EFFECT OF NUCLEOSIDE DIPHOSPHATE-LINKED MOIETY X MOTIF AND THIOPURINE METHYL TRANSFERASE GENE POLYMORPHISM ON 6- MERCAPTOPURINE HEMATOPOIETIC TOXICITY IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is responsible for nearly one third of all childhood cancers and can be cured with combination chemotherapy alone. 6- Mercaptopurine (6-MP) is one of the most commonly prescribed chemotherapeutic agents to treat ALL. The main toxicity of this 6-MP is myelosuppression that usually results in interruption or even discontinuation of effective anticancer therapy, contributing to an increased incidence of late relapse.6-MP intolerance is commonly associated with a deficiency in the activity of the enzymes thiopurine S-methyltransferase (TPMT) and NUDT15 (Nucleoside diphosphate-linked moiety X motif 15) that metabolize 6-MP.

Objectives:

1. To determine the pattern of TPMT and NUDT15 gene polymorphism in patients of acute lymphoblastic leukemia
2. To estimate the serum concentration of 6MMP in patients on maintenance therapy of 6 MP at 24 weeks/ during maintenance therapy
3. To find the association between gene polymorphism with the myelosuppression due to 6 MP in ALL patients (treatment related toxicity)

Methodology: Eligible patients on maintenance phase of 6-MP (daily 6MP, weekly oral methotrexate (MTX) along with an intrathecal MTX at 3-monthly intervals) will be recruited. 3ml of venous blood sample for genotyping for TPMT and NUDT15 will be drawn prior to starting maintenance therapy. Myelosuppression, Absolute neutrophil count (ANC) , platelet count, Episodes of febrile neutropenia, Hepatotoxicity, cumulative dose of 6MP and duration of treatment interruptions will be recorded for 24 weeks.

conclusion: The study will help us to estimate the pattern of gene polymorphism prevalent in ALL patient in our population. This may enable the oncologist to determine the dose adjustments needed to increase the therapeutic efficacy and minimize the treatment related toxicity (TRT) of 6-MP in ALL patients.

Keywords: NUDT15 gene, TPMT gene, Acute Lymphoblastic leukemia, Polymorphism

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Background:

Acute lymphoblastic leukemia (ALL) is responsible for nearly one third of all childhood cancers and can be cured with combination chemotherapy alone [1]. 6- Mercaptopurine (6-MP) is one of the most commonly prescribed chemotherapeutic agents to treat ALL. [2-4] The main toxicity of this 6-MP is myelosuppression that usually results in interruption or even discontinuation of effective anticancer therapy, contributing to an increased incidence of late relapse.6-MP intolerance is commonly associated with a deficiency in the activity of the enzymes thiopurine S-methyltransferase (TPMT) that metabolizes 6-MP.[5]

TPMT causes S-methylation of Azathioprine (AZA), 6-MP. Although thiopurines are widely used, adverse drug reactions (ADRs) like gastrointestinal intolerance, pancreatitis, hypersensitivity and myelosuppression are observed in up to 30% of patients. [6,7] Individuals with normal TPMT activity can be given full thiopurine doses, whereas TPMT-deficient patients have to be initiated with drastically reduced doses. Drug metabolites can accumulate in high-risk patients resulting in severe ADR, if they are given standard thiopurine doses. Severe myelosuppression cases with a reduced blood cell production can occasionally be fatal, causing increased susceptibility to infections, clotting abnormalities and anemia. [8].

Hypoxanthine phosphoribosyltransferase (HPRT), Xanthine Oxidase (XO) and Thiopurine Methyltransferase (TPMT) compete to break down 6MP into various metabolites. HPRT is the primary anabolic route of 6MP, leading to pharmacologically active 6TGN. [9].

On the other hand, TPMT methylation diverts a proportion of available substrate away from HPRT pathway to form 6-methylmercaptopurine (6MMP). Therefore, TPMT enzyme activity is inversely related to 6TGN levels, subsequently, to the drug toxicity. Deficiency in TPMT activity will result in more substrate following the HPRT route and the higher concentration of 6TGN produced [10]. On the contrary, a high TPMT activity will result in less 6TGN being produced, posing a potential treatment failure. High 6MMP levels in patients with an increased TPMT activity can inhibit purine biosynthesis and cause liver damage.

The four major TPMT alleles that contain point mutations leading to amino acid substitutions are TPMT*2(238G>C), *3A(460G>A),*3B(460G>A), and *3C(719A>G) .This will account for 80- 95% of people with decreased TPMT activity, and the rest of the alleles are very rare [11-15] TPMT wild-type genotype is associated with an increased risk of relapse and higher levels of residual leukaemia in ALL patients [16-18]. Homozygous and heterozygous individuals, on the other hand, demonstrated higher risks of life-threatening toxicity, myelosuppression, and leukopenia from standard thiopurine doses [19-21].

NUDT15 (Nucleoside diphosphate-linked moiety X motif 15) is hypothesized to dephosphorylate the thiopurine active metabolites TGTP and TdGTP, thus preventing their incorporation into DNA and negatively affecting the desired cytotoxic effects of thiopurines. Recent genome-wide association studies have shown a missense variant in the NUDT15 gene (rs116855232, referred to as the c.415C>T or the p.Arg139Cys variant hereafter) is strongly associated with thiopurine-related myelosuppression in patients with inflammatory bowel disease. [22] and in children with ALL [23]. Individuals homozygous for the risk allele at p.Arg139Cys were exquisitely sensitive to MP and tolerated only 8% of the standard dose, and this NUDT15 variant alone explained 22% of variance in MP tolerance [23].

Most studies have shown that the incidence of NUDT15 allelic mutations in the Asian populations is 8.5–16% [24,25] whereas it is less than 1% in Caucasian populations. [23] Recent studies have also found that NUDT15 c.415C>T variants were associated with thiopurine-induced leukopenia, particularly in Asian populations [24, 26-28]

Preliminary data by using bioinformatics tools:

Testing of the genotype is thus a potential diagnostic tool for identifying individuals prone to poor thiopurine metabolism, and preventing ADR. The overall TPMT heterozygous carrier allele frequency in Indian, Asian and other population vary significantly ranging from 3-15%. [29]. The toxicity of 6-MP in ALL has not fully explained by the TPMT polymorphism. NUDT15 gene polymorphism has shown greater prevalence in the north Indian population. These patients tolerate lower dose of 6-MP. To best of our knowledge there are limited data from south

Indian patient population on gene polymorphism of TPMT and NUDT15and its effect in ALL patients.

In silico analysis of genes using bioinformatics tools avoids screening of a huge number of individuals to detect an association between the gene and the disease at a reasonable level of statistical significance. In other words, these tools help in the pre-selection of SNPs.

Before taking up wet lab-based approaches, if disease-associated SNPs can be identified from neutral SNPs, it would be of great use. In silico analyses are useful when the disease associations could not be established by subsequent independent studies. Hence, independent evidence of functionality of SNPs obtained by using prediction tools could also serve as additional resources to discriminate true associations from false positives.

In silico analysis of the SNPs using bioinformatics tools, SIFT, Polyphen 2 and I Mutant 3 showed that all the SNPs were deleterious (Table 1and 2). Hence selection of these SNPs is justifiable.

In missense mutations of **TPMT gene**, 4 SNPs were selected, Y240C, Y240S, A154T and C70C (table 1). Three SNPs were found to be Damaging with SIFT Score of 0.02, 0.05 and 0.01. These SNPs were analyzed by the PolyPhen tool with score of 1.00, 1.00, 0.993, and 1.00 respectively all of them being probably damaging. I-mutant 3.0 was used to predict the effects of single point mutation on the protein stability. DDG values of binary classification showed values of <0 implying a decreased stability of the protein as a result of the gene polymorphism.

Table 1: In silico analysis of the TPMT gene using bioinformatics tools

Common Allele ID	Codons	Substitution	dbSNP	SNP type	Prediction	SIFT Score	Polyphen Score	Sensitivity	Specificity	Prediction	SVM 2 Prediction effect	DDG value Prediction
6,18130918,1,T/C	TAT-TgT	Y240C	rs1142345:C	Nonsynonymous	Damaging	0.02	1.00	0.00	1.00	Probably damaging	-0.85 Kcal/mol	Decrease
6,18130918,1,T/G	TAT-TcT	Y240S	Novel	Nonsynonymous	Damaging	0.05	1.00	0.00	1.00	Probably damaging	-0.85 Kcal/mol	Decrease
6,18139228,1,C/T	GCA-aCA	A154T	rs1800460:T	Nonsynonymous	Damaging	0.01	0.930	0.81	0.94	Probably damaging	-0.87 Kcal/mol	Decrease
6,18148077,1,G/A	TGC-TGt	C70C	rs186214874:A	synonymous	N/A	1.00	1.00	0.00	1.00	Probably damaging	-0.09 Kcal/mol	Decrease

In missense mutations of **NUDT gene**, 2 SNPs were selected, R139C and R139H (table 2). Both the SNPs were found to be Tolerated with SIFT Score of 0.2 and 0.55. These SNPs were analyzed by the PolyPhen tool with score of 0.995 and 1.00 respectively all of them being probably damaging. I-mutant 3.0 was used to predict the effects of single point mutation on the protein stability. DDG values of binary classification showed values of <0 implying a decreased stability of the protein as a result of the gene polymorphism. Hence it is justifiable to find its association with the effect of drug and therapeutic outcome.

Table 2: In silico analysis of the NUDT gene using bioinformatics tool

Common Allele ID	Codons	Substitution	dbSNP	SNP type	Prediction	SIFT Score	Polyphen Score	Sensitivity	Specificity	Prediction	SVM 2 Prediction effect	DDG valuePrediction
13,48619855,1,C/T	CGT-tGT	R139C	rs116855232:T	Nonsynonymous	TOLERATED	0.2	0.995	0.68	0.97	Probably damaging	-0.74 Kcal/mol	Decrease
13,48619856,1,G/A	CGT-CaT	R139H	rs147390019:A	Nonsynonymous	TOLERATED	0.55	1.00	0.00	1.00	Probably damaging	-0.99 Kcal/mol	Decrease

Research question/ Hypothesis:

TPMP and NUDT15 gene polymorphism is associated with myelosuppression and hepatotoxicity due to 6-Mercaptopurine maintenance therapy phase in patients of acute lymphoblastic leukemia in Indian population.

Specific Objectives:

1. To determine the pattern of TPMT and NUDT15 gene polymorphisms in patients of acute lymphoblastic leukemia receiving 6MP
2. To estimate the serum concentration of 6MP in

patients on maintenance therapy of 6 MP at 24 weeks/ during maintenance therapy
3. To find the association between gene polymorphism with the myelosuppression due to 6 MP in ALL patients (treatment related toxicity)

Methodology

Study Design: Observational Study

Study Setting: Medical Oncology Dept of JKSHCH, Mangalore & KMC Attavar Central Research Laboratory, KSHEMA

Study Period: 3 years from IEC approval

Inclusion Criteria: Patients of either gender diagnosed as ALL, entering to maintenance therapy phase of the Indian Childhood Collaborative Leukemia Group 2015 (ICICL) protocol.
Patients willing to participate in the study.

Exclusion criteria: The patients who are CNS positive and being treated for a relapse.

Sample Size: 65
Formula used :4pq/d2 p- Prevalence of NUDT15 polymorphism – 10%, q= 1-p, d=5 % of error

Methodology:
Clinical Work: Eligible patients on maintenance phase of 6-MP (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 will be started at a 6MP dose of approximately 60 mg/ m2 /day and MTX dose of 20 mg/m2 /week (adjusted to the tablet strength, liquid preparation not being available in the country).

Sample collection: 3ml of venous blood sample for genotyping for TPMT and NUDT15 was drawn prior to starting maintenance therapy. The baseline parameters such as CBC, (Hb%, TC, DC, ESR, Platelet count, LFT were performed and followed with a complete blood count done every 2 weeks and liver function tests done monthly. The patient will be clinically evaluated for signs of gastrointestinal, hemopoietic and hepatotoxicity in addition to routine oncology evaluation for ALL.
Parameters observed: Myelosuppression: Absolute neutrophil count (ANC) , platelet count, Episodes of febrile neutropenia, Hepatotoxicity, cumulative dose of 6MP and (5) duration of treatment interruptions; were recorded for 24 weeks. Dose adjustment, if any, was done as per routine in the pediatric oncology clinic to maintain an ANC between 750–1500/cumm.
Definitions: Myelosuppression ANC<750 cumm and or thrombocytopenia<75000cumm 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 (Temperature higher than 38 °C with ANC).

Laboratory work
Blood sample collection for genetic analysis: 3 ml of venous blood samples will be collected in EDTA (2%) vial. EDTA blood will be utilized for DNA extraction and genotyping. DNA will be extracted from leukocytes by using DNA extraction mini kit.
Quality analysis of the extracted DNA: The quality of the DNA will be checked by electrophoresis on 0.8% Agarose gel, containing ethidium bromide (0.5 µg/ml) in TAE buffer.

Quantification of genomic DNA: The quantification and purity of DNA will be checked by the spectrophotometer (ratio of OD260 / OD280). DNA concentration was calculated using the following formula:
Concentration (µg/ml) of DNA in original solution= Absorbance x 100 x 50 µg/ml.

Amplification and Genotyping of the gene polymorphism: Genotyping of all genes will be confirmed by PCR-RFLP.
Genotyping: 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 will be determined according to the method described by Yates [11]
Allele-specific PCR amplification will be used to detect the c.238 G>C transversion in exon 5 using suitable forward and reverse primers for wild-type and r mutant variant. PCR amplification and restriction enzyme digestion (PCR-RFLP) will be used to detect the c.460 G>A and c.719 A>GSNPs in exon 7 and 10 respectively. RFLP for the variants c.460 G>A and c.719 A>G will be detected using enzymes MwoI (Thermo Fisher Sci.) and ACCI (Thermo Fisher Sci.), respectively.
NUDT15: The c.415 CT (R139C) SNP will be genotyped by amplifying genomic DNA using the suitable primers. The endonuclease TaaI (Thermo

Fisher Sci.) will be used to cut the mutant C>T allele.
6MP will be estimated either at 24 weeks (those who do not manifest with any of the above mentioned TRT or at the time of TRT (Myelosuppression, hepatotoxicity, grade 3 or 4 GIT toxicity. (diarrhoea)

Ethical Considerations: IEC approval and ISC approval will be obtained. Patients will be recruited with the written informed consent/ascent.

Statistical Analysis: Statistical analysis will be performed using IBM SPSS software version 23.0. Allelic and genotypic frequencies, disequilibrium coefficients and the associated standard error for co-dominant traits will be calculated. Hardy Weinberg equilibrium (HWE) will be calculated for each polymorphism studied. The association between 6MP dose along with adverse effects and gene polymorphisms will be tested using Fisher's exact test for comparison between groups and ANOVA for comparison between the subgroups. Odds ratios and 95% confidence intervals will be calculated. P value < 0.05 is considered as statistically significant.

Significance of the study
The study will help us to estimate the pattern of gene polymorphism prevalent in ALL patient in our population. This may enable the oncologist to determine the dose adjustments needed to increase the therapeutic efficacy and minimize the toxicity related toxicity (TRT) of 6-MP in ALL patients**.**

Conclusion: Genetic polymorphisms of TPMP and NUDT15 may influence 6 MP metabolism and hence the steady-state concentration of the drug in plasma may be altered. This may demand an increased or reduced dose of 6MP in ALL patients to maintain the therapeutic levels as well as for minimizing the adverse drug reactions. The large inter-individual variability may reflect functional consequences of genetic polymorphisms in genes encoding drug-metabolizing enzymes. The concept of "individualized medicine" is evolving and there has been a paradigm shift from the concept of "one drug fits all" to "right drug for the right patient at the right dose and time." Hence it is very important to investigate the possible roles of genetic polymorphisms in the metabolism of 6MP. There are only a few studies on the effect of TPMP and NUDT 15 gene polymorphism on 6MP metabolism, especially in Indian settings to the best of our knowledge.

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