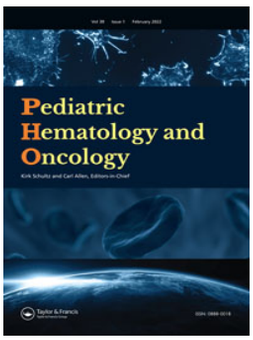

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The effects of *NUDT15* and *TPMT* variants on mercaptopurine treatment in Vietnamese pediatric acute lymphoblastic leukemia patients

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ABSTRACT

6-mercaptopurine (6-MP) plays a critical role in the treatment of pediatric acute lymphoblastic leukemia (ALL). *NUDT15* and *TPMT* gene variants have been strongly associated with myelotoxicity caused by using 6-MP. Therefore, the purpose of this study is to investigate the frequency of *NUDT15* and *TPMT* polymorphisms, as well as the impact of *NUDT15* variants on the use of 6-MP to treat pediatric ALL in Vietnam. Sanger sequencing was applied to detect *NUDT15* and *TPMT* gene variants in 70 pediatric ALL patients. Duration of drug interruption, level of neutropenia, and 6-MP tolerance dose were recorded. *NUDT15* variants were detected from 23 out of 70 (32.9%) patients. Three well-known haplotype variants were identified as *NUDT15* *2 (p.V18_V19insGV and p.R139C), *3 (p.R139C), and *6 (p.V18_V19insGV); besides, a novel *NUDT15* p.R11Q was not previously reported. The *NUDT15* wild-type, heterozygous variant, and homozygous variant genotypes were 67.1%, 30.1%, and 2.8%, respectively. Two *TPMT* heterozygous polymorphisms were *TPMT**3C and *6, accounted for 2.8%. Patients with intermediate and low activity NUDT15 were given the median 6-MP tolerance dose of 55.2 and 37.2 versus 69.5 mg/m^2/day of patients with NUDT15 normal activity ($p = 0.0001$). Patients with homozygous variant diplotype were drastically sensitive to 6-MP, with an average dose intensity of 49.6%, compared to 73.6% and 92.7% of those with heterozygous and wild-type diplotype, respectively ($p = 0.0001$). Our results suggest that 6-MP dose adjustment should be based on *NUDT15* variants in pediatric Vietnamese ALL patients.

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Introduction

Maintenance therapy plays a crucial role in acute lymphoblastic leukemia (ALL) treatment, especially in childhood ALL, with strong evidence showing that the opportunity for a long-lasting remission is significantly improved. In general, ALL protocols

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comprise an induction regimen and subsequent several months of consolidation therapy with three or four types of antileukemic drugs. After achieving complete remission, the patient goes through the maintenance phase with daily 6-mercaptopurine (6-MP) and weekly methotrexate. 6-MP is a fundamental agent in the ALL treatment, but it can also lead to severe adverse drug reactions (ADRs), such as leucopenia and hepatotoxicity. 6-MP is a prodrug, which demands intracellular activation by a complex multi-enzymatic process to form the sufficient cytotoxic component thioguanine nucleotide (TGN).[1] TGN is a purine antagonist that is incorporated into the DNA of leukocytes and inhibits their DNA synthesis.[2] However, the massive insertion of TGN could cause catastrophic adverse results such as myelosuppression, implying the need for personalized adjustment to the medication. One of the main enzymes of the 6-MP metabolite is thiopurine methyltransferase (*TPMT*). The rare genetic polymorphism of the *TPMT* gene has been depicted regarding the *TPMT* activity. Patients with heterozygous or homozygous for *TPMT* deficiency are at high risk of potential myelotoxicity with thiopurine therapy.[3] For the Caucasian population, approximately 10% are heterozygous, and 0.5% are homozygous, resulting in intermediate enzyme activity and low enzyme activity, respectively.[4] The most prevalent variant alleles are *TPMT**3B, *TPMT**3C, *TPMT**2, and *TPMT**6, corresponding to amino acid change A154T, Y240C, A80P, and Y180F, respectively. Nevertheless, the allelic frequency may differ significantly among ethnic groups. The frequency of *TPMT* polymorphism is remarkably lower in Asian populations compared to populations of European descent. For instance, 3.3% in Korean,[5] 2.1% in Japanese,[6] and 1.3% in Chinese[7] versus 10%. However, such studies in Asian populations revealed a comparable frequency of serious myelosuppression requiring a reduction in 6-MP dose or protocol disruption to the European population studied. Thus, it is feasible for additional candidate genes other than *TPMT* variants to be involved in 6-MP induced myelotoxicity in Asian patients.

Recently, some studies have determined that the nucleoside diphosphate-linked moiety X motif 15 (*NUDT15*) enzyme is a novel factor in thiopurine metabolism. *NUDT15* polymorphism is illustrated to be related to intolerance in patients with ALL or inflammatory bowel diseases (IBD), most notably in Asians and Hispanics.[8–10] As a purine-specific nucleotide diphosphatase, the *NUDT15* enzyme transforms the active thiopurine metabolite thioguanine-triphosphate (TGTP) into an inactive thioguanine monophosphate (TGMP), thereby negatively regulating the cytotoxic effects of thiopurine. Thus, individuals with *NUDT15* variants that cause low enzyme activity have decreased thiopurine metabolism, resulting in accumulated DNA destruction; therefore, they required a reduction or interruption of 6-MP. Some genome-wide association studies have identified different genetic variations of *NUDT15* (e.g., p.R139C, p.R139H, p.V18I, p.V18_V19insGV,…) and various haplotypes (e.g., *1, *2, *3, *6, etc.). The degree of NUDT15 activity in patients with different diplotypes was determined by combining variant or wild-type proteins. Moriyama et al., for instance, divided patients into three diplotypic groups: normal activity (*1/*1), intermediate activity (*1/*2, *1/*3, *1/*4 and *1/*5), and low activity (*2/*3, *3/*3 and *3/*5); in conclusion, individuals with malfunctioning NUDT15 alleles experienced excessive thiopurine active metabolites and toxicity.[9] Therefore, we conducted this study to (1) figure out the prevalence of *NUDT15* and *TPMT* variants in Vietnamese pediatric ALL; (2) investigate the

association between *NUDT15* and *TPMT* deficient activity phenotypes and their effects upon drug-inducing complications.

Materials and methods

Patient and treatment

A total of 70 Vietnamese pediatric ALL patients who underwent the maintenance phase of the FRALLE 2000 protocol, including 96 treatment weeks, were recruited into this study at Blood Transfusion and Hematology Hospital, Ho Chi Minh city. For the first year, patients were given a 1.5 mg/m^2/4-week dose of intravenous vincristine, as well as a 75 mg/m^2/day dose of oral 6-MP and a 25 mg/m^2/week dose of oral methotrexate. Patients were examined and had a complete blood count test every four weeks; however, if any adverse events occurred, it was done every week. The absolute neutrophil count (ANC) was used to adjust the 6-MP dose, i.e., 0.5×10^9/L: postpone (1 week), $0.5–0.8 \times 10^9$/L: reduce by one-third of the dose, $> 0.8 \times 10^9$/L: no change. Neutropenia was characterized as grade 3 or grade 4 based on the Common Terminology Criteria for Adverse Events CTCAE version 5.0. The mean of ANC for each group was recorded and compared between the three groups. The total number of weeks of therapy comprises drug-taking weeks and interruption weeks due to any adverse events. To analyze the correlation between drug effects and NUDT15 phenotype on myelotoxicity, we observed the clinical data for patients who ended 48-week maintenance therapy. The daily average 6-MP dose (mg/m^2/day) was calculated by dividing the total cumulative 6-MP taken by the number of maintenance days. The 6-MP tolerance dose was defined as the average stable 6-MP dosage (mg/m^2/day) over four weeks during the maintenance therapy. Clinical data were collected from the patient's medical records retrospectively. This study was approved by the Ethics Committees of the University of Medicine and Pharmacy at Ho Chi Minh City (number 67/ĐHYD-HĐĐĐ).

NUDT15 and TPMT genotyping

Two hundred microliters of peripheral blood in EDTA coagulation were collected, and genomic DNA was extracted using ReliaPrepTM Blood gDNA Miniprep System (Promega, USA), following the manufacturer's instruction. Exon 1 and exon 3 of the *NUDT15* and exon 3 to exon 10 of the *TPMT,* including exon-intron boundaries, were amplified by polymerase chain reaction (PCR) using primers as shown in Table 1. PCR was performed using 2720 Thermal Cycler (Life Technologies, USA) and TaKaRa Taq^{TM} HotStart Polymerase (Takara, Japan). The annealing temperature of PCR reactions was at 60 °C. The length of PCR products was shown in Table 1. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) and subjected to a cycle sequencing using a Bigdye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were run on the ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Variants were analyzed on SeqScape Software version 2.6 (Thermo Fisher, Scientific, Waltham, MA, USA) and compared with the reference sequence of *NUDT15* (NG_047021.1) and *TPMT* (NG_012137.3).

Table 1. Primers for the polymerase chain reaction of *NUDT15* and *TPMT* genes.

Primer	Sequence 5′ → 3′	Size
NUD-1F	AGTGAGCGCGTCACTTCCTG	198 bp
NUD-1R	AGATGACCTCCAGGGAGTTG	
NUD-3F	GGTTGGGAGTGGGTTCCTTG	158 bp
NUD-3R	CAAATCTTCTCGGCCACCTA	
TPMT-3F	ACGTAGGCACGGAAGACATA	1597 bp
TPMT-4R	CCCATTGGCTCCAAACTGAT	
TPMT-5F	AATATAGATCTGCTTTCCTGCATGTTC	390 bp
TPMT-5R	AGGAACCATCGGACACATGA	
TPMT-6F	GGTGCCAATAAAGTGCAGTA	726 bp
TPMT-7R	CTTACACCCAGGTCTCTGTA	
TPMT-8F	TCTGGACCAATTCCCAGCTT	413 bp
TPMT-8R	GCAGTATGCTTCCTATGAGA	
TPMT-9F	ACATGCCACATCATCACCTA	1696 bp
TPMT-10R	TTCATCCATTACATTTTCAGGCTTT	

Statistical analysis

To compare predicted enzyme activity between phenotype groups, we designated the *1/*1 diplotype as a *NUDT15* phenotype with normal activity coded group A, a heterozygous at a single variant with one prototype allele (e.g., *1/*3) as intermediate activity coded group B, and one with both variant alleles (e.g., *3/*3) as low activity coded group C.[9]

Data were collected and analyzed using Stata version 12 (StataCorp LLC, USA). Data was presented as absolute percentages (genotype frequencies), means ± standard deviations (SDs) (weeks of therapy, ANC, average dose, and tolerance dose). The Kruskal Wallis non-parametric and ANOVA tests were used for statistical analysis. A two-sided *p*-value less than or equal to 0.05 was considered statistically significant.

Results

We investigated 70 Vietnamese pediatric ALL patients, achieving complete remission after the consolidation phase and undergoing the maintenance stage of the FRALLE 2000 protocol. The median age was six years old with patients ranging from 2 to16 years old, and 38/70 were male. The treatment was categorized by B cell or T cell lymphoblast lineage, in which B-group accounted for the majority of the protocol with 97%; and T-group was 3% of the selecting treatment.

Genotype and allele frequencies of NUDT15 and TPMT

Out of 70 patients, 23 patients (32.9%) had one or two *NUDT15* variant alleles. Three well-known alleles were detected, namely *2, *3, and *6 at 7.2%, 18.6%, and 5.7%, respectively. In particular, we found a novel *NUDT15* genetic variant c.32G > A (R11Q) (Figure 1), which accounted for 1.4%. The *NUDT15* diplotypes included wild-type (*1/*1), heterozygote (*1/*2, *1/*3, *1/*6, *1/R11Q), and homozygote (*3/*3, *6/*6), occurring in 67.1%, 30.1%, and 2.8% of the studied patients, respectively (Table 2).

Regarding *TPMT*, two heterozygous variants (2.8%), *TPMT**3C and *6 alleles, were detected. Noticeably, one of them showed digenic heterozygous variants of *TPMT* *6 and *NUDT15* *3 (Table 2).

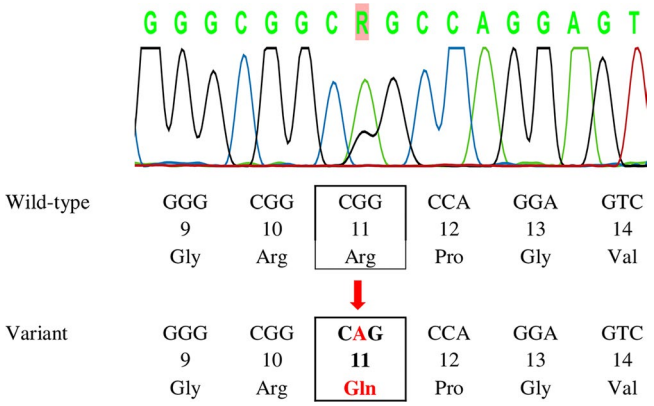

Figure 1. DNA sequencing of the *NUDT15* genotype R11Q.

Table 2. Allele frequency of *NUDT15* and *TPMT* genes in pediatric ALL according to genotypes (n = 70).

NUDT15		*TPMT*	
Diplotypes	Frequency (%)	Diplotypes	Frequency (%)
*1/*1	47 (67.1)	*1/*1	68 (97.2)
*1/*2	5 (7.2)	*1/*3 C	1 (1.4)
*1/*3	12[a] (17.2)	*1/*6	1[a] (1.4)
*1/*6	3 (4.3)		
*1/R11Q	1 (1.4)		
*3/*3	1 (1.4)		
*6/*6	1 (1.4)		

NUDT15 *1: wild-type; *2: p.V18_V19insGV and R139C; *3: R139C; *6: p.V18_V19insGV. TPMT *1: wild-type; *3 C: p.Y240C; *6: Y180C. [a] Case showed both variant NUDT15 and TPMT.

Association between NUDT15 phenotypes and thiopurine-induced myelotoxicity

Because of the very low incidence of *TPMT* variants, we only evaluated the effect of *NUDT15* phenotype groups on myelosuppression during the treatment. In this study, 51 patients finished at least 48 weeks of maintenance therapy. Due to myelotoxicity, therapy interruption was observed in all patients. Patients in groups A, B and C required 57, 62 and 77 weeks, respectively, to complete the standard maintenance therapy duration (48 weeks) ($p = 0.0027$) (Table 3). The treatment interruptions in groups A, B, and C were 9, 14, and 29 weeks, respectively.

The association of *NUDT15* polymorphism and neutropenia circumstances was shown in Table 3. *NUDT15* risk variants were related to a higher risk of myelotoxicity than wild-type ($p < 0.0001$). Group A denoted as grade 3 neutropenia, at 0.61×10^9/L, while group B and group C showed grade 4 neutropenia with the ANC were 0.45 and 0.36×10^9/L.

The daily average dose of 6-MP was 69.7 ± 4.6, 55.2 ± 11.6, and 38.9 ± 17.2 mg/m^2/day for group A, B, and C, respectively ($p = 0.0001$) (Figure 2A). Groups B and C were roughly 50% and 73% effective, respectively, when compared to the standard dosage of 75 mg/m2. In comparison, wild-type group A consumed approximately 93% of the prescribed dosage.

Table 3. Association between *NUDT15* phenotypes and clinical data in 48 weeks of maintenance therapy. (n = 51).

	Normal activity (n = 35)	Intermediate activity (n = 14)	Low activity (n = 2)	*p*-value
Total weeks of therapy [median (range)] (weeks)	56 (51-63)	61 (55-71)	77 (66-88)	0.0027*
Absolute neutrophile count (mean ± std) (x 10^9/L)	0.61 ± 0.12	0.45 ± 0.10	0.36 ± 0.06	0.0001=

*Kruskal Wallis non-parametric test. =One-way ANOVA test.

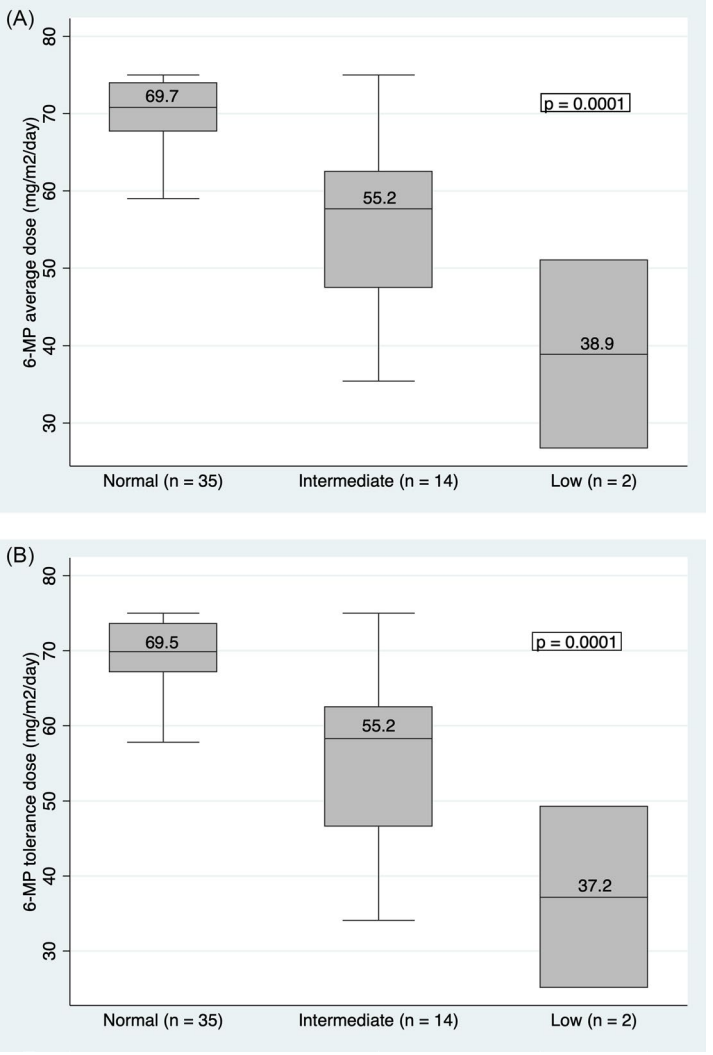

Figure 2. Box plot of correlation between *NUDT15* activity phenotypes and the 6-MP daily average dose (A) and the 6-MP tolerance dose (B).

The maximal tolerable 6-MP dose was 69.5 ± 4.8, 55.2 ± 11.8, and 37.2 ± 17.0 mg/m^2/day for the group A, B, and C, respectively ($p = 0.0001$) (Figure 2B). Compared to the standard dose, the median cumulative 6-MP tolerance dose was 92.7% in patients with normal *NUDT15* activity group A, while only 73.6% and 49.6% were required in patients with *NUDT15* deficient active heterozygote group B and homozygous group C.

In a novel variant *NUDT15* R11Q, the patient postponed five weeks of therapy due to descent of ANC, which level was 0.47×10^9/L during 48-week taking 6-MP of the maintenance treatment.

Discussion

A large number of children suffer from acute lymphoblastic leukemia annually, accounting for one-quarter of all pediatric malignancies and forcing an extended and precise plan of a chemotherapy regimen in distinct stages. The maintenance phase is the longest and lasts 2–3 years,[11] and it consists of daily 6-MP and weekly methotrexate taken orally. According to *TPMT* insufficient activity variants, the initial dosage reduction is applied to diminish severe adverse effects induced by 6-MP, especially neutropenia. However, the prevalence of *TPMT* variants in the Asian population is low, which cannot explain the high incidence of ADRs caused by thiopurine.

In this study, after analyzing 70 DNA samples of pediatric ALL using Sanger sequencing, we found that 32.9% and 2.8% of cases carried *NUDT15* and *TPMT* variants, respectively. Our findings were consistent with other Asian reports.[8,12,13] The most common variant was *NUDT15**3, which accounted for 18.6% of all variant distributions. Several studies in Asian ALL populations have revealed relatively high *NUDT15**3 frequencies (19–25%).[12,14,15] Furthermore, by testing a diverse set of *NUDT15* variants, we emphasized the presence of other heterozygous (combined *1 with *2, *6, or the novel variant R11Q) and homozygous (*3/*3, *6/*6) deficient variants in the Asian population. Despite the low frequency of such variants (Table 2), our study highlighted the requisite of performing *NUDT15* sequencing in ALL patients, particularly Asian patients, prior to the maintenance treatment to detect all possible *NUDT15* variants, not only *NUDT15**3. The well-known *NUDT15* variants, consisting of heterozygous and homozygous, was found in 28.7% and 2.8%, respectively. The result of allele frequencies in this study was similar to those shown in the previous reports.[12,14–17] In addition, a minor incidence of *TPMT* variants was recorded at 2.8%, comparable to other studies in the Asian community, at around 4%.[13] The disparity in frequency between *NUDT15* and *TPMT* polymorphisms where *NUDT15* was predominant elucidated the direct link between *NUDT15* polymorphisms and 6-MP induced myelosuppression, as opposed to *TPMT* polymorphisms and toxicity caused by the drug, in the Asian population.

Furthermore, to clarify the correlation between *NUDT15* risk variants and adverse drug reactions, the taken drug dosage was recorded. Although the initially planned dose was 75 mg/m^2/day, all patients could not be administered the total drug dose due to myelosuppression. The pediatric ALL patients were examined and given outpatient maintenance treatment every four weeks unless there were any adverse events. Our findings revealed that the average daily dosage of 6-MP fell from the usual dose by only around 50% for homozygous genotypes (e.g., 38.9 mg/m^2/day) and 73% for

heterozygous genotypes (e.g., 55.2 mg/m^2/day) (Figure 2A), which were relatively similar to the tolerance dose (Figure 2B). Although the 6-MP dose was reduced, the ANC of the heterozygous and homozygous groups were still significantly reduced compared to the wild-type group (Table 3). Thus, the 6-MP tolerance at 49.6% and 73.6% of the planned dose were shown to be appropriate to avoid excessive toxicity in patients with *NUDT15* homozygous low activity and heterozygous intermediate activity phenotype ($p = 0.0001$). Moriyama et al., 2016. also found that the decreased dosage could be beneficial in eliminating ADRs in patients with *NUDT15* risk variant for Guatemalan ($p = 0.021$), Singaporean ($p = 2.1 \times 10^5$), and Japanese ($p = 0.0054$);[9] for instance, in Japanese patients, the tolerated 6-MP dosage for the intermediate and the low activity groups were approximately 50% and 10% of the standard dose.[9] The 6-MP dose fell to 37% in Yang's study[8] and even to 50% in Liang's study.[14]

In the case of a novel variant *NUDT15* R11Q, the patient recoded a five-week pause in therapy due to ANC descent. The degree of ANC reduction was comparable to that of other *NUDT15* variants. Regarding the *TPMT**3C variant case, the patient administrated a reduction of 6-MP dosage, in which both average daily dose and tolerance dose were around 31 mg/m^2/day (e.g., 41.3% of the planned dose). Such treatment-related toxicity was shown in other reports.[18,19] In addition, a patient carrying both *NUDT15**3 and *TPMT**6 heterozygous variant also interrupted seven weeks of therapy and received 63 mg/m^2/day of 6-MP (e.g., 84% of the planned dose). In Zhou's study, they showed one patient with a heterozygous variant of *NUDT15* and *TPMT*, being also sensitive to 6-MP with approximately 75% of the standard dose.[20]

In conclusion, our results are consistent with other Asian studies that the *NUDT15* genetic variants are the most common polymorphisms and significantly affect 6-MP induced toxicity. Furthermore, we will carry out routine *NUDT15* variants tests on all ALL patients and follow the guidelines for thiopurine-adjusted dosage based on *NUDT15* polymorphism before undergoing maintenance therapy.

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Disclosure statement

The authors report no conflicts of interest

Availability of data and material

Data and materials supporting the findings of this study are available from the corresponding author upon reasonable request.

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