International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 9, Issue 2, 2017

Original Article

PRE-SCREENING TPMT STATUS OF LIVER TRANSPLANT PATIENTS FOR AZATHIOPRINE THERAPY–A SINGLE CENTRE EXPERIENCE FROM SOUTH INDIA

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Received: 12 Oct 2016 Revised and Accepted: 07 Dec 2016

ABSTRACT

Objective: To assess azathioprine-induced bone marrow toxicity and its correlation with thiopurine methyltransferase (TPMT) mutation in liver transplant patients who develop myelosuppression while on azathioprine therapy.

Methods: A prospective observational study was conducted from 1st September 2014 to 30thJune 2015 on 60 liver transplant patients who were tested for *TPMT* allele activity prior to receiving azathioprine. Haemoglobin levels, platelet counts and white blood cell counts of the patients were monitored for the occurrence of myelotoxicity. Patients who underwent liver transplant during the retrospective period from 1st September 2011 to 31st August 2014 and who developed myelosuppression while on azathioprine therapy were also tested for *TPMT* genotype.

Results: A total of 76 liver transplant patients were tested for *TPMT* mutation. Prevalence of *TPMT* mutation in the study patients was 3.95%. The heterozygous *TPMT*1/*3C* genotype was traced in 2.63% of the patients while 1.32% of patients were homozygous for *TPMT*3C* allele. Interestingly 43.4% of patients with wild allele also showed azathioprine-induced myelosuppression. Azathioprine dose of 100 mg showed a higher degree of myelotoxicity than lower doses. Haematological indices of 42.1% of patients normalised on cessation of azathioprine therapy.

Conclusion: Myelosuppression following the introduction of azathioprine was observed in patients with both 'mutant' and 'wild-type' alleles. Therefore a cautious approach has to be taken in pre-screening liver transplant recipients for *TPMT* allele determination in our population. The absence of *TPMT* mutation does not ensure freedom from myelosuppression. Hence regular monitoring of haematological indices of such patients receiving thiopurine therapy should be continued.

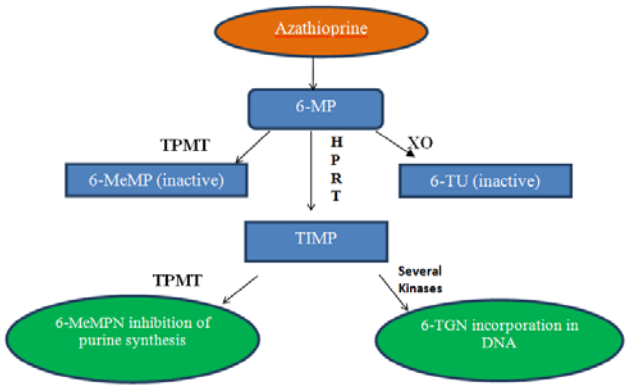
Keywords: Azathioprine, Immunosuppressive regimens, Liver transplantation, Myelosuppression, Thiopurine methyltransferase, *TPMT* genotype testing

DOI: http://dx.doi.org/10.22159/ijpps.2017v9i2.15630

INTRODUCTION

Azathioprine is an immunosuppressant used in patients with organ transplant and autoimmune diseases. It is used to prevent rejection following liver transplantation. Though mycophenolate mofetil has replaced azathioprine in some liver transplant (LT) centres as an immunosuppressant, the clinical benefit of mycophenolate mofetil over azathioprine is questionable. Azathioprine has still a role in maintaining immunosuppression in liver transplant patients especially those taking tacrolimus-based regimens [1].

Azathioprine is a prodrug of mercaptopurine and thiopurine s-methyltransferase (TPMT) is one of the main enzymes that inactivate mercaptopurine. Azathioprine is metabolised in an anabolic pathway to cytotoxic 6-thioguanine and 6-methylmercaptopurine (fig. 1). With a true deficiency of TPMT, there is an increased chance of accumulation of cytotoxic metabolites leading to bone marrow toxicity and myelosuppression. Hence there is a risk of bone marrow suppression in all patients receiving azathioprine. This adverse effect is dose-dependent and can be managed by dose reduction or discontinuation of azathioprine [2, 3].



TPMT: Thiopurine-S-methyltransferase, 6-MP: 6-Mercaptopurine, XO: Xanthine oxidase, 6TU: 6-Thiouric acid, 6-TGN: 6-thioguanine, 6-MeMPN: 6-Methylmercaptopurine, TIMP: 6-Thioinosine 5'-monophosphate, HGPRT: Hypoxanthine phosphoribosyl transferase

Fig. 1: Metabolism pathway of azathioprine

Variation in TPMT activity is related to three distinct *TMPT* mutations and can be identified by TPMT genotyping based on a polymerase chain reaction. Patients with high TPMT activity (wild-type) are found to have two normal alleles. Patients with two non-functional alleles (homozygous) have low or absent TPMT activity and those with one non-functional allele (heterozygous) have intermediate activity [4]. The following alleles are related to decreased levels of TPMT**:** *TPMT*2*, *TPMT*3A*, *TPMT*3B*, *TPMT*3C*. Hence it is suggested that determination of TPMT genotype in patients on azathioprine therapy may improve our ability to manage these patients more effectively and safely by reducing the dose or discontinuing the therapy [3]. The practice of monitoring thiopurine metabolism has been well established in several western countries where the national guidelines suggest that TPMT genotype and/or phenotype determination should be considered for all patients prior to initiation of azathioprine treatment. But there are also reports [5, 6] that non-TPMT determinants may also play a role in azathioprine toxicity.

Though a number of studies [7-14] are available on polymorphism of *TPMT* gene, data on liver transplant patients are scarce from India. Hence the objective of our study was to assess the bone marrow toxicity induced by azathioprine immunosuppressive therapy and to identify any correlation with *TPMT* mutation and myelosuppression in LT patients receiving azathioprine.

MATERIALS AND METHODS

A prospective observational study was conducted on LT patients admitted to the department of gastrointestinal surgery, Amrita Institute of Medical Sciences and Research Centre Kochi, India during 1st September 2014 to 30th June 2015 and who underwent TPMT genotyping. Additionally, patients who underwent LT during the retrospective period from 1st September 2011 to 31st August 2014 and who developed myelosuppression while on azathioprine (AZA) therapy were tested for TPMT genotype. The study was done with the approval of the Institutional Review Board (Pharma/2015/04 dated 01.09.2014). A study information sheet regarding the purpose, risks and benefits of the study was distributed to each of the participants and informed signed consent was obtained from each patient prior to the study. Patients who died within six months of LT were excluded. LT patients of all age groups were included. The demographic details of the patients, social habits, co-morbid conditions, laboratory values and details of immune-suppressive regimens were collected from the medical records and by direct interaction with patients/their caregivers and also from digital hospital information system. All the LT patients were tested for TPMT allele in the molecular biology laboratory of the hospital using Vienna Lab PGX-TPMT Strip Assay® kit. The kit was capable of detecting the genetic variants of TPMT alleles *1, *2, *3A, *3B and *3C. Bone marrow suppression was defined as a white blood cell count of less than 3.5×10^9/l, or a platelet count of less than 150 $\times10^9$/l or a haemoglobin level below 11g/dl.

TPMT activity definitions [4]

High/normal TPMT activity>13.7 units/ml RBC (Red blood corpuscles); Intermediate TPMT activity between 5 and 13.7 units/ml RBC; Low TPMT activity<5 units/ml RBC. The post-transplant status, the introduction of azathioprine therapy, baseline haematological parameters and adverse effects of immune-suppressive regimens were assessed in all the LT patients.

RESULTS

A total of 76 patients, consisting of 60 prospective and 16 retrospective patients, were tested for TPMT genotype. There were 132 patients who were on azathioprine therapy during the retrospective period and severe myelosuppression was observed in 16 of these patients. Hence these 16 patients were tested for TPMT activity. The mean age of the 76 patients tested for TPMT genotype was 43.0±17.1 y (Median age 49 y, Range 1-68 y). The majority (47, 61.8 %) of patients were in the age group 41-60 y. Males constituted 59 (77. 6 %) of the study patients. Thirty-one (40.8 %) patients were alcoholics and 24 (31.6%) were smokers. Indications for LT were a chronic liver disease in 64 (84.2%) patients and acute liver disease in the remaining 12 (15.8%) patients. The co-morbid conditions of LT patients included diabetes in 25 (32.9 %), hypertension in 9 (11.8%), tuberculosis in 3 (3.9%), asthma in 4 (5.3%) and dyslipidaemia in 4 (5.3%) patients. Out of the 16 retrospective patients tested for TPMT activity, 15 had wild-type TPMT allele. TPMT*2, *3A, *3B alleles were absent in our study patients. Distribution of genotype frequencies of TPMT in the liver transplant patients are shown in table 1.

Table 1: Distribution of allele/genotype frequencies of *TPMT* in the liver transplant patients

Type of allele	**No. (%) of alleles (n=152)**
TPMT*1	148 (97.37)
TPMT*3C	4 (2.63)
Genotype	**No. (%) of patients (n=76)**
Homozygous wild	
TPMT*1/TPMT*1	73 (96.1)
Mutant genotypes	3 (3.9)
Homozygous mutant	
TPMT*3C/TPMT*3C	1(1.3)
Heterozygous mutant	
TPMT*1+TPMT*3C	2(2.6)

Out of 76 patients tested for TPMT, normal activity (TPMT *1, wild allele) was detected in 73 (96.1%) patients. Three patients had mutant alleles of which 1 patient (1.3 %) had low levels of enzyme activity and 2 patients (2.6%) had intermediate enzyme activity. Decrease in haematological counts was observed more with an immunosuppressive regimen containing 100 mg dose of azathioprine than lower doses (table 2). Azathioprine was used in combination with tacrolimus and steroids. The median dose of azathioprine was 75 mg daily.

Table 2: Hematological adverse effects of various doses of azathioprine as combination therapy in the study patients (n=44)

AZA dose	**No. of patients with decrease in Hb count(<11g/dl)**	**No. of patients with decrease in WBC count (<3.5K/ul)**	**No. of patients with decrease in platelet count(<150 K/ul)**
25 mg	1	1	1
50 mg	5	5	6
75 mg	0	2	1
100 mg	6	7	9

The onset of adverse effects in patients on azathioprine combination therapy is shown in table 3. The majority of patients showed signs of bone marrow suppression within 1 to 5 mo of starting azathioprine combination therapy, though some patients exhibited more than one type of haematological adverse effects.

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

Onset of adverse effects	No. (%) of patients with decreased Haemoglobin levels	No.(%) of patients with decreased WBC counts	No.(%) of patients with decreased Platelet counts	Total No. (%) of patients
<1month	5(11.3)	3(6.81)	7(15.9)	15 (33.3)
1-5months	7(15.9)	8(18.2)	12(27.3)	27 (60.0)
6-12 mo	0	0	3(6.8)	3 (6.7)

Thirty-nine patients on azathioprine regimen were converted to other immunosuppressive therapy and haematological profiles of 32(42.1%) patients normalised on discontinuing azathioprine therapy. The maximum decrease in haematological counts occurred during 1-5 mo after starting azathioprine. Thirty-three (43.4%) patients with 'wild allele' showed bone marrow suppression after azathioprine therapy. The incidence of leucopenia was 40.8% with azathioprine therapy and 26.3% with mycophenolate mofetil therapy. Table 4 shows the mean variation in haematological counts from baseline. There was 69.7% decrease in WBC counts from mean baseline value and 9.65% decrease in platelet (PLT) counts from the mean baseline platelet count in patients on azathioprine therapy.

Table 4: Change in hematological counts from baseline of patients on azathioprine therapy

Parameters	Hb level (g/dl) (<11)	WBC count(K/ul) (<3.5)	PLT count (K/ul) (<150)
Mean baseline count (2weeks prior to AZA therapy)	9.93±1.5	10.9±9.3	150.2±146.4
Mean variation from baseline	10.7±1.9	3.3±0.8	135.7±54.7
% change from baseline	**7.75%** ↑	69.72% ↓	9.65% ↓

Data expressed as mean±SD (n=27 for Hb, 31 for WBC and 29 for PLT)

Fig. 2 represents the time of introduction of azathioprine therapy in post-transplant patients. Azathioprine was introduced in 11 (34.3%) patients 1 mo after transplantation and 10 (31.2%) patients were started on azathioprine in less than one month of LT and the remaining 39 (65%) patients received azathioprine 2-6 mo after LT.

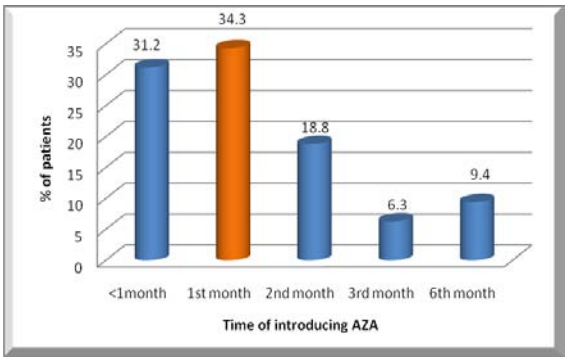


Fig. 2: Time of introduction of AZA in LT patients (n=60) in the prospective study

DISCUSSION

Azathioprine immunosuppressive therapy is used in LT patients for prevention of rejection by inhibiting the proliferation of lymphocytes. However, azathioprine has a generalised effect on bone marrow, inhibiting production of blood-forming cells leading to leucopenia and thrombocytopenia [15]. Genetic variation in TPMT enzyme has been related to the occurrence of toxicity with thiopurine treatment. More than 27 mutations are now documented, but the clinical relevance of some of them remains unclear [16]. TPMT*3A, TPMT*3C, and TPMT*2 represent the most prevalent mutant alleles in Caucasians and Asians, resulting from genetic polymorphism; 6-11% of mutations account for 'intermediate' and 0.3% 'low' thiopurine methyltransferase activity [16]. TPMT*3A, the most common variant, has 5% frequency in Caucasians. The second most frequent variant, TPMT*3C occurs at a frequency of 2% in Asian populations [17, 18]. The present study aimed to evaluate the utility of TPMT allele determination in reducing bone marrow toxicity of azathioprine when used in post-LT patients.

Our study revealed that there is an increased prevalence of TPMT mutation (3.9%) in the study patients. Intermediate TPMT activity was seen in 2.6% and low activity in 1.3% of our patients (table 1). A previous study [8] conducted on 326 healthy individuals from southern India reported a prevalence of 97.2% for the 'wild-type' allele and 2.76% for heterozygous mutation with the absence of any homozygous mutation. TPMT*3A was absent in the study patients and the heterozygous variants were TPMT*1/*2, *1/*3B and *1/*3C. The prevalence of 'wild-type' allele in our LT patients was almost comparable (96.1%), but there was one homozygous mutation and TPMT*3C was the only mutant allele.

When the adverse effects were looked into, 33 (43.42%) patients with 'wild allele' had the propensity to cause decreased haematological counts while on azathioprine therapy revealing that myelosuppression was more often caused by factors other than TPMT mutation. When azathioprine immunosuppressive therapy was compared with non-azathioprine immunosuppressive therapy, leucopenia was relatively two-fold higher in patients on azathioprine therapy and thrombocytopenia was equally observed in both immunosuppressive therapies. In the retrospective design, we selected those 16 patients who exhibited myelotoxicity while on azathioprine specifically to assess TPMT mutation in which one patient was detected with 'intermediate' TPMT activity. In our study, azathioprine or mycophenolate mofetil was used as an adjunct to tacrolimus therapy in post-LT patients. But azathioprine was found to be 4 times cheaper than mycophenolate mofetil and was equally effective.

An observational study was done by Gisbert *et al.* [19] suggests that bone marrow suppression can occur at any time after commencing treatment with azathioprine and more frequently during the first month. Contrary to the above study, our study reveals that the occurrence of bone marrow toxicity was predominantly between 1 and 5 mo after the introduction of azathioprine therapy.

In the present study, the risk of bone marrow suppression was relatively high in patients on azathioprine therapy when compared to patients on other immunosuppressive regimens specifically those containing mycophenolate mofetil. The incidence of leucopenia was 40.8% with azathioprine therapy and 26.3% with mycophenolate mofetil therapy. But these results are contradictory to randomised double-blind comparative study [20] by Wiesner *et al.* which showed that the incidence of leucopenia was more in patients on mycophenolate mofetil (10.1%) compared to patients on azathioprine (7.3%).

It could be speculated that the cause of bone marrow suppression in patients with 'wild' and 'mutant' alleles was azathioprine therapy as the haematological indices normalised on discontinuation of azathioprine. There are studies [21-24] on the relationship between 'low' TPMT activity and the adverse effects of azathioprine and other thiopurines. Some of these studies [23, 24] show a notable increase in adverse effects such as bone marrow toxicity in patients with a mutated TPMT gene. On the other hand, other studies [5, 6] suggest that azathioprine-induced adverse effects can be caused by factors other than TPMT mutations. Our results are in agreement with the study [21] which suggests that myelosuppression during azathioprine therapy in LT patients are caused by factors other than mutations leading to reduced *TPMT* activity. This study from UK suggested that TPMT genotyping do not predict bone marrow suppression in LT patients. Non-TPMT determinants were suggested as a cause of azathioprine-induced myelosuppression in inflammatory bowel disease patients taking the drug [5]. The adverse effects caused by azathioprine may also be influenced by the genotype of the donor since the liver is the principal site of 6-MP methylation [21].

Though the frequency of *TPMT* mutation is much lower in Asians as compared to Caucasians the incidence of myelosuppression is similar to that seen in patients of European heritage. A recent study [25] on Korean patients with Crohn's disease treated with thiopurines found that myelosuppression was associated more with a mutation in *NUDT15* than *TPMT*. *NUDT15* polymorphism was also related to mercaptopurine intolerance in Taiwan Chinese children with acute lymphoblastic leukaemia [26]. *NUDT15* variants are very rare in individuals of European and African ancestry, but are relatively common in people of Asian descent [6, 27]. This could be the reason for the high frequency of thiopurine intolerance in Asian populations despite the low frequency of *TPMT* variants. Hence *NUDT15* can be a target for future studies involving our LT patients who require azathioprine. NUDT15 encodes a nucleoside diphosphatase and is a safeguard mechanism in mammalian cells to minimise DNA damage and avoid subsequent repair and apoptosis [28].

In our study, the test kit for TPMT allele determination was capable of detecting only five TPMT variants (*1, *2, *3A, *3B and *3C). As more than 27 variants have been identified for TPMT gene, it may be possible that the variants not detected in our study might have also been involved in causing myelotoxicity. Even though it is clear that patients with severe deficiency should not receive azathioprine because of the high risk of adverse effects, it is also well known that TPMT deficiency explains only a portion of all azathioprine-related adverse effects [5]. Consequently, opinion is still divided as to whether TMPT activity determination and TPMT genotyping are needed prior to starting azathioprine or 6-MP therapy in Asian patients. Based on our study TPMT allele testing should not be made mandatory in LT patients as a pre-screening test considering the high cost (Rs 5500 per test) of TPMT genotyping. But regular monitoring of haematological indices of LT patients receiving thiopurine therapy should be continued.

CONCLUSION

The prevalence of TPMT mutation among liver transplant patients treated with azathioprine was 3.95%, the majority being heterozygous genotype (TPMT*1/TPMT*3C). However, azathioprine-induced myelo-suppression occurred in 43% of patients with 'wild type' TPMT as well. Therefore a cautious approach has to be taken in pre-screening liver transplant recipients for TPMT allele determination in South Indian population prior to transplantation. Further genetic studies in this population are needed to evaluate other mutations involved.

ACKNOWLEDGMENT

We are grateful to the patients for their participation in the study. Our sincere appreciation to Dr. Prem Nair, Medical Director, Amrita Institute of Medical sciences and Dr. Shanthi Nair, Dean of Research, Amrita Vishwa Vidyapeetham, Amrita University for providing the facilities to carry out the study.

CONFLICTS OF INTERESTS

Declared none

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How to cite this article

- Sagi Sasidharan, Emmanuel James, 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(2):84-88.