PREVALENCE OF THIOPURINE S-METHYLTRANSFERASE (*TPMT*) GENE VARIANTS IN THAI PATIENTS SUFFERING TOXICITY FROM THIOGUANINE-CONTAINING CHILDHOOD LEUKEMIA PROTOCOLS: FIRST REPORT OF *TPMT*3A* IN THAIS

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Abstract. Thiopurine S-methyltransferase (TPMT) is a cytoplasmic enzyme that catalyzes anticancer thiopurine drugs into less active metabolites. As a result, acute leukemia patients with TPMT deficiency will likely be at increased risk for developing toxicity. TPMT activity can be indirectly assessed by genotyping polymorphisms of *TPMT*, an encoding gene. Since the frequency of *TPMT* polymorphisms differs by ethnicity, it is important to determine the prevalence of *TPMT* variants in each ethnic population. The aim of this study was to determine the prevalence of 5 common polymorphisms of *TPMT* gene in Thai children with acute lymphoblastic leukemia/lymphoblastic lymphoma or acute non-lymphoblastic leukemia who experienced toxicity during treatment regimens that contained thiopurine drugs. A total of 164 patients were evaluated using allele-specific polymerase chain reaction (AS-PCR) for *TPMT*2*, and PCR-restriction fragment length polymorphism (RFLP) for *TPMT*3A*, **3B*, **3C*, and **6* detection. Seven of 164 (4.26%) patients had *TPMT* variants. Six patients had *TPMT*3C/*1* and one patient had *TPMT*3A/*1*. This is the first report of *TPMT*3A* polymorphism in Thai population. The percentage frequencies of genotypes *TPMT*3C/*1* and *TPMT*3A/*1* were 3.65% and 0.61%, respectively. The allele frequencies of *TPMT*3C* and **3A* were 1.82, and 0.30, respectively. Prevalence of the 5 common *TPMT* variants in Thai children with thiopurine-associated toxicity in this study was low. This finding indicates the necessity of further phamacogenomic study of other candidate-genes to completely justify the risk of thiopurine-related toxicities in Thai population.

Keywords: TPMT, toxicity, acute leukemia, chemotherapy

INTRODUCTION

Acute leukemia is the most common cancer among children, and the most frequent cause of cancer-related death in people younger than 20 years of age (Smith *et al*, 2010). Control of disease relapse and treatment-related morbidity/mortality are key factors that affect treatment outcome in this pediatric population. Progressive improvements in the efficacy of multiagent chemotherapy regimens has significantly reduced disease relapse (Hunger and Mullighan, 2015). Thiopurine drugs, such as 6-mercaptopurine (6-MP) and 6 thioguanine (6-TG), have been an integral component of childhood acute lymphoblastic leukemia (ALL), lymphoblastic lymphoma (LBL), and acute non-lymphoblastic

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leukemia (ANLL) chemotherapy protocols for many decades.

Thiopurine S-methyltransferase (TPMT) is a cytoplasmic enzyme that catalyzes anticancer thiopurine drugs (6-MP and 6-TG) into less active pharmacologic metabolites via the methylation process (McLeod *et al*, 2000; Zaza *et al*, 2010). As a result, acute leukemia patients with TPMT deficiency that are treated with a standard dose of thiopurine drugs will be at increased risk for developing hematopoietic toxicity, because most thiopurine drugs will be transformed into highly active cytotoxic metabolites [*ie*, thioguanine nucleotides (TGN)] at low or no level of TPMT activity. *TPMT* genetic variation is the primary determinant of TPMT enzyme activity (Tamm *et al*, 2016). Sensitivity to the cytotoxic effects of thiopurines increases in patients who carry specific single nucleotide polymorphisms (SNP) (Salavaggione *et al*, 2005). By genotyping the encoding gene *TPMT*, heterozygous or homozygous individuals having one or two non-functional variants will have decreased TPMT activity in the intermediate or low/absent level, respectively, as compared to wild type allele (*TPMT*1/*1*) (DiPiero *et al*, 2015). *TPMT* genotype analysis enables the physician to tailor the dosage of thiopurine drugs to the specific needs of the patient and/or to alter drugs in order to avoid toxicity (DiPiero *et al*, 2015).

Of approximately 40 reported alleles (Appell *et al*, 2013), the top five *TPMT* variants associated with deficient enzyme activity are *TPMT*2*(G238C, Ala80Pro), **3A*(G460A+A719G, Ala154Thr, and Tyr240Cys), **3B*(G460A, Ala154Thr), **3C*(A719G, Tyr240Cys), and **6*(A539T, Tyr180Phe). These variants have been studied in many different populations, and their frequency was found to vary significantly among ethnic groups. Several studies reported *TPMT*3C* to be the most prevalent allele among Africans and Asians, while *TPMT*3A* was found to be the most prevalent allele in Caucasians (Collie-Duguid *et al*, 1999; Hon *et al*, 1999; McLeod *et al*, 1999). In Southeast Asians, *TPMT*3C* predominance has most often been reported (Hongeng *et al*, 2000; Chang *et al*, 2002; Kham *et al*, 2002; Srimartpirom *et al*, 2004; Kham *et al*, 2008; Lee *et al*, 2008). However, in the multiracial population of Singapore, three heterozygous *TPMT*6* (2 Chinese, 1 Malay) variants and four heterozygous *TPMT*3A* (2 Indians, 1 Chinese, 1 Malay) variants were found in addition to *TPMT*3C* (Kham *et al*, 2002; Kham *et al*, 2008). In Thai population, only *TPMT*3C* has been reported (Hongeng *et al*, 2000; Srimartpirom *et al*, 2004). Previous studies reported frequency of *TPMT* polymorphisms by testing blood either from normal population (Srimartpirom *et al*, 2004) or from any ALL patient who was or was not suffering from thiopurine toxicity (Hongeng *et al*, 2000). Based on our review of the literature, no data has been reported on the prevalence of *TPMT* polymorphisms in Thai pediatric population that developed treatment-related toxicity while undergoing thiopurine therapy.

The aim of this study was to determine the prevalence of 5 different *TPMT* variants in Thai children with ALL/LBL or ANLL who experienced toxicity during treatment regimens that contained thiopurine drugs.

MATERIALS AND METHODS

The protocol for this study was approved by the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University [approval no. 440/2556(EC1)]. This retrospective chart review was conducted in ethnic Thai children with ALL/LBL or ANLL that developed drug toxicities during treatment with chemotherapy regimens that contained thiopurine drugs during the 1 November 2004 to 31 October 2015 study period. All included patients were treated at the Department of Pediatrics, Siriraj Hospital – Thailand's largest university-based national tertiary referral center. Peripheral blood samples of all patients were investigated for 5 *TPMT* variants (*TPMT*2, *3A,*

Table 1
Demographic data of 7 children with *TPMT* variants.

Case no.	Gender	Age (months)	Diagnosis	Treatment protocol	Relapse	*TPMT* variant	Toxicity (organ, grading)
1	Male	48	ALL	ALL standard risk	No	*TPMT*3C/*1*	Blood, grade 4; infection, grade 2
2	Female	28	ALL	ALL high risk	Yes	*TPMT*3C/*1*	Blood, grade 4
3	Female	53	ALL	ALL standard risk	No	*TPMT*3C/*1*	Blood, grade 4; hepatic, grade 3
4	Female	25	ALL	ALL standard risk	No	*TPMT*3C/*1*	Blood, grade 4; hepatic, grade 3
5	Female	49	ALL	ALL standard risk	No	*TPMT*3C/*1*	Hepatic, grade 3
6	Female	75	ALL	ALL standard risk	No	*TPMT*3C/*1*	Hepatic, grade 2
7	Male	120	LBL	ALL high risk	No	*TPMT*3A/*1*	Infection, grade 2

ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; blood grade 4, absolute neutrophil count <500/mm^3; hepatic grade 2, AST or ALT >2.5 - 5 x upper limit of normal (ULM); hepatic grade 3, AST or ALT >5 - 20 x ULM; infection grade 2, moderate, localized infection.

**3B, *3C*, and **6*). DNA was extracted from whole blood samples using the salting out procedure (Miller *et al*, 1988). *TPMT*2* was detected using allele-specific polymerase chain reaction (AS-PCR), and *TPMT*3A, *3B, *3C*, and **6* were detected using PCR-RFLP, as previously described (Otterness *et al*, 1997; Yates *et al*, 1997; Kham *et al*, 2002).

In patients with the *TPMT*3A* variant (which contains two nucleotide transition mutations) that were baring both *TPMT*3B* and *TPMT*3C* alleles on the same chromosome, we further investigated their parents to confirm whether these *TPMT*3B* and *TPMT*3C* alleles were *TPMT*3A* or compound heterozygote *TPMT*3B/*3C.* All cases in which *TPMT* variants were detected by PCR methods were confirmed by direct PCR product sequencing. Ten negative *TPMT* variant cases were also randomly selected to be sequenced as negative controls. Alleles without any one of these five alleles were assumed to be wild type (*TPMT*1*).

Baseline demographic and clinical characteristics, such as age at diagnosis, treatment protocol, risk classification, drug toxicity, and drug doses were collected and analyzed using SPSS Statistics version 16.0 (SPSS, Chicago, IL). Characteristics of patients that carried the SNPs of interest were described. Data are presented as percentage and 95% confidence interval. Toxicity grade was classified using National Cancer Institute Common Toxicity Criteria (CTC) version 2 [Online] (1999).

RESULTS

Of the 164 pediatric patients that were included, seven had *TPMT* variants. Of those 7 patients, five were females and two were males, and the median age at diagnosis of hematologic malignancies was 49 months (range: 25-120) (Table 1). The prevalence of *TPMT* variants was 4.27%. Six patients were heterozygous for *TPMT*3C (TPMT*3C/*1)* and one patient was heterozygous for *TPMT*3A* (*TPMT*3A/*1*). The percentage frequencies of genotypes *TPMT*3C/*1* and *TPMT*3A/*1* were 3.65%

Table 2
Frequencies of *TPMT* genotypes in 164 Thai children with toxicity during thiopurine drug treatment.

Genotype	No. of patients (*n*=164)	Genotype frequency (95% CI)
**1/*1*	157	95.73% (92.60-98.86)
**3A/*1*	1	0.61% (-0.59-1.81)
**3C/*1*	6	3.65% (0.75-6.56)
Allele	**No. of alleles (*n*=328)**	**Allele frequency (95% CI)**
**1*	321	97.86% (96.29-99.43)
**2*	0	0% (0-0)
**3A*	1	0.30% (-0.29-0.90)
**3B*	0	0% (0-0)
**3C*	6	1.82% (0.37-3.29)
**6*	0	0% (0-0)

CI, confidence interval.

and 0.61%, respectively (Table 2). *TPMT*3C* was the most prevalent variant allele (6 of 7 patients; 85.71%). The percentage allele frequencies of *TPMT*3C* and **3A* were 1.82% and 0.30%, respectively (Table 2). Homozygous *TPMT*3C* and *TPMT*3A* were not found, including *TPMT*2, TPMT*3B,* and **6*.

DNA sequencing for *TPMT*3B* and *TPMT*3C* in all 10 negative controls and 6 patients with positive *TPMT* variants (6 *TPMT*3C/*1*) yielded 100% accuracy when compared with the results of PCR-based method.

One patient in this study was found to be heterozygous for *TPMT*3A*. Given that this is the first reported finding of *TPMT*3A* in Thai population, both of the *TPMT*3B* and *TPMT*3C* alleles, which are components of *TPMT*3A*, were further investigated by DNA sequencing. The results confirmed nucleotide changes at codon 460 719 in *TPMT*3A* (Fig 1). Moreover, an analysis of this patient's parents revealed that *TPMT*3A* was inherited from his father, which suggests that both nucleotide alterations are linked on the same haplotype, which confirms a genuine *TPMT*3A* (Fig 2).

Using PCR-RFLP method, we also investigated a nucleotide change at G292T to exclude *TPMT*3D* (G460A, A719G and G292T) from *TPMT*3A* (G460A and A719G) (Chang *et al*,

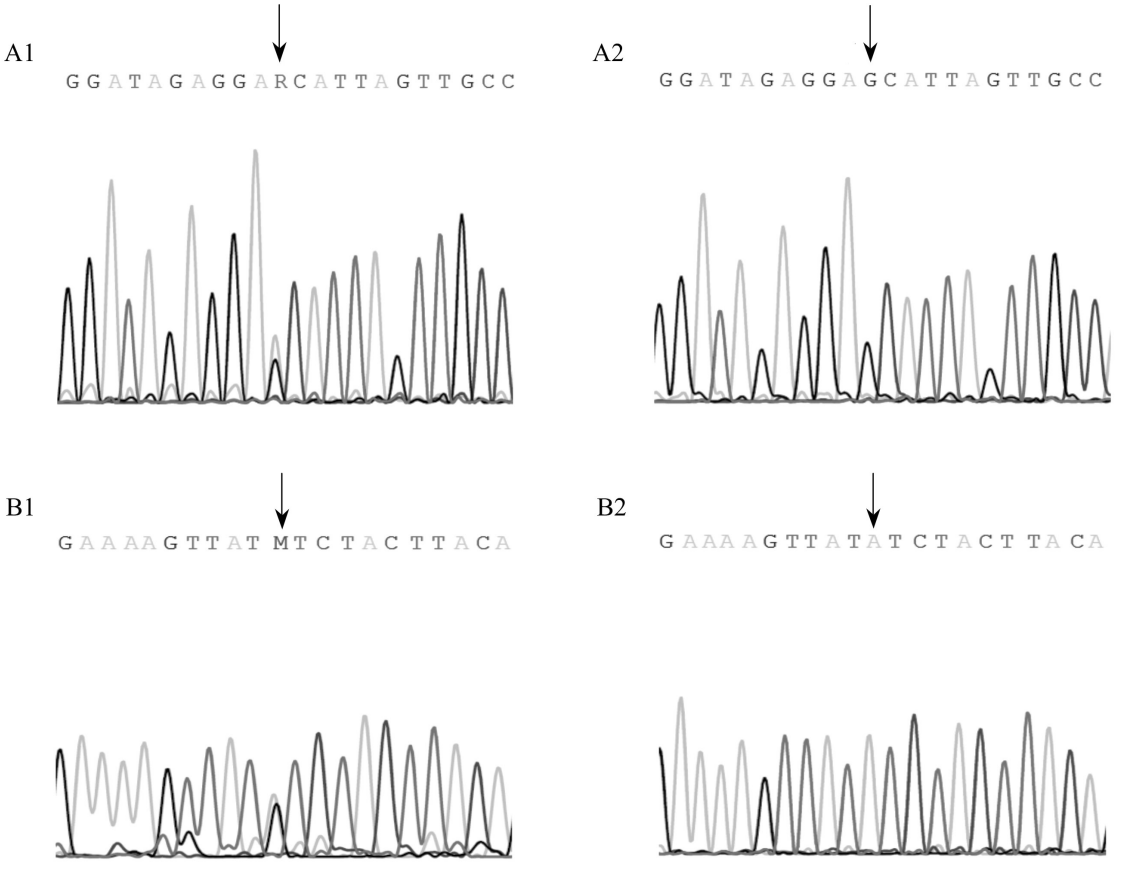

Fig 1– DNA sequence chromatograms of the index patient with positive heterozygous *TPMT*3A* showing (A1) heterozygous G460A compared with (A2) wild type G460; and, (B1) heterozygous A719G compared with (B2) wild type A719.

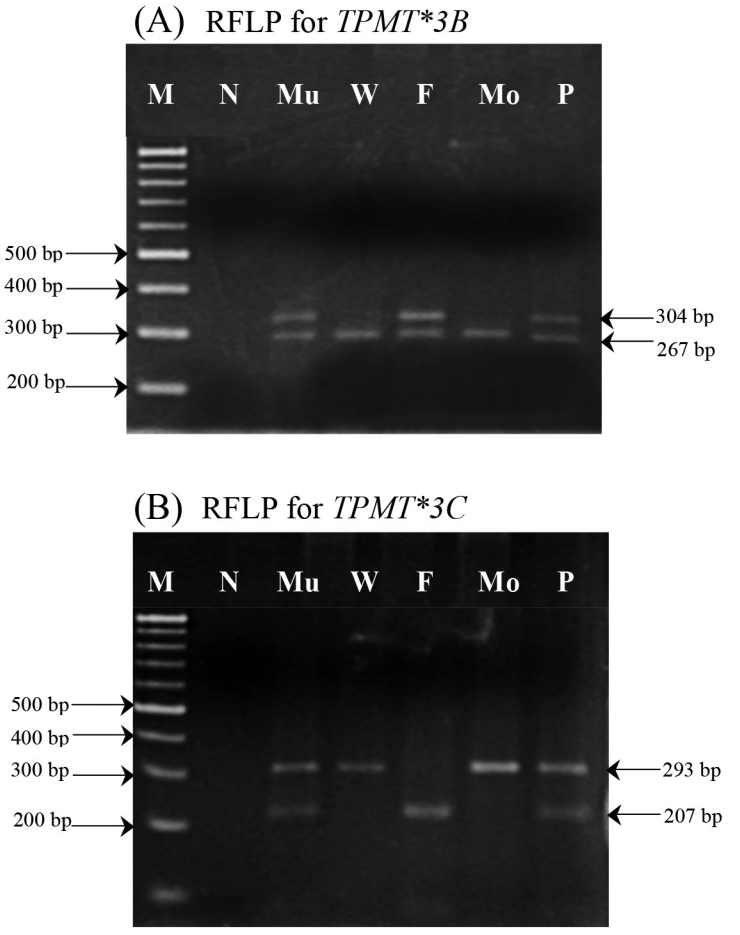

Fig 2– RFLP-PCR-based genotyping assay for (A) *TPMT*3B* and (B) *TPMT*3C* within the family of a patient baring *TPMT*3B* and *TPMT*3C* clearly demonstrated that the father carried mutant alleles at both nucleotide 460 and 719 as heterozygous and homozygous, respectively. The mother carried wild type allele at both nucleotides. This result suggests that the patient (P) was compound heterozygous for *TPMT*3A/*1*, instead of for *TPMT*3B/3C*. Lane A6 and B6 shows that both of the patient's *TPMT*3B* and *TPMT*3C* alleles were inherited from his father (not de novo), and confirmed that the patient was *TPMT*3A/*1*, not heterozygous *TPMT*3B/*3C*. N, negative control; Mu, control mutant; W, wild type; F, father: *TPMT*3A/*3C*; Mo, mother: *TPMT*1/*1*; P, patient: *TPMT*3A/*1*; M, standard DNA 100 bp ladder marker.

2002), after which DNA sequencing was performed to verify a correct result. G292T was not found, so we concluded that the alleles were *TPMT*3A*, not *TPMT*3D*.

Of the 6 patients who had *TPMT*3C,* four (66%) developed grade 4 blood toxicity, four (66%) developed grade 2-3 hepatic toxicity, and one (16%) developed grade 2 infection during full dose of 6-mercaptopurine (Table 1). Six of 7 patients with *TPMT* variants (85%) required dose reduction. However, all 6 patients were able to continue 6-MP-containing chemotherapy with

no severe hematopoietic toxicity complications after reduction in dose of 6-mercaptopurine of 25-75% (median 50%) from the original dose of 50 mg/m^2/day.

DISCUSSION

To the best of our knowledge, this is the first study to report the prevalence of *TPMT* polymorphisms in Thai pediatric patients that developed treatment-related toxicity while undergoing thiopurine therapy for childhood leukemia. Interestingly, our findings revealed that the 5 evaluated SNPs were rare in this population. The prevalence of *TPMT* variants in Thais who developed drug toxicity was only 4.26%.

According to reports describing the prevalence of *TPMT* variants in Southeast Asian populations, *TPMT*3C* is the most prevalent variant allele (Table 3). No subjects from any Southeast Asian population have yet been identified as having the *TPMT*2*, *3B,* or *6 variants. From previous studies in Thai population, only *TPMT*3C* was reported, with allele frequencies of 5.3 in children with acute leukemia (Hongeng *et al*, 2000), 3.2 in patients underwent kidney transplantation (Vannaprasaht *et al*, 2009) and 5.0 in healthy volunteer subjects (Srimartpirom *et al*, 2004). In this study, *TPMT*3C* was found with allele frequency of 1.82, which is quite low compared to previous studies, even though our studied population had experienced thiopurine toxicity nor healthy subjects or leukemia patient at first diagnosis. This population should have a higher frequency of *TPMT* variants, however we did not observe that speculation in our study. Overall, the frequency of *TPMT*3C* in Thai population appears to be similar to the frequency of *TPMT*3C* in other Asian populations (Table 3). In addition, *TPMT*3A* was detected in a *TPMT*3A/*1* male patient with an allele frequency of 0.30. This is the first reported occurrence of *TPMT*3A* in Thai population. Apart from the present study, heterozygous *TPMT*3A* was also found in a specimen from a different Thai patient from another hospital that was sent to our laboratory for routine genotyping of *TPMT*. *TPMT*2, TPMT*3B*, and *TPMT*6* were not found in this study, which confirms that *TPMT*2* and *3B* are very rare alleles in non-Caucasian populations. However, all patients that tested negative for the 5 studied *TPMT* variants and that were assumed to have TPMT*1/*1 should be further studied for possible presence of unknown variant alleles. In addition, a genotype of *TPMT*3A* is recommended to Thai subjects as shown in this study in order to provide a correct *TPMT* genotype.

Since patients with negative test for common *TPMT*-deficient variants still experience excessive toxicity from thiopurine-containing regimens, genotyping of the 5 common *TPMT* variants prior to starting thiopurine may not be sufficient to predict clinical outcome in Thai patients. Severe toxicities requiring thiopurine dose reduction in Thais that are comparable to those reported in European populations are still being observed. As such, the next step is to study the so called *TPMT*1/*1* wild type genotype using in-depth genomic resequencing in order to identify heretofore unrecognized TPMT variants. In addition, factors other than *TPMT* variants that may affect 6-MP toxicity in Thai patients should be considered. Several candidate genes have been identified, including nucleoside diphosphate-linked moiety X motif 15 (*NUDT15*), which has been reported to be responsible for thiopurine intolerance in those with East Asian ancestry (Yang *et al*, 2015; Zhu *et al*, 2016), and inosine triphosphate pyrophosphatase (*ITPA*), a gene encoding enzyme for neutralizing the toxic effects of 6-MP metabolites that has been identified and reported in several Asian populations, including Thai population (Glomglao *et al*, 2014; Uchiyama *et al*, 2009; Wan Rosalina *et al*, 2012).

This study has some mentionable limitations. First and consistent with the retrospective nature of this study, some patient data may have been missing or incomplete. Second, we could not

Table 3
Allele frequencies of *TPMT* variants in Southeast Asian populations.

Population	Ethnicity	No. of subjects	*2	*3A	*3B	*3C	*6	Reference
Workers in Taiwan	Indonesian	100	0	0	0	1.0	0	(Chang *et al*, 2002)
	Filipino	100	0.5[a]	0	0	1.0	0	
	Thai	100	0	0	0	1.0	0	
Healthy blood donors	Chinese Singaporean	153	NA	0	0	1.31	0	(Kham *et al*, 2008)
	Malay Singaporean	163	NA	0.31	0	1.84	0	
	Indian Singaporean	163	NA	0	0	0.92	0	
Childhood ALL	Chinese Singaporean	220	NA	0	0	1.82	0.45	(Kham *et al*, 2008)
	Malay Singaporean	160	NA	0	0	1.88	0	
	Indian Singaporean	38	NA	0	0	3.95	0	
Unrelated cord blood samples	Chinese Singaporean	200	0	0	0	3.0	0	(Kham *et al*, 2002)
	Malay Singaporean	200	0	0	0	2.3	0.3	
	Indian Singaporean	200	0	0.5	0	0.8	0	
Childhood ALL	Chinese Singaporean	71	0	0.7	0	2.1	0	(Kham *et al*, 2002)
	Malay Singaporean	17	0	0	0	3.0	0	
General population	Vietnamese	159	0	0	0	2.83	0	(Lee *et al*, 2008)
Childhood ALL	Vietnamese	127	NA	0	0	4.33	NA	(Hoang *et al*, 2015)
Childhood acute leukemia	Thai	75	0	0	0	5.33	NA	(Hongeng *et al*, 2000)
Kidney transplant recipients	Thai	139	NA	NA	NA	3.2	NA	(Vannaprasaht *et al*, 2009)
General population	Thai	200	0	0	0	5.0	0	(Srimartpirom *et al*, 2004)
Childhood leukemia with treatment toxicity	Thai	164	0	0.3	0	1.82	0	This study

NA, not available; ALL, acute lymphoblastic leukemia.
[a]This patient's Caucasian parent was found to be heterozygous for the *TPMT*2* allele.

perform TPMT enzyme activity in erythrocytes to confirm deficient stage, as the majority of patients had received blood transfusion.

In conclusion, the prevalence of the 5 common *TPMT* variants in Thai children with thiopurine-associated toxicity in this study was low. Further pharmacogenomic study is needed to identify inherited variants in other candidate genes that elucidate thiopurine-related toxicities.

CONFLICTS OF INTEREST

The authors hereby declare no conflicts of interest regarding any aspect of this study.

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