Pharmacogenomics and Personalized Medicine

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ORIGINAL RESEARCH

NUDT15 c.415C>T Polymorphism Predicts 6-MP Induced Early Myelotoxicity in Patients with Acute Lymphoblastic Leukemia Undergoing Maintenance Therapy

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Purpose: Severe myelosuppression in patients with acute lymphoblastic leukemia (ALL) undergoing 6-MP-based maintenance therapy is attributed to *TPMT* gene polymorphisms, which is rare in Asian populations. This study aims to evaluate the role of selected polymorphisms in *NUDT15, ITPA*, and *MRP4* genes in addition to *TPMT* in predicting 6-MP intolerance during ALL maintenance therapy.

Patients and Methods: We screened for the presence of *NUDT15*3* (c.415 C>T, rs116855232); *MRP4* c.2269 C>T (rs3765534), *ITPA* c.94 C>A (rs1127354) polymorphisms in addition to *TPMT* *2 (rs1800462), *3A (*3B and *3C; rs1800460 and rs1142345) in ALL patients with documented severe neutropenia (cohort-1; n=42). These polymorphisms were then screened in a prospective cohort of ALL patients (cohort-2; n=133) and compared with 6-MP dose reduction, early/late myelotoxicity.

Results: Nineteen (45%) patients in cohort-1 and 18 (14%) in cohort-2 had *NUDT15* c.415 C>T variant while 4 (3%) patients in cohort-2 had *TPMT*3C* variant. Five (12%) in cohort-1 and 30 (24%) in cohort-2 had *ITPA* c.94 C>A variant while 9 (22%) and 15 (12%) had *MRP4* c.2269 C>T variant in cohorts-1 and 2, respectively. All in cohort-1 and 36 (27%) in cohort-2 had severe myelotoxicity. Twenty-eight patients (66.6%) in cohort-1 and 40 (30%) patients in cohort-2 had significant 6-MP dose reduction. *NUDT15* c.415 C>T variant explained severe myelotoxicity in 63% and 33% in cohort 1 and 2. *TPMT**3C and *ITPA* c.94 C>A variants also explained myelotoxicity in cohort-2 (Median ANC: 376 vs 1014 mm^3; p=0.04 and 776 vs 1023 mm^3; p=0.04 respectively). *NUDT15* c.415 C>T polymorphism explained significant myelotoxicity (507 vs 1298 mm^3; p<0.0001) in the multivariate analysis as well (β=−0.314, p<0.0001).

Conclusion: *NUDT15* c.415 C>T (15*3), TPMT*3C, as well as ITPA c.94 C>A and MRP4 c.2269 C>T polymorphisms explain hematotoxicities. Preemptive genotype-based (*NUDT15*3, TPMT, ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy.

Keywords: leukemia, mercaptopurine, myelotoxicity, pharmacogenomics

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and also occurs in adults.[1] The last phase of the treatment of ALL involves maintenance therapy with daily oral 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) for nearly 2 years in most treatment protocols. However, the significant dose-limiting toxicity is life-threatening myelosuppression[2] owing to the narrow

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Received: 23 June 2021
Accepted: 2 September 2021
Published: 2 October 2021

therapeutic indices of these drugs and a wide inter-individual variation in drug response. These toxicities often lead to extended hospitalization resulting in increased cost of treatment. Genetic polymorphisms in drug-metabolizing enzymes and transporters contribute significantly to this variability in response to drugs. It is now well acknowledged that polymorphisms in Thiopurine Methyltransferase (*TPMT*)[3] and Nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*)[4–6] genes explain 6-MP mediated cytopenia.

We have reported previously that *TPMT* variants are rare in our population and do not fully explain the severe myelosuppression occurring in patients with ALL undergoing maintenance therapy.[7] Further screening for *NUDT15* c.415 C>T polymorphism in a cohort of patients with severe myelosuppression (but lacking *TPMT* variants) showed that only ~53% of these patients carried *NUDT15* c.415 C>T[8] suggesting that additional genetic factors could play a role. Polymorphisms in Multidrug resistance protein-4 (MRP4 c.2269 C>T)[9,10] and Inosine triphosphate pyrophosphatase (ITPA c.94 C>A)[11–13] have also been shown to explain toxicity to 6-MP in specific ethnic populations. We have previously reported that *TPMT* polymorphisms are rare in our populations and do not entirely explain the variation in 6-MP toxicity.[7] We have recently reported that *NUDT15 c.415C>T* polymorphism is a significant determinant of myelosuppression related to the intake of thiopurines in patients with Immune thrombocytopenic purpura and autoimmune hemolytic anemia.[14] A recent report from the Clinical Pharmacogenetics Implementation Consortium (CPIC) has recommended preemptive genotyping of *NUDT15* apart from *TPMT* variant testing before 6-MP dosing.[6]

Although previous studies in the Indian population have highlighted the role of *NUDT15 c.415 C>T* polymorphism associated with 6-MP toxicity,[15–17] there are no comprehensive reports on all the identified genetic polymorphisms related to 6-MP intolerance and early hematological toxicities in patients with ALL. This study aims to evaluate the role of selected polymorphisms in *ITPA*, and *MRP4* genes in addition to CPIC recommended *NUDT15* and *TPMT* testing in predicting 6-MP intolerance/6-MP dose reduction and toxicities during ALL maintenance therapy.

Patients and Methods

Patients

Patients with ALL who experienced severe clinical thiopurine-related myelotoxicity requiring dose-reduction referred for *TPMT* genetic testing from 2009 to 2017 were included (cohort-1). In addition, all consecutive patients diagnosed with ALL undergoing maintenance therapy between September 2018 and March 2020 in the Department of Hematology, Christian Medical College, Vellore, India, were prospectively enrolled (Cohort-2). The purpose of retrospective cohort 1 is to identify genetic polymorphisms (in addition to *TPMT*) that could explain myelotoxicity, while prospective cohort-2 is to study the influence of genetic polymorphisms on 6-MP intolerance, toxicities, and survival. Patients with less than six-month follow-up or lost follow-up, who underwent consolidation therapy with 6-MP, and those who refused to consent, were excluded from the analysis. Written informed consent was obtained from the patients/parents. This study was approved by the Institutional Review Board [IRB (EC)-ER-1-23-07-2014]. The initial doses of 6-MP and MTX for maintenance therapy were 50 mg/m^2 daily and 20 mg/m^2 weekly, respectively. The treating physicians adjusted the 6-MP/MTX doses to maintain a white blood cell (WBC) count of 3.0×10^9/L and avoid infections and hepatotoxicity.

Genotyping

Before starting maintenance therapy, peripheral blood was collected in EDTA tubes, and DNA was extracted using the Qiagen Gentra kit. The samples were screened for selected polymorphisms: *NUDT15*3* (c.415 C > T, rs116855232, p.Arg139Cys), *ITPA* (c.94 C > A, rs1127354, p.Pro32Thr) and *MRP4* (c.2269 G>A, rs3765534, p.Glu757Lys) by bidirectional sanger sequencing[14] and *TPMT**3A [*3B (460 G>A, rs1800460, p.Ala154Thr) and *3C (719 A > G, rs1142345, p.Tyr240Cys)] by Restriction Fragment Length Polymorphism (RFLP) and [*2(238G > C, rs1800462, p.Ala80Pro)] by PCR using allele-specific oligonucleotides (ASO) as reported previously.[7] Additionally, polymorphisms in *NUDT15* exon1 were screened by Sanger sequencing as reported previously.[18]

Clinical Outcomes

Clinical data such as dose reduction, WBC/ANC counts, and ALT/AST levels were monitored and documented longitudinally throughout the maintenance therapy. %6-MP dose intensity was defined as the ratio between clinician prescribed 6-MP dose to protocol dose (%) and was captured monthly for the first 6-months since the start of 6-MP. We calculated the average 6-MP dose intensity (up to 6 months) for association analysis in the present study. In addition, information on the total direct hospital costs incurred during the first 6 months of

maintenance therapy was obtained for all the patients. Therapy interruption was defined as the cessation of medicine administration resulting from cytopenia, infections, or hepatotoxicity. Hepatotoxicity (Grade 3 and above) was defined based on the Common Terminology Criteria for Adverse Events version 5.0) (CTCAE5.0)[19] (ALT/AST levels >5.0–20.0 x ULN if the baseline was normal; >5.0–20.0 x baseline if the baseline was abnormal at any time point during maintenance therapy). Severe myelotoxicity/neutropenia was defined as ANC (Absolute Neutrophil Count) below 500/mm^3. Early and late myelotoxicity were documented during 1–3 and 4–6 months, respectively, after maintenance therapy. Overall survival (OS) was calculated from the start of maintenance therapy to the date of death or the last follow-up as applicable. Event Free Survival (EFS) was defined as the percentage of patients who were alive without relapse or death at last follow-up through maintenance therapy. Relapse Free Survival (RFS) was defined as the percentage of patients who had no relapse event at last follow-up from the start of maintenance therapy. Relapse and survival were documented for the prospective cohort only.

Statistics

All statistical analyses were performed using SPSS (IBM SPSS statistics version 21.0, Armonk, NY) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA); p-value <0.05 was used for significance testing. Comparison of clinical response indices such as myelotoxicity (ANC), leukotoxicity (WBC), hepatotoxicity (ALT/AST levels), and %6-MP dose intensities with genotype was done using one-way ANOVA or Mann–Whitney *U*-test. Associations between genotypes and clinical responses were evaluated using a linear regression model, and multivariate analysis was performed with significant covariates from univariate linear regression model. Haplotype analyses for all genes were performed using SNPStats programs (Institut Català d'Oncologia, Barcelona, Spain). We used Firth logistic regression to test the association between polymorphism and outcomes (R Statistical software version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria). Log-rank Cox regression was used for the survival analysis, and the Kaplan– Meier curves were generated for OS, EFS, and RFS.

Results

Patient Demographics

The baseline characteristics of patients included in cohort-1 (n=42) are listed in Table 1. During the study period, 241 patients with ALL underwent maintenance therapy in our center. Of these, patients who gave consent to participate in the study and those with regular follow-up were prospectively enrolled (cohort-2; n=133). The study design is illustrated in Figure 1. There was no significant difference in demographics between the patients enrolled in the study and those not enrolled (Table S1). Most of the patients had B-ALL (82.5%) and belonged to intermediate cytogenetic risk (58%) based on stratification reported previously.[20,21]

Genotype

None of the patients carried *TPMT* polymorphisms in cohort-1, while four patients (3%) were heterozygous for the *TPMT**3C variant in cohort-2. Nineteen patients in cohort-1 had *NUDT15 c.415C>T* variant (14 heterozygous and five homozygous), while 18 in cohort-2 were heterozygous for this variant. None of the patients included in the study had polymorphisms in exon 1 of the *NUDT15* gene. The allelic and genotypic frequencies are tabulated in Table S2.

6-MP Dose

In cohort-1, eight patients (19%) required drastic 6-MP/MTX dose reduction (less than 50%), 20 (47.6%) received 50 −80% of the total dose, and 14 (33.4%) received more than 80% of the planned dose of 6-MP.

The majority of the patients in cohort-2 (n=93; 70%) received more than 80% of the planned dose while 36 (27%) received 50–80% of the full dose, and four (3%) patients received less than 50% of the total planned dose of 6-MP.

Incidence of Myelotoxicity and Hepatotoxicity

Although all the 42 patients in cohort-1 had severe myelotoxicity, 16 patients (38%) had severe neutropenic episodes [median ANC<500 (range 32–504/mm^3)], while remaining 26 (62%) had a median ANC of 1100 (range: 528–1760/mm^3) during the study period. Three patients (7%) had a high ALT level (Grade 3), and one patient (2%) had a high AST level (Grade 3) based on NCI CTCAE criteria.

Thirty-six patients (27%) in cohort-2 had severe neutropenic episodes [median ANC<500 (range: 36–507/mm^3)]. Twelve patients (9%) had high ALT levels (Grade 3), and two patients (2%) had high AST levels (Grade 3).

Further outcome analysis was carried out only in cohort-2, where 21 patients (16%) relapsed during or after maintenance therapy, and the 3-year RFS in this cohort was 84.2% at a median follow-up of 18 (6–38) months. One hundred and twenty-eight patients were

Table 1 Baseline Characteristics of Patients

Patient Parameters	Cohort-1 (n=42) N (%)	Cohort-2 (n=133) N (%)
Median Age at Diagnosis (range)	16.5 (2–56)	17 (1–63)
Sex		
Male	35 (83.3)	88 (66)
Female	07 (16.7)	45 (34)
BSA (m^2)	1.5 (0.5–2.2)	1.5 (0.4–2.1)
Immunophenotype		
B cell	31 (73.8)	111 (83.4)
T cell	09 (21.4)	20 (15)
MP-ALL	–	1 (1)
NA	2 (4.7)	1 (1)
Cytogenetics Risk		
Favourable	6 (14.2)	25 (18.7)
Intermediate	25 (59.5)	81 (61)
Poor	7 (16.7)	7 (5.3)
NA	4 (9.6)	20 (15)
ALL Risk		
Standard Risk	17 (40.4)	20 (15)
Intermediate Risk	20 (47.6)	70 (52.6)
High Risk	5 (12)	5 (3.8)
NA	–	38 (28.6)
Polymorphisms		
TPMT variants		
***2A**		
Homozygous reference	42 (100)	130 (100)
Not available	0	3 (-)
***3A (*3B&C)**		
Homozygous reference	39 (100)	123 (96.8)
Heterozygous variant	–	4 (3.2)
Not available	3 (-)	6 (-)
ITPA c.94 C > A		
Homozygous reference	37 (88)	97 (76.4)
Heterozygous variant	5 (12)	28 (22)
Homozygous variant	–	2 (1.6)
Not available	–	6 (-)
MRP4 c.2269G>A		
Homozygous reference	32 (76.2)	107 (80)
Heterozygous variant	9 (21.4)	13 (10)
Homozygous variant	1 (2.4)	2 (2)
Not available	–	11 (8)
NUDT15 c.415C>T		
Homozygous reference	23 (54.7)	115 (86)
Heterozygous variant	14 (33.3)	18 (14)
Homozygous variant	5 (12)	–

alive at the time of the last follow-up. The 3-year OS and EFS were 96.2 and 83.5%, respectively, at a median follow-up of 18 (6–38) months.

NUDT15, TPMT, ITPA, MRP4 Polymorphisms, and 6-MP Dose Reduction/Therapy Intervention

Cohort-1

Although all the patients in cohort-1 had neutropenic episodes and required dose reduction, the *NUDT15 c.415C>T* was identified only in 19 patients and *ITPA* c.94C > A in 3 and *MRP4* c.2269G>A in 2 patients, respectively. While five patients homozygous for *NUDT15* c.415C>T polymorphism were highly sensitive to 6-MP and tolerated only 25% of the median dose intensity (range 14–34% of the protocol dose), those with heterozygous (n = 14) or wild-type genotype (n = 23), tolerated an average dose intensity of 58% (31–94) and 77% (46–96), p= 0.040 and 0.0005, respectively (Figure 2A).

With respect to therapy intervention, patients in cohort-1 who were homozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy [median: 80 (25–94) days] compared to patients who were heterozygous or wild type [21 (7–54) and 28 (7–70) days, p=0.080 and 0.009, respectively] (Figure 2B).

Cohort-2

None of the patients in cohort-2 were homozygous for *NUDT15* c.415C>T polymorphism. Patients heterozygous (n=18 (14%)) for *NUDT15* c.415C>T polymorphism were sensitive to 6-MP compared to those with wild type (n = 115) genotype [%6-MP dose intensity: 82% (45–96) vs 90% (27–100), p=0.034] (Figure 2C).

None of the other polymorphisms screened (*TPMT**3C, *ITPA* c.94 C > A, and *MRP4* c.2269 G>A) were significantly associated with 6-MP dose intensity. With respect to therapy intervention, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy compared to those who were wild type [23 (0–67) vs 14 (0–67) days, respectively, p=0.033] in cohort-2 (Figure 2D).

In the multivariate analysis, there was no significant association between NUDT15 c.415C>T polymorphism and 6-MP dose reduction (Table 2).

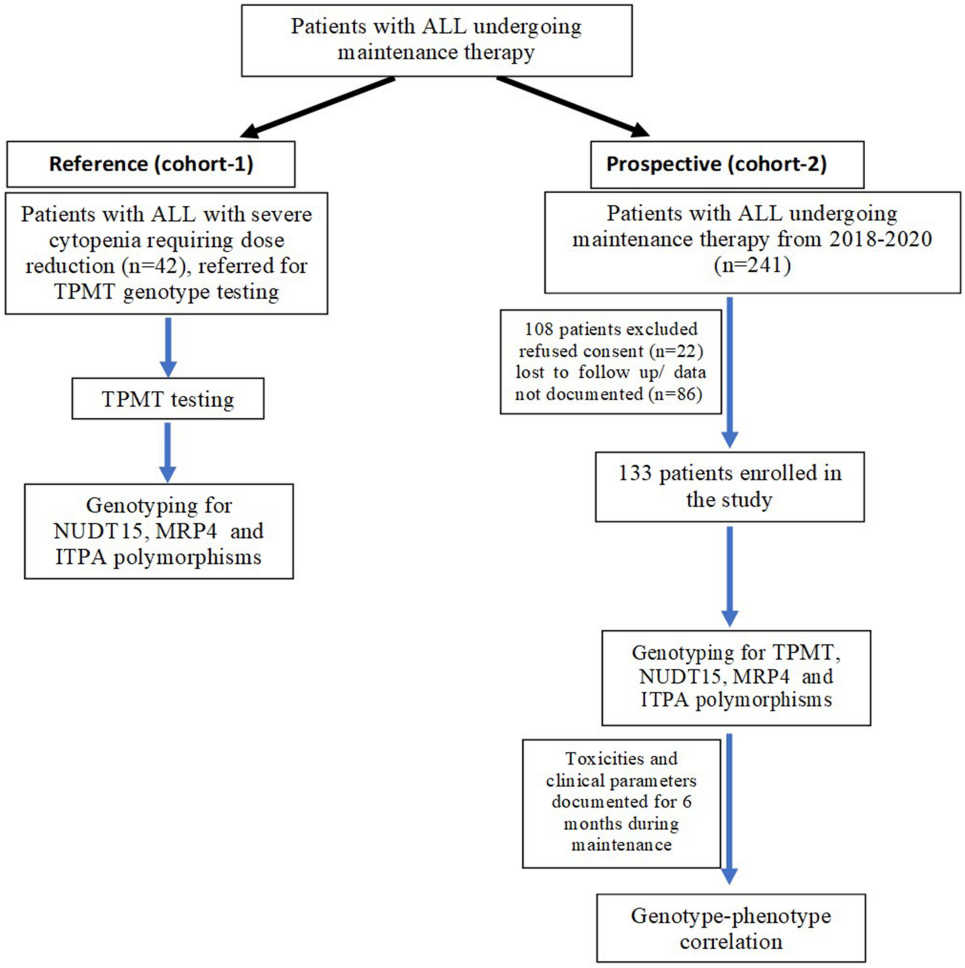

Figure 1 Study design.

NUDT15, ITPA, and TPMT Polymorphisms Explain Myelotoxicity but Not Hepatotoxicity

We then evaluated the association between these polymorphisms and myelotoxicity, hepatotoxicity, relapse, and survival. In cohort-1, patients who were homozygous/heterozygous for *NUDT15* c.415C>T polymorphism had significantly lower ANC [Median ANC-504 (32–1443) mm^3] compared to patients with wild-type genotype [988 (280–1760) mm^3, p=0.006].

In cohort-2, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had significantly higher early (Median ANC: 507 vs 1298 mm^3; p<0.0001) and late myelotoxicity (Median ANC: 982 vs 1517 mm^3; p=0.015) compared to patients carrying wild-type genotype (Figure 3). Additionally, *ITPA* c.94C > A and *TPMT**3C polymorphisms also contributed to myelotoxicity; patients who had risk allele for *TPMT**3C and *ITPA* c.94C > A polymorphism had significantly lower ANC (Median ANC: 376 vs 1014 mm^3; p=0.04 and 776 vs 1023 mm^3; p=0.04 respectively) during maintenance therapy (Figure S1). Upon multivariate analysis (cohort-2), although *NUDT15* c.415C>T, and *TPMT**3C (β=−0.209, p=0.02) were significantly associated with myelotoxicity, *NUDT15* c.415C>T was the most significant variable associated with both early (β=−0.314, p<0.0001) and late myelotoxicity (β=−0.197, p=0.018) (Table 2). None of the polymorphisms tested, including *NUDT15*, was associated with hepatotoxicity, relapse (RFS), and survival (OS & EFS) (Figure S2).

Interestingly, a significant proportion of patients (10 of the 36 patients with severe myelotoxicity in cohort 2 (28%)) carried no variants for any of the screened polymorphisms but still had severe neutropenia [Median ANC-214 (88–420 mm^3)]. Also, 53% of patients (n=21) who received ≤80% of the planned dose of 6-MP [Median % 6-MP intensity-67 (45–79)] did not bear any genetic polymorphisms.

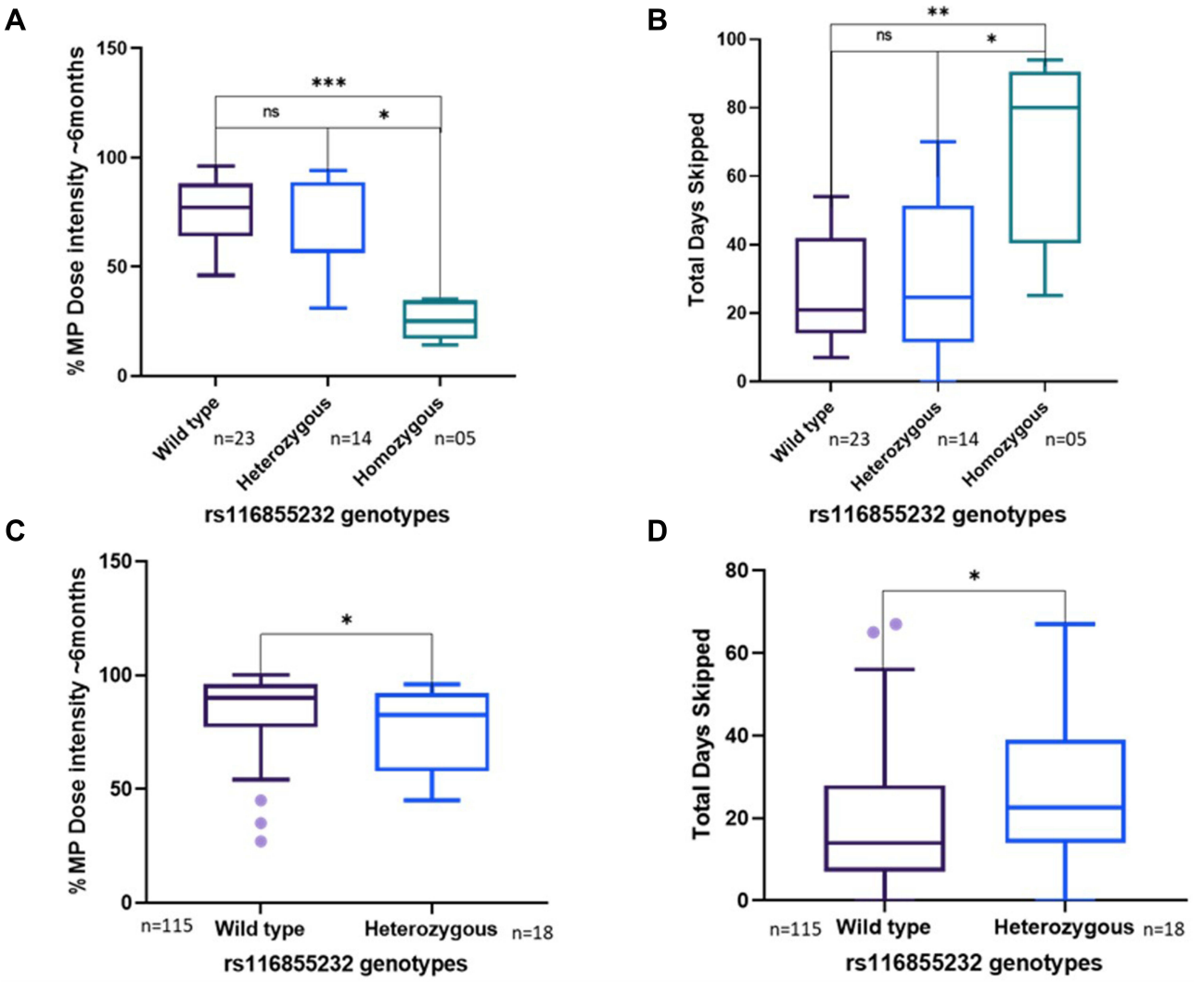

Figure 2 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and % 6-MP dose intensity (**A**) and therapy interruption (**B**) in cohort-1 and cohort-2 (**C**) and (**D**). *Asterisks indicate the level of the significance (p-value); *Means p< 0.05, **Means p< 0.01, ***Means p< 0.001 and ns-not significant.

Combined Effect of Genotypes on 6-MP Intolerance and Toxicities

To evaluate the combined effects of *TPMT, NUDT15, ITPA*, and *MRP4* variants on 6-MP intolerance and toxicities, the patients in cohort-2 with different genotype combinations were grouped-

Group 1- patients wild type for NUDT15, TPMT, ITPA, and MRP4 (n=66);

Group 2- patients with *MRP4* c.2269G>A alone (n=11);

Group 3- patients with *ITPA* c.94 C > A alone (n=21) and

Group 4- patients with *NUDT15* c.415C>T alone (n=12).

Patients carrying the risk allele for *NUDT15* c.415C>T polymorphism alone (Group 4) had increased myelotoxicity (OR-7.33; 95% CI-0.72–3.33; p=0.002) compared to Group 1. Similarly, patients harboring the risk allele for *ITPA* c.94 C > A polymorphism alone (Group 3) also had increased myelotoxicity (OR-3.49; 95% CI-0.12–2.31; p=0.030) compared to patients in Group 1.

Cost Analysis

We then compared the total direct hospital costs incurred during the first 6 months of maintenance therapy between patients with wild-type genotype and those with heterozygous or homozygous for *NUDT15* c.415C>T polymorphism among all patients (Cohort-1 + Cohort-2). The median total direct hospital cost incurred during the first 6 months of maintenance therapy was higher for patients with heterozygous or homozygous compared to patients with wild-type genotype for *NUDT15* c.415C>T polymorphism [20245 (IQR: 12192 to 28746 INR) vs 14356 (IQR: 9939 to 19894 INR), p=0.07]

Table 2 Association Analysis of Genetic Variants and Clinical Response in Prospective Patients with ALL Undergoing Maintenance Therapy

Clinical Response/ Toxicities	Association Analysis*			
Covariates	**Univariate Linear Regression**		**Multivariate Regression**	
	β	**p value**	**β**	**p value**
% MP Dose intensity				
Age	−0.195	0.063	−0.044	0.760
ALL risk group	−0.228	0.027	−0.169	0.134
BSA	−0.191	0.028	−0.166	0.265
MRP4 c.2269G>A	−0.178	0.050	−0.029	0.789
NUDT15 c.415C>T	−0.173	0.047	0.138	0.203
ITPA c.94 C > A	0.155	0.081	0.137	0.200
Myelotoxicity				
Early				
Age	0.162	0.063	−0.062	0.590
BSA	**0.245**	**0.005**	**0.263**	**0.024**
NUDT15 c.415C>T	**−0.328**	**<0.001**	**−0.314**	**<0.001**
Late				
*TPMT**3A	**−0.181**	**0.042**	**−0.209**	**0.025**
NUDT15 c.415C>T	**−0.191**	**0.028**	**−0.197**	**0.018**

Notes: *Amongst covariates tested in both univariate and multivariate regression, only significant variables are listed above. Bold values denote statistical significance at the p ≤ 0.05 level.

Discussion

The present CPIC guidelines[6] recommend *NUDT15* and *TPMT* testing in patients before thiopurine therapy to prevent dramatic myelotoxicity. The frequency of these genetic polymorphisms is noticeably lower in the Indian population than in other populations (Table S3). Therefore, we aimed to explore the role of additional genetic variants over and above CPIC recommended genetic variants. This is the first single centre study to explore the association between *NUDT15* (c.415C>T), *TPMT* (G238C, G460A & A719G), *ITPA* (c.94C>A), and *MRP4* (2269 G>A) polymorphisms vs early and late myelotoxicity to 6-MP in Indian patients with ALL on final maintenance therapy.

Similar to previous reports from other Asian studies[22] and ours,[7] except for the rare occurrence of the *TPMT**3C variant, other *TPMT* polymorphisms were not identified in this cohort. The frequency of the *NUDT15* c.415T allele in the present study is similar to previous studies in India,[15–17] but higher than that of the West Asian population,[23] and lower than East Asian population[24–26] (Table S3).

Patients belonging to Asian ancestry with *NUDT15* c.415C>T polymorphism have been shown to be poor metabolizers of 6-MP and may show intolerance to the drug.[27] Patients homozygous for *NUDT15* c.415C>T polymorphism in the present study tolerated ~25% of the total planned 6-MP dose, which is higher than the previously reported studies[4,5] and the current recommended CPIC guidelines on 6-MP dosing.[4–6] This discrepancy in 6-MP dose intensity (25% in the present study vs 8%[4,5] as per previous reports) can be attributed to different treatment practices. Apart from the *NUDT15*3*, we failed to identify additional polymorphisms in *NUDT15* exon 1 that has been reported to play role in 6-MP intolerance.[28,29] It is possible that additional confounding genetic/non-genetic factors could also contribute to increased tolerance,

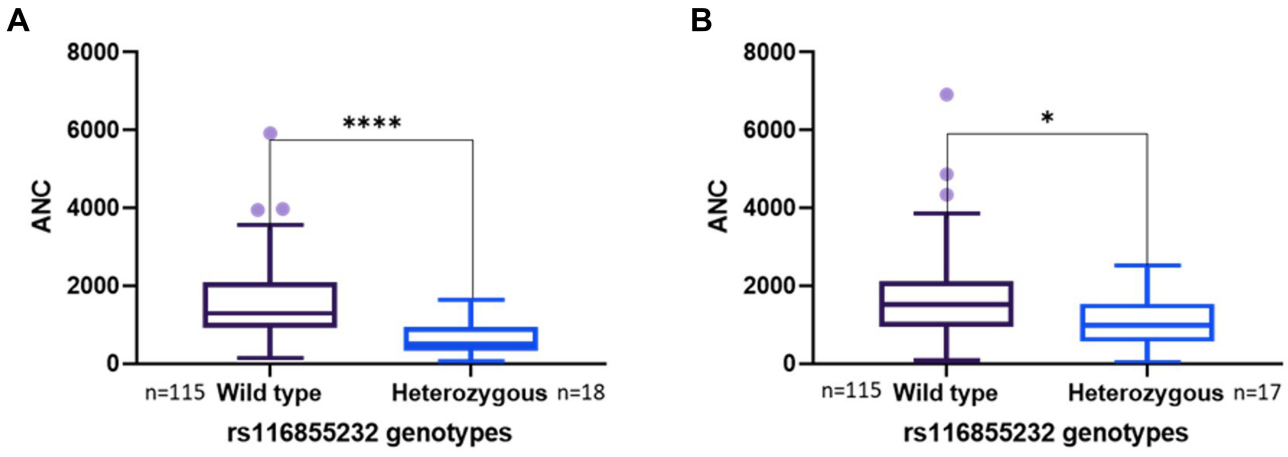

Figure 3 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and (**A**) Early, (**B**) Late Myelotoxicity. *Asterisks indicate the level of the significance (p-value); *Means p< 0.05 and ****Means p<0.0001.
Abbreviation: ANC, absolute neutrophil count.

especially in patients homozygous for *NUDT15* c.415C>T polymorphism in the Indian population compared to other ethnicities, which needs to be further evaluated.

Myelotoxicity is a common adverse drug reaction during 6-MP maintenance therapy that may result in frequent febrile neutropenic episodes affecting treatment outcomes, risk of relapse, and quality of life.[30] Similar to previous studies,[15,25–27,31,32] patients who were homozygous for *NUDT15* c.415C>T polymorphism experienced a significantly higher incidence of marrow toxicities and, as a result, experienced considerably more therapy interruptions/6-MP dose reduction in comparison to patients who were wild-type or heterozygous for this polymorphism (cohort-1). Although there were no patients with homozygous genotype for *NUDT15* c.415C>T polymorphism in the prospective cohort, patients with heterozygous genotype experienced moderate/severe neutropenia leading to considerable dose reduction (Table 3). Although the *TPMT* polymorphisms were rare in our study cohort, patients who were heterozygous for *3C polymorphism experienced significantly increased neutropenic episodes similar to previous studies,[33–35] but no significant 6-MP dose reduction.

ITPA is another crucial enzyme in thiopurine detoxification whose enzyme activity is genetically determined.[11] A missense variant (c.94 C > A) was observed to be a significant determinant of mercaptopurine metabolism and severe febrile neutropenia.[35–37] Consistent with previous reports,[9,10,22] we observed that patients with variant genotype for ITPA c.94 C > A polymorphism experienced neutropenic episodes due to low ANC. Unlike previous studies[13,38] that showed *ITPA* c.94 C > A polymorphism to be associated with lower EFS, we did not find this association in the present study.

A missense polymorphism (c.2269G>A) identified in the *MRP4*/ATP binding cassette subfamily C member-4 (*ABCC4*) has been shown to dramatically enhance 6-MP sensitivity, especially in the Asian population.[39] Tanaka et al[10] have reported a high frequency of 6-MP dose reduction in Japanese children with ALL bearing homozygous variant allele for this polymorphism. These authors subsequently reported a higher incidence of leukopenia in children with risk allele for *MRP4* c.2269G>A polymorphism but not with %6-MP dose intensity.[24] Similar to these reports,[10,24] we observed that patients carrying risk allele for *MRP4* c.2269G>A polymorphism experienced leukopenia (data not shown), albeit no 6-MP dose reduction. Our data suggest the limited clinical utility of *MRP4* pharmacogenetics as compared to *NUDT15* /*TPMT* in ALL.

In addition, we also identified a significant proportion of patients with severe neutropenia yet did not bear any genetic variants screened in this study, suggesting that additional genetic polymorphisms related/unrelated to the thiopurine metabolic pathway could explain 6-MP intolerance/toxicities. It is now well acknowledged that severe neutropenia can also be attributable to methotrexate intolerance[15,40] during maintenance therapy, which was not evaluated in the present study. An exploratory approach to identify additional genetic variants in patients who required dose reduction due to toxicities with no polymorphisms in *NUDT15, ITPA, TPMT*, or *MRP4* genes is ongoing in our laboratory.

Table 3 Influence of *NUDT15* c.415C>T Polymorphism in Early 6-MP Induced Toxicities (Cohort-2)

Clinical Response	Patients with *NUDT15* c.415C>T Polymorphism		
	Wild Type, N=/115 (%)	Heterozygous, N=/18 (%)	Odds Ratio (95% CI), p-value*
MP dose reduced (<100%)	99 (86)	18 (100)	1.6 (−0.56–1.46), 0.361
% MP Dose intensity			
>80%	66 (57)	11 (61)	
50–80%	30 (26)	6 (33)	
<50%	3 (3)	1 (6)	
Neutropenia (ANC <1500 mm^3)	84 (73)	18 (100)	**13.79 (0.57–7.48), 0.005**
Severe neutropenia (ANC <500 mm^3)	24 (21)	12 (67)	**7.18 (0.95–3.07), 0.0001**
Dose interruption	98 (85)	17 (94)	2.07 (−0.75–2.97), 0.373

Notes: *ORs and p-values were calculated using penalized likelihood test-Firth logistic regression method using R. Bold values denote statistical significance at the p ≤ 0.05 level.

Neutropenia and other thiopurine-induced toxicities culminate in increased cost of maintenance therapy. We observed that patients with heterozygous or homozygous NUDT15 c.415C>T polymorphism had higher maintenance therapy-related costs. Therefore, pre-emptive genotype-based (*NUDT15* c.415C>T, TPMT) dosing could benefit Indian patients by decreasing the cost of maintenance therapy, minimizing hospital visits and therapeutic interventions.

The major limitation of the study is its retrospective nature, wherein none of the patients received tailored dosing based on genotype, including *NUDT15* c.415C>T polymorphism. Other limitations include small sample size, wide window of the study period, and lack of validation cohorts. MTX is administered along with 6-MP, which also possesses similar myelotoxicity and hepatotoxicity.[41] However, the present study did not address the role of MTX pharmacogenetics, which again is a major limitation.

Conclusion

Our results suggest that *NUDT15*3, TPMT*3C*, as well as *ITPA* c.94 C > A and *MRP4* c.2269 C>T polymorphisms explain hematological toxicity to 6-MP in patients with ALL undergoing maintenance therapy. Genotyping for *NUDT15, TPMT,* and *ITPA* is routinely performed in ALL patients undergoing maintenance therapy as well as in patients on thioguanine therapy for other conditions including AHA, ITP, inflammatory bowel disease (IBD), and autoimmune disorders at our centre. Preemptive genotype based (*NUDT15*3, TPMT, ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy. Further whole-genome sequencing in patients with severe myelotoxicity but not carrying *TPMT* or *NUDT15* variants is ongoing to identify potential genetic risk for intolerance to maintenance therapy in ALL.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Approval was obtained from the Institutional Review Board [IRB (EC)-ER-1-23-07-2014] of Christian Medical College, Vellore, India. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed Consent

Informed consent was obtained from all individual participants/legal guardians included in the study.

Acknowledgments

We thank Dr Partha Majumdar (NIBMG, Kalyani) and Dr Ram Prasad (MedGenome Laboratories, Bengaluru) for providing the variant allele frequency data of the selected polymorphisms in the Indian population. Technical assistance provided by Ms Sangeetha is gratefully acknowledged. We thank all the medical and nursing staff who managed the outpatients.

Author Contributions

All authors contributed to data analysis, drafting, and revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This study is funded by the Indian Council of Medical Research Centre for Advanced Research grant 70/14/14-CAR to Dr Poonkuzhali Balasubramanian. AAP is supported by a Senior Research Fellowship from DBT, India. VM and PB are supported by Wellcome DBT India Alliance (IA/CPHS/18/1/503930 and IA/S/15/1/501842), respectively. UK is supported by an early career fellowship program of Wellcome DBT India Alliance (IA/CPHE/17/1/503351).

Disclosure

The authors report no conflicts of interest in this work.

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