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ORIGINAL ARTICLE

Prevalence of *TPMT, ITPA* and *NUDT 15* genetic polymorphisms and their relation to 6MP toxicity in north Indian children with acute lymphoblastic leukemia

Sanjeev Khera[1] · Amita Trehan[1] · Prateek Bhatia[1] · Minu Singh[1] · Deepak Bansal[1] · Neelam Varma[2]

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Abstract

Purpose Toxicity of 6-Mercaptopurine (6MP) is related to single nucleotide polymorphism (SNP) in genes coding for metabolizing enzymes, with *TPMT* analysis being recommended prior to maintenance therapy. However, *ITPA* and *NUDT15* polymorphisms appear more important in the Asian population.

Method In this study 63 consecutive patients with ALL, entering maintenance phase of therapy, were evaluated for *TPMT, ITPA* and *NUDT15* polymorphisms by PCR RFLP and confirmed by sequencing. Hematological and hepatic toxicities were monitored for 36 weeks. The groups with and without any of the three studied polymorphisms (Risk SNP + and Risk SNP-) were compared.

Results Eighteen (28.6%) patients had major polymorphisms, 17 being heterozygous. *ITPA(198CA)*: 11(17.5%); *NUDT (415CT)*: 6(9.5%) *and TPMT*3C*: in 2(3.1%). Mean cumulative dose of 6MP was lower: 10927 mg/m^2 in group with one of the polymorphisms compared to 12533 mg/m^2 in the group without a polymorphism ($p = 0.009$). The group with Risk SNP + tolerated lesser weeks of full-dose 6MP chemotherapy (20.81 vs 30.40 weeks; $p = 0.001$). Risk of neutropenia > 3 weeks was pronounced in Risk SNP + group. The individual *TPMT, ITPA* and *NUDT15* polymorphism subgroups had similar cumulative 6MP dose and chemotherapy interruptions. There was no difference in the average cumulative dose of methotrexate in the two groups. No significant hepatotoxicity was noted.

Conclusion Polymorphisms in *ITPA* and *NUDT15* have a greater prevalence in the north Indian population. Patients with these SNPs tolerate lower doses of 6MP.

Keywords ALL · 6 Mercaptopurine · Single neucleotide polymorphism · Myelosuppression · Drug dose

Abbreviations

6 MP	6-Mercaptopurine
SNP	Single nucleotide polymorphism
ALL	Acute lymphoblastic leukaemia
TPMT	Thiopurine methyltransferase
ITPA	Inosine triphosphate pyrophosphatase
NUDT15	Nucleoside diphosphate linked moiety-type motif 15
ICICLE	Indian Childhood Collaborative Leukaemia Group 2015
MTX	Methotrexate
PCR-RFLP	PCR amplification and restriction enzyme digestion
ALT	Alanine transferase
FN	Febrile neutropenia
ANC	Absolute neutrophil count
HWE	Hardy Weinberg equilibrium
ANOVA	Analysis of variance
6TGN	6-Thioguanine
TLC	Total leucocyte count
CBC	Complete blood count
PCR	Polymerized chain reaction

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✉ Amita Trehan
trehanamita@hotmail.com; trehan.amita@pgimer.edu.in

[1] Division of Pediatric Hematology and Oncology, Department of Pediatrics, Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

[2] Department of Hematology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Introduction

Thiopurine drugs like 6-Mercaptopurine (6MP) are the cornerstone of maintenance therapy in acute lymphoblastic leukaemia (ALL). However being cytotoxic, these drugs are associated with haematological and hepatic toxicities requiring dose modifications and treatment interruptions [1].

Germ line genetic variations determine the inter-individual variability in drug dosing, tolerance, side effect profile and susceptibility of therapeutics commonly used in ALL [2, 3]. Pharmacogenomics when applied in childhood ALL have implicated toxicity of 6MP to single nucleotide polymorphisms (SNP's) in genes encoding 6MP metabolizing enzymes with thiopurine methyltransferase (*TPMT*) being the most researched polymorphism [4]. *TPMT* is inherited as an autosomal co-dominant trait. Individuals homozygous for alleles are *TPMT* deficient while heterozygotes have intermediate enzyme activity, leading to unexpected myelosuppression when patients are treated with recommended doses of 6MP [4]. *TPMT*3A* SNPs have been observed to have a greater prevalence in the Caucasian population [5] with their frequencies being less than 5% in populations of Asian ethnicity [6, 7]. The application of *TPMT* pre-emptive testing in patients with ALL as recommended in European and North American population is conceivably of limited clinical utility in population with Asian ancestry. Recently, SNPs of inosine triphosphate pyrophosphatase *(ITPA198 CA)* and nucleoside diphosphate linked moiety-type motif 15 (*NUDT15415 CT)* have been linked with myelosuppression and possibly are one of the unidentified reasons of high toxicity despite the low prevalence of polymorphism in *TPMT* genes in the Asian population [8–10].

Extrapolating pharmacogenomics into clinical decision making may prove to be a cost-effective way of pre-emptively genotyping all patients who need the drug. Guidelines have already been framed for pre-emptive testing of *TPMT* genotype and their clinical utility in tailoring 6MP doses at start of maintenance therapy in patients with ALL, which will minimize the therapeutic interruptions and subsequent chances of relapse with a resultant influence on the outcome of childhood ALL [5].

This study was performed to evaluate the prevalence of *TPMT, ITPA* and *NUDT15* gene polymorphism in children with ALL in a tertiary care hospital in north India and assess the relationship of these polymorphisms with the doses and subsequently incurred toxicities of 6MP.

Materials and methods

This was aprospective Mendelian randomisation study conducted in a tertiary care hospital in north India from 01 January 2016 to 30 April 2017.

Patients and treatment

All children treated for ALL, entering the maintenance therapy phase of the Indian Childhood Collaborative Leukemia Group 2015 (ICICLE) protocol (the protocol for ALL being followed in the unit) from 01 January 2016 to 30 April 2017, were consecutively enrolled in the study. The patients who were CNS positive and being treated for a relapse were excluded. Informed consent was obtained from all individual participants (or their guardians) included in the study.

As per protocol, maintenance phase includes daily 6MP, weekly oral methotrexate (MTX) along with an intrathecal MTX at 3-monthly intervals. Vincristine and dexamethasone pulses are not given during maintenance therapy. All children were started at a 6MP dose of approximately 60 mg/m^2/day and MTX dose of 20 mg/m^2/week (adjusted to the tablet strength, liquid preparation not being available in the country). A blood sample for genotyping for *TPMT, ITPA* and *NUDT15* was drawn prior to starting maintenance therapy. The enrolled patients were followed with a complete blood count done every 2 weeks and liver function tests done monthly. Side effect profile included; (1) myelosuppression: absolute neutrophil count (ANC) and platelet count, (2) occurrence of febrile neutropenia, (3) hepatotoxicity, (4) cumulative dose of 6MP and (5) duration of treatment interruptions; were recorded for 36 weeks.

Dose adjustment, if any, was done as per routine in the pediatric oncology clinic to maintain an ANC between 750–1500/cumm. The patients, treating clinicians and observers were blinded to the results of gene polymorphism.

Definitions

Myelosuppression ANC < 750/cumm, and/or thrombocytopenia (platelets count < 75,000/cumm).

Hepatotoxicity An increase of the liver enzyme alanine transferase (ALT) to at least 5 times(> 44 U/L) and /or rise of serum bilirubin beyond 3 times the upper limit of normal.

Febrile Neutropenia (FN) Temperature higher than 38 °C with ANC < 500/cumm or ANC showing a falling trend to reach < 500/cumm.

Genotyping

Approximately 2 ml of peripheral blood was collected from study participants in tubes containing sodium EDTA. DNA was extracted using the QIAmp DNA blood kit (Qiagen Inc.) as per the manufacturer's instructions.

(i) *TPMT*: Three sites of known *TPMT* gene polymorphisms[*c.238 G > C(TPMT*2), c.460 G > A(TPMT*3A & 3B)*, and *c.719A > G (TPMT*3A & *3C)*] causing *TPMT* deficiency were determined

according to the method described by Yates [11]. Briefly, allele-specific PCR amplification was used to detect the *c.238 G > C* transversion in exon 5 using primer P2W& P2C for wild-type and P2M & P2C for mutant variant (Supplementary table S1). PCR amplification and restriction enzyme digestion (PCR-RFLP) were used to detect the *c.460 G > A* and *c.719 A > G*SNPs in exon 7 and 10 respectively, using the primers listed in Supplementary table I. RFLP for the variants *c.460 G > A* and *c.719 A > G* were detected using enzymes MwoI (Thermo Fisher Sci.) and ACCI(Thermo Fisher Sci.), respectively. The condition of RFLP enzymes is detailed in Supplementary table S2.

(ii) *ITPA*: The *c.198 CA (P32T)*SNP was genotyped as described previously [12]. Briefly, the genomic DNA was amplified using the primers for exon 2 and 3 (Supplementary table S1). For the *198 CA* SNP, the 328-bp product was digested with enzyme NspI(Thermo Fisher Sci.).

(iii) *NUDT15*: The *c.415 CT (R139C)* SNP was genotyped by amplifying genomic DNA using the primers shown in Supplementary table S1. The endonuclease TaaI (Thermo Fisher Sci.) was used to cut the mutant C > T allele (Supplementary table S2).

The SNP positive cases were further confirmed by Sanger's sequencing using ABI 3130 genetic analyzer (Applied Biosystems) and classified as heterozygous or homozygous.

Author SK recorded the doses received, treatment interruptions and other morbidities in the cohort under study. The study was approved by the hospital ethics committee and departmental review board. A written consent was obtained from parents or guardians.

Statistical analysis

Statistical analysis was performed using IBM SPSS software version 21.0. Allelic and genotypic frequencies, disequilibrium coefficients and the associated standard error for co-dominant traits were calculated. Hardy Weinberg equilibrium (HWE) was calculated for each polymorphism studied. The association between 6MP dose along with adverse effects and gene polymorphisms was tested using Fisher's exact test for comparison between groups and ANOVA for comparison between the subgroups. Odds ratios and 95% confidence intervals were calculated. *P* value < 0.05 was considered as statistically significant.

Results

A total of 67 children with ALL entering the maintenance phase of therapy were enrolled. Four children were excluded (as per criteria) and 63 underwent analysis for gene polymorphisms. Fifty-eight children had a complete follow-up for a period of 36 weeks of maintenance chemotherapy (Fig. 1).

Prevalence of studied gene polymorphisms

Sixty-three children underwent genotyping. Nineteen SNPs were identified in 18(28.5%) children. *ITPA(198CA)* polymorphism was, seen in 11(17.5%) cases, with 10 being heterozygous and one having homozygosity. *NUDT15(415CT)* polymorphism was noted in 6 (9.5%) children, all being heterozygous. *TPMT* polymorphism was seen in only two cases (3.1%). Both *TPMT* polymorphisms were *TPMT*3C(c.719A > G)* and were heterozygous. One case had co-existing *ITPA* and *TPMT*3C* SNP's (Figs. 2, 3).

Hardy Weinberg equilibrium analysis was applied for the three polymorphisms to ensure that the study sample did not violate the assumption of HWE and target population for current study was in HWE at all three loci *p* value [*TPMT* ($p = 0.89$), *ITPA* ($p = 0.53$) and *NUDT* ($p = 0.69$)].

The 58 patients who completed the study were divided into two groups for analysis (Risk SNP + and Risk SNP-). Risk SNP+: group with any of the three studied gene polymorphism

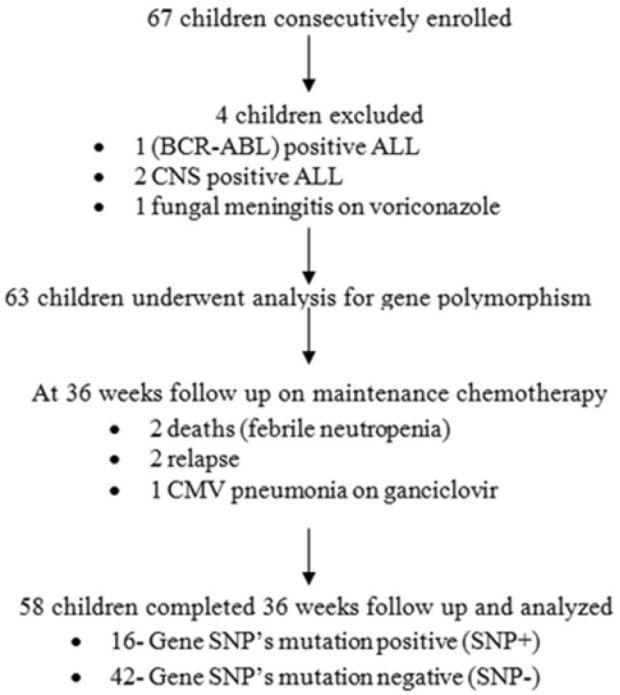

Fig. 1 Patient flow sheet

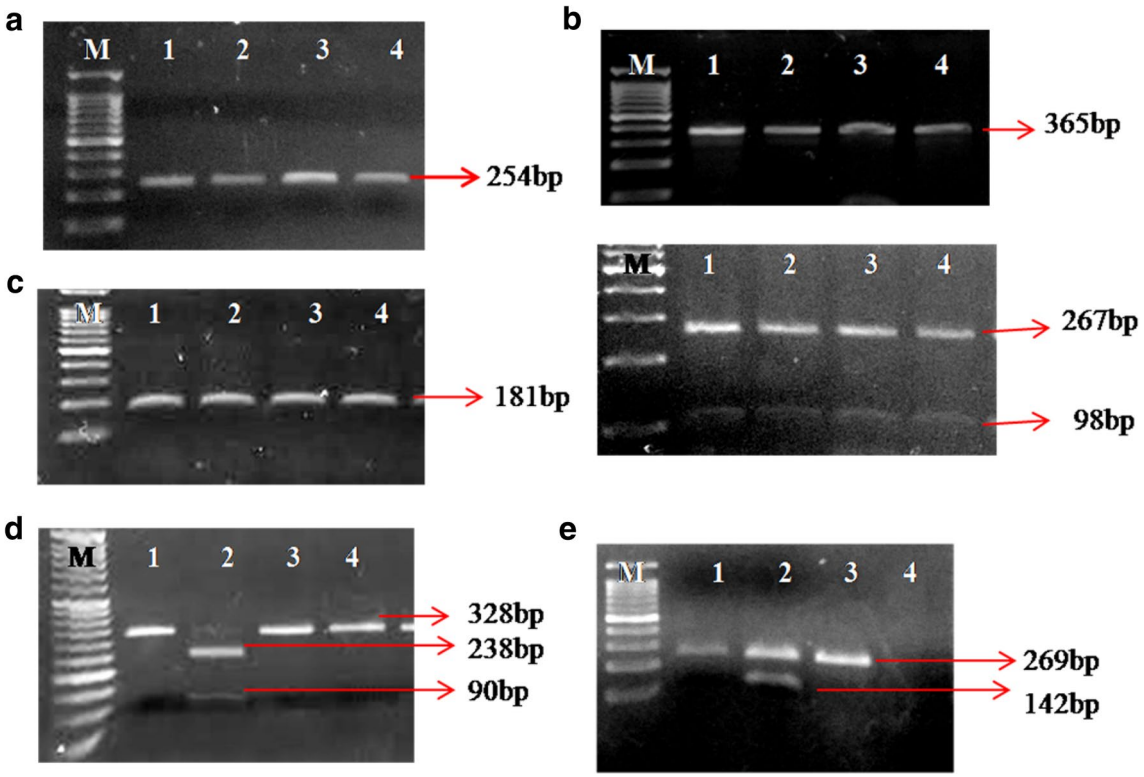

Fig. 2 Screening of genetic variants in *TPMT, ITPA and NUDT15* in representative patient samples (Lane 1–4, M-50 bp marker). **a** Allele-specific PCR showing wild-type *TPMT*2* gene (exon 5); **b** PCR (upper panel) followed by restriction digestion (lower panel) using MwoI showing wild-type *TPMT*3B* (exon 7); **c** Wild-type *TPMT*3C* (exon 10) variants in different patient samples following PCR-RFLP using enzyme AccI; **d** Restriction digestion using NspI of PCR product of *ITPA* (exon 2,3) showing homozygous mutation at c.198 CA (P32T) in lane 2; **e** Restriction digestion using TaaI of PCR product of *NUDT15* (exon 1) showing heterozygous mutation at c.415 CT (R139C) in lane 2

being positive ($n=16$); Risk SNP-group: group with all the three studied gene polymorphism being negative ($n=42$) (Fig. 1).

Comparison of two groups (Risk SNP + and Risk SNP –)

The baseline characters in the two groups including the demographic characters, along with the mean 6MP and MTX dose at start of maintenance were comparable (Table 1).

Cumulative doses of 6MP

The mean cumulative dose of 6MP was 10,927 mg/m^2 (9857–11,997), equivalent to 43 mg/m^2/day in the Risk SNP + group and 12,533 mg/m^2 (11,874–13,191) equivalent to 50 mg/m^2/day in the Risk SNP- group ($P=0.011$) (Fig. 4). The mean cumulative dose of 6MP in the three polymorphisms identified was (1) *NUDT*(415CT) ($n=6$): 10,690 mg/m^2 (7722–13,658), (2) *ITPA(198CA)* ($n=10$) : 10,840 mg/m^2(9569–12,211) and (3) *TPMT**3C ($n=2$) 10,962 mg/m^2 (8946–12,978), respectively, (Supplementary table S3). We analyzed cumulative doses of 6 MP per pair of loci. It was observed that, combined NUDT + ITPA SNP group had significantly lower tolerated dose (10790 mg/m^2) ($p=0.01$), while other groups like NUDT + TPMT, ITPA + TPMT had tolerated 11,072 mg/m^2 and 11,035 mg/m^2, respectively (Supplementary table S3).The mean cumulative dose of MTX was higher in the Risk SNP-group 480 mg/m^2 (451–510) compared to Risk SNP + group 425 mg/m^2 (377–473) ($p=0.051$).

Chemotherapy interruptions

Children with polymorphisms tolerated significantly fewer mean weeks of full-dose 6MP therapy (20.81 vs 30.40 weeks; $p=0.001$). The Risk SNP + group tolerated full-dose chemotherapy for 57% of the time as compared to Risk SNP- group who received full dose for 85% of time in the 36-week follow-up period of the study. On individual SNP analysis, patients with *ITPA(198CA)* tolerated full dose of the drug for 58% of the time and those with *NUDT(415CT)* polymorphism tolerated the drug for 47% time (Supplementary table S3). The Risk SNP + group had significantly

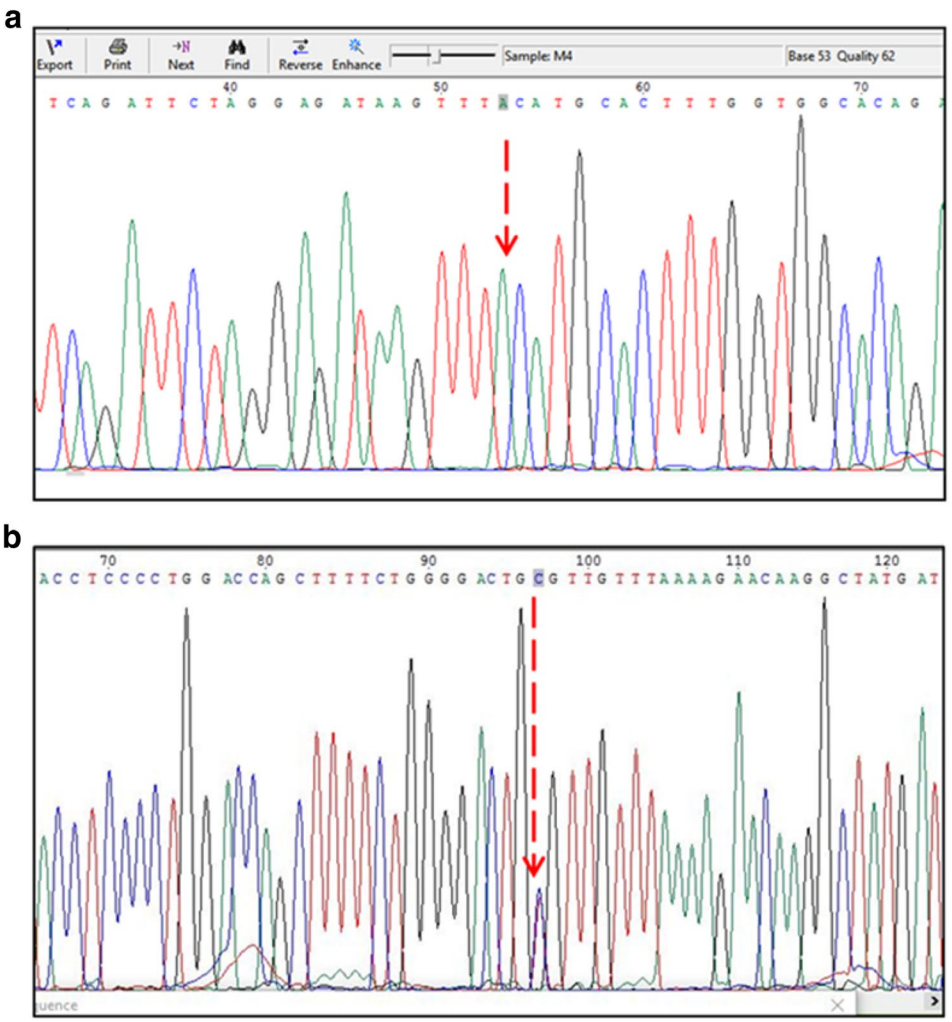

Fig. 3 Representative chromatograms of *ITPA c.198 CA* (P32T) homozygous variant (**a**), and *NUDT15 c.415 CT* (R139C) heterozygous variant (**b**) in Risk SNP + patients. Red arrow denotes site of mutation

Table 1 Baseline characteristics of patients evaluated (Risk SNP+ and Risk SNP-)

Characteristics		Risk SNP + ($n=16$)	Risk SNP – ($n=42$)	Total	*P* value
Age (years)		5.81 (4.0-7.63)	5.24 (4.4–6.07)		0.502
Sex	M	14	34	48	0.551
	F	2	8	10	
Immunophenotype (PreB/ T-ALL)	PreB	12	35	47	0.292
	T	4	7	11	
ALL risk group	SR[1]	7	21	28	0.626
	IR[2]	2	8	10	
	HR[3]	7	13	20	
Mean 6MP dose at start of maintenance ($mg/m^2/day$)(95%CI)		53 (49.85–56.15)	55.69 (54.31–57.07)		0.065
Mean MTX dose at start maintenance ($mg/m^2/wk$)(95%CI)		14.88 (14.08–15.67)	14.67 (13.96–15.35)		0.728

SR[1]- Standard risk, IR[2]- Intermediate risk, HR[3]- High risk

higher mean weeks of 6MP therapy interruptions (4.75 vs 2.79 weeks; $p=0.016$) compared to the Risk SNP- group (Table 2).

The Risk SNP + group had significantly higher mean weeks of neutropenia (4.63 vs 2.69 weeks; $p=0.024$). The risk of prolonged neutropenia (> 3 weeks) was

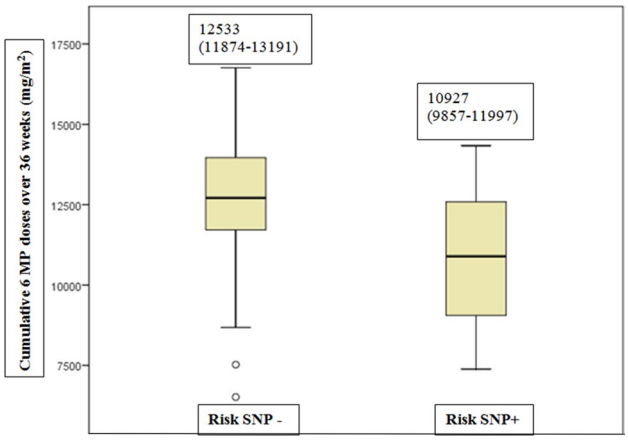

Fig. 4 Mean cumulative 6 MP dose over 36 weeks in two groups ($p = 0.011$)

pronounced in children with polymorphisms with an odds ratio of 6. FN was encountered more frequently (62.5% vs 47.6%) in Risk SNP + group as compared to the Risk SNP- group. However, there was no difference in the mean weeks of FN and thrombocytopenia between the two groups ($p = 0.1$) (Table 2).

Other side effects Hepatotoxicity was evident by increased transaminases in 18.9% of our cohort with the median alanine transferase (ALT) in the cohort being 66.5 IU/L (17–712 IU/L). Serum bilirubin was abnormal in only three cases. However, no case required maintenance therapy to be paused as a result of hepatotoxicity.

Discussion

Inter–patient variability in the doses of 6MP and MTX required to maintain the TLC and ANC in the desired range makes the maintenance phase of therapy in ALL a challenging exercise. It is estimated that 20–25% of children who are treated for a malignancy experience severe drug-related adverse effects [13]. Research in heritable genetic variations in drug metabolism are giving rise to precision medicine/individualized therapy and currently thiopurines are the only drug where there is sufficient evidence for pharmacogenomics to be incorporated into clinical practice with pre-emptive testing for the *TPMT* polymorphism being recommended to avoid serious adverse effects [5].

Polymorphisms in the *TPMT, NUDT 15* and *ITPA* enzymes which metabolize 6MP have been associated with a decreased tolerance to 6MP and these polymorphisms have been noted to have a varying prevalence across different ethnic populations across the globe. *TPMT(*2, *3A and *3C)* is to date the most widely studied SNP for 6MP metabolism. The frequency and pattern of various *TPMT* alleles vary with ethnicity. The incidence is around 10–15% in the Caucasian population (commonly *TPMT*3A*) where currently pre-emptive testing is recommended, with the homozygous variants tolerating only 10% of the recommended doses while the heterozygotes have been observed to be having up to four times risk of neutropenia necessitating a 50% decrease in dose [5]. However, in people of south east Asian ethnicity, literature uniformly reports that the *TPMT* allele(commonly *TPMT*3C*) accounts for less than 5% prevalence, thereby having a limited role in 6MP toxicity. In the data published from the Indian sub-continent, *TPMT* polymorphisms was

Table 2 Comparison of treatment interruptions and side effect profile in Risk SNP + versus and Risk SNP − groups

Events		Risk SNP+ ($n = 16$) (95%CI)	Risk SNP− ($n = 42$) (95%CI)	OR (95% CI)	p value
Treatment interruptions	Full dose (mean weeks)	20.8 (14.7–26.9)	30.4 (27.7–33.0)		0.001
	Full dose (% weeks of follow-up)	57	85		
	Off chemotherapy (mean weeks)	4.7 (3.1–6.3)	2.7 (1.9–3.5)		0.016
Side effect profile(mean weeks)	Neutropenia	4.6 (3.0–6.2)	2.6 (1.9–3.4)		0.024
	FN[1]	2.1 (0.7–3.5)	1.1 (0.5-1.6)		0.10
	Thrombocytopenia	0.50 (−0.3 to 1.3)	0.14 (−0.07 to 0.3)		0.10
Neutropenic events	Nil	2/16 (12.5%)	8/42 (19%)	1.6 (0.3–8.7)	0.710
	> 1 week	13/16 (81.3%)	27/42 (64.3%)	2.4 (0.5–9.8)	0.212
	> 2 weeks	13/16 (81.3%)	25/42 (59.5%)	0.9 (0.7–11.9)	0.120
	> 3 weeks	11/16 (68.7%)	11/42 (26.2%)	6.2 (1.7–21.8)	0.003
FN[1] events		10/16 (62.5%)	20/42 (47.6%)	1.8 (0.5–5.9)	0.3
Hepato-toxicity		1/16 (6.2%)	10/42 (23.8%)	0.2 (0.02–1.8)	0.158

FN[1] Febrile neutropenia

seen in < 5% of patients, with *TPMT *3C* being the predominant allele [14–16].

ITPA & NUDT15 polymorphisms are the other enzymes involved in the metabolism of 6MP. Sanitization of the cellular nucleotide pools from mutagenic base analogues is necessary and *ITPA*/*NUDT* help by removing oxidised deoxyneucleoside triphosphates (dNTP's) responsible for 6MP toxicity [17]. South east Asia reports prominence of *ITPA* polymorphism. The ITPA94CA & *198CA* are the most relevant and widely studied polymorphisms. In south-east Asia, quoted *ITPA* polymorphism prevalence ranges from 20 to 40%. A prevalence of 11–22% has been reported in patients of Indian origin [18]. This polymorphism has been seen in a bare 2% population of Hispanic and European origin, with a 3–5% prevalence reported from South America. Of late, *NUDT15* polymorphisms have also been identified to be associated with intolerance to 6MP. *NUDT15(415CT)* frequency varied from 15% in patients with Thai ancestry to 23% in the Korean population [19]. The prevalence of *NUDT15* polymorphisms is reported to be less than 1% in Europe. Studies from the Middle East and Central America also report a frequency of 1–3% while South America has reported 9% prevalence. Most of these SNPs were heterozygous barring a few homozygous mutations which were seen in less than 5% of cases. In our cohort the prevalence of *TPMT*3C (c.719A > G), ITPA (198 CA)* and *NUDT15 (415 CT)* was observed to be 3.1%, 17.5% and 9.5%. All polymorphisms except one were heterozygous. This distribution is similar to what has been observed from other south east Asian countries.

Germ line genetic variations are important determinants of inter-patient variability in susceptibility, drug response and toxicities of ALL therapy and affect survival and relapse rates [5]. The level of erythrocyte-6TGN is responsible for the tolerated doses and subsequent side effect profile [20]. In our study the mean cumulative dose of 6MP which was tolerated was significantly lower in cases with a detectable polymorphism as compared to wild type (10,927 mg/m^{2}equivalent to 43 mg/m^2/day vs 12,533 mg/m^2 equivalent to 50 mg/m^2/day). Independently, the mean cumulative dose of 6MP in children with the *ITPA* and *NUDT15* polymorphism was lower, though not statistically significant. On analysing various SNP group combinations, NUDT + ITPA was found to tolerate even lower doses of 6MP compared to risk SNP + group. There is conclusive evidence of the relationship of the dose of 6MP and toxicity in individuals with the *NUDT415CT* variant allele with a number of studies, done mostly in Asian countries, establishing these SNP's as significant in the Asian population [3, 8, 10, 21]. There are conflicting reports regarding the effect of *ITPA198CA* SNP on 6MP toxicity (hepatic & myelosuppression) implying the need for more research on the toxicity profile of this allele with the dose of 6MP [10, 18, 22, 23]. We have observed patients with this allele to receive lower mean doses (10,840 mg/m^2 vs 12,533 mg/m^2; $p = 0.05$) of 6MP. No difference was observed with MTX dosing, confirming that the 6MP metabolizing enzymes contribute to differential tolerated dose.

Pauses in therapy during the maintenance phase in ALL have an impact on the outcome of therapy. In our study, patients with any of the polymorphisms tolerated significantly fewer weeks of full-dose 6MP chemotherapy (20.81 vs 30.40 weeks; $p = 0.001$) and had significantly higher mean weeks of chemotherapy interruptions (4.75 vs 2.29 weeks; $p = 0.01$). Prolonged neutropenia (> 3 weeks) was significantly higher in Risk SNP + groups. Our findings are consistent with reports from literature where polymorphisms of *ITPA & NUDT15* were associated with increased neutropenic events with resultant therapy interruptions [3, 8, 10, 17, 19, 21, 23].

There is a definite role of polymorphisms (*TPMT & NUDT15*) in the pharmacogenetics and pharmacokinetics of 6MP. It is clear from literature that polymorphisms differ with ancestry. Research is required for studying promoter polymorphism, respective weightage of each polymorphism in light of racial differences, role of environmental factors and standardizing a method for genotype assessment. The effect of ITPA on toxicity associated with 6MP as yet needs elucidation. Studies with larger sample size and across various ethnicities are required to define the correlation if any, between the variants of *ITPA*/*NUDT15* gene and the long-term survival in ALL, their effect at the molecular level and whether it can be used in prediction of relapse; as seen in case of *TPMT*.

This was a prospective Mendelian randomization study. There are very few prospective blinded studies in literature where all the three prominent gene polymorphisms implicated in 6MP metabolism have been tested with a 6-month follow-up period. The SNP's in gene polymorphisms detected by PCR-RFLP were confirmed with gene sequencing. Our shortcoming is that this is a single centre study with a limited number of patients which precludes adequate statistical inference. Further large geographic area based pharmacogenetic studies will help establish a robust association with pharmacokinetic effects on 6MP before pre-emptive testing of these polymorphisms may be advocated across various ethnic groups.

Compliance ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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