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The Effects of Inherited *NUDT15* Polymorphisms on Thiopurine Active Metabolites in Japanese Children with Acute Lymphoblastic Leukemia

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Abstract

Thiopurines (e.g., mercaptopurine [MP]) are widely used chemotherapeutic agents in the treatment of pediatric acute lymphoblastic leukemia (ALL) with dose-limiting hematopoietic toxicity. Recently, germline variants in *NUDT15* have been identified as a major genetic cause for MP-related bone marrow suppression, and there is increasing interest in clinical implementation of *NUDT15* genotype-guided MP dose individualization. Therefore, we sought to evaluate the effects of *NUDT15* on thiopurine metabolism and identify pharmacologic markers to inform *NUDT15* genotype-guided MP dosing. In 55 Japanese children with ALL, we simultaneously measured both thioguanine nucleotides (TGN) in red blood cells and DNA-incorporated thioguanine (DNA-TG) in white blood cells. TGN levels were significantly lower in patients with *NUDT15* deficiency,

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Conflicts of Interest

The authors declare no conflicts of interest.

likely due to toxicity-related MP dose reduction. In contrast, when exposed to the same dose of MP, DNA-TG accumulated more efficiently *in vivo* with increasing number of risk alleles in *NUDT15* ($P = 4.0 \times 10^{-9}$). Cytosolic TGN and nuclear DNA-TG were positively correlated with each other across genotype groups ($P = 6.5 \times 10^{-4}$), but the ratio of DNA-TG to TGN was significantly higher in *NUDT15* deficient patients ($P = 3.6 \times 10^{-9}$), consistent with excessive MP activation. In conclusion, our results suggest that DNA-TG is a more relevant MP metabolite than TGN to inform *NUDT15* genotype-guided dose adjustments.

Keywords

NUDT15; DNA-TG; Acute Lymphoblastic Leukemia; 6-Thiopurine; individualized therapy

Introduction

Thiopurines (e.g., 6-mercaptopurine [MP]) are widely-used purine antimetabolites [1, 2]. In acute lymphoblastic leukemia (ALL), patients are exposed to daily MP for more than a year to achieve a long-term cure [3–6]. However, frequent MP dose adjustment is needed due to severe myelosuppression that can lead to life-threatening infections [3]. Thiopurines are prodrugs that require enzymatic conversion into thioguanine nucleotides (TGN), which are in turn incorporated into DNA (forming DNA-TG) to induce cell apoptosis [1]. Therefore, active metabolites of thiopurines (i.e., TGN and DNA-TG) are hypothesized to be the pharmacologic determinants of drug efficacy and toxicity. Previous studies have reported that thiopurine toxicity can be influenced by inherited genetic polymorphisms in the *TPMT* and *NUDT15* genes, which encode key enzymes involved in thiopurine metabolism particularly the formation of TGN and DNA-TG, respectively [7–12].

Preemptive *TPMT*-guided thiopurine dose adjustments have been clinically implemented to mitigate drug toxicity, as a prototype of pharmacogenetics-driven precision medicine [13–15]. However, the *TPMT*-based algorithm is not universally informative because *TPMT* risk alleles are uncommon in certain ancestral groups (e.g., East Asians). In contrast, *NUDT15* variants are the primary genetic cause for thiopurine toxicity in Asians and Hispanics for whom *NUDT15*-based dosing can be of particular relevance. In fact, in Japanese population, one in every 4 people carries at least one copy of the known damaging alleles in *NUDT15*, and one in every 50 people is homozygous or compound heterozygous for these defective variants. When thiopurine dosing is adjusted based on *TPMT* genotype, the premise is to decrease thiopurine dose for patients with *TPMT* deficiency such that they are exposed to a TGN level comparable to that in patients with wild-type alleles who receive the standard thiopurine dose. Although we hypothesized that this principle could be adopted for MP dose reduction in patients with *NUDT15* risk alleles [12, 16], it remains unclear which MP metabolites should be monitored to direct dose modification. Herein, as a part of the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) ALL B-12 protocol, we simultaneously determined TGN and DNA-TG during MP therapy, and evaluate the effects of *NUDT15* genotype on these metabolites.

Patients and therapy

A total of 55 pediatric patients with newly-diagnosed ALL and treated on standard risk (N = 47), intermediate risk (N = 7), or high risk arms (N = 1) of the JPLSG ALL B-12 protocol (<http://www.umin.ac.jp/UMIN000009339>) were included in this study. Of these, 32 were described previously [12]. The standard MP dosage during the maintenance phase of treatment was 50 mg/m² per day, and the dosage was adjusted to a target white blood cell (WBC) count of between 2.0×10^9 and 3.0×10^9 cells/L according to a standardized guideline in the ALL B-12 protocol. Peripheral blood collection was scheduled for the 13th and 41st weeks after the initiation of maintenance therapy and all patients in this study received at least 14 days of MP at a stable dose before blood draw. The 14-day average dosage was used to define MP tolerance (i.e., the tolerated MP dosage). Patients were selected on the basis of the availability of germline DNA, thiopurine metabolite data, and MP dosage history (i.e., at least 14 days of stable MP dosage at the time of sample collection). This study was approved by the respective institutional review boards, and informed consent was obtained from the parents, guardians, and/or patients, as appropriate.

NUDT15 and *TPMT* genotyping

Germline DNA was extracted from peripheral blood collected in EDTA tubes from patients who were in clinical remission. Coding regions (exons 1, 2, and 3) of the *NUDT15* gene were first amplified by polymerase chain reaction (PCR) from germline DNA, then Sanger sequencing was performed as described previously [12]. Sequence alignment and comparison were performed using the CLC Genomics Workbench (CLC Bio, QIAGEN, Redwood City, CA) to identify genetic variants. Common *TPMT* risk variants (rs1800462, rs1800460, and rs1142345, defining the *2, *3A, *3B, and *3C alleles) were genotyped by using Sanger sequencing [17].

Quantification of leukocyte DNA-TG and erythrocyte TGN

DNA was extracted from nucleated cells in peripheral blood within 72 h of blood collection. The levels of DNA-TG were quantified using LC-MS/MS, as described in previous reports [16, 18–20]. DNA-TG stability was evaluated and we observed no significant variation within the 72 h window. Erythrocyte TGN was quantified in the hydrolyzed cell lysate by using HPLC [21].

Statistical analysis

Each patient was assigned an *NUDT15* “genetic score” of 0, 1, or 2, indicating normal, intermediate, or low *NUDT15* activity, respectively, on the basis of 4 known non-functional variants [12]. The correlation between the *NUDT15* genetic score and MP tolerance and thiopurine metabolites (TGN, TGN/dosage, DNA-TG/dosage, and DNA-TG/TGN) was tested using the linear regression model. Correlation between the tolerated TGN and DNA-TG was determined by the Spearman rank test. All statistical tests were two-sided and were chosen as appropriate according to the data distribution; the threshold for statistical significance was defined as a *P*-value less than 0.05. In the majority of patients, MP dosage and metabolite levels were measured at more than one time points for each patient and these values were included in the analyses as independent measurements. We also tested the

genotype-phenotype association after assigning a MP dosage and metabolite value for each patient using the average of values from multiple time points, and observed similar results. R software (version 3.0; <http://www.r-project.org/>) was used for all analyses unless indicated otherwise.

Results

A total of 55 children enrolled on the JPLSG B-12 frontline ALL protocol were included in this study and 74 peripheral blood samples (1.3 per patient) were collected for thiopurine metabolites (TGN and DNA-TG) measurement during daily MP treatment as part of the maintenance therapy (Table 1). The cumulative frequency of *NUDT15* risk alleles (*2, *3, or *5) was 10.9% in this cohort. The intermediate- or low-activity *NUDT15* diplotypes were observed in 10 (18.2%) and 1 (1.8%) of 55 subjects, and we did not observe any patients with *TPMT* risk variants. The mean MP dosages were 48.0 (± 21.2), 34.1 (± 17.0), and 3.2 (± 1.2) mg/m² for the normal-, intermediate-, and low-activity *NUDT15* groups, respectively ($P = 4.8 \times 10^{-4}$).

The TGN and DNA-TG were successfully quantified for 69 and 74 samples, respectively, with 68 pairs of TGN and DNA-TG collected in the same patients and at the time points. The mean TGN and DNA-TG levels were 134.1 pmol/ 4×10^8 RBC (range, 0.46–315.5 pmol/ 4×10^8 RBC) and 442.8 fmol/ μ g DNA (range, 78.1–1054.0 fmol/ μ g DNA), respectively. TGN level was negatively correlated with the number of *NUDT15* risk alleles ($P = 5.3 \times 10^{-6}$) and this association remained significant after adjusting for MP dosage ($P = 1.7 \times 10^{-6}$). In contrast, patients with the low-activity *NUDT15* diplotypes had the highest DNA-TG/dosage ratio, followed by the intermediate-activity diplotype group, and lowest in patients with wild-type *NUDT15* ($P = 4.0 \times 10^{-9}$). Importantly, we observed that cytosolic TGN and nuclear DNA-TG levels were highly correlated with each other, consistent with the fact that TGN is the precursor metabolite of DNA-TG during thiopurine activation ($P = 6.5 \times 10^{-4}$; $R^2 = 0.16$, Fig 1). However, the regression slope of DNA-TG over TGN was significantly higher for patients with the intermediate-activity *NUDT15* diplotype (the green line) than those with the normal-activity *NUDT15* diplotype (the black line in Fig 1). Consistently, the ratio of DNA-TG to TGN (i.e., the percent of TGN converted to DNA-TG) was significantly higher in *NUDT15* deficient patients ($P = 3.6 \times 10^{-9}$, Fig 2), suggesting that the lack of functional *NUDT15* directly resulted in more robust conversion of TGN to DNA-TG, and therefore elevated risk of toxicity.

Discussion

It is now well established that *NUDT15* is a major negative regulator of thiopurine activation and polymorphisms in this gene strongly influence hematopoietic toxicity related to this class of drugs [10–12, 22, 23]. Therefore there is a pressing need to develop *NUDT15*-based dosing algorithm to individualize thiopurine therapy. To that end, we set out to evaluate how *NUDT15* genotype affects two main active metabolites of thiopurines (TGN and DNA-TG) in a cohort of Japanese pediatric ALL patients. In patients with *TPMT* deficiency, because thiopurines cannot be inactivated by methylation, the prodrug is converted to TGN in excess. In fact, in spite of 90% dose reduction, TGN level remains significantly higher in patients

with homozygous *TPMT* deficiency compared to those with wild-type *TPMT* receiving full dose of thiopurine [24]. In contrast, the absolute level of TGN was significantly lower in patients with *NUDT15* risk variants, most likely reflecting the reduced dose received. This is consistent with our previous report that the point of action of *NUDT15* is distinct from *TPMT* in thiopurine metabolism, and also suggests that TGN is not responsible for the excessive toxicity in patients with *NUDT15* deficiency [12]. Instead, *NUDT15* alters the ratio of TGTP to TGMP (but not the total TGN), which in turn increases incorporation into DNA (DNA-TG) in deficient patients, as shown in our prior studies *in vitro* [12]. If this holds true in patients, cytosolic TGTP and nuclear DNA-TG in WBC would be the most relevant metabolite to be monitored in patients with *NUDT15* deficiency. This strategy has a number of potential advantages over the current erythrocyte TGN assay: 1) TGTP and DNA-TG would be measured from WBC which represent cell populations directly affected by myelosuppression, as opposed to TGN measured in red blood cells as a proxy marker, 2) because TGTP and DNA-TG are more directly related to DNA damage, they are thus possibly better metabolite markers for thiopurine cytotoxicity compared to TGN. It should be noted that methylated thiopurine metabolites have also been shown directly or indirectly influence DNA-TG conversion[20]. Finally even though there was a significant difference in DNA-TG/TGN ratio by *NUDT15* genotype, there was also notable variation in MP metabolites within each genotype group, pointing to possible effects of other yet unknown genetic variants.

In conclusion, our results suggest that DNA-TG levels were strongly influenced by *NUDT15* genotypes in children with ALL and point to this metabolite as a potential therapeutic monitoring endpoint for *NUDT15* genotype-based thiopurine dosing algorithms.

Acknowledgments

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Fig. 1.

Association of erythrocyte TGN with mononucleated cells DNA-TG. Cytosolic TGN and nuclear DNA-TG levels were highly correlated with each other, consistent with the fact that TGN is the precursor metabolite of DNA-TG during thiopurine activation ($P=6.5 \times 10^{-4}$, $R^2 = 0.16$). However, the regression slope of DNA-TG over TGN was significantly higher for patients with the intermediate-activity *NUDT15* diplotype (the green line) than those with the normal-activity *NUDT15* diplotype (the black line).

Fig. 2. Association of DNATG/TGN with *NUDT15* genotypes. The ratio of DNA-TG to TGN (i.e., the percent of TGN converted to DNA-TG) was significantly higher in *NUDT15* deficient patients ($P = 3.6 \times 10^{-9}$), suggesting that the lack of functional *NUDT15* directly resulted in more robust conversion of TGN to DNA-TG, and therefore elevated risk of toxicity.

Table 1

Patients summary in the studied cohort

Gender	
Male	30
Female	25
Age at diagnosis	4.8 (range 1–14)
<i>NUDT15</i> diplotypes	
*1/*1	44
*1/*2	3
*1/*3	6
*1/*5	1
*2/*3	1
<i>TPMT</i> diplotype	
*1/*1	55