the most common TPMT mutation prevalent in our population as different mutations have a different potential of decreasing TPMT enzyme activity. Depending upon the answer to this question, one can take a decision about the utility of the genetic tests for TPMT polymorphism in our population prior to starting AZA therapy. Data generated in form of prevalence, will help us to know the frequency of different alleles of TPMT gene in Indian adult patients with immunological disorders and whether pre-treatment TPMT testing should be recommended in Indian patients before starting azathioprine as recommended by US FDA[7] for Western population; where high prevalence of TPMT mutation is present.[8]

Material and Methods

It was a single arm prospective; clinical and laboratory based study. The study was conducted in Department of Hematology at Sir Ganga Ram Hospital, New Delhi over a period of one and a half year, from September 2015 to March 2017. The study was started after obtaining ethical clearance from the Institutional Ethics Committee.

Subjects: The study included total 176 adult patients of immunological disorders; requiring azathioprine therapy, coming to outpatient department at Sir Ganga Ram hospital, from September 2015 to March 2017. Patients receiving azathioprine for non-autoimmune diseases like malignancies or transplant recipients were excluded from analysis. Informed written consent was obtained from all the participants. Patients were counselled about the type of study, its importance, and the possible benefits. Their proper detailed clinical history was taken and thorough physical examination was conducted, along with other relevant investigations pertaining to the diagnosis and was noted down sequentially.

Molecular analysis: Approximately 3 to 5 ml peripheral blood was collected from all the subjects in EDTA vacutainer before starting azathioprine therapy. Molecular analysis for the most common TPMT mutations: TPMT*2 (G238C), TPMT*3A (G460A, A719G), TPMT*3B (G460A) and TPMT*3C (A719G) was carried out in molecular hematology unit, SGRH. Analysis of TPMT*2 (G238C) mutation was done by Amplification refractory mutation system (ARMS-PCR). The other mutations TPMT *3A (G460A, A719G), TPMT *3B (G460A) and TPMT *3C (A719G) were characterized by Polymerase Chain Reaction (PCR) and restriction fragment length polymorphism (RFLP). Further validation of results was done by retesting all the samples by reverse dot blot method, using Vienna Lab PGX-TPMT Strip Assay® kit (Austria).

Briefly, DNA was extracted from peripheral blood using QIAamp DNA Blood Mini kit (Hilden, Germany) as per kit protocol. Obtained DNA was directly used in PCR, after measuring concentration on a Nanodrop spectrophotometer (Thermofisher) at 260 nm, for assessing adequacy. All PCR reactions were performed with 50-80 ng/ul of DNA in 25 μl of the total reaction mixture containing 1.5 mM dNTPs, 10 pmol of each primer and 1 unit of Taq DNA polymerase. Analysis was performed on thermal cycler (Biorad, USA) by using gene specific forward and reverse primers. The amplified products were run on the agarose gel electrophoresis containing ethidium bromide and bands were visualized under UV light. The presence of expected base pair band was indicative of proper amplification. The amplified PCR product was digested by polymorphism specific restriction enzymes and suitable buffer Restriction enzymes used were MwoI, and AccI for TPMT*3B and TPMT*3C, respectively. Digestion products were electrophoresed on 2% agarose gel stained with ethidium bromide. Fragment analysis was done by analysing the size of the digested product. For TPMT*3B, PCR amplicon generated was 365bp in length. The wild-type allele contained the MwoI restriction site; however, the restriction site was absent in the mutant allele. Similarly for TPMT*3C, the amplified PCR amplicon was 293bp in length and products were digested by AccI restriction enzyme. However, in this case the mutant type allele had AccI restriction site; which was absent in the wild-type allele. On the basis of different sized fragments generated, patients were divided into wild type, homozygous or heterozygous mutants. For TPMT*2 mutation, DNA was amplified using a common reverse primer, and two forward primers with the single allele discriminating base at their 3' end. The presence of expected band with either mutant or wild type primer or both were indicative of homozygous mutant, wild type or heterozygous state respectively.

The same DNA was used in reverse dot blot analysis for further validation of the results. The procedure for Reverse dot blot analysis included steps: (1) PCR amplification using biotinylated primers (2) Hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized, as an array of parallel lines (3) Bound biotinylated sequences detected using streptavidin-alkaline phosphatase (Conjugate) and color substrates. PCR amplification was done with 15 **µl** Amplification Mix, 5 **µl** diluted Taq DNA polymerase (1U), 5 **µl** DNA template. Thermocycling conditions were, Pre-PCR: 94°C for 2 minutes followed by thermocycling at 94°C for 15 sec, 58°C for 30 sec and 72°C for 30 seconds (30 cycles) and then final extension of 72°C for 3 minutes. Amplification products were analyzed by gel electrophoresis (e.g. 3% agarose gel) and bands were visualized under ultra violet light. Next hybridisation of PCR products; was done by incubation (45°C) in shaking water bath; with test strips containing immobilized allele specific probes for 30 minutes. After incubation, washing was done two times followed by addition of conjugate solution and incubation again for 15 minutes at room temperature. At the end of incubation, color developer

was added and incubation was done for 15 minutes, at room temperature, in dark on a rocker or orbital shaker. If the reaction comes positive, a purple staining would appear. Genotype was determined by comparing the test strip, with the comparison strip given by the manufacturer (Figure 1) along with kit. This assay covered the three most common polymorphic loci in the TPMT gene: G238C (TPMT*2), G460A (TPMT*3B), and A719G (TPMT*3C).

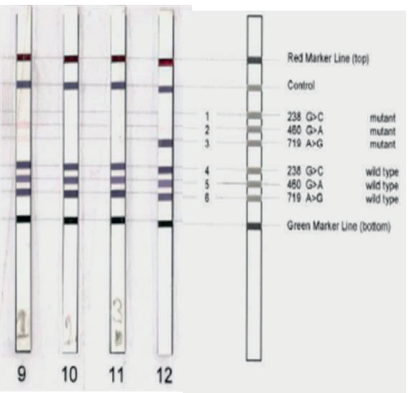

Fig. 1: Representative test strip by reverse dot blot analysis showing positivity for TPMT*3C. Test strips 9, 10 and 11 shows bands in wild type loci only. No band visible in mutant loci. Test strip 12 shows bands in wild type as well as in mutant locus -3 (719 A>G). Hence sample is heterozygous for TPMT*1/*3C. Test strip on extreme right for comparison (supplied by kit manufacturer)

Follow up: Patients who came positive for TPMT mutations; were not put on azathioprine therapy due to ethical reasons and were excluded from further analysis. Patients with wild type normal TPMT genotype were followed for the development of myelosuppression whenever possible. Bone marrow suppression was defined as a white blood cell count of less than 3000/cumm, or a platelet count of less than 50,000/cumm or haemoglobin level below 8g/dL as per disease modifying anti-rheumatic drugs toxicity criteria for azathioprine.[13]

Results

Total 176 patients were studied. All patients studied were Indian ethnicity, belonging to North India. The median age of the patients in our study was 39 years with age range of 18 to 85 years. On sub- grouping of patients as per the age, the majority of patients were in age group 21-60 years. Males and Females were present in equal proportions (0.9:1). Almost half of the patients with immunological disorders were having Neurological etiology (47%) followed by Rheumatology (27%). Rest of the 26% patients covered Gastroenterology, Ophthalmology, Dermatology and Hematology groups (Table 1 describes all the etiologies of the patients of autoimmune disorders recruited in the study).

TPMT genotyping: Out of total 176 patients investigated, majority of the patients (173) were found to be homozygous for wild type normal TPMT*1/*1 (98.29%) genotype. Only 3 patients were characterized as positive for TPMT mutation. Two patients were heterozygous for TPMT*1/TPMT*3C genotype. One patient was heterozygous for TPMT*1/*3A genotype. Other TPMT mutant alleles (TPMT*2, and TPMT*3B) were not identified in any of the patients studied. The genotypic frequency of TPMT mutation in our analysis of 176 patients was 1.7% {TPMT*1/*3C (1.13%), TPMT*1/*3A (0.56%)} (Table 2). No homozygous TPMT mutants were identified. Allele frequency of TPMT*3A was 0.28% and TPMT*3C was 0.56% (Table 2) These three patients were found to be positive by reverse dot blot analysis also while 173 patients were negative; depicting 100% concordance between the two methods. Agarose gel electrophoresis images representative of genotyping experiment results by PCR-RFLP and ARMS-PCR are shown in Figure 2. Test strips of reverse dot blot analysis along with comparison strip are shown in Figure 1. Both the positive patients for TPMT*1/*3C are of myasthenia gravis. The third one with heterozygous

Table 1: Distribution of the patients as per their clinical diagnosis (supplementary material)

Broad speciality (N)	Diagnosis	Number of cases (%)
Neurology (82)	Myasthenia gravis (MG)	38 (46.3)
	Multiple sclerosis (MS)	26 (31.7)
	Neuromeylitis optica (NM)	8 (9.7)
	Transverse myelitis (TM)	1 (1.2)
	Polymyositis	6 (7.3)
	Chronic inflammatory demeylinating polyneuropathy (CIDP)	3 (3.6)
Rheumatology (47)	Systemic Lupus Erythematosus (SLE)	27 (57)
	Vasculitis	8 (17)
	Wegener granulomatosis(WG)	3 (6.3)
	Behcet's Disease	1 (2.1)
	Rheumatoid arthritis	5 (10.6)
	Mixed connective tissue disorder	2 (4.2)
	Eosinophilic granulomatosis with polyangiitis	1 (2.1)
Gastroenterology (27)	Ulcerative colitis	10 (37)
	Crohn's disease	9 (33.3)
	Autoimmune hepatitis	8 (29.6)
Ophthalmology (16)	Geographic helicoid peripapillary choroidopathy (GHPC)	6 (37.5)
	Posterior Uveitis	10 (62.5)
Dermatology (2)	Pemphigus vulgaris	2 (100)
Hematology (2)	Autoimmune haemolytic anemia	2 (100)

Table 2: Individual genotypic and allele frequencies for TPMT variants

TPMT genotype	Genotypic frequency	TPMT alleles	Allele Frequency
Genotype	Number of patients (%)	Alleles	No. of alleles (%)
TPMT*1/TPMT*1	173 (98.29)	TPMT*1	349 (99.1)
TPMT*1/TPMT*2	0	TPMT*2	0
TPMT*1/TPMT*3A	1 (0.56)	TPMT*3A	1 (0.28)
TPMT*1/TPMT*3B	0	TPMT*3B	0
TPMT*1/TPMT*3C	2 (1.13)	TPMT*3C	2 (0.56)
Total	176		352

Table 3: Onset of haematological adverse effects in patients on azathioprine therapy

Onset of adverse effects	Patients with low Hb levels (<8gm/dl)	Patients with low WBC counts	Patients with decreased neutrophil counts	Patients with decreased platelet counts	Total no. patients with myelosuppression
<1 month	0	1	1	1	1(5)
1-5 months	6	8	7	6	12(60)
6-12 months	2	3	3	0	5(25)
12-14 months	1	1	1	0	2(10)
Total patients followed n=114	9 (7.8)	13 (11.4)	12 (10.5)	7 (6.1)	20(17.5)

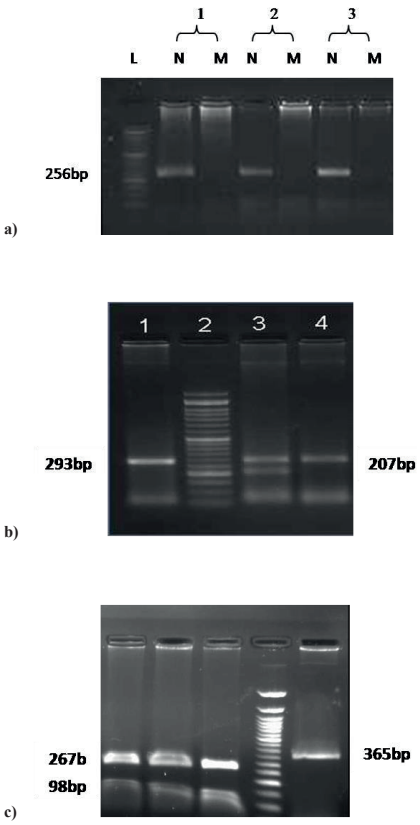

Fig. 2: (a) Representative gel picture showing ARMS PCR for TPMT*2 mutation. Lane 1-3 representing different samples, showing normal wild type genotype. No band is present with mutant primer. (b) Representative gel picture showing PCR-RFLP products for TPMT*3C mutation. Lane 1: Undigested PCR Product, Lane 2: DNA Ladder (50bp), Lane 3: Heterozygous for TPMT*3C and Lane 4: wild type. (c) Representative gel picture showing PCR- RFLP Products for TPMT*3B on 2% Agarose gel. Lane 1-3: Wild type, Lane 4: DNA Ladder (50bp), Lane 5: Undigested PCR product

TPMT*1/*3A genotype was a case of autoimmune hepatitis. All three positive patients were not put on azathioprine because of ethical reasons and hence were excluded from further analysis. Rest 173 patients having wild type TPMT genotype, on azathioprine therapy were followed for development of myelosuppression.

Out of these 173 patients, follow-up was available in 114 patients. In rest 59 patients follow-up was not available despite repeated attempts of communication by emails, telephonic conversation etc. Follow-up ranged from 28 days to 1.5 years with a median of 5 months. On follow-up; 17.5% (20/114) of patients developed myelosuppression. One patient developed severe leucopenia (Total leucocyte count-200/cumm) with very severe neutropenia (ANC-100/cumm) after 28 days of therapy. Majority of patients (60%) developed myelosuppression within 1-5 months of therapy. Leucopenia along with neutropenia was the most common presentation (11.4%) followed by anaemia (7.8%) and thrombocytopenia (6.1%) (Table 3).

Discussion

Azathioprine has been used commonly in autoimmune disorders mainly as a steroid sparing drug during maintenance therapy. However, azathioprine has a generalized effect on bone marrow, inhibiting production of blood-forming cells, which leads to myelosuppression including leucopenia, thrombocytopenia, and anemia. Majority of studies on TPMT mutation prevalence comes from western literature where high prevalence of TPMT mutation has been observed[8]. In Indian scenario, few isolated studies have been done on TPMT mutation prevalence; in different diseases like inflammatory bowel disease, acute lymphoblastic leukemia[12] but none of the researcher have focused collectively on autoimmune disorders as a group where azathioprine is used commonly. To the best of our knowledge, this is the first study from North India on the prevalence of TPMT mutations in autoimmune disorders requiring azathioprine therapy.

We did genotypic analysis for most common TPMT mutations (TPMT*2, TPMT*3A, TPMT*3B, TPMT*3C) by PCR-RFLP and ARMS-PCR. Results were further validated by reverse dot blot analysis of all samples. In the present study, out of total 176 patients, only 3 patients came positive for TPMT mutation giving a very low prevalence of 1.7%. All the three patients were heterozygous, two for TPMT*3C (TPMT*1/*3C) and one for TPMT*3A (TPMT*1/*3A). None of the other variant alleles, which were identified in other studies (TPMT*2, TPMT*3B) were observed. This is in contrast to the Western studies which have reported higher prevalence (10.1%) of TPMT mutations with TPMT*3A as the predominant allele[8]. However, studies done in African and Asian populations have reported lower prevalence of 1.63% and 4.7% respectively[8,14] with TPMT*3C as the predominant allele similar to our study. Table 4 shows frequency distribution of TPMT variant alleles (*2, *3A, *3B and *3C) reported in different regions of the world, in comparison to our study. Indian data on TPMT prevalence varies, with few studies showing higher prevalence (4.9 and 10% in two North Indian studies)[11,12] and others reporting lower prevalence (2.7% in a South Indian study[10]). In North India, Kapoor et al in 2010 studied prevalence of TPMT polymorphism in 71 patients of acute lymphoblastic leukemia.[12] They reported a very high prevalence of 10% with equal frequencies of TPMT*2 (4.2%) and TPMT*3C (4.2%) and a low frequency of TPMT*3A (1.4%). None of the other studies done in India, have shown such a high prevalence and particularly prevalence of TPMT*2 allele which is rare in Asians and has been mainly reported in Caucasians.[8] Another study, from the same institution in 2009 by same author, has reported lower prevalence of 4.9%[11]. Studies done in South India have shown variable prevalence of TPMT polymorphism, ranging from 2.7% to 3.95 % with TPMT*1/*3C as the predominant allele.[10,15] A study done by Murugesan et al in South India, on 326 healthy individuals reported genotypic prevalence of 2.76%. TPMT*1/*3C was the most common variant genotype; present in 1.53% of cases.[10] Our study also showed findings similar to this study with prevalence of 1.7%, and TPMT*1/*3C as the predominant genotype (1.13%). Table 5 shows genotypic frequency of TPMT polymorphisms in different parts of India, in comparison to present study.

We also followed patients with wild type TPMT genotype for development of myelosuppression. As patients with TPMT mutations were not given azathioprine in our study, we were unable to address the question whether myelosuppression was more common in patients having mutant genotype as compared to patients with wild type TPMT genotype. In our study, 17.5% of patients with wild type TPMT genotype developed myelosuppression. A south Indian study done on liver transplant patients has reported myelosuppression

Table 4: Frequencies (%) of thiopurine S-methyltransferase (TPMT)*2, *3A, *3B and *3C alleles reported in different populations of the world in comparison to our study

Population	Number of participants (n)	TPMT*2	TPMT*3A	TPMT*3B	TPMT*3C
Indians (present study)	176	0.0	0.28	0.0	0.56
British Caucasians	199	1	8.5	N/D	0.5
French Caucasians	469	3.0	0.7	N/D	0.4
Polish	358	0.4	2.7	0.00	0.1
African Americans	248	0.4	0.8	N/D	2.4
Brazilians	204	2.2	1.5	0.2	1.0
Kenyans	101	0.0	0.0	0.0	5.4
Libyans	296	0.0	0.61	0.0	1.02
Egyptians	200	0.0	0.3	0.0	1.3
South east Asian	300	0.0	0.0	0.0	1.3
Thai	200	0.0	0.0	0.0	5

N/D – Not determined

Table 5: Frequencies (%) of thiopurine S-methyltransferase (TPMT)*2, *3A, *3B and *3C genotypes reported in different parts of India

Study centre in India	Total no. of participants (n)	Total % Prevalence	TPMT *2	TPMT *3A	TPMT *3B	TPMT *3C	TPMT *2/*3C
Delhi (Present study)	176	1.70	0.0	0.56	0.0	1.13	0
Delhi	120	4.9	0.0	0.8	0.0	4.1	0
Delhi	71	10	4.2	1.4	0.0	4.2	0
Mumbai	225	1.56	0.0	0.0	0.67	0.89	0
Manipal	326	2.76	0.61	0.0	0.61	1.53	0
Vellore	98	6.1	0.0	--	--	5.1	1.02

in almost half (43.2%) of their patients with wild type TPMT genotype.[15] This is in contrast to our study where it was observed in lesser number of patients. However, we defined myelosuppression on basis of DMARD criteria[13] which included patients with moderate to severe myelosuppression as compared to this study which used liberal criteria and included all the patients, even showing mild myelosuppression (White blood cell count < 3500/cumm, Platelet count < 1.5lakh/cumm or haemoglobin level < 11g/dl). Also; patients in their study were on other immunosuppressants like tacrolimus which might be a confounding factor, for assessment of myelosuppression. Moreover they included liver transplant patients and liver itself is a site for thrombopoietin production, which could be a further confounding factor in causing thrombocytopenia in their study.

In present study, majority of the patients (60%) with wild type TPMT genotype, developed myelosuppression within 1-5 months of therapy. This is in contrast to the study done by Gisbert et al. which reported myelosuppression more frequently during the first month of starting azathioprine therapy[16]. However, they did not subdivide patients on the basis of TPMT status, for assessment of myelosuppression. Whereas, in the Sasidharan et al study[15] majority of patients (60%) with normal wild type TPMT genotype developed decrease in blood counts between 1-5 months of therapy similar to our study (60%). These studies along with ours shows that myelosuppression could occur even in patients having wild type TPMT genotype; underscoring the importance of TPMT mutation as the only factor governing side effects of azathioprine therapy. Perhaps; factors other than TPMT; like drug-drug interactions, other pathways of metabolism or other unknown genetic variants could also be associated with myelosuppression, as not all the cases with myelosuppression can be explained by decrease in TPMT activity alone. Moreover though the frequency of TPMT mutation is much lower in Asians as compared to Caucasians, the incidence of myelosuppression is similar in both the ethnicities,[17,18] suggesting the need to look out for other factors, which might be responsible for myelosuppression.

Recently studies have shown importance of NUDT15 gene, in predicting myelosuppression in patients on azathioprine therapy. NUDT15 encodes a nucleoside diphosphatase and is a safeguard mechanism in mammalian cells to minimize DNA damage and avoid subsequent repair and apoptosis. In few studies, patients having NUDT15 polymorphisms, even with wild type TPMT genotypes, have showed myelosuppression and tolerated azathioprine doses, much lesser as compared to patients with normal allele. Studies have shown higher prevalence of this gene mutation in Asians as compared to individuals of European and African ancestry.[19] This could be one of the reasons for the high frequency of thiopurine intolerance in Asian populations despite the low frequency of TPMT variants.

Though prevalence of TPMT mutation is low in Indian setup, it is almost indisputable that patients having TPMT mutations shows severe myelosuppression.[20] Hence our recommendation is to do pretesting for TPMT polymorphism, before starting azathioprine therapy, so that starting doses can be modified based on results and subsequent doses can be adjusted based on routine monitoring of blood counts. Reverse dot blot analysis is a newer upcoming method for detection of TPMT mutations. We observed 100% concordance between PCR-RFLP, ARMS-PCR and reverse dot blot assays. As compared to PCR-RFLP and ARMS-PCR, this assay is easier to perform and less labour intensive and results are available earlier as all the three mutations can be detected in single run. In our study, we observed myelosuppression (17.5%) even in patients with normal wild type TPMT genotype. Studies should be done on other variant genes like NUDT15, which in preliminary studies have shown higher prevalence in Asian population and have been linked with azathioprine induced myelosupression.[19] During our study tenure, we were able to include only 176 patients for calculating prevalence of TPMT mutations. Hence further larger studies are required, for assessing TPMT prevalence in Indian setup.

Acknowledgement

Authors are thankful to Sir Ganga Ram Hospital, New Delhi for providing necessary infrastructure and Mrs Preeti Bhatt for technical assistance for carrying out the molecular tests.

References

1. De Boer NK, Mulder CJ, van Bodegraven AA. Myelotoxicity and hepatotoxicity during azathioprine therapy. *Neth J Med* 2005; 63:444-6.
2. Lennard L, Van Loon JA, Weinshilboum RM. Pharmacogenetics of acute azathioprine toxicity:relationship to thiopurine methyltransferase genetic polymorphism. *Clin Pharmacol Ther* 1989; 46:149-54.
3. Zaza G, Cheok M, Krynetskaia N, Thorn C, Stocco G, Hebert JM et al. "Thiopurine pathway". *Pharmcogenet Genomics*

2010; 20:573–4.

4. Black AJ, McLeod HL, Capell HA, Powrie RH, Matowe LK, Pritchard SC et al. Thiopurine methyltransferase genotype predicts therapy limiting severe toxicity from azathioprine. *Ann Intern Med* 1998; 129:716-8.
5. Schaeffeler E, Eichelbaum M, Reinisch W, Zanger UM, Schwab M. Three novel thiopurine S-methyltransferase allelic variants (TPMT*20, *21, *22) - association with decreased enzyme function. *Hum Mutat* 2006; 27:976.
6. Yates CR, Krynetski EY, Loennechen T, Fessing MY, Tai HL, Pui CH et al. Molecular diagnosis of thiopurine-S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann Intern Med* 1997; 126:608-14.
7. Relling MV, Gardner EE, Sandborn WJ, Schmiegelow K, Pui CH, Yee SW et al. Clinical pharmacogenetics implementation consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin Pharmacol Ther* 2011; 89:387-91.
8. Collie-Duguid ES, Pritchard SC, Powrie RH, Sludden J, Collier DA, Li T et al. The frequency and distribution of thiopurine methyltransferase alleles in Caucasian and Asian populations. *Pharmacogenetics* 1999; 9:37-42.
9. Hon YY, Fessing MY, Pui CH, Relling MV, Krynetski EY, Evans WE. Polymorphism of the thiopurine S-methyltransferase gene in African-Americans. *Hum Mol Genet* 1999; 8:371-6.
10. Murugesan R, Vahab SA, Patra S, Rao R, Rao J, Rai P et al. Thiopurine S-methyltransferase alleles, TPMT(*)2, (*)3B and (*)3C, and genotype frequencies in an Indian population. *Exp Ther Med* 2010; 1:121-7.
11. Kapoor G, Maitra A, Somlata, Brahmachari V. Application of SNaPshot for analysis of thiopurine methyltransferase gene polymorphism. *Indian J Med Res* 2009; 129:500-5.
12. Kapoor G, Sinha R, Naithani R, Chandgothia M.Thiopurine Smethyltransferase gene polymorphism and 6mercaptopurine dose intensity in Indian children with acute lymphoblastic leukemia. *Leuk Res* 2010; 34:1023-6.
13. Australian department of human services. Azathioprine toxicity criteria and severity descriptors for the listing of biological agents for rheumatoid arthritis [INTERNET]. http://www.combinedrheumatology.com.au/pdf/azathioprine- toxicity-criteria.
14. Zeglam HB, Benhamer A, Aboud A, Rtemi H, Mattardi M, Saleh SS et al. Polymorphisms of the thiopurine S-methyltransferase gene among the Libyan population. *Libyan J Med* 2015; 10:27053.
15. Sasidharan S, James E, Sudhindran S. PRE-screening TPMT status of liver transplant patients for azathioprine therapy–A single centre experience from South India. *Int J Pharm Pharm Sci* 2017; 9:84-88.
16. Gisbert JP, Gomollon F. Thiopurine-induced myelotoxicity in patients with inflammatory bowel disease: a review. *Am J Gastroenterol* 2008; 103:1783-800.
17. Bhatia S, Landier W, Hageman L, Kim H, Chen Y, Crews KR et al. 6MP adherence in a multiracial cohort of children with acute lymphoblastic leukemia: a Children's Oncology Group study. *Blood* 2014; 124:2345-53.
18. Cooper SC, Ford LT, Berg JD, Lewis MJ. Ethnic variation of thiopurine S-methyltransferase activity: A large, prospective population study. *Pharmacogenomics* 2008; 9:303-9.
19. Yang JJ, Landier W, Yang W, Liu C, Hageman L, Cheng C et al. Inherited NUDT15 Variant Is a Genetic Determinant of Mercaptopurine Intolerance in Children with Acute Lymphoblastic Leukemia. *J Clin Oncol* 2015; 33:1235-42.
20. Relling MV, Hancock ML, Rivera GK, Sandlund JT, Ribeiro RC, Krynetski EY et al. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J Natl Cancer Inst* 1999; 91:2001-8.