NUDT15 Variants are Associated with Myelotoxicity and Reduction of 6-Mercaptopurine Dose Intensity in Thai Childhood Acute Lymphoblastic Leukemia

การกลายของยีน *NUDT15* สัมพันธ์กับภาวะเป็นพิษต่อเซลล์และการลดขนาดยา 6MP ในโรคมะเร็งเม็ดเลือดขาวชนิดลิมโฟบลาสแบบเฉียบพลันในเด็กไทย

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ABSTRACT

NUDT15 variants altered the enzyme activity and caused thiopurine-induced myelotoxicity. Therefore, our study investigated *NUDT15* polymorphisms and association between *NUDT15* and myelotoxic effects in Thai children with acute lymphoblastic leukemia (ALL). Fisher's exact tests indicated that all *NUDT15* variants were significantly associated with neutropenia ($P = 3.12x10^{-5}$) in week 1-8 and thrombocytopenia ($P = 0.027$) in week 9-24 of maintenance phase. Moreover, the three types of *NUDT15* polymorphisms: wild-type, heterozygous and homozygous variants tolerated 66.67%, 48.8% and 16.67% of 6-mercaptopurine (6MP) protocol dose, respectively. This results concluded that *NUDT15* variants affected 6MP-induced myelotoxicity and dose adjustment should be concerned in each of *NUDT15* diplotypes.

บทคัดย่อ

การกลายของยีน *NUDT15* มีผลต่อระดับการทำงานของเอนไซม์และทำให้เกิดภาวะเป็นพิษต่อเซลล์ไขกระดูกเมื่อได้รับยากลุ่ม thiopurine ดังนั้นจุดประสงค์ของงานวิจัยคือ การศึกษาความสัมพันธ์ระหว่างการกลายของยีน *NUDT15* กับภาวะเป็นพิษต่อเซลล์ในคนไข้เด็กมะเร็งเม็ดเลือดขาวชนิดลิมโฟบลาสแบบเฉียบพลัน ผลการวิจัยนี้พบว่าการกลายของยีน *NUDT15* ทุกแบบสัมพันธ์กับภาวะเม็ดเลือดขาวชนิดนิวโตรฟิลต่ำ ($P = 3.12x10^{-5}$) ในช่วงสัปดาห์ที่ 1-8 และเกล็ดเลือดต่ำ ($P = 0.027$) ในช่วงสัปดาห์ที่ 9-24 ของการรักษาระยะ maintenance อย่างมีนัยยะสำคัญทางสถิติ นอกจากนี้คนไข้ที่มียีน *NUDT15* แบบปกติ, heterozygous และ homozygous variants สามารถใช้ยา 6MP ได้ 66.67%, 48.8% และ 16.67% ของระดับยามาตรฐานของการรักษา ตามลำดับ ดังนั้นจึงสรุปได้ว่า การกลายของยีนมีผลต่อภาวะเป็นพิษต่อเซลล์เมื่อใช้ยา 6MP และมีผลต่อการปรับลดระดับยาให้เหมาะสมกับการกลายของยีน *NUDT15* แต่ละแบบ

Keywords: *NUDT15* polymorphisms, Myelotoxicity, 6MP

คำสำคัญ: ความหลากหลายของยีน *NUDT15* ภาวะเป็นพิษต่อเซลล์ ยา 6MP

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Introduction

6-mercaptopurine (6MP) is the anti-cancer agent which has been used in the maintenance phase of acute lymphoblastic leukemia (ALL) treatment (Timmer et al., 2016). It can improve the event-free survival (EFS) in ALL patient (Relling et al., 1999). 6MP-induced myelotoxicity discontinues the treatment and associates with the opportunistic infections (Relling et al., 1999; Inaba et al., 2017). Non-adherence of treatment could increase the relapse risk (Toyoda et al., 2000). 6MP is a specific substrate of thiopurine methyltransferase (TPMT) that balances the metabolized form of thiopurine drugs (Chouchana et al., 2012). Patient carrying *TPMT* variants could have the higher risk of myelotoxic effects and 6MP dose intolerance (Pogorzelski et al., 2011). Thai population has been found 5% allele frequency of *TPMT*3C* which presents the decrease enzyme function (Srimartpirom et al., 2004; Mcleod et al., 1999; Szumlanski et al., 1996). However, 6MP-induced myelotoxicity has been observed in ALL patient although *TPMT* is wild-type.

Recently, genome-wide association study (GWAS) reported that Nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) polymorphism (rs116855232) associated with 6MP dose intensity reduction in ALL patients, and the heterozygous and homozygous variants of T allele indicated the dosage effect (Yang et al., 2015). *NUDT15* is located on the germ line human chromosome and showed the defective function to catalyze the thioguanine triphosphate (TGTP) to thioguanine monophosphate (TGMP) (Moriyama et al., 2016; Valerie et al., 2016). TGTP is the active metabolite that acts as the nucleotide analog to block DNA replication and inhibited the cell proliferation (Fairchild et al., 1986). Moriyama et al. (2016) found that the six diplotypes with the different of the functional levels. *NUDT15*1* (wild-type) is the normal activity, (compound) heterozygous variants are the intermediate activity and homozygous variants are the low activity.

Chiengthong et al. (2016) presented the association between only *NUDT15:rs116855232* and 6-MP induced neutropenia in ALL children. The medians of cumulative 6-MP dose of patient carrying heterozygous and homozygous variants were lower than those wild-type. Allele frequency of *NUDT15:rs116855232* from this study was 9%. Interestingly, many studies in Asian population reported only rs116855232 minor allele frequency of 10.2%, 10.4%, 7.2% and 12.1% in Japanese, Korean, Indian and Chinese, respectively (Tanaka et al., 2015; Yang et al., 2014; Shah et al., 2017; Zhu et al., 2016) but the other *NUDT15* genotypes have not yet investigated in Thai

Objective of this study

Our objectives were to genotype the *NUDT15* in Thai children with ALL, and investigated the association between *NUDT15* variants and myelotoxicity including to compare the 6-MP dose intensity between *NUDT15* wild-type and variants.

Materials and Methods

Patients and treatment

One hundred Thai children with ALL were taken part in this retrospective study; the patients were treated at Ramathibodi Hospital, Mahidol University, they received the chemotherapy according to RAMA ALL001 protocol

between 2004- 2017. The duration of treatment for girls was two years and a half, boys was three years and a half. For RAMA ALL001 protocol, the risk groups were classified to three groups: low, standard and high risk groups. For the low risk group, the initial dose of 6-MP and methotrexate (MTX) were 75 mg/m^2/day daily and 40 mg/m^2 weekly, respectively. Vincristine (VCR) was intravenous 2 mg/m^2 and prednisolone is oral 40 mg/m^2 monthly. Whereas, the standard and high risk group also received intravenous cyclophosphamide 300 mg/m^2 and cytarabine 300 mg/m^2 monthly. The 6-MP dose adjustment based on the CBC and clinical symptoms. 6-MP dose intensity that based on dose adjustment was the ratio between administrated dose and protocol dose (%).

Toxicity definition

All patients were assessed for the presence of toxicity according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 within the first 6 months of maintenance phase. A patient was classified as having toxicity based on either grade 3 thrombocytopenia or grade 4 neutropenia. Neutropenia (ANC< 500 cells/mm^3) was defined as grade 4, thrombocytopenia (Plt count< 50,000/ mm^3) was defined as grade 3. The lowest points of ANC and Plt count were clarified to myelotoxicity. Developing myelotoxicity within the first 8 weeks was termed as early myelotoxicity and week 9-24 was the late myelotoxicity.

***NUDT15* Genotyping**

Genomic DNA (gDNA) was extracted from buffy coat separated by 3ml peripheral blood using Easy Blood Genomic DNA Purification kit according the manufacturer's protocol (GMbiolab, Taichung City, Taiwan).Total gDNA was quantified by Multiskan GO Microplate Spectophotometer (ThermoFisher Scientific, Massachusetts, USA) and prepared to 30 ng/µl.

We genotyped *NUDT15* by Sanger sequencing. Forward and reverse primers for genotyping *NUDT15*5* (rs186364861, c.52G>A) and *NUDT15*6* (rs554405994, c.36_37insGGAGTC) were F1: 5'-GTC ACT TCC TGC CGC TGC CAG-3' and R1: 5'-GCT CAC CCG AAC TCC AGA TGA CC-3'. PCR was performed on CFX96 TouchTM Real-Time PCR detection System (BIORAD, California, USA). The PCR was performed as followed: 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 45 sec, and final extension at 72°C for 10 min. Forward and reverse primers for genotyping *NUDT15*3* (rs116855232, c.415C>T) and *NUDT15*4* (rs147390019, c.416G>A) were F2: 5'-GCA AAG CAT CAC TAT GAG TTT-3' and R2: 5'-GCC ACC TAG AGA TGA TTT CCT-3'. The PCR reaction used 57°C of the annealing temperature. The 50 µl PCR was fulfilled by using 1X PCR buffer, 2.6 mM $MgCl_2$, 0.4 mM dNTPs, 10pmol of primers, and 1.5 units of Taq DNA polymerase (Invitrogen, Massachusetts, USA). The amplified PCR product of 204 bp from F1R1 and 287 bp from F2R2 were electrophoresed on a 2% agarose gel and selected those PCR products to purify and sequence (ABI3730XL DNA analyzer, ThermoFisher Scientific, USA). *NUDT15* diplotypes were classified according to Moriyama et al. (2016).

Statistical analysis

Data were analyzed using SPSS version 18. The samples were evaluated for the Hardy-Weinberg equilibrium using Chi-square test. Statistical associations between categorical variables were evaluated using the chi-squared or Fisher's exact tests. The strength of the association was expressed as odd ratios (ORs) with 95% confidence interval (CI) using the genotype, with only the common allele as a reference. Independent Mann-Whitney U-test was compared

the median of dose intensity, ANC and platelet count (Plt) between *NUDT15* wild-type and variants. A two sided test was considered statistically significant with *P*-value less than 0.05.

Results

Characteristic of patients

One-hundred children with ALL had the median age of 5.9 years and 6-MP mean initial dose was 58.16±20.58 mg/m^2/day. ALL patients were *TPMT* wild-type. B-cell ALL patients were the most found in this study as shown in Table 1. The minor allele frequency of rs116855232 was 5% and rs554405994 was 6%. These genotypes distribution were in Hardy- Weinberg equilibrium. The diplotype frequencies of *NUDT15*1/*3*, **1/*6*, **1/*2* and **2/*2* were 4%, 6%, 4% and 1%, respectively (Table 2).

Table 1 Characteristics of ALL children on 6-MP therapy

Characteristics	**Subject (n=100)**
Age (years)	5.9 (1-14)
Female/Male	46/54
Hematologic malignancy types	
- T cell ALL	10
- B cell ALL	88
- Lymphoblastice lymphoma	2
Risk groups	
- Low	42
- Standard	44
- High	14
6-MP initial dose (mg/m^2/day)	58.16±20.58

Table 2 Allele and genotype distributions of *NUDT15* in ALL children

SNPs	**Genotypes**	**n (100)**	**Allele frequency (%)**	**Hardy–Weinberg P-value**
rs116855232 (c.415C>T)	CC CT TT	91 8 1	5	0.114
rs554405994 (c.36_37insGGAGTC)	-/- -/ins ins/ins	89 10 1	6	0.257
***NUDT15* diplotypes**		**n (100)**		
*1/*1 (wild-type)		85		
*1/*3 (rs116855232)		4		
*1/*6 (rs554405994)		6		
*1/*2(rs116855232,rs554405994)		4		
*2/*2		1		

Association between *NUDT15* variants and myelotoxicity

We retrospectively analyzed the myelotoxicity in the first 6 months of maintenance phase. The results showed that *NUDT15*3* and *NUDT15*6* associated with neutropenia with *P*-value = 0.045 (OR: 10.421; 95% CI: 1.024-106.04) and 0.034 (OR: 6.947; 95% CI: 1.181-40.887) whereas *NUDT15*2* associated with neutropenia with *P* - value = $9.67x10^{-4}$ (OR: 37.531; 95% CI: 1.986-708.724) and thrombocytopenia with *P*-value = 0.009 (OR: 16.714; 95% CI: 2.38-117.386) in the early myelotoxicity (Table 3). In the late myelotoxicity, *NUDT15*6* associated with neutropenia with *P*-value = 0.012 (OR: 15.304; 95% CI: 0.836-280.253) (Table 4). Taken all diplotypes, we found that all *NUDT15* diplotypes were strong associated with neutropenia in the first 8 weeks with *P*-value = $3.12x10^{-5}$ (OR: 13.895; 95% CI: 3.551-54.363), and thrombocytopenia in week 9-24 with *P*-value = 0.027 (OR: 5.818; 95% CI: 1.354-25.005).

Table 3 Association between *NUDT15* diplotypes and myelotoxicity in week 1-8 of maintenance phase

Myelotoxicity (week 1-8)	**Yes**	**No**	**OR (95% CI)**	***P*-value***
NUDT15*3:rs116855232				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*3* *NUDT15*1/*1*	3 (75%) 19 (22.4%)	1 (25%) 66 (77.6%)	10.421 (1.024-106.040)	**0.045***
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*3* *NUDT15*1/*1*	0 (0%) 7 (8.3%)	4 (100%) 78 (91.7%)	1.163 (0.057-23.743)	1.000
NUDT15*6:rs554405994				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*6* *NUDT15*1/*1*	4 (66.7%) 19 (22.4%)	2 (33.3%) 66 (77.6%)	6.947 (1.181-40.887)	**0.034***
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*6* *NUDT15*1/*1*	1 (16.7%) 7 (8.3%)	5 (83.3%) 78 (91.7%)	2.229 (0.228-21.834)	0.434
NUDT15*2 (rs116855232,rs554405994)				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*2,*2/*2* *NUDT15*1/*1*	5 (100%) 19 (22.4%)	0 (0%) 66 (77.6%)	37.513 (1.986-708.724)	**$9.67x10^{-4}$***
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*2,*2/*2* *NUDT15*1/*1*	3 (60%) 7 (8.3%)	2 (40%) 78 (91.7%)	16.714 (2.380-117.386	**0.009***
all *NUDT15* diplotypes				
Neutropenia grade 4 (ANC < 500/ mm^3)				
variants wild-type	12 (80%) 19 (22.4%)	3 (20%) 66 (77.6%)	13.895 (3.551-54.363)	**$3.12x10^{-5}$***
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
variants wild-type	4 (26.7%) 7 (8.3%)	11 (73.3%) 78 (91.7%)	4.052 (1.018-16.125)	0.058

Table 4 Association between *NUDT15* diplotypes and myelotoxicity at week 9-24 of maintenance phase

Myelotoxicity (week 9-24)	**Yes**	**No**	**OR (95% CI)**	***P*-value***
NUDT15*3:rs116855232				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*3*	0 (0%)	4 (100%)	0.131	0.128
*NUDT15*1/*1*	39 (45.9%)	46 (54.1%)	(0.007-2.505)	
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*3*	1 (25%)	3 (75%)	5.333	0.247
*NUDT15*1/*1*	5 (5.9%)	80 (94.1%)	(0.466-61.000)	
NUDT15*6:rs554405994				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*6*	6 (100%)	0 (0%)	15.304	**0.012**
*NUDT15*1/*1*	39 (45.9%)	46 (54.1%)	(0.836-280.253)	
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*6*	2 (33.3%)	4 (66.7%)	8.000	0.066
*NUDT15*1/*1*	5 (5.9%)	80 (94.1%)	(1.170-54.726)	
NUDT15*2 (rs116855232,rs554405994)				
Neutropenia grade 4 (ANC < 500/ mm^3)				
*NUDT15*1/*2,*2/*2*	2 (40%)	3 (60%)	0.782	1.000
*NUDT15*1/*1*	39 (45.9%)	46 (54.1%)	(0.125-4.948)	
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
*NUDT15*1/*2,*2/*2*	1 (20%)	4 (80%)	4.000	0.298
*NUDT15*1/*1*	5 (5.9%)	80 (94.1%)	(0.374-42.803)	
all *NUDT15* diplotypes				
Neutropenia grade 4 (ANC < 500/ mm^3)				
variants	8 (53.4%)	7 (46.6%)	1.348	0.780
wild-type	39 (45.9%)	46 (54.1%)	(0.449-4.051)	
Thrombocytopenia grade 3 (Plt < 50,000/ mm^3)				
variants	4 (26.7%)	11 (73.3%)	5.818	**0.027***
wild-type	5 (5.9%	80 (94.1%)	(1.354-25.005)	

Comparison of ANC, platelet count, dose intensity and *NUDT15* polymorphisms

Patients were grouped into two groups according to *NUDT15* wild-type (WT) and variants. *NUDT15* variants (Var) had statistic significantly lower the absolute neutrophil count (ANC) and platelet count (Plt) with *P*-value = $2.13x10^{-4}$ and 0.009 respectively in week 1- 8 of maintenance phase (Figure 1).

In the first 8 weeks of the maintenance phase, dose intensity of *NUDT* wild-type, heterozygous variants (*NUDT15*1/*3*, *NUDT15*1/*6* and *NUDT15*1/*2*) and homozygous variant (*NUDT15*2/*2*) did not statistically significant difference. However, myelotoxicity severally occurred during 8 weeks of maintenance phase then 6MP was adjusted after that on the basis of myelotoxicity and clinical symptoms. The dose reduction was prescribed at the discretion of the treating clinicians. Comparing the dose intensity during week 9-24 of the maintenance phase, the homozygous variant *NUDT15*2/*2* had lowest 6MP dose intensity (Figure 2). However, the results showed the trend for the difference of 6MP dose intensity among 3 types of *NUDT15* variants (*P*-value = 0.056).

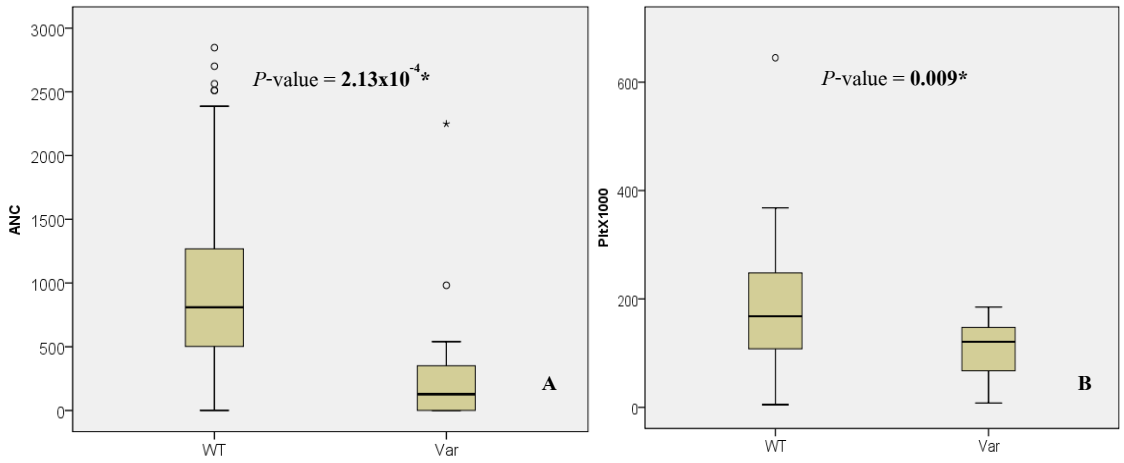

Figure 1 Box plot of *NUDT15* wild-type (WT, n = 85) and variants (Var, n = 15) shows the median of absolute neutrophil count (ANC) (A), and platelet count (Plt) (B) in the week 1-8 of the maintenance phase.

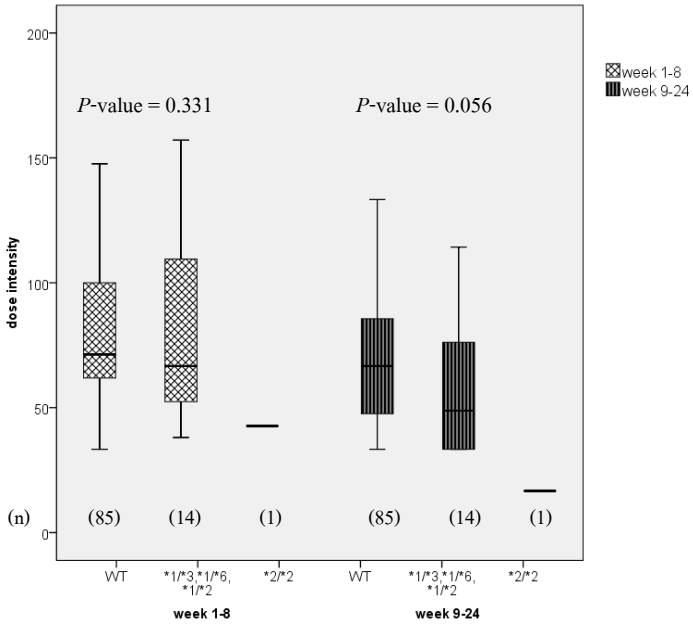

Figure 2 Comparison of the dose intensity between *NUDT15* wild-type (n = 85), heterozygous variants (n = 14) and homozygous variant (n = 1) in the week1-8 and week 9-24. **P*-values were calculated using the Kruskal-Wallis test.

Discussion

This was the first report of *NUDT15* diplotypes in Thai children with ALL and the results of association between *NUDT15* and myelotoxicity, and 6-MP dose intensity. *TPMT* has been the germ line polymorphism of candidate gene for thiopurine immunosuppressant and anti-cancer drug (Vannaprasaht et al., 2009; Booth et al., 2011). However, myelotoxicity incidence was high in Asians even though the frequency of *TPMT* polymorphisms was less found compared with Europeans (Kham et al., 2008; Cooper et al., 2008). Our study showed that *NUDT15* variants conferred 6MP myelotoxicity and were susceptible to 6MP protocol dose in the patient with *TPMT* wild-type. According to Yang et al. (2015), they revealed that *NUDT15:rs116855232* was the genetic determinant of 6-MP intolerance in ALL children. They observed minor allele frequency of 9.8%, 3.9%, 0.2% and 0% in East Asians Hispanics, Europeans and Africans, respectively. Chiengthong et al. (2016) demonstrated that *NUDT15:rs116855232* increased the 6-MP induced myelotoxicity in the maintenance phase of ALL children, and allele frequency of this genotype was 9%. Similarly to Japanese children with ALL, *NUDT15:rs116855232* significantly associated the leukopenia (WBC < 2,000cells/mm^3) during first 2 months of maintenance phase. The allele frequency is 16% of Japanese (Tanaka et al., 2015). Our study detected the minor allele frequency of rs116855232 and rs554405994 were 5% and 6%, respectively. Therefore, the genotyping of rs554405994 can improve the predictive value. Our results showed that *NUDT15*1/*3* (rs116855232) variant was associated with neutropenia grade 4 in the first 8 weeks of maintenance phase similarly to Chingthong et al. (2016). Furthermore, *NUDT15*1/*6* (rs554405994) associated with

grade 4 neutropenia in the first 8 weeks in maintenance phase. Whereas, *NUDT15*1/*2* (rs116855232, rs554405994) and *NUDT15*2/*2* were 100% neutropenia grade 4 and developed thrombocytopenia grade 3 in the first 8 weeks. Especially, a patient carrying *NUDT15*2/*2* had pancytopenia with the lowest points of white blood cell count (WBC): 920/mm^3, platelet (Plt) count: 8,000/mm^3 and 10% Hematocrit before 6-MP dose was adjusted. All association results suggested that genotyping of rs554405994 (c.36_37insGGAGTC) could be done to avoid thrombocytopenia and neutropenia after 8 weeks of maintenance phase. Thus, the detection of rs554405994 and rs116855232 should be considered for pre-emptive working for 6MP administration in ALL patients.

The median of absolute neutrophil count and platelet count from routine monitoring showed that *NUDT15* variants had markedly lower than those wild-type, consistent with Yi et al. (2017). An analysis of dose intensity in week 9-24, *NUDT15* wild-type, heterozygous and homozygous diplotypes could be tolerant 6MP 66.67% (50 mg/m^2/day), 48.8% (36.6 mg/m^2/day) and 16.67% (12.5 mg/m^2/day) dose intensity, respectively. Remarkably, even if a patient with *NUDT15*2/*2* received the lowest dose of all subjects along the maintenance phase; therapy was several interrupted and the myelotoxicity still presented until the treatment ended. It was possible that 6MP 12.5 mg/m^2/day was not suitable for ALL children with homozygous variant of *NUDT15*. The tolerant dose in this study was higher than Japanese children with ALL that had the average dose 42.2, 18 and 6 mg/m^2/day of *NUDT15:rs116855232* wild-type, heterozygous and homozygous variants, respectively (Tanaka et al., 2015). While Yang et al. (2015) revealed the significantly diminished 6MP dose intensity in ALL East Asians according to rs116855232 genotype. Their 6MP dose intensity were 75% (56.25 mg/m^2/day), 47.5% (35.62 mg/m^2/day) and 10% (7.5 mg/m^2/day) of *NUDT15:rs116855232* wild-type, heterozygous and homozygous variants, respectively. We did not find the association between *NUDT15* variants and hepatotoxicity. It could be possible that NUDT15 could metabolize TGTP which plays an important role in the inhibition of cell proliferation then intracellular TGTP accumulation causes the myelosuppression (Valerie et al., 2016). On the other hand, hepatotoxicity correlated with higher 6-methylmercaptopurine ribonucletides (6-MMPR) level (Nygaard et al., 2004). Taken together, *NUDT15* variants could develop the sensitivity of TGTP, but may not accumulate 6-MMPR.

Considering the association between *NUDT15* all diplotypes and neutropenia in Table 3, the positive predictive value (PPV) and negative predictive value (NPV) of severe neutropenia was 80% PPV and 77.6% NPV. Thrombocytopenia was predicted with 26.7% PPV and 94.1% NPV, respectively.

Conclusion

Our study was the first report the association of *NUDT15* diplotypes with 6MP-induced myelotoxicity and 6MP dose reduction. Furthermore, we developed the genotyping of *NUDT15* polymorphisms and clarify the *NUDT15* diplotypes in Thai children with ALL. *NUDT15* genotyping could be the pre-emptive the 6MP prescription in Thai and other East Asians.

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