An international retrospective study for tolerability of 6-mercaptopurine on *NUDT15* bi-allelic variants in children with acute lymphoblastic leukemia

6-mercaptopurine (6-MP) is one of the essential chemotherapeutic agents for treatment of acute lymphoblastic leukemia (ALL) in children and adults.[1] Bone marrow suppression is the main dose-limiting toxicity of 6-MP, and the sensitivity to 6-MP is strongly affected by germline variants in genes regulating thiopurine metabolism.[2] Recently, the *NUDT15* variant c.415C>T has been identified as a genetic cause for 6-MP intolerability,[3] which could explain the majority of thiopurine-induced myelosuppression in Asians that are also common in Hispanics.[2] So far, multiple *NUDT15* haplotypes with various combination of variants are known to exist (Figure 1A). Several researchers have reported that these variants had decreased NUDT15 activity,[4, 5] and bi-allelic variants caused extremely intolerance to 6-MP.[6] However, individual studies included a limited number of patients with bi-allelic variants, which significantly hindered the comprehensive analysis of the exact clinical course of 6-MP toxicity and development of evidence-based recommendations. Therefore, in this international collaborative study, we comprehensively evaluated the actual 6-MP tolerable dose, frequencies of 6-MP-induced toxicity, and outcomes in ALL patients with bi-allelic variants of *NUDT15*.

We asked collaborators from Japan, Singapore, Malaysia, Taiwan, China, and Thailand, about their experience of cases with *NUDT15* bi-allelic variants, which led to the identificationof 37 ALL cases, most of the which were genotyped due to intolerance to 6-MP. Clinical information of the cases was retrospectively collected, focusing on 6-MP dosing and toxicity. Patients with *NUDT15* bi-allelic variants were enrolled in this study, including some patients in prior case reports or small case series.[6, 7] *NUDT15* was genotyped by Sanger sequencing[4]. Thiopurine methyltransferase (*TPMT*) genotype information was available for 20 cases, and no case had hypomorphic variants which also confer 6-MP sensitivity. The treatment of maintenance therapy typically started with 40 to 60 mg/m^2/day of 6-MP (*Online Supplementary Table S1*) and 20 to 40 mg/m^2/week of methotrexate (MTX); these dosages were adjusted to maintain the target leukocyte count at 1,500 to 3,000/μL. Toxicities were graded by the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0, and those rates were estimated by cumulative incidence. The tolerated dosages of 6-MP and MTX were defined as the average (mean) of the doses per day or per week, respectively, during the entire duration of maintenance therapy. The dose for bi-allelic variant was compared with the dose for wild-type and mono-allelic variant in our previous report.[6, 7]

The average dose in each *NUDT15* genotype was estimated by the Kruskal-Wallis test. The interruption duration between 6-MP initial doses was estimated by the Mann Whitney *U*-test. Four-year overall survival (OS) and event-free survival (EFS) from start of maintenance therapy were estimated by the log-rank test. The statistical analysis was conducted using R statistical software (version 3.4.1; http://www.r-project.org/).

Patient characteristics for the 37 cases are shown in Table 1. Patients with bi-allelic variant had intolerance to 6-MP, and reduction was required mainly due to myelosuppression (*Online Supplementary Figure S1*). The average 6-MP dose of these patients during maintenance therapy was 5.2 (range, 1.1–25.6) mg/m^2/day, and the 6-MP dose by each diplotype is shown in Figure 1B. Comparatively, the average MTX dose was 10.4 (range, 1.9–44.6) mg/m^2/week (*Online Supplementary Figure S2*). This 6-MP dose was significantly lower compared with the average dose for the *NUDT15* wild-type (n=138, 41.7 mg/m^2, P=3.9×10^{-14}) and mono-allelic variant (n=47, 33.6 mg/m^2, P=2.7×10^{-13}) in Japanese patients reported previously (Figure 2).[6, 7] Most of the cases showed intolerance to 6-MP, and 10 mg/m^2 or less was sufficient to maintain the target leukocyte range for 32 (86.4%) of the 37 cases. The median 6-MP average dose for *2/*2, *2/*3, and *3/*3 (poor metabolizer [PM]) were 5.2 mg/m^2/day, and the average dose was not different among these three diplotypes (P=0.29, Figure 1B). *NUDT15* haplotypes other than PM showed heterogeneous sensitivity to 6-MP, although the average 6-MP dose as a group was not statistically different from PM (*Online Supplementary Table S2*, P=0.53).

Table 1. Patient characteristics

	Japan	Singapore	Taiwan	China	Thailand
Total, n	20	7	6	3	1
Male/Female, n	9/11	3/4	5/1	1/2	1/0
Median age, years (range)	6 (3-15)	6 (3-14)	9 (3-16)	6 (4-7)	5
Immunotype (BCP/T), n	18/2	7/0	5/1	2/1	1/0
Median 6-MP initial dose, mg/m^2 (range)	17.2 (1.9-51.3)	10.9 (3.5-17.5)	11.4(5.0-39.5)	20.6 (4.3-29.8)	24.4
NCI/Rome criteria Standard/High risk, n	15/5	3/4	4/2	2/1	1/0
NUDT15 genotype, n					
*2/*2	1	0	3	0	0
*2/*3	5	1	0	1	0
*2/*5	1	0	0	0	0
*2/*6	0	2	0	0	0
*2/*7	0	0	1	0	0
*3/*3	10	4	2	1	1
*3/*5	2	0	0	1	0
*5/*5	1	0	0	0	0

BCP: B-cell precursor; T: T-cell; 6-MP: 6-mercaptopurine; n: number; 6-MP: 6-mercaptopurine; NCI: National Cancer Institute.

Thirty-two of the 37 patients (86.5%) required interruption of maintenance therapy, and the median duration of interruption for all patients was 47 days (range, 0– 148 days). In patients with a 6-MP initial dose <10 mg/m^2, the days of interruption during whole maintenance therapy was significantly shorter than in patients with a 6-MP initial dose of 10 mg/m^2 or more (P=0.042) (*Online Supplementary Figure S3*). When limited to the interruption within the first 8 weeks of maintenance therapy,[8] the effect of the initial dose was more remarkable (Figure 1C).

In terms of toxicities, 36 of the 37 patients were observed to have grade 3 or worse neutropenia. Grade 4 leukopenia and grade 4 neutropenia were observed in 16 (43.2%) and 32 (86.4%) patients, respectively, and the median observation times of leukopenia and neutropenia were 33 days (range, 19–662 days) and 37 days (range, 9–139 days), respectively, from start of the maintenance therapy (Figure 1D). We, thus, confirmed that the dose-limiting toxicity of 6-MP in patients with *NUDT15* bi-allelic variant was neutropenia. Moreover, during the consolidation therapy (most of the protocol adopted early consolidation with 6-MP, so called "IB"), severe myelosuppression was observed in 21 of these patients (*Online Supplementary Table S3*). Conversely, grade 3 or worse liver enzyme elevation was observed in only 10 patients.

The median duration of follow-up was 1,398 days (range, 84–5,357 days) from the start of maintenance therapy. One patient relapsed during maintenance therapy and five patients relapsed at 772 to 2,659 days from the start of maintenance therapy. Three of these six patients died at 499 to 720 days after relapse. The causes

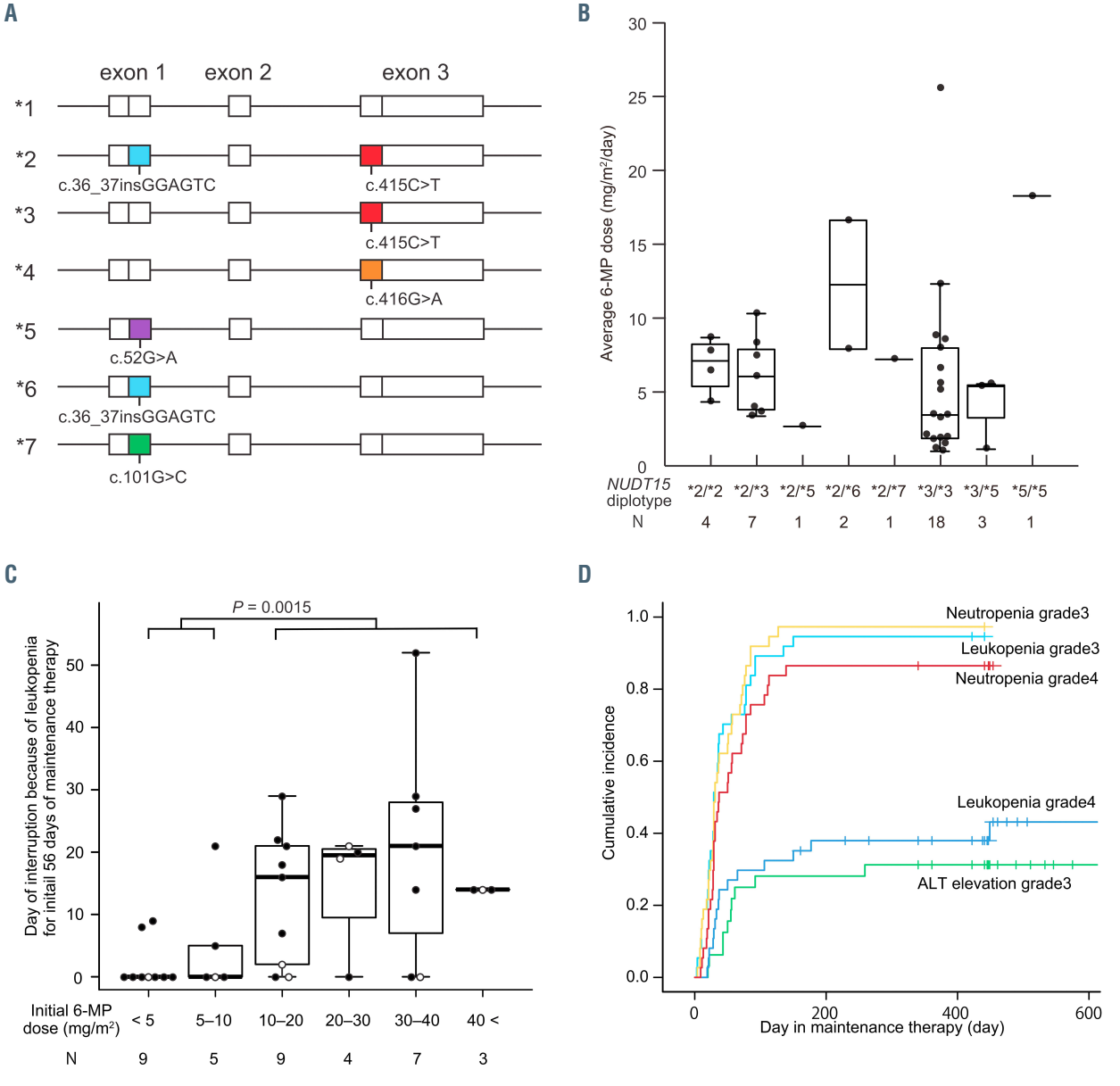

Figure 1. Tolerability and efficacy for patients with *NUDT15* bi-allelic variants. (A) Major haplotypes of *NUDT15*. (B) Average 6-mercaptopurine (6-MP) dose in each *NUDT15* bi-allelic variant. (C) The association between initial 6-MP dose and therapy interruption for 56 days for start of therapy in maintenance therapy in patients with *NUDT15* bi-allelic variant. Black circles and white circles show starting dose for patients with bi-allelic variant of exon 3 and others, respectively. (D) Toxicity during maintenance therapy.

of death were relapse of leukemia, second malignancy, or complications related to bone marrow transplantation. OS and EFS were 91%± 6% and 82%±7%, respectively (*Online Supplementary Figure S4*).

This Asian international study showed that most patients with NUDT15 PM required a reduced 6-MP dose to <10 mg/m^2 during maintenance therapy. These findings were concordant with the recommendations by the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines.[2] *NUDT15* c.52G>A and c.36_37insGGAGTC are defined as an uncertain function allele in the CPIC guidelines,[2] and a patient with *5/*5 can tolerate as high as 18.3 mg/m^2. However, three cases with *3/*5 had intolerance to 6-MP at <10 mg/m^2, pointing to a compound heterozygous effect. Additionally, cases with bi-allelic variant with *6 (only c.36_37insGGAGTC) might be more tolerant to 6-MP than those with c.415C>T. Moriyama *et al.* defined *3 as low, and *5 and *6 as intermediate activity *in vitro*.[4] Our results demonstrate that diplotypes of intermediate/intermediate tolerate moderate intensity, but that intermediate/low is extremely sensitive to 6-MP. These heterogeneous sensitivities in bi-allelic variants of *NUDT15* highlight the importance of precise diplotyping analysis.

Twenty-seven patients started maintenance therapy with the reduced 6-MP dose to less than 30 mg/m^2, mainly because they experienced severe toxicities during consolidation and their *NUDT15* variants had already been genotyped. As shown in the *Online Supplementary Figure S1*, typical cases with *NUDT15* bi-allelic variants showed a sudden crash of the leukocyte count after an approximately 2-week exposure to 6-MP, and required a long time to attain recovery of leukocyte counts. These observations are concordant with the findings of previous reports.[8, 9] Accordingly, adjustment of the 6-MP dose is often difficult in most cases as the 6-MP dose fluctuated dramatically and treatment interruption was common. With a reduced starting dose of 6-MP, dose fluctuation was not observed and maintenance therapy could be given continuously. However, some researchers reported that patients with the *NUDT15* c.415C>T variant developed thiopurine-induced leukopenia within 2 months from initiation of therapy.[7, 10]

Regarding tolerability to MTX, some studies reported that the average MTX dose was not different in *NUDT15* genotypes.[6, 11] However, some cases had reduced MTX dose, probably due to myelosuppression caused by 6-MP and, thus, the optimal MTX dose in *NUDT15* bi-allelic cases needs to be established in future studies.

Patients with the *NUDT15* variant experienced thiopurine-induced hematological toxicity for several months regardless of the disease or race.[9] The majority of patients with *NUDT15* bi-allelic variant experienced grade 4 neutropenia. This finding was in line with previous reports that *Nudt15*$^{-/-}$ mice, which demonstrated significantly decreased neutrophil counts upon thiopurine exposure.[12] Neutrophils were more sensitive than other leukocytes to thiopurine with deficient *NUDT15*. For patients with bi-allelic variants, neutrophil counts should be carefully monitored, as well as total leukocyte counts, during 6-MP treatment. Given the risk of severe infectious complications, pre-emptive *NUDT15* genotyping for all patients with ALL should be performed and dose modification in cases with bi-allelic variants must be considered.

This study has some limitations. First, *TPMT* genotype information is insufficient because routine screening for TPMT variants, another determinant of 6-MP sensitivity, was not performed. However, considering variant distribution of NUDT15 and TMPT[2], variant allele frequency

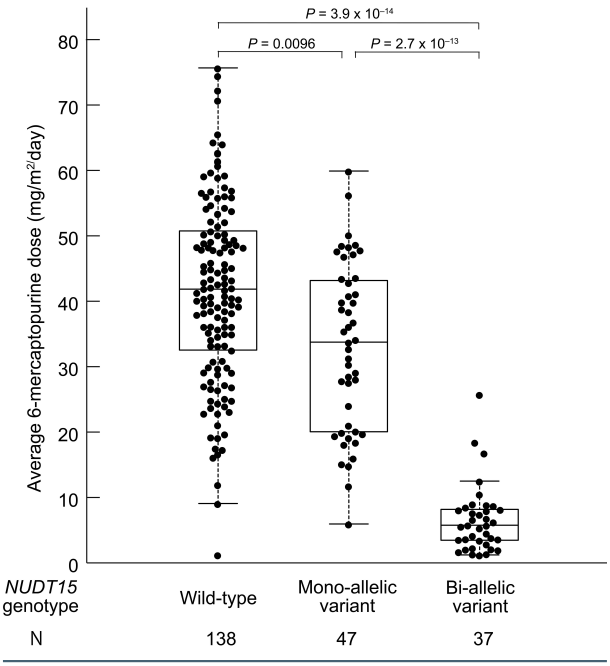

Figure 2. Average 6-mercaptopurine dose during maintenance therapy.

of TPMT in those with NUDT15 bi-allelic variant is extremely low as observed in our limited data. Therefore, we can select, according to each racial background, which of the two major genetic determinants of 6-MP should be genotyped. However, considering recent racial mixture and advances in genomic analysis technology, comprehensive genotyping information responsible for drug sensitivity for all cases should be obtained to provide a precise medical approach. Second, most of our cases were identified as having NUDT15 variants because of their intolerance to 6-MP, and , thus, the tolerable dose of NUDT15 bi-allelic cases may be overestimated, which underpins the importance of upfront genotyping. Third, the number of cases with some haplotypes (such as *6 or *7) were small, and tolerability of those patients with these rare haplotypes still needs to be determined by future studies.

In conclusion, bi-allelic *NUDT15* variants conferred extreme intolerance to 6-MP. Pre-emptive *NUDT15* genotyping for all patients with ALL should be performed and dose modification in cases with bi-allelic variants must be considered. Precise upfront genotyping and a reduction of the 6-MP dose to less to than 10 mg/m^2 is recommended to avoid the risk of severe complications and therapy interruption.

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