Indian Journal of Biochemistry & Biophysics
Vol. 61, June 2024, pp. 371-379
DOI: 10.56042/ijbb.v61i6.9285

In vitro cytotoxicity analysis on patient-derived PBMCs to predict 6-mercaptopurine therapy response in children with acute lymphoblastic leukemia

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Received 04 March 2024; *revised 12 March 2024*

6-Mercaptopurine (6-MP) is a critical medication used in the remission-maintenance phase of pediatric acute lymphoblastic leukemia (ALL). Mercaptopurine has a narrow therapeutic index with treatment-related toxicities and resistance as major challenges. In addition to well-documented pharmacogenetic determinants, various other molecular mechanisms are known to influence 6-MP adherence. A predictive therapy response analysis encompassing all these molecular factors would help in accomplishing personalized 6-MP therapy goals to mitigate relapse and toxicity. We aim to study *in vitro* 6-MP cytotoxicity results in correlation with the erythrocyte 6-MP metabolite profile to predict survival in pediatric ALL. Peripheral blood samples were collected from 56 children with ALL and 10 healthy individuals. *In vitro* cytotoxicity was performed on patient-derived peripheral blood mononuclear cells (PBMCs) (n=30) using the WST-1 assay. Erythrocyte methyl-mercaptopurine (MMP) and thioguanine (TG) levels were measured using UPLC-MS/MS. Thiopurine methyl transferase (*TPMT*) pharmacogenetic variants (rs1800460, rs1800462, and rs1142345) were analyzed using TaqMan probe-based genotyping assay. Subjects were stratified into sensitive, responders, and non-responder groups based on the cytotoxicity assay results. Non-responders showed higher erythrocyte MMP levels (P = 0.023), and the sensitive group had higher TG levels (P = 0.008). Non-responders had a diminished overall survival compared to sensitive and responder groups (P = 0.043). *In vitro* cytotoxicity assay on patient-derived PBMCs provides a plausible platform for predicting 6-MP therapy response and relapse/ death in children with ALL.

Keywords: 6-thioguanine, ALL, Methyl-mercaptopurine, Myelotoxicity, Thioguanine nucleotide

Acute lymphoblastic leukemia represents the most prevalent childhood hematological malignancy, characterized by the uncontrolled proliferation of lymphoblasts[1]. It constitutes approximately 25% of all pediatric cancers occurring within the age range of 1-19 years[2]. In maintenance therapy of pediatric ALL, 6-mercaptopurine drug (6-MP) is employed as daily oral administration to maintain complete remission[3]. Mercaptopurine is a prodrug formulation of thiopurine antimetabolite; once inside the cell, it gets metabolized into an active purine nucleoside analog known as thioguanine nucleotide (TGN)[4]. TGN is a structural analog of guanine that gets incorporated into the newly synthesized DNA and RNA of the rapidly proliferating lymphoblasts. The cytotoxicity of 6-MP is majorly attributed to DNA and RNA damage leading to apoptosis[5]. However, a comprehensive understanding of 6-MP cytotoxicity modalities is still an area of research interest.

The optimal drug response depends on various molecular mechanisms that impact pharmacokinetics and pharmacodynamics. Pre-emptive pharmacogenetic analysis of *TPMT* (thiopurine methyl transferase) and therapeutic drug monitoring for 6-MP metabolites in RBC are clinically utilized to assess the therapy response[6,7]. Mercaptopurine metabolites are generally accumulated in the erythrocytes, and their levels are assessed using either HPLC or LC-MS/MS[8-10]. Therapeutic drug monitoring of 6-MP and its key metabolites methyl-mercaptopurine (MMP) and thioguanine (TG) represents the therapeutic efficacy[11]. MMP and TG metabolite levels are required in the therapeutic range for adherence to 6-MP treatment during the maintenance phase. However, a significant number of patients do not show optimal therapy response, thereby developing either toxicity or resistance towards 6-MP[12].

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The existing literature supports the role of pharmacogenetics, pharmaco-transcriptomics, and drug metabolism in influencing the safety and efficacy of thiopurine therapy[13-15]. *In vitro* cytotoxicity assay represents the effects of all these factors specific to the individual and helps in predicting the drug response[16].

About 20-25% of patients show either early or late relapse making it the major cause of death in pediatric ALL[17]. Optimizing thiopurine therapy requires a balance between reducing the risk of relapse and minimizing treatment-related toxicities. A combined assessment of 6-MP pharmacogenetics, TDM of mercaptopurine metabolites in RBC, and *in vitro* cytotoxicity assay on patients' PBMCs helps in achieving the personalized therapy goals. So far, no comprehensive studies exist on the *in vitro* 6-MP cytotoxicity and metabolomics in conjugation with therapeutic outcomes in children with ALL. Cytotoxicity assay on patient-derived peripheral blood mononuclear cells (PBMCs) provides a real-time information on the patient's response to the drug. Determining the *in vitro* 6-MP IC_{50} allows for individual-specific dose adjustments for enhanced treatment effectiveness. Hence, this study aims to assess the *in vitro* response towards 6-MP in the PBMCs and correlate with erythrocyte 6-MP metabolite profile and clinical outcomes in children with ALL (Fig. 1).

Materials and Methods

Study Participants

Whole blood samples (5 mL) were collected in EDTA-K2 anticoagulant tubes from 56 children with ALL during the maintenance phase of the treatment and 10 age-matched healthy subjects. The written informed consent was obtained from all the study participants/guardians as per the declaration of Helsinki. This study was approved by the Institutional Ethics Committee of Nizam's Institute of Medical Sciences (EC/NIMS/2307/2019) and Apollo Institute of Medical Sciences and Research (AIMSR/001/2017).

PBMC and RBC preparation

PBMCs were isolated following the manufacturer's protocol. Briefly, a 3 mL of whole blood was

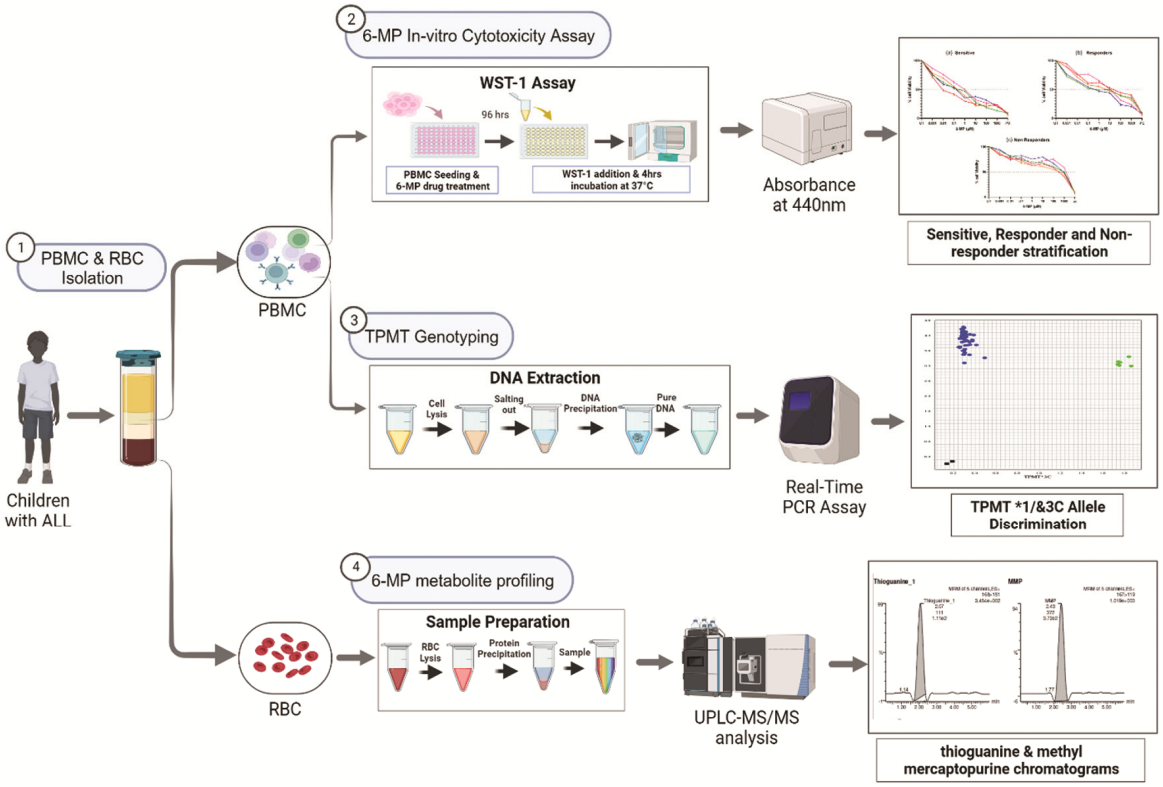

Fig. 1 — Workflow: Methodology adopted in the study design (1) Sample collection, peripheral blood mononuclear cells and erythrocytes isolation using density gradient centrifugation (2) *In vitro* cytotoxicity assay on patient-derived PBMC and stratification of subjects into sensitive, responder, and non-responder groups. (3) Thiopurine methyltransferase single nucleotide variants genotyping using TaqMan genotyping assay kits on a Real-Time PCR platform (4) Measurement of erythrocyte thioguanine and methyl-mercaptopurine levels using Ultra Performance Liquid Chromatography-Mass Spectrometry

overlayed carefully above the Lymphoprep gradient medium (STEMCELL™ Technologies) and subjected to centrifugation at 1800 rpm for 30 min at room temperature[18]. The interphase rich in lymphocytes was collected, washed with Dulbecco's Phosphate-buffered saline (PBS), and proceeded for cytotoxicity assay. The packed RBC fraction was washed twice with PBS, counted, and stored at -80°C until further LC-MS analysis.

DNA isolation by the salting-out method

One part of PBMCs was used to isolate the DNA following the salting-out method[19]. Briefly, 1 million PBMCs were lysed with TKM2 buffer and 10% SDS at 37°C for 5 min. The proteins were salted out using a 5M NaCl solution, and DNA was precipitated from the supernatant using ice-cold ethanol. The DNA was washed twice with 70% molecular grade ethanol and dissolved in TE buffer (pH-8.0). The purity and concentration of the DNA were analyzed using a NanoDrop™ spectrophotometer.

TPMT genotyping

Single nucleotide variants (SNVs) of the TPMT gene were evaluated using Taqman Genotyping assays for the rs1800460, rs1800462, and rs1142345 following the manufacturer's protocol. Briefly, 10 ng of genomic DNA was used per reaction consisting of TaqMan™ Genotyping Master Mix and SNV-specific assay mix (Assay ID: C_30634116_20, C_12091552_30, C_19567_20) on a QuantStudio™ 5 real-time PCR system.

Cell line and *Ex vivo* PBMC culture

Molt-3 cell line and PBMCs isolated from study participants were cultured in a complete RPMI-1640 medium with 10% fetal bovine serum[20], 1X Gibco™ MEM Non-Essential Amino Acids and 1X antibiotic-antimycotic, at 5% CO_2 and 37ºC temperature in a humidified incubator.

Cell proliferation and 6-MP treatment

The cell proliferation was induced with anti-CD3 followed by anti-CD28 antibodies (BioLegend®) following the manufacturer's protocol. Briefly, 100 µl of anti-CD3 antibody preparation (10 µg/mL) was added to the flat-bottomed 96-well plates and incubated for an hour at 37ºC for antibody adherence to the well surface. Later, the solution was aspirated out, and molt-3 cells or PBMC fraction in complete RPMI medium with anti-CD28 antibody (2.5 µg/mL) was added to the wells. After 4 h of activation, the cells were treated with 6-MP (10 nM-100 µM) dissolved in 0.01 N NaOH. The control wells were treated with 0.01 N NaOH to compensate for the vehicle effect on cell proliferation.

***In vitro* WST-1 cytotoxicity assay**

After 96 h of the 6-MP treatment, the cytotoxicity assay was carried out using the WST-1 cell proliferation assay kit (Takara Bio) following the manufacturer's protocol. Briefly, 10 µL of WST-1 premix was added into each well and incubated for 4 h in a 5% CO_2 incubator. The absorbance was measured at 440 nm, and IC_{50} values were derived. Among the 56 samples, 26 samples had low PBMC counts and did not respond to proliferation induction. The cytotoxicity assay was performed successfully on 30 patient samples. Erythrocyte metabolite levels were quantified in these 30 study participants using UPLC-MS/MS.

Erythrocyte 6-MP metabolite estimation by UPLC-MS/MS

All the patients were on maintenance therapy for at least eight weeks at the point of sample collection, as a minimum of 4 weeks of 6-MP administration is required to achieve steady-state metabolite levels[21]. Samples were analyzed on Quattro Premier XE MS/MS coupled Acquity™ UPLC (Waters, Milford, MA, USA). The sample preparation and analysis followed the method described elsewhere[8] and was modified to the packed RBC samples. Briefly, 20 µL of ISTD (Verapamil-5 µg/mL) and 300 µL of Acetonitrile were added to 200 µL erythrocyte suspension (8 x 10^8 RBC); Vortexed and centrifuged at 4000 rpm, for 10 min. The supernatant layer of 200 µL was transferred into the ria-vial, and a 10 µL sample was injected into LC-MS/MS system. A straight-line fit was made through the data points by 1/X weighting method. The method was found to be linear between the range of 5 ng/mL to 1000 ng/mL for 6-MP (r^2=0.99) TG (r^2=0.98) and MMP (r^2=0.99) (Fig. 2).

Statistical analysis

Statistical analyses were done using Jamovi™ and GraphPad 9.4 (GraphPad Software, Inc., San Diego, California, USA). Descriptives are presented as frequencies and percentages or median and interquartile ranges where applicable. One-way ANOVA (Kruskal-Wallis) was used to analyze the differences between the groups. Log-Rank (mantel-cox) test was performed to analyze overall-survival. A p-value of less than 0.05 was considered statistically significant.

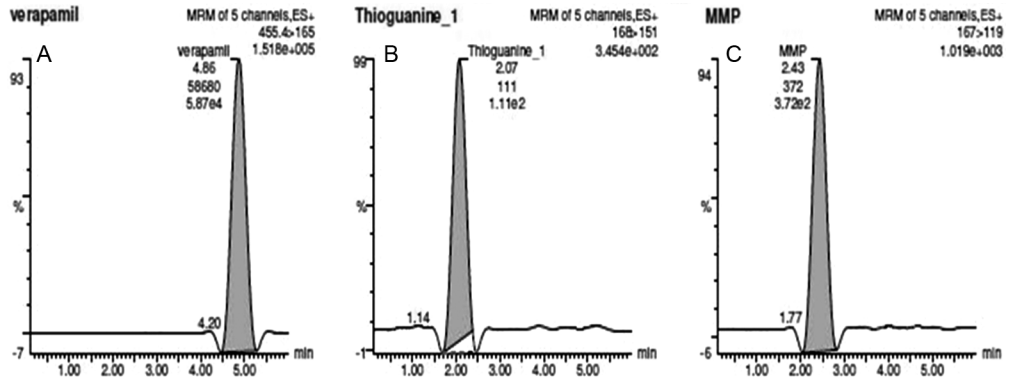

Fig. 2 — Chromatograms of (A) internal standard; Verapamil; (B) thioguanine; and (C) methyl-mercaptopurine detected by Ultra Performance Liquid Chromatography-Mass Spectrometry

Table 1 — Patient Demographics and clinical characteristics

Characteristic	Data
Age (years), median (range)	5.5 (1-15)
Sex, n (%):	
Male	16(53.3)
Female	14 (46.7)
Immuno-phenotype, n (%):	
B-ALL	28 (93.3)
T-ALL	02 (06.7)
Overall Survival:	
Months, median (range)	65.5 (11-80)
Mortality, n (%)	05 (16.7)

Results

Patient Characteristics

Among the 30 participants 16 were male and 14 were female children, with a median age of 5.5 years at the diagnosis. Immunophenotyping showed that 28 children exhibited B-ALL, while 02 children presented with T-ALL (Table 1). All the children achieved remission and were undergoing the maintenance phase of treatment at the time of sample collection. The outcomes revealed that 16.7% of the children experienced a relapse followed by death.

TPMT genotyping

We evaluated the frequency of rs1800460, rs1800462, and rs1142345 SNVs of the *TPMT* gene in the 30 study participants with ALL. Among the 30 individuals, 27 subjects showed wild type TPMT*1/TPMT*1 genotype, and only three patients showed TPMT*1/TPMT*3C heterozygous genotype (Fig 3). No significant association was found between the heterozygous TPMT*1/*3C genotypes and other variables.

In vitro cytotoxicity assay

The cytotoxicity assay was carried out on a total of 30 ALL and 10 healthy subjects PBMCs. 6-MP *in vitro* dose-response and IC_{50} was initially established on the Molt-3 cell line and PBMC isolated from age-matched healthy subjects. In molt-3 cell line and healthy PBMC, the mean (+/- S.D) IC_{50} concentration of 6-MP was found to be 10 (+/- 2 μM). Only 30 ALL samples had adequate cell numbers and responded to the cell proliferation and cytotoxicity protocols. The subjects with % cell viability ≈50% (48.6 ±3.2 μM) represent the expected response to the IC_{50} dose and were stratified into responder groups. Subjects with <50% cell viability (28.7±6.2 μM) at IC_{50} represent toxicity and are grouped as sensitive. While subjects showing >50% cell viability (69.2±6.2 μM) at IC_{50} represent resistance phenotype and were stratified into the non-responder group. Among the 30 samples, 14 samples were identified to be responders, 9 samples were identified as non-responders, and 7 samples were identified as sensitive to 6-MP at IC_{50} (10 μM) (Fig. 4).

Association of MMP and TG metabolites with cytotoxicity

To identify the association of *in vitro* cytotoxicity with the patient's drug response, we estimated erythrocyte 6-MP metabolite levels using UPLC-MS/MS. 6-MP was lower than the detectable limits in all except one sample, whereas MMP was detected in all the 30 samples, and TG was detected in 14 samples. The MMP median level was found to be 277 pmol/10 x 10^8 RBC (range: 70 – 7903 pmol/10 x 10^8 RBC), and the TG median was 114 pmol/10x10^8 RBC (range: 15 – 880 pmol/10 x 10^8 RBC). MMP (P = 0.023) and TG (P = 0.008) showed a significant association with the sensitive, responders, and non-responders stratification of cytotoxicity assay (Fig. 5).

Overall survival

This study was carried out from 2017 to 2023, and the follow-up data was collected from the patients at the end of the maintenance phase of the last subject

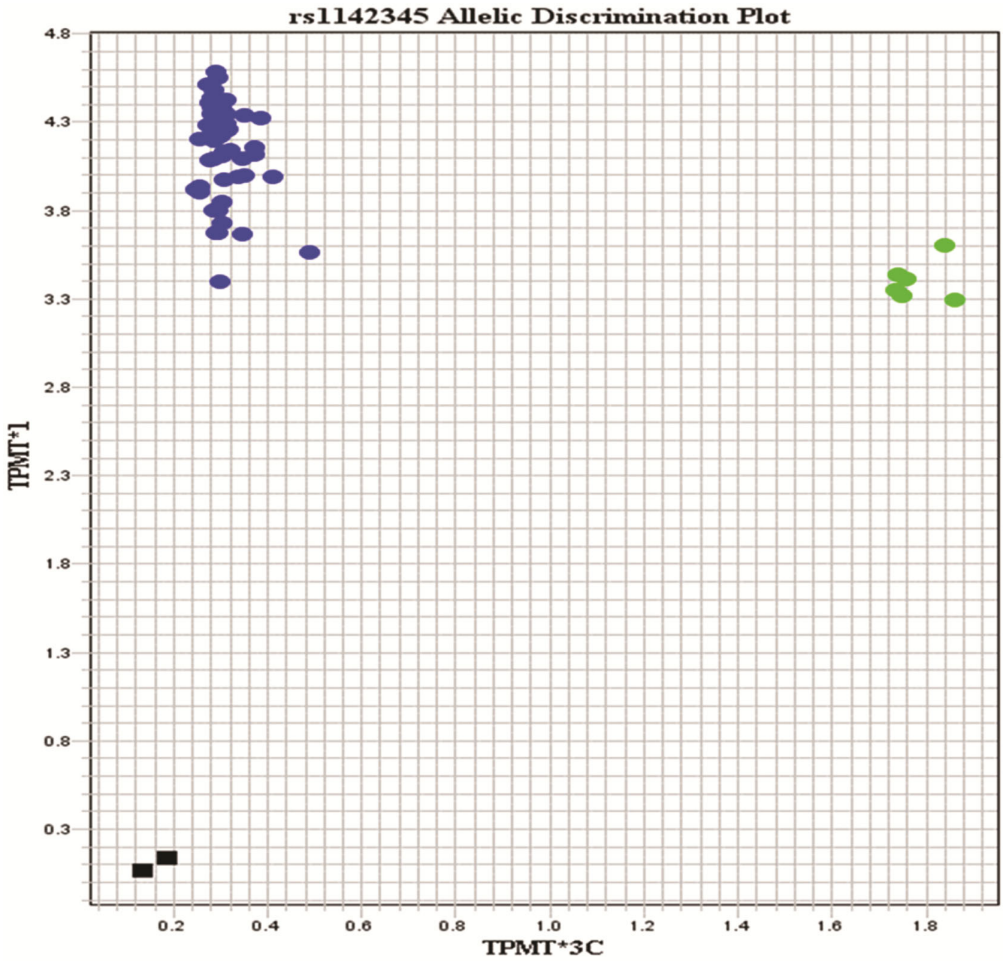

Fig. 3 — Allelic discrimination plot of TPMT *1/*3c evaluated by genotyping of SNV rs1142345. Samples in blue (n=27) represent TPMT *1/*1 genotype and samples in green (n=3) represent heterozygous TPMT *1/*3c genotype. All the samples were genotyped in technical duplicates

recruited. The median survival period was identified as 65.5 months, ranging from 11 to 80 months. Out of 30 patients, five children were found to have relapsed and subsequently died. Of the five patients, four had B-ALL and one had T-ALL immunophenotype. The Kaplan-Meier survival analysis showed a decreased overall survival rate in 6-MP non-responders compared to the sensitive and responder groups (Logrank test P = 0.043). Due to the small sample size, a statistically significant association was not found between the erythrocyte MMP or TG levels with survival in our study cohort [Log-rank test MMP (P = 0.739) and TG (P = 0.832)] (Fig. 6).

Discussion

Molecular mechanisms underlying the interindividual variability towards 6-MP therapy are not fully understood[22]. Pharmacogenetics of TPMT and therapeutic drug monitoring of 6-MP metabolites, MMP, and TG are currently incorporated into the clinical decision-making of dose modifications[23]. However, children with ALL still face challenges in 6-MP therapy due to toxicity or resistance to the drug. Overdosage of 6-MP leads to myelotoxicity or hepatotoxicity, while low dosage can result in resistant lymphoblasts, which can lead to disease relapse[24]. Despite pharmacogenetics and TDM of 6-MP metabolites, still a significant proportion of children (10-25%) face either early or late-stage relapse due to unexplainable reasons[25]. We hypothesize that *in vitro* cytotoxicity assay, while accounting for well-documented pharmacogenetics, also represents other molecular mechanisms behind the therapy response specific to each individual. Predictive *in vitro* analysis of cancer chemotherapeutic agents in longer therapy phases helps in identifying drug resistance[26].

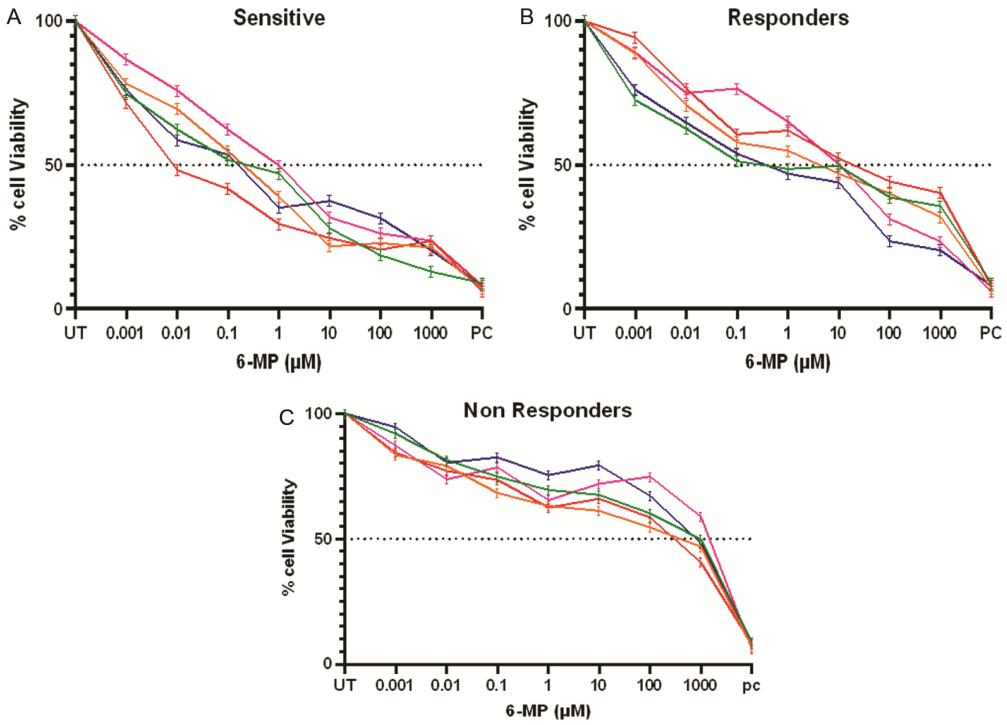

Fig. 4 — Representative dose-response curves of five patients each stratified into sensitive, responders and non-responders based on *In vitro* 6-MP cytotoxicity assay on patient-derived PBMCs. (A) Sensitive: subjects with <50% cell viability – 28.7±6.2 at 10 µM (IC_{50}); (B) Responders: subjects with 50% cell viability – 48.6±3.2 at 10 µM; and (C) Non-responders: subjects with >50% cell viability – 69.2±6.8 at 10 µM

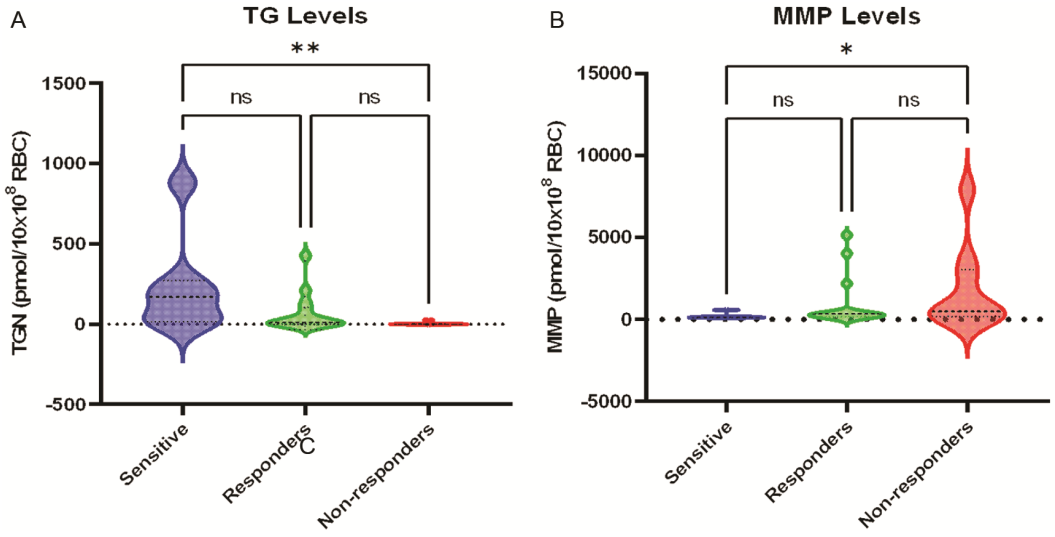

Fig. 5 — (A) Comparison of Thioguanine (left panel); (B) methyl mercpatopurine (right panel) levels between sensitive, responders, and non-responder groups (n=30) *P< 0.05, **P< 0.005

We evaluated the TPMT pharmacogenetics to account for the confounding effect; notably, 27 (90%) subjects carried the wild-type genotype, and only 3 (10%) subjects showed the TPMT*1/*3C genotype. Different studies conducted in Indian and other populations detected the TPMT*1/*3C frequency to be between 1.73% – 11%[27–29]. Our study showed a minor allele frequency of 5% for TPMT rs1142345 SNV.

Various *in vitro* studies observed the IC_{50} of 6-MP on PBMCs ranging from 100 nM to 200 µM[30–33]. In our study, IC_{50} of 6-MP in MOLT-3, JM1 cell lines,

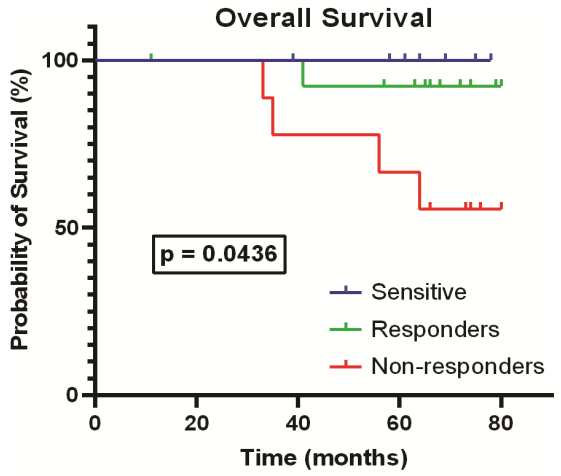

Fig. 6 — The Kaplan-Meier curve of overall survival comparison of patients stratified into sensitive (blue), responders (green) and non-responders (red) groups, Mantel-Cox log-rank test P = 0.046 (n=30)

and age-matched healthy subjects was 10 µM. The advancement of the *in vitro* MTT assay on patient-derived cells greatly simplifies investigations into drug resistance among individuals with leukemia[34]. The current study demonstrates a strong association between *in vitro* 6-MP drug sensitivity and erythrocyte metabolite levels and the overall survival in children with ALL.

Subjects with high cell viability at IC_{50} (non-responders) showed a positive association with erythrocyte MMP levels. This observation is explained by the hypermethylation or MMP shunting, resulting in lower TG levels and non-responsiveness to 6-MP[35,36]. Also, there was a lower overall survival in non-responders compared to the responders and sensitive groups. Out of five deaths during the follow-up period, four subjects were observed to be in the non-responder category based on cytotoxicity assay. This observation explains the role of oral mercaptopurine adherence in disease relapse and death[37].

Conversely, *in vitro* cell death significantly higher than 50% at IC_{50} was positively associated with erythrocyte TG levels. However, no significant association was found between the erythrocyte TG levels and mortality. This observation is in tandem with an earlier study showing no significant association with erythrocyte TG levels and relapse [38].

The limitations of the present study are: i) the impact of methotrexate drug administered in parallel with 6-MP during the maintenance phase, and ii) exploratory study with a small sample size. Future studies on large cohorts of ALL patients are warranted to substantiate these observations further and translate them to a clinical setting.

Conclusion

In vitro cytotoxicity assay on patient-derived PBMCs provides insights into the variability in 6-MP drug response. The cytotoxicity assay was used to stratify subjects into sensitive, responder and non-responder categories. This might help in predicting the therapy response, toxicity and death in children with ALL. *In vitro* 6-MP cytotoxicity analysis, in conjugation with pharmacogenetics and TDM, helps to understand the real-time status of mercaptopurine therapy. It facilitates personalized drug dose modifications to achieve better clinical outcomes.

Acknowledgement

This study was funded by DST-SERB, grant number EMR/2016/003512/HS (Ver-1). We express our sincere gratitude to Dr. Vijay Kumar Kutala and Dr. Gunjan Sharma from multidisciplinary research unit (MRU), Department of Health Research, NIMS for their inputs and support. The data visualization figures were made using Graph-pad Prism; (Fig. 1) was created using BioRender.com.

Conflict of interest

All the authors declare no conflict of interest.

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