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Diplotype analysis of *NUDT15* variants and 6-mercaptopurine sensitivity in pediatric lymphoid neoplasms

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Thiopurines are key to the treatment of acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma (LBL) in children and adults, and 6-mercaptopurine (6-MP) is commonly used in consolidation and maintenance therapy–. Leukopenia is a dose-limiting toxicity of 6-MP partly explained by hypomorphic variants of *TPMT*– and clinical importance of *TMPT* genotyping is well established. Recently, *NUDT15* was identified as a novel thiopurine regulator conferring 6-MP sensitivity most prominently in Asians and

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Author contributions

M. Kato is the principal investigator and takes primary responsibility for the paper, designed this study, interpreted data, wrote the manuscript, and gave final approval; S.T., T.O. collected data, analyzed data, interpreted data, and wrote the manuscript; T.O. evaluated patients, collected data, analyzed data, interpreted data, and wrote the manuscript; M.U., R.S., T.M., R.N., Y.Y., T.U., N.K., and J.J.Y. collected data, analyzed data, interpreted data. K.Kudo, M.S., Y.A., K.T., S.I., K.Koh, J.T., E.I., and D.T. evaluated patients, and collected data; All authors discussed the results and critically reviewed the manuscript. No author has a conflict of interest to disclose.

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Hispanics–. Patients with bi-allelic *NUDT15* variants are extremely sensitive to 6-MP, and only 5–10% of the standard dose is sufficient to maintain the target leukocyte count·.

Thus far, a total of seven variants in *NUDT15* with low diphosphatase activity resulting in excess myelosuppression by 6-MP have been identified·, and haplotypes with different combinations of variants are known to exist (Figure 1A). Given the clinical importance of *NUDT15* genotyping for individualized dosing of 6-MP·, the diplotype should be precisely determined, especially for those with heterozygous genotype at multiple variants. For example, a case with heterozygous c.36_37insGGAGTC and c.415C>T, which is the most frequent combination, should be determined as compound heterozygosity (*3/*6) or mono-allelic variants (*1/*2), because the two diplotypes have significantly different impacts on total NUDT15 activity.

However, a convenient method to determine the diplotype of *NUDT15* has not been established, and as shown in the previous study, the diplotype is currently inferred using a public catalogue of human variants. Herein we demonstrated a method for identifying the diplotype using droplet digital PCR (ddPCR), and confirmed the results by wild-type specific PCR by a restriction enzyme.

In total 138 Japanese children with ALL or LBL were enrolled (Supplementary Table 1). Patients were treated at the National Center for Child Health and Development, the University of Tokyo Hospital, the Hirosaki University Hospital or the Saitama Children's Medical Center. For maintenance therapy, the 6-MP and MTX dosages were adjusted to maintain a leukocyte count of 1,500–3,000/μl by at least monthly blood tests. In this study, a tolerable 6-MP and MTX dose was defined as the average of the doses per day (per week for MTX) administered during the first 6 months of maintenance therapy. When maintenance therapy was interrupted during the 6 months and 6-MP/MTX was held, the period was calculated as 0 mg/m^2.

This study was approved by the institutional ethics board of the National Center for Child Health and Development (#1035), and informed consent was obtained from the patients or guardians.

Germline DNA and RNA were extracted from peripheral blood during remission. We genotyped the seven known variants of *NUDT15* loci (c.36_37insGGAGTC, c.37_42delGGAGTC, c.52G>A, c.101G>C, c.103A>G, c.415C>T, and c.416G>A) by Sanger sequencing, as reported previously·. For all cases, *TMPT* variants were also genotyped, for c.280G>C (rs1800462), c.460G>A (rs1800460), and c.719A>G (rs1142345). The primer sequences are listed in Supplementary Table 2.

The diplotype of each case was assessed by ddPCR utilizing characteristics of droplets that could separate template DNA molecules·. PCR reaction using fluorescent probes specific to the variants was performed for each droplet to determine the allelic configuration of *NUDT15* (Figure 1B).

The results of diplotyping were validated by restriction enzyme-PCR (RE-PCR). The synthesized cDNA was digested by HpyCH4~, which specifically digests the c.415C>T

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allele. PCR was performed using the digested cDNA as a template, followed by analysis with capillary sequencing to provide a wild-type allele-specific nucleotide sequence for cases with heterozygous c.415C>T (Figure 1C).

The genomic *NUDT15* was subcloned into pUC19 cloning vector, and the diplotyping results were also confirmed by Sanger sequencing of the vectors.

The details of *NUDT15* diplotyping and the primer sequences are shown in the Supplemental Method section.

The genotyping results are shown in Supplementary Table 1. Thirty-eight (27.5%) cases had one or more variants in the *NUDT15 gene*, and three cases (2%) had a homozygous c.415C>T variant. Twenty cases (14.4%) had one heterozygous variant of c.36_37insGGAGTC (n = 1), c.52G>A (n = 7) or c.415C>T (n = 12). Fifteen cases were heterozygous at two variants, 14 patients had c.36_37insGGAGTC and c.415C>T, and one case had c.52G>A and c.415C>T. Three cases had *TPMT* heterozygous variants of c.719A>G, whose *NUDT15* was wild-type.

Because c.415C>T and c.36_37insGGAGTC can be located either on the same allele (i.e., *1/*2) or on different alleles (*3/*6), we sought to define diplotype of the 14 patients carrying the two variants (i.e., c.415C>T and c.36_37insGGAGTC). As confirmed that both ddPCR and RE-PCR were capable of detecting all the combinations with or without c.36_37insGGAGTC or c.415C>T (*1, *2, *3, and *6) using an artificial mixture of subcloned vectors (Figure 1B and 1C), these two methods consistently demonstrated that c.36_37insGGAGTC exists on the same allele of c.415C>T in all of the 14 cases, plausibly determining their diplotypes as *1/*2. Considering the allele frequencies of *2, *3 and *6 in our cohort (5.1%, 6.9% and 0.4%, respectively), there is still a possibility to encounter a patient showing compound heterozygosity (i.e., *3/*6) who is at higher risk for excessive 6-MP toxicity compared to patients with *1/*2. Thus, our methods can be informative to distinguish *1/*2 from *3/*6, and eventually mitigate 6-MP toxicity.

We were unable to validate the diplotyping of the case with the two heterozygous variants of c.52G>A and c.415C>T by either ddPCR or RE-PCR, although a reason for this failure is still unclear. We then cloned the whole genomic region of *NUDT15* to the TA-cloning pUC19 vector confirming that the two variants were located on different alleles with compound heterozygotes for *3/*5 (Supplementary Figure 1A and 1B).

Based on these results, the allele frequency for one or more hypomorphic variants of *NUDT15* in our cohort was estimated at 15.2% (Table 1). We next evaluated the average dose of 6-MP required to maintain the target leukocyte count in 103 patients receiving maintenance therapy for more than 6 months. The median of the tolerated dose in all cases was 39.4 mg/m^2 (1.1–75.5 mg/m^2). The tolerated 6-MP doses were extremely low among the three patients with homozygous c.415C>T variants (1.4, 2.0, and 5.6 mg/m^2), and the patient with compound heterozygous variants required significant reduction of 6-MP (2.5 mg/m^2). On the other hand, even though 14 cases had two heterozygous variants (c.36_37insGGAGTC and c.415C>T), they were able to tolerate a significantly higher dose of

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6-MP than those with bi-allelic variants because the two variants were located on the same allele (Figure 1D).

Using a combination of methods (e.g., ddPCR, TA cloning), we were able to resolve *NUDT15* haplotype in 100% of the cases in this cohort. As all four variants detected in our cohort were previously reported to have lower enzymatic activity of NUDT15 at the same degree, we classified the children into three groups based on diplotype: the normal activity (normal/normal), intermediate activity (normal/low), and low activity (low/low) groups. Sensitivity of the low activity group (n = 4) was significantly higher than that of the intermediate activity cases (n = 27) and the normal activity cases (n = 72), and the median tolerable dosage was 2.3 mg/m^2, 36.7 mg/m^2, and 43.5 mg/m^2, respectively (Supplementary Figure 2A).

Even though we reproduced high sensitivity to 6-MP is conferred by the *NUDT15* variants, the tolerable dose for Asian cases without the *NUDT15* variants were significantly low, and it was concordant to the previous reports. Especially, 25% of cases had sensitive to 6-MP (~30 mg/m^2) and some cases showed extremely high sensitivity (<5 mg/m^2) even without having known variants of either *TPMT* or *NUDT15*. Extremely sensitive cases are also found in non-Asian population. More comprehensive approaches including whole genome sequencing should be done to identify potential novel variants responsible for high sensitivity to 6-MP.

Patients with low *NUDT15* activity are able to tolerate the standard dose of MTX (Supplementary Figure 2B), and Asian population can tolerate approximately 20 mg/m2, which is almost identical to that seen in North American patients. Therefore, preemptive genotyping of *NUDT15* can help to avoid not only excess myelosuppression and serious complications such as infection, but also any unnecessary reduction in concomitant MTX during maintenance therapy.

Our study has some limitations. First, the number of cases is still small to identify optimal doses for each allelic genotype including rare variants and diplotypes, and to find statistical difference between intermediate activity cases and normal activity cases. Second, in this study, we only included newly diagnosed cases, and it should be noted that relapsed patients would likely not tolerate comparable doses of 6-MP. Further studies with larger cohort are required to solve these issues.

In conclusion, we established a novel method of *NUDT15* diplotype analysis by ddPCR which was validated by allele-specific sequencing. Thiopurines are used not only hematologic malignancies, but also auto-immune diseases such as inflammatory bowel disease, thus clinical importance of *NUDT15* genotyping is widely recognized. The diplotype information for *NUDT15* is essential for predicting 6-MP sensitivity, and its possible clinical applications should be considered.

Supplementary Material

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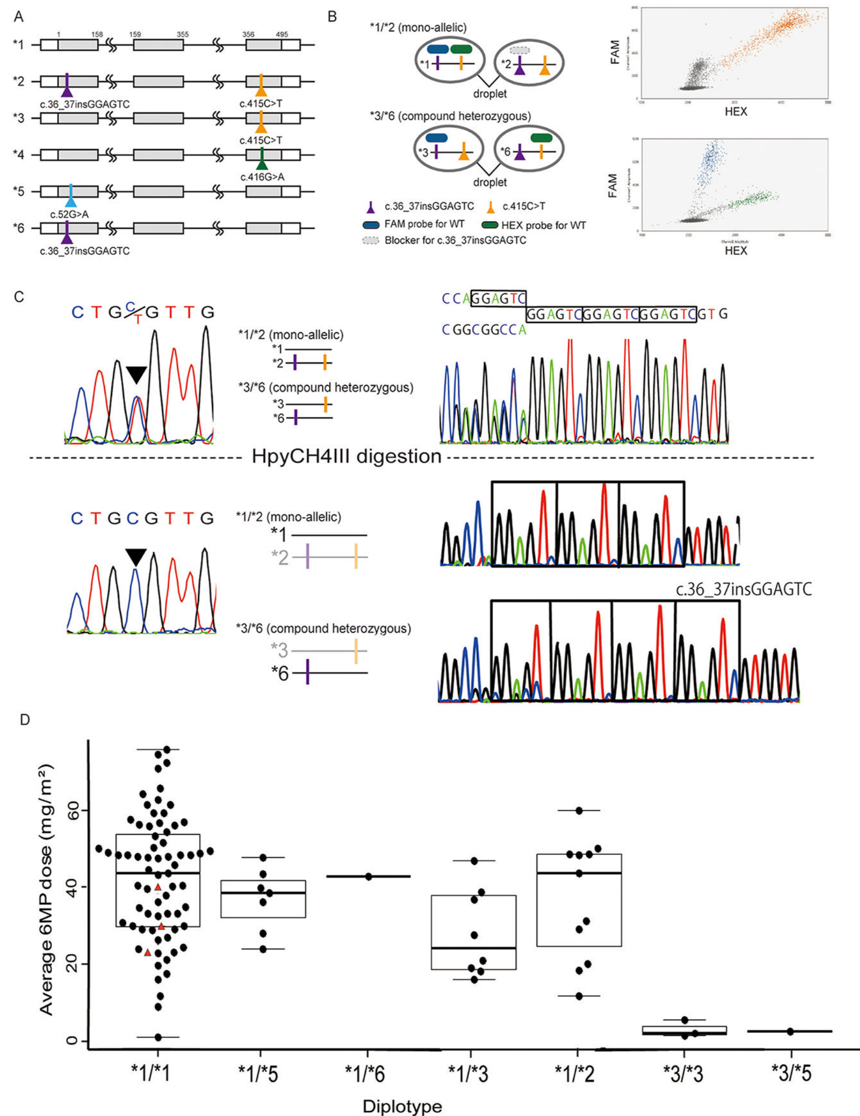

Figure 1. Diplotyping of *NUDT15* variants and tolerated 6-MP dose

(A) Major haplotypes of *NUDT15*. (B) Diplotype analysis by droplet digital PCR. Variant-specific probes (FAM for c.36_37insGGAGTC and HEX for c.415C>T) were able to distinguish compound heterozygous variants (*3/*6) and mono-allelic variants (*1/*2). (C) Diplotype analysis by restriction enzyme-PCR. They were cleaved with *HpyCH4III* and PCR was performed to confirm the results of ddPCR. (D) Average 6MP doses according to diplotype. Three cases without a *NUDT15* variant (*1/*1) had a *TPMT* heterozygous variant (c.719A>G), shown in red triangles.

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Table 1.

Distribution of diplotypes of *NUDT15*

***NUDT15* diplotype**	**Number of patients**
No variant	
*1/*1	100 (72.5)
Mono-allelic variants	
*1/*2	14 (10.1)
*1/*3	12 (8.7)
*1/*5	7 (5.1)
*1/*6	1 (0.7)
Bi-allelic variants	
*3/*3	3 (2.2)
*3/*5	1 (0.7)

Diplotypes of the 138 cases determined in our study are shown.