Interplay between IL6 and CRIM1 in thiopurine intolerance due to hematological toxicity in leukemic patients with wild-type NUDT15 and TPMT

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NUDT15 and TPMT variants are strong genetic determinants of thiopurine-induced hematological toxicity. Despite the impact of homozygous CRIM1 on thiopurine toxicity, several patients with wild-type NUDT15, TPMT, and CRIM1 experience thiopurine toxicity, therapeutic failure, and relapse of acute lymphoblastic leukemia (ALL). Novel pharmacogenetic interactions associated with thiopurine intolerance from hematological toxicities were investigated using whole-exome sequencing for last-cycle 6-mercaptopurine dose intensity percentages (DIP) tolerated by pediatric ALL patients (N = 320). IL6 rs13306435 carriers (N = 19) exhibited significantly lower DIP (48.0 ± 27.3%) than non-carriers (N = 209, 69.9 ± 29.0%; p = 0.0016 and 0.0028 by t test and multiple linear regression, respectively). Among 19 carriers, 7 with both heterozygous IL6 rs13306435 and CRIM1 rs3821169 showed significantly decreased DIP (24.7 ± 8.9%) than those with IL6 (N = 12, 61.6 ± 25.1%) or CRIM1 (N = 94, 68.1 ± 28.4%) variants. IL6 and CRIM1 variants showed marked inter-ethnic variability. Four-gene-interplay models revealed the best odds ratio (8.06) and potential population impact [relative risk (5.73), population attributable fraction (58%), number needed to treat (3.67), and number needed to genotype (12.50)]. Interplay between IL6 rs13306435 and CRIM1 rs3821169 was suggested as an independent and/or additive genetic determinant of thiopurine intolerance beyond NUDT15 and TPMT in pediatric ALL.

Abbreviations
ALL Acute lymphoblastic leukemia
DIP Dose intensity percentage
GVB Gene-wise Variant Burden
CPIC Clinical Pharmacogenetics Implementation Consortium
PGx Pharmacogenetic
PM Poor metabolizer
IM Intermediate metabolizer
NM Normal metabolizer

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Despite improvements in combination drug therapy and risk stratification, approximately 20% of pediatric patients with acute lymphoblastic leukemia (ALL) still experience drug resistance and treatment failure due to drug toxicities. In European populations, about 50% of thiopurine-induced cytotoxic adverse reactions, such as severe neutropenia and leukopenia, are explained by NUDT15 and TPMT genetic variants. The Clinical Pharmacogenetics Implementation Consortium (CPIC) publishes practical guidelines for the implementation of pharmacogenetic (PGx) testing of thiopurine by using traditional star (*) allele-based molecular phenotyping for NUDT15 and TPMT.

According to the established guideline, the thiopurine dose is pharmacogenetically titrated based on the known risk variants of NUDT15 and TPMT. However, a substantial proportion of patients with leukemia presenting no genetic variation in NUDT15 or TPMT still experience life-threatening toxicities, which may result in dose reduction and/or discontinuation of thiopurine, resulting in therapeutic failure and relapse of leukemia. In an attempt to overcome the PGx gap, CRIM1 rs3821169 homozygote has been identified in East Asians as a novel risk variant of thiopurine-induced hematological toxicities. Heterozygotes of the variant have revealed only mild effect on thiopurine toxicity with an unknown clinical impact. However, its high prevalence (T = 0.066, Phase 3 of the 1000 Genomes Project) and remarkable inter-ethnic variability might have severely confounded previous PGx studies assessing thiopurine toxicity. Therefore, investigating PGx interactions of novel genes/variants, other than NUDT15 and TPMT variations, is urgently needed for preventing thiopurine intolerance due to hematological toxicities and improving pediatric ALL care.

The categorical nature of the traditional star allele haplotype-based method can complement the quantitative nature of gene-wise variant burden (GVB) method for evaluating the complex interplay of multiple genes/variants. For instance, designating three categories (i.e., poor (PM), intermediate (IM), and normal (NM) metabolizers) per gene creates an exponentially increasing complexity of $3^N$ for a drug with N-gene PGx interactions. NUDT15 and TPMT have been assigned nine PGx subgroups for thiopurine, which will increase exponentially following new PGx discoveries across different ethnic groups. GVB quantitates the cumulative variant burden of one or more genes into a single score with dimensionality reduction, thus providing a reliable frame for multiple gene-interaction analysis.

In the present study, we aimed to identify novel PGx interactions associated with thiopurine toxicity in pediatric ALL patients carrying both wild-type (WT) NUDT15 and TPMT (and not carrying homozygous CRIM1 rs3821169) by using whole-exome sequencing (WES) technology. Our investigation of the effect of novel candidate PGx variants on the last-cycle 6-mercaptopurine (6-MP) dose intensity percentage (DIP) tolerated by pediatric patients with ALL, revealed clinically significant hematological toxicities and thiopurine intolerance. Our results provide not only the measures of clinical validity but also the measures of population impact (or clinical utility), including relative risk (RR), population attributable fraction (PAF), number needed to treat (NNT), and number needed to genotype (NNG), for preventing thiopurine toxicity.

**Methods**

**Subjects.** As described in our previous study, we recruited 320 Korean pediatric patients with ALL, who underwent maintenance therapy with 6-MP at three teaching hospitals, Seoul National University Hospital (SNUH), Asan Medical Center (AMC), and Samsung Seoul Medical Center (SMC), located in Seoul, South Korea. All subjects conformed with the exclusion criteria (i.e., relapse of the disease, stem cell transplantation, Burkitt’s lymphoma, mixed phenotype acute leukemia, infant ALL, or very high-risk of ALL). Patients were assigned to the standard-risk group if they were 1–9 years of age at the time of diagnosis with a white blood cell (WBC) count less than 50 × 10⁹/L, and all other patients were assigned to the high-risk group. Patients underwent hematopoietic stem cell transplantation if they met one or more of the following criteria: age younger than 1 year, hypodiploidy, the presence of t(9;22), a WBC count equal to or greater than 200 × 10⁹/L, or the 11q23 rearrangement. Patients allocated to the standard-risk group were treated with Children’s Cancer Group (CCG)-18919, CCG-195217, or Children’s Oncology Group (COG) AALL-0331 regimens. In high-risk groups, CCG-188219, 0601, or 1501 protocols for Korean multicenter studies were employed. In Korea, the planned dose of 6-MP was modified from 75 to 50 mg/m², as several patients who had been administered the same dose...
under the original Western protocol exhibited moderate to severe toxicities during 6-MP administration\textsuperscript{15,21}. The 6-MP doses during maintenance therapy were adjusted to maintain a WBC count of 2.0–3.5 × 10^9/L, with an absolute neutrophil count (ANC) of over 500/μL. Hepatotoxicity-related dose modifications were primarily based on the COG guidelines; however, they were also performed at the discretion of the treating physician as this study was not undertaken per the uniform prospective protocols. Hematotoxicity as the clinical endpoint was estimated by the tolerated last-cycle 6-MP DIP (%). The percentage of the actual prescribed amount to the planned dose (50 mg/m^2) was defined as the last-cycle 6-MP DIP using the recorded 6-MP dose per meter body surface area over the last-cycle (12-week) of maintenance. Doses employed for the last maintenance cycle were considered, as dose modification of 6-MP was mainly adopted during the early phase of maintenance. Further detailed descriptions of patients and measurements have been summarized in our previous study\textsuperscript{3,15,21}. The present study was approved by the SNUH, AMC, and SMC Institutional Review Boards. Written informed consent was obtained from each participant. For the participants under the age of 18 years, informed consent was obtained from a parent and/or legal guardian. All experiments and methods were performed in accordance with the relevant guidelines and regulations.

Whole-exome sequencing and pharmacogenomic subgrouping. WES data were obtained for pediatric patients with ALL patients and analyzed in a bioinformatics pipeline as previously described\textsuperscript{4,5,11}. CPIC provides major PGx genes with haplotype definitions and molecular function annotations based on star (* ) nomenclature. We classified patients with ALL into PM, IM, and NM groups for each gene, NUDT15 and TPMT, according to CPIC classifications\textsuperscript{13,14}. For both genes, we considered NMs as WTs. As in our previous study, data regarding DIP and the relative frequency of neutropenia (ANC < 500 μL) was available for the discovery cohort (N = 244)\textsuperscript{8}. The relative frequency of neutropenia was defined by the ratio of frequencies of complete blood cell counts (CBC) with neutropenia from among the total tested CBCs. However, data regarding the frequency of neutropenia was not collected for the replication cohort (N = 76) at the time of this analysis. Therefore, we conducted a variant selection process using the discovery cohort. First, we performed multivariate linear regression, adjusting age, sex, and body surface area. Of 14,931 genes with GVB scores, 10 genes were identified with statistical significance at both DIP and the relative frequency of ANC < 500 μL. Next, we identified 45 variants with SIFT (sorting intolerant from tolerant) scores from among 156 variants of genes that passed multivariate linear regression with GVB score. Among the 45 variants, 3 variants passed the multivariate linear regression cutoff for SNPs\textsuperscript{32}. Finally, we selected IL6 rs13306435 as a novel candidate, which was only a missense variant among 3 variants (Fig. 1, Supplementary Table S1). We observed that no star name had been designated to the novel (or candidate) PGx genes. Thus, for the purpose of the present study, we defined non-carriers of CRIM1 rs3821169 and IL6 rs13306435 as WT carriers (WTs) of CRIM1 and IL6, respectively (Table 2). Haplotypes were determined using PHASE 2.1.1\textsuperscript{23,24}.

Gene-wise variant burden for evaluating single- and multi-gene effects. GVB analysis was performed to evaluate the aggregated impact of both common and rare variants\textsuperscript{5,15}. For each individual, the GVB score was calculated for each gene independently in the coding region, where GVB\textsuperscript{G} denotes the GVB score of gene G [range 0.0–1.0]. The more deleterious the variant burden, the lower the score. First, we included NUDT15 and TPMT in GVB analysis because these genes are clinically recognized to be related to thiopurine-induced toxicity and have clinical guidelines like CPIC guidelines. Additionally, we included CRIM1 rs3821169, which was identified in our previous study to determine conditional GVB\textsuperscript{C}. Finally, we included IL6 rs13306435 following the selection process stated above. The multi-gene effect was evaluated by defining GVB\textsuperscript{M} as the geometric mean of GVB\textsuperscript{G} scores, GVB\textsuperscript{P} and GVB\textsuperscript{B} [range 0.0–1.0]. Gene-variant interaction was considered by defining conditional GVB\textsuperscript{G,(variant)} as the GVB score of gene G, depending on the presence or absence of the specified variant. For example, GVB\textsuperscript{G,B} equals GVB\textsuperscript{CRIM1} when rs13306435 is present, vanishing to a WT score of 1.0 when absent.

Inter-ethnic variability of allele frequencies and molecular phenotypes. Using the 2504 whole-genome sequences with multiple ethnicities provided by the 1000 Genomes Project phase 3\textsuperscript{5}, we investigated inter-ethnic distributions of PGx alleles and haplotypes, along with their molecular phenotypes associated with thiopurine intolerance due to hematological toxicities (Table 2).

Statistical analysis. The last-cycle 6-MP DIPs (%) according to different PGx groups were assessed using Student’s t test or one-way ANOVA with post hoc Tukey test. Multiple linear regression was also applied to adjust for confounding clinical variables. The powers of GVB\textsuperscript{NUDT15}, GVB\textsuperscript{TPMT}, GVB\textsuperscript{CRIM1}, and GVB\textsuperscript{IL6} and their combinations for predicting 6-MP DIPs, were systematically evaluated by analyzing ROC (receiver operating characteristic) curves across eight different DIP cutoffs (i.e., 10%, 15%, 25%, 35%, 45%, 60%, 80%, and 100%) in terms of AUCs (areas under the ROC curves) (Figs. 3, 4). An ROC curve is a two-dimensional depiction of classification performance integrating all sensitivity and specificity values at all cutoff levels\textsuperscript{25}. All statistical analyses were performed using the R statistical package (version 3.5.1). R package ‘PROC’ was used for calculating AUC values\textsuperscript{26}. The optimal cutoff for the GVB score was determined by maximizing Youden’s index\textsuperscript{27}.

GVB\textsuperscript{CRIM1*(rs3821169)} was applied to control the potential confounding effect of the impressively high carrier frequency in East Asians [43.7% (220/504)], compared with other ethnicities (0.2–9.4%), and the mild effect of heterozygous expression on thiopurine intolerance attributed to hematological toxicities. GVB\textsuperscript{CRIM1*(rs3821169)} denotes a conditional GVB score of CRIM1 dependent on the presence or absence of homozygous rs3821169 variant (denoted as rs3821169*). It equals GVB\textsuperscript{CRIM1} when the subject carries homozygous rs3821169 variant and otherwise vanishes to 1.0.
Clinical validity parameters. We calculated and assessed clinical validity for each statistical parameter as follows.

Positive predictive value (PPV) implies the probability of an event when the genetic variant is present. In contrast, negative predictive value (NPV) means the probability of no event when the genetic variant is absent.

NNT is the inverse of the absolute of intervention, that is, the difference between the proportion of events in the control group and the proportion of events in the case group, which can be written as:

\[ \text{NNT} = \frac{1}{P_c - P_i} \]  

If NNT is 20, it implies that 20 patients are needed to prevent an event like death or an adverse effect. NNG is the number of patients who must be genotyped to avoid one patient from experiencing an adverse event, which can be predicted based on following formula

\[ \text{NNG} = \frac{1}{P_c} \]  

For example, an NNG of 33 means that one adverse event was avoided for every 33 patients genotyped.

RR is the relative ratio of the proportion of events in the control group and the proportion of events in the case group, which is calculated by following formula

\[ \text{RR} = \frac{P_i}{P_c} \]  

Odds ratio (OR) is calculated based on the comparison of the relative odds of an event in each group, which can be determined as

\[ \text{OR} = \frac{P_i}{P_c} \]
### Table 1. Clinical characteristics of 320 pediatric ALL patients with 6-MP maintenance therapy according to their pharmacogenetic subgroupings of NUDT15, TPMT, CRIM1, and IL6 genes. ALL acute lymphoblastic leukemia, 6-MP 6-mercaptopurine, WT wild-type, SD standard deviation. † One subject showed non-WT characterization for both NUDT15 and TPMT genes. Values are the number of subjects (percentage) unless specified. Age means the age at the start of 6-MP maintenance therapy.

| Characteristics          | All WTs N = 115 | NUDT15 or TPMT Non-WT N = 80† | NUDT15 and TPMT both WTs N = 125 |
|--------------------------|----------------|-------------------------------|----------------------------------|
| **No. of subjects**      | 115 (35.94%)  | 72 (22.50%)                  | 94 (39.17%)                      |
| **Age, median (range, year)** | 5.3 (1.2–19.4) | 4.6 (1.7–15.8)              | 5.7 (1.1–17.0)                  |
| **Sex**                  |                |                               |                                  |
| Male                     | 58             | 43                            | 63                               |
| Female                   | 57             | 29                            | 31                               |
| **Risk group**           |                |                               |                                  |
| Standard-risk            | 73             | 46                            | 35                               |
| High-risk                | 42             | 26                            | 15                               |
| **Last-cycle 6-MP dose intensity percentage, (%)** |                |                               |                                  |
| ~ 10                     | 2 (1.7%)       | 9 (12.5%)                    | 0 (0.0%)                         |
| 10–15                    | 1 (0.9%)       | 3 (4.2%)                     | 1 (1.1%)                         |
| 15–25                    | 4 (3.5%)       | 9 (12.5%)                    | 1 (1.1%)                         |
| 25–35                    | 5 (4.4%)       | 7 (9.7%)                     | 9 (9.6%)                         |
| 35–45                    | 5 (4.4%)       | 9 (12.5%)                    | 5 (5.3%)                         |
| 45–60                    | 26 (22.6%)     | 11 (15.3%)                   | 16 (17.0%)                       |
| 60–80                    | 31 (27.0%)     | 14 (19.4%)                   | 32 (34.0%)                       |
| 80–100                   | 23 (20.0%)     | 7 (9.7%)                     | 13 (13.8%)                       |
| **Total**                | 115 (100.0%)   | 72 (100.0%)                  | 94 (100.0%)                      |
| **Average ± SD (%)**     | 71.3 ± 29.55   | 47.1 ± 30.48                 | 68.0 ± 28.39                     |

In the above Eqs. (1),(2),(3),(4), \( P_c \) is the proportion of events in the control group and \( P_p \) is the proportion of events in the case group. PAF is the proportion of events that would be eliminated from the population if exposure to the risk factor were eliminated, which can be assessed as

\[
P(Y = 1) - P(Y = 1|X = 0)
\]

\[
P(Y = 1)
\]

In Eq. (5), Y is an event development and X is a binary risk factor.

**Results**

**IL6 rs13306435 as a novel pharmacogenetic variant for thiopurine intolerance due to hematological toxicities.** We classified patients into three groups according to the variant status of NUDT15 and TPMT to identify new variants not confounded by the two most critical PGX genes associated with thiopurine intolerance. Table 1 describes the clinical characteristics of 320 pediatric patients with ALL according to their PGX subgroups, presenting 80 patients who were non-WTs (i.e., IMs or PMs) of NUDT15 and/or TPMT (N = 80), 115 patients who were all WTs (WT carriers of all the four genes), and 125 who were WTs of both genes, NUDT15 and TPMT (both WTs) and carried CRIM1 rs3821169 and/or IL6 rs13306435 variants. Of the 125 patients with WT characterization for both NUDT15 and TPMT, 94, 12, 11, and 8 patients belonged to the heterozygous CRIM1, heterozygous IL6, homozygous CRIM1, and IL6 and CRIM1 variant groups, respectively (Table 1). We used patients with all WTs (N = 115) as a control group for the following analysis. The average of the tolerated 6-MP DIPs of non-WTs for NUDT15 (47.1 ± 30.5%, N = 72) and/or for TPMT (56.6 ± 33.6%, N = 9) were significantly lower than that of all WTs (71.3 ± 29.6%, N = 115) (p < 0.001, Table 1). The patients with homozygous CRIM1 (dark blue circle in Fig. 3) tolerated significantly lower 6-MP DIP than the patients with all WTs before (N = 16, 44.6 ± 35.2%) or after (N = 11, 42.3 ± 35.0%) controlling the five subjects with NUDT15 (59.76 ± 37.24%) or IL6 (9.77%) variants.

To rule out the PGX effect of NUDT15, TPMT, and homozygous CRIM1 on thiopurine intolerance, we extracted 228 samples of non-carriers for these variants for the further discovery of novel PGX variants. We observed that carriers of IL6 rs13306435 (N = 19, 48.0 ± 27.3%) exhibited significantly lower 6-MP DIPs than non-carriers (N = 209, 69.9 ± 29.0%), as evaluated by Student’s t test (p = 0.0016) and multiple covariate linear regression (p = 0.0028). Furthermore, of the 19 carriers, 7 patients with both IL6 rs13306435 and CRIM1 variants demonstrated significantly lower 6-MP intolerance, with a DIP of 24.7 ± 8.9% when compared with the DIP of...
12 patients harboring only IL6 rs13306435 variant (61.6 ± 25.1%; orange circle in Fig. 3). The potential interplay between IL6 and CRIM1 variants was suggested, which was further supported by the finding that seven patients with both IL6 and CRIM1 variants showed significantly lower 6-MP DIPs (24.7 ± 8.9%) than 94 heterozygous CRIM1 carriers (68.1 ± 28.4%; light blue circle in Fig. 3).

**Interplay of IL6 and CRIM1 variants in thiopurine toxicity.** Figure 2 exhibits the distributions of the last cycle 6-MP DIPs (%) of 115 all WT (Fig. 2a), carriers of only heterozygous CRIM1 (N = 94, Fig. 2b), carriers of only heterozygous IL6 (N = 12, Fig. 2c), carriers of only homozygous CRIM1 (N = 11, Fig. 2d), and carriers of both IL6 and CRIM1 variants (N = 8, Fig. 2e). Homozygous CRIM1 and IL6 and CRIM1 groups showed significantly lower 6-MP DIPs (44.6 ± 35.2% and 24.7 ± 8.9%, respectively, Fig. 2d,e) than all WTs and heterozygous CRIM1 groups (71.3 ± 29.6% and 68.1 ± 28.4%, respectively, Figs 2a,b) by one-way ANOVA (p < 0.0001; adj. p < 0.05 post hoc Tukey). Furthermore, the IL6 and CRIM1 group showed significantly lower 6-MP DIPs (44.6 ± 35.2%) than the heterozygous IL6 group (61.6 ± 25.1%; adj. p < 0.05, post hoc Tukey) (Fig. 2c,e). All 10 patients with both IL6 and CRIM1 variants with any NUDT15 or TPMT status (red numbers in Fig. 3) exhibited the lowest DIPs (9.77–32.68%) among all subgroups of the whole PGx groups. Thus, a significant interplay between IL6 and CRIM1 in thiopurine intolerance was suggested.

Notably, it was more clinically relevant to evaluate the magnitude of the actual decrease in 6-MP DIP (%) tolerated by patients than the sheer statistical significance affected by the study sample size and biomarker prevalence. Table 1 shows that more than one-quarter of the patients with homozygous CRIM1 (36.4%) and IL6 and CRIM1 (50.0%) tolerated less than 25% of the planned DIP, increasing the risk of thiopurine therapeutic failure. The DIPs of our cohorts were comparable with those of the recommended 6-MP doses published in the current CPIC guideline when TPMT variants were involved. Furthermore, when we raised the DIP cutoff from 25 to 35%, the proportions of homozygous CRIM1 and IL6 and CRIM1 groups increased to 54.6% (6/11) and 87.5% (7/8), respectively, which far exceeded 38.9% and 33.3% of NUDT15 (28/72) and TPMT (3/9) non-WTs, respectively. Notably, only 6.1% (7/115) and 10.5% (12/115) of all WTs tolerated less than 25% and 35% of the planned DIP (Table 1).

**Inter-ethnic variabilities in carrier frequencies and molecular phenotypes.** Both NUDT15 and TPMT show wide inter-ethnic variabilities. Table 2 exhibits inter-ethnic variabilities of the PGx variants and molecular phenotypes of the four thiopurine pharmacogenes computed from among 2504 subjects of the 1000 Genomes Project. NUDT15 non-WT (i.e., IM or PM) is common in East (22.6%) and South (13.9%) Asians but rare in Europeans and Africans (<1%). In contrast, TPMT non-WT is common in Europeans (8.0%) and Americans (13.3%) but relatively rare in Asians (<5.0%).

Novel PGx variant, CRIM1 rs3821169, demonstrates remarkably high minor allele frequency (T = 0.255) and carrier prevalence (43.7%, 220/504) in East Asians. Table 2 also shows that 6.5% of East Asians harbor homozygous CRIM1 rs3821169 variant, which can hardly be detected in other populations (<1.0%). In contrast, IL6 rs13306435 is widely distributed with the highest carrier frequency of 15.0% in Americans and 3.0% among Asian and European populations; It is rare in South Asian and African populations (<1.0%). The carrier frequencies of both IL6 and CRIM1 variants were 2.0% and 1.2% for East Asian and American populations, respectively.

**Single- and multi-gene prediction performances of IL6 and CRIM1.** We performed ROC analysis of GVB-based single- and multi-gene models to predict the last-cycle 6-MP DIPs (%) using 240 both WTs for NUDT15 and TPMT to control their long-known PGx effects. Figure 4 demonstrates that (b) GVB^CRIM1 outperformed (a) GVB^IL6 in predicting DIPs at all cutoff levels, probably due to the higher variant frequency of CRIM1 over IL6 in the study population. Two-gene model GVB^IL6,CRIM1 (Fig. 4c) consistently outperformed each of the single-gene models (GVB^IL6 and GVB^CRIM1) at all cutoffs.

For a comprehensive evaluation of all PGx interactions among NUDT15, TPMT, IL6, and CRIM1, we performed comprehensive ROC analysis using data of all 320 pediatric patients with ALL (Fig. 5). Among the four single-gene models in Fig. 5a,b,d), GVB^NUDT15 outperformed others at all cutoffs, probably due to the high prevalence of NUDT15 variants and the strong metabolic impact of on thiopurine toxicity. Two-gene models (Fig. 5c,f) consistently outperformed each of the corresponding single-gene counterparts, i.e., the order of their AUCs was GVB^NUDT15,TPMT > GVB^NUDT15 > GVB^TPMT and of GVB^IL6,CRIM1 > GVB^CRIM1 > GVB^IL6 at all cutoff levels. Three-gene models created by adding IL6 or CRIM1 to the traditional NUDT15 and TPMT model also consistently improved the prediction accuracy (Fig. 5g,h). The final four-gene model in Fig. 5i outperformed all other models in predicting DIPs at all cutoff levels. Moreover, it is worth noting that the ROC curves across eight DIP cutoffs in Fig. 5 exhibited ‘dose–response relationships’, i.e., GVB score’s prediction power (measured by AUC) increases as a function of the severity of thiopurine intolerance (measured by DIP). That is, the final four-gene model’s AUC increased as a function of decreasing DIP (%) (i.e., AUC^c^35% = 0.757, AUC^c^25% = 0.748, AUC^c^20% = 0.711, AUC^c^15% = 0.716, AUC<^10%^ = 0.646, and AUC<^5%^ = 0.592 in a descending order, Fig. 5i).

**Evaluation of the clinical validity and utility of the star allele and GVB methods.** We systematically compared the clinical utility as well as clinical validity of traditional star (*) allele-based and GVB-based methods for preventing thiopurine toxicity. Table 3 demonstrates the measures of clinical validity and potential population impact along with the pharmacogenetic association of the different prediction models. Because the designated star alleles for IL6 or CRIM1 were not available, star allele-based molecular phenotyping was not applicable for these novel genes. GVB^NUDT15,TPMT slightly outperformed STAR^NUDT15,TPMT, the classical star (*) allele-based molecular phenotyping (Table 3a,b). Three-gene models (i.e., GVB^NUDT15,TPMT,IL6 and GVB^NUDT15,TPMT,CRIM1) also outperformed the
two-gene models (Table 3c,d). The four-gene interplay model, GVB\textsuperscript{NUDT15,TPMT,IL6,CRIM1}, presented the best performance for all the eight measures of clinical validity and potential population impact (except specificity) (marked in bold numbers in Table 3g).

Figure 2. Distribution of the average of the tolerated last-cycle 6-MP DIPs (%) of the CRIM1 rs3821169 and/or IL6 rs13306435 variant carrier vs. non-carrier subgroups among 240 pediatric patients with ALL presenting both NUDT15 and TPMT WT characterization. Of the 320 pediatric patients with ALL, we excluded 80 carriers presenting either NUDT15 or TPMT variants to obtain 240 subjects with both NUDT15 and TPMT WT. Both (a) non-carrier group of CRIM1 rs3821169 or IL6 rs13306435 (N=115, 47.92%, i.e., “All WTs” in Table 2 and Fig. 3) and (b) carrier group of heterozygous CRIM1 rs3821169 only (N=94, 39.17%) showed significantly higher thiopurine tolerance than the carrier groups of (d) homozygous CRIM1 rs3821169 (N=11, 4.38%) (adj. \( p < 0.05 \), posthoc Tukey) and of (e) both IL6 rs13306435 and CRIM1 rs3821169 (N=8, 3.33%) (adj. \( p < 0.0005 \), posthoc Tukey) by one-way ANOVA (\( p = 0.0001 \)). (c) Carrier group of IL6 rs13306435 heterozygous variant only (N=12, 5.00%) showed significantly higher thiopurine intolerance than (c) that of the hetero/homozygous variant of both IL6 and CRIM1 (adj. \( p < 0.05 \), posthoc Tukey). No carrier of homozygous IL6 was detected, and only one subject carried both heterozygous IL6 and homozygous CRIM1 variants (DIP = 9.7%). Thiopurine intolerance was measured by the last-cycle 6-MP DIP (%) among 240 pediatric patients with ALL presenting both NUDT15 and TPMT WT genes to control their effect on thiopurine intolerance. *\( p < 0.05 \) and **\( p < 0.01 \), posthoc Tukey test after one-way ANOVA. ALL acute lymphoblastic leukemia, WT wild-type, DIP (%) dose intensity percentage, 6-MP 6-mercaptopurine.
Figure 3. Distribution of the last-cycle 6-MP DIP for pediatric patients with ALL according to NUDT15, TPMT, CRIM1, and IL6 pharmacogenetic subgroups (N = 320). Green circles depict NUDT15 and TPMT metabolism phenotypes, and blue and orange circles represent CRIM1 rs3821169 and IL6 rs13306435 genotype subgroups, respectively. Of the 320 patients, 115 with no pharmacogenetic variants exhibited higher 6-MP DIPs (71.31%) than 72 NUDT15 (47.14%), 9 TPMT (56.56%), 147 CRIM1 (57.89%), and 25 IL6 (DIP = 44.47%) non-WTs. Subjects with both CRIM1 and IL6 variants (N = 10, 3.13%) exhibited the lowest DIPs (9.77–32.68%, in red numbers). Numbers are the number of subjects and 6-MP DIPs (mean ± SD).

Table 2. Inter-ethnic variability of thiopurine toxicity-associated pharmacogenetic variants. Whole-genome sequences of multiple ethnic groups were obtained from the 1000 Genomes Project (N = 2504). Haplotypes and diplotypes were determined by the CPIC allele-definition tables and molecular phenotypes by the CPIC diplotype-phenotype matching tables. NM normal metabolizer, IM intermediate metabolizer, PM poor metabolizer, WT wild-type, non-WT non-wild-type (i.e., poor- or intermediate thiopurine-metabolizing subgroups of NUDT15 and TPMT carriers), DIP dose intensity percentage, SD standard deviation.
traditional GVB-based model than with the traditional star (*) allele-based method [23 patients in Table 3a].

In the group of all the patients with 6-MP toxicities (DIP < 25%), we could expect eight patients more with the GVB-based model than with the traditional star (*) allele-based model [23 patients in Table 3a].

In the present study, the interplay between IL6 and CRIM1 variants in thiopurine intolerance due to hematological toxicity was investigated in 320 pediatric patients with ALL.

### Discussion

In the present study, the interplay between IL6 and CRIM1 variants in thiopurine intolerance due to hematological toxicity was investigated in 320 pediatric patients with ALL.

IL6 has been known to modulate hematopoiesis and neutrophil trafficking, especially possessing a role in anti-apoptosis38. In patients with osteomyelitis, IL6 was correlated with longer neutrophil survival apart from other cytokines; this anti-apoptotic effect was blocked using anti-IL6 antibodies and reversed with anti-IL629.

In this regard, patients with a heterozygous variant of rs13306435 might have decreased anti-apoptosis29. In patients with osteomyelitis, IL6 and neutrophil survival.

CRIM1 is a cell-surface transmembrane protein that resembles developmentally important proteins which are known to interact with bone morphogenetic proteins (BMPs). A role of CRIM1 in drug resistance has been...
suggested by previous studies\textsuperscript{34,35} revealing that the level of mRNA expression of CRIM1 is high in resistant leukemic cells. This affects the levels of BMPs, suggesting that CRIM1 regulates the growth and differentiation of hematopoietic cells. The rs3821169 heterozygous cases revealed lower mRNA expression levels than the WT cases, which indicated that subjects carrying this variant might display drug-sensitive responsiveness\textsuperscript{8}. Although we could not clarify the detailed mechanism underlying the interplay between IL6 and the CRIM1 variant, the presence of a negative feedback loop between IL6 and the BMP pathway was reported, in which increased levels of IL6 induced BMP pathway activities resulting in the suppression of IL6\textsuperscript{36}. Based on our findings, it can be suggested that the interplay between IL6 and CRIM1 in thiopurine intolerance due to hematological toxicity may represent a pharmacodynamic effect leading to an adverse reaction, while the well-known NUDT15 and TPMT are pharmacokinetic enzymes for metabolizing thiopurines.

The present study presented several limitations that need to be acknowledged, including the possible confounding effects from concomitant medications (methotrexate or vincristine) and the absence of serum level measurements of drugs or metabolites. Moreover, not all patients with thiopurine toxicity were explained by pharmacogenetic analysis. Seven (6.1\%) of the 115 all-WT patients experienced thiopurine toxicity. Supplementary Table S2 lists further candidate variants determined by analyzing the all WTs (N = 115, p < 0.05 by one-sided Student’s t test). Of the three carriers of FSIP2 rs191083003, two (66.7\%) exhibited DIP < 25\% (8.82, 21.88, and 48.54\%, N = 3). We observed one more FSIP2 rs191083003 carrier in the homozygous-CRIM1 group, who exhibited the lowest DIP of 6.94\% within the entire ALL cohort (N = 320). The low frequency (1.25\%, 4/320) of FSIP2 rs191083003 prohibited any conclusion, necessitating further elucidation. Overall, the interplay between IL6 and CRIM1 in thiopurine intolerance due to hematological toxicity may represent a pharmacodynamic effect leading to an adverse reaction, while the well-known NUDT15 and TPMT are pharmacokinetic enzymes for metabolizing thiopurines.

Figure 5. Prediction accuracy profile of single- and multi-gene models for thiopurine intolerance in pediatric patients with ALL (N = 320). Single-gene prediction models of (a) NUDT15 and (b) TPMT were outperformed by (c) the two-gene combined model and those of (d) IL6 and (e) CRIM by (f) the IL6-CRIM1 combined model. Three-gene models of NUDT15, TPMT, and (g) IL6 and (h) CRIM1 were outperformed by (i) all four-gene combined models. Overall the final (i) four-gene combined models outperformed other models for predicting thiopurine intolerance at all DIP levels in pediatric patients with ALL (N = 320). AUCs for predicting the last-cycle 6-MP DIP (%) were measured at 8 cutoff levels (≤ 10\%, ≤ 15\%, ≤ 25\%, ≤ 35\%, ≤ 45\%, ≤ 60\%, ≤ 80\%, and ≤ 100\%). ALL acute lymphoblastic leukemia, DIP dose intensity percentage, AUC area under the receiver operating characteristics curve, GVB gene-wise variant burden.

\textsuperscript{34,35}
Table 3. Contingency tables for predicting thiopurine intolerance (DIP < 25%) of two-, three-, and four-gene models in pediatric patients with ALL (N = 320). ALL acute lymphoblastic leukemia, DIP (%) the last-cycle 6-MP dose intensity percentage, PAF population attributable fraction, PPV positive predictive value, NNT number needed to treat, NNG number needed to genotype, OR odds ratio, M poor metabolizer, IM intermediate metabolizer, STAR\textsuperscript{NUDT15, TPMT} classical star (*) allele-based haplotyping of NUDT15 and TPMT genes according to the CPIC guideline, GVB gene-wise variant burden, GVB\textsuperscript{CRIM1} \textsuperscript{(rs13306435, rs3821169*)} \textsuperscript{GVB} of CRIM1 dependent on IL6 rs13306435 or CRIM1 rs3821169 homozygote, GVB cutoff value of 0.3 was selected as maximized Youden's index. Bold number means True Positive (TP) group, True Negative (TN) group and patients with thiopurine intolerance (DIP<25%). For example, In Table 3a, 23 means TP group and 219 means TN group. 44 indicates that number of patients with thiopurine intolerance(DIP<25%) is 44. Additionally, the most strongest statistical parameter numbers related to clinical validity at the bottom of the table was highlighted in bold. For example, prediction model (g) and (e) showed the best effectiveness in number needed to trat (NNT, 3.673) and number needed to genotype (NNG, 12.503).

Reportedly, Americans present the highest allele frequency of IL6 rs13306435 (A = 0.078) among all ethnic groups (Global A = 0.020, the 1000 Genomes Project, Phase 3\textsuperscript{39}). This high inter-ethnic variability may partially explain why rs13306435 has not yet been identified as a biomarker for thiopurine intolerance. Current research is mostly biased towards Europeans\textsuperscript{37}. NUDT15 rs116855232 variant, which was recently discovered in the Korean population as a strong predictor of thiopurine toxicity\textsuperscript{9}, shows the highest allele frequency in East Asians (T = 0.095) among all ethnic groups (Global T = 0.040). Pharmacogenes, by definition, unlike pathogenic disease genes, do not have an overt phenotype unless exposed to drugs. The absence of detrimental phenotypic effect attributed to pharmacogenes may have permitted wide inter-ethnic variability and/or diversity across different genes, according to the CPIC guideline, haplotyping of TPMT and NUDT15, TPMT, IL6, CRIM1\textsuperscript{(rs13306435, rs3821169*)}.

**Conclusion**

In summary, our results suggest an independent and/or additive effect of the interplay between IL6 rs13306435 and CRIM1 rs3821169 on thiopurine intolerance attributed to hematological toxicity in pediatric ALL.
Data availability

All data generated or analyzed during this study are included in this article. If any additional information is required, it may be obtained by request from the corresponding author.

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References

1. Schaeffeler, E. et al. Impact of NUDT15 genetics on severe thiourowel-related hematotoxicity in patients with European ancestry. Genet. Med. 21(9), 2145–2150 (2019).
2. Yang, J. J. et al. Inherited NUDT15 variant is a genetic determinant of mercaptopurine intolerance in children with acute lymphoblastic leukemia. J. Clin. Oncol. 33(11), 1235 (2015).
3. Yang, S.-K. et al. A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leukemia. Nat. Genet. 46, 1017–1020 (2014).
4. Moriyama, T. et al. NUDT15 polymorphisms alter thiopurine metabolism and hematopoietic toxicity. Nat. Genet. 48(4), 367–373 (2016).
5. Relling, M. V. & Klein, T. E. CPIC: Clinical pharmacogenetics implementation consortium of the pharmacogenomics research network. Clin. Pharmacol. Ther. 89(3), 464–467 (2011).
6. Relling, M. V. et al. Clinical pharmacogenetics implementation consortium guidelines for thiourowel methyltransferase genotype and thiopurine dosing: 2013 update. Clin. Pharmacol. Ther. 93(4), 324–325 (2013).
7. Relling, M. V. et al. Clinical pharmacogenetics implementation consortium guideline for thiopurine dosing based on TPMT and NUDT15 genotypes: 2018 update. Clin. Pharmacol. Ther. 105(5), 1095–1105 (2019).
8. Park, Y. et al. A CRIM1 genetic variant is associated with thiopurine-induced neutropenia in leukemic patients with both wild-type NUDT15 and TPMT. J. Transl. Med. 18(1), 1–13 (2020).
9. Genomes, P.C. et al. A global reference for human genetic variation. Nature 526(7571), 68–74 (2015).
10. Lee, K. H. et al. Genome sequence variability predicts drug precautions and withdrawals from the market. PLoS One 11, e0162135-e1162115 (2016).
11. Park, Y. et al. Star allele-based haplotyping versus gene-wise variant burden scoring for predicting 6-mercaptopurine intolerance in pediatric acute lymphoblastic leukemia patients. Front Pharmacol. 10, 654 (2019).
12. Park, Y., Soo, H., Bye, B. Y. & Kim, J. H. Gene-wise variant burden and genomic characterization of nearly every gene. Pharmacogenomics 21(12), 827–840 (2020).
13. Park, J. et al. Gene-wise burden of coding variants correlates to noncoding pharmacogenetic risk variants. Int. J. Mol. Sci. 21(9), 3091 (2020).
14. Tonk, E. C. M., Gurwitz, D., Maitland-van der Zee, A.-H. & Janssens, A. C. J. W. Assessment of pharmacogenetic tests: Presenting required, it may be obtained by request from the corresponding author.
15. Yang, J. J. et al. Interleukin-6 as a keystone cytokine in health and disease. J. Transl. Med. 33(11), 1–13 (2020).
16. Park, J. et al. Star allele-based haplotyping versus gene-wise variant burden scoring for predicting 6-mercaptopurine intolerance in pediatric acute lymphoblastic leukemia patients. Front Pharmacol. 10, 654 (2019).
17. Matloub, Y. et al. In vivo interleukin-6 protects neutrophils from apoptosis in osteomyelitis. Infect. Immun. 72(7), 3823–3828 (2004).
18. Asensi, V. et al. Interleukin-6 rescues lymphocyte from apoptosis and exhaustion induced by chronic hepatitis C virus infection. Viral Immunol. 31(9), 624–631 (2018).
19. Fawcett, T. An introduction to ROC analysis. Pattern Recogn. Lett. 27(8), 861–874 (2006).
20. Robin, X. et al. pROC: An open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinform. 12(1), 77 (2011).
21. Iwata, N. Index for rating diagnostic tests. Cancer 3(1), 32–35 (1950).
22. Relling, M. V. et al. Clinical pharmacogenetics implementation consortium guideline for thiopurine dosing based on TPMT and NUDT15 genotypes: 2018 update. Clin. Pharmacol. Ther. 105(5), 1095–1105 (2019).
23. Hunter, C. A. & Simon, A. J. IL-6 as a keystone cytokine in health and disease. J. Investig. 102(7), 1360–1370 (1998).
24. Renkert, M., Uggla, B., Tideloft, U. & Strid, H. CRIM1 is expressed at higher levels in drug-resistant than in drug-sensitive myeloid hematopoietic leukemia IL60 cells. Anticancer Res. 30(10), 4157–4161 (2010).
25. Ziliak, D. et al. Genetic variation that predicts platinum sensitivity reveals the role of miR-193b* in chemotherapeutic susceptibility. Mol. Cancer Ther. 11(9), 2054–2061 (2012).
26. Hagen, M. et al. Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. Am. J. Pathol. Lung Cell. Mol. Physiol. 292(6), L1473–L1479 (2007).
27. Sirugo, G., Williams, S. M. & Tishkoff, S. A. The missing diversity in human genetic studies. Cell 177, 26–31 (2019).
28. Bansal, V., Libiger, O., Torkamani, A. & Schork, N. J. Statistical analysis strategies for association studies involving rare variants. Nat. Rev. Genet. 11(11), 773–785 (2010).
39. Witte,. Rare genetic variants and treatment response: Sample size and analysis issues. Stat. Med. 31(25), 3041–3050 (2012).

**Author contributions**
H.K., S.Y., H.J.I., H.J.K., and J.H.K. designed the model and the framework. H.K., J.Y.C., Y.M., K.H.Y., H.J.I., and H.J.K. collected samples and clinical data. S.Y., Y.P., and S.Y. analyzed the data and carried out the implementation of the idea. S.Y. performed the calculations. S.Y. and J.H.K. wrote the manuscript. K.T.H., K.N.K., K.H.L., H.Y.S., and S.L. reviewed the manuscript. J.H.K., H.J,K, and H.J.I. conceived the study and were in charge of its overall direction and planning. All authors read and approved the final version of the manuscript.

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