## Combination of common and novel rare *NUDT15* variants improves predictive sensitivity of thiopurine-induced leukopenia in children with acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer in the world, with treatment outcomes improving dramatically in the past several decades due to the combined usage of multiple drugs and sophisticated therapeutic protocols.[1] Thiopurine (e.g., 6-mercaptopurine) is primarily used in ALL treatment, but can induce severe adverse drug reactions (ADR), including leukopenia and hepatotoxicity.[1,2] Inherited predispositions to both ALL susceptibility and treatment outcomes have been noticed.[3-7] Pharmacogenetics studies have indicated that cases of leukopenia can be largely explained by single nucleotide polymorphisms (SNPs) in *TPMT* and *NUDT15* with varied variant allele frequencies among different ethnicities.[2,8,9] In particular, the common missense variant in *NUDT15* (i.e., rs116855232) has been reported to be consistently associated with thiopurine-induced leukopenia, either through genome-wide association studies or candidate replications in several independent patient cohorts, made up for the most part by East Asians and Hispanics.[8-10] Subsequently, several less frequent and rare variants have also been identified that negatively impact on *NUDT15* function, and which can further increase the predictive sensitivity of the eventual dosage of thiopurine.[9,11-13] As a consequence, it is necessary to sequence the full length of *NUDT15* in order to determine the initial clinical dosage of thiopurine, which, unlike *TPMT*, has had a limited number of functional variants identified thus far.[14]

In the study herein, which is a CCCG-ALL-2015 protocol based trial of Chinese pediatric ALL patients aged from one to fifteen years old (N = 188), we systematically investigated the association of thiopurine-induced ADRs with all *NUDT15* variants in the West China Second Hospital between 2015 and 2016. 6-mercaptopurine (6-MP) was used in multiple phases of ALL therapy: remission induction (standard dosage of 60mg/m$^2$ in the last two weeks), consolidation (25mg/m$^2$), and maintenance (50mg/m$^2$). Forty-eight patients in total experienced thiopurine-induced leukopenia, requiring an obligatory reduction of the dosage of 6-MP, an interruption of the therapy, and/or the prescription of human granulocyte colony-stimulating factor (G-CSF). Thirty-seven patients experienced thiopurine-induced hepatotoxicity, which was characterized by an increase in aspartate transaminase and/or alanine transaminase by more than five times following the introduction of thiopurine.

Firstly, we estimated the correlation of thiopurine-induced leukopenia/hepatotoxicity events with clinical characteristics, and found no significant association (Table 1). Next, the impact of the reported *NUDT15* and *TPMT* SNPs on leukopenia was evaluated, including rs116855232 (p. R139C), rs186364861 (p. V18I), and rs554405994 (p. V18_V19insGV) in *NUDT15*, and rs1142345 (inducing p. Y240C) in *TPMT*. Not surprisingly, the genotypes of rs116855232, rs554405994 and rs1142345 were significantly associated with 6-MP-induced leukopenia (Table 1). However, rs554405994 completely lost its significance in a multivariate model after adjusting for rs116855232, due to the linkage dise-

**Table 1.** Association of clinical characteristics and genetic variants with 6-MP-induced toxicity.

| Features | Leukopenia with (N=48) | Leukopenia without (N=140) | *P* | Hepatotoxicity with (N=37) | Hepatotoxicity without (N=149) | *P* |
|---|---|---|---|---|---|---|
| | median ± s.d. | | | median ± s.d. | | |
| Initial WBC ($10^9$/L) | 12.2 ± 95.1 | 10.3 ± 92.8 | 0.92 | 15.8 ± 154.5 | 9.6 ± 68.9 | 0.07 |
| age at diagnosis | 4.8 ± 2.9 | 4.5 ± 3.2 | 0.86 | 4.8 ± 3.3 | 4.6 ± 3.0 | 0.92 |
| No. of males (%) | Number of patients (%) | | | Number of patients (%) | | |
| | 41 (56.3%) | 68 (48.6%) | 0.4 | 21 (56.8%) | 74 (49%) | 0.36 |
| Genetic Factors* | Number of risk allele carriers (risk allele frequency) | | | Number of risk allele carriers (risk allele frequency) | | |
| *NUDT15* | | | | | | |
| rs116855232 (C/T) | 33 (40.6%) | 4 (1.4%) | $5.1 \times 10^{-12}$ | 6 (9.5%) | 31 (12.1%) | 0.56 |
| rs554405994 (wt/ins) | 11 (14.6%) | 5 (1.8%) | $1.5 \times 10^{-4}$ | 4 (6.8%) | 12 (4.7%) | 0.51 |
| rs186364861 (C/T) | 1 (1.0%) | 2 (0.7%) | 0.76 | 1 (1.4%) | 2 (0.7%) | 0.56 |
| rs149436418† (C/G) | 1 (1.0%) | 0 (0%) | 0.99 | 0 (0%) | 1 (0.3%) | 0.99 |
| rs761191455† (-/G) | 1 (1.0%) | 0 (0%) | 0.99 | 0 (0%) | 1 (0.3%) | 0.99 |
| rs751671087† (A/G) | 1 (1.0%) | 0 (0%) | 0.99 | 0 (0%) | 1 (0.3%) | 0.99 |
| *TMPT* | | | | | | |
| rs1142345 (A/G) | 7 (8.3%) | 1 (0.4%) | 0.005 | 0 (0%) | 3.0% | 0.17 |
| SNP combination‡ | Number of patients (%) | | | Number of patients (%) | | |
| | 39 (81.3%) | 10 (7.1%) | $3.5 \times 10^{-16}$ | 8 (21.6%) | 41 (27.2%) | 0.47 |

*Including reported and new SNPs in *NUDT15* and *TPMT*. The risk alleles are underlined. †Indicates the new SNPs we identified in *NUDT15*. ‡Combined six listed SNPs in *NUDT15* and one SNP in *TPMT*. Patients were classfied into two groups in terms of taking any risk alleles of these 6 SNPs in *NUDT15* (excluding rs554405994 due to redunancy) and *TPMT*. 6-MP: 6-mercaptopurine; SNP: single nucleotide polymorphism; WBC: white blood cell.

quilibrium between these two SNPs. No significance was observed for rs186364861, probably because only a small number of patients (N=3) carried the variant allele of this SNP in our cohort. Notably, the cases with the homozygous genotype of either rs116855232 of *NUDT15* (N = 6) or rs1142345 of *TPMT* (N = 1), or heterozygous for both SNPs (N = 3), have 100% sensitivity and specificity to predict thiopurine-induced leucopenia in our cohort. For all risk allele carriers, the sensitivity and specificity reached 75% (36/48) and 96.4% (135/140), respectively, after the combination of rs116855232 and rs1142345, compared with 68.8% (33/48) and 97.1% (136/140), respectively, for rs116855232 alone.

Moreover, one patient experienced a much earlier occurrence and more severe myelosuppression than the rest of our patients, with the lowest level of white blood cells (WBC = 1 × $10^9$/L), absolute neutrophil counts (ANC = 0.01 × $10^9$/L), platelets (PLT = 19 × $10^9$/L), and hemoglobin (HB = 65g/L) after two weeks of standard 6-MP treatment in the consolidation stage (Figure 1A). The treatment was stopped for a total of 16 days in the consolidation stage, with a continuous prescription of G-CSF, and 6-MP usage was gradually decreased to 2.5% of the standard dosage in the maintenance stage (i.e., 2.5 mg/$m^2$ every two days, Figure 1A), which was far lower than that of any other patient. Interestingly, this patient was genotyped and exhibited as a very rare case, carrying homozygous variant alleles of rs116855232 and heterozygous for rs1142345 (*NUDT15*$^{risk/risk}$*TPMT*$^{wt/risk}$). Considering the different mechanisms of *NUDT15* and *TPMT* involved in thiopurine metabolism, our findings indicate the cumulative effects of gene deficiency in thiopurine-induced myelosuppression, suggesting that variants of both *NUDT15* and *TPMT* should be prospectively genotyped and prescribed a very low initial dose of thiopurine for rare cases, such as those described above.

Of consequence, three novel SNPs were identified in the coding region of *NUDT15* (rs149436418, rs761191455, rs751671087), the carriers of which all developed leukopenia (Table 1 and Figure 1B), with final dose reductions of 20%, 33%, and 50%, respectively. Variant allele frequencies of these SNPs are very low (e.g., 0.05% of rs761191455 in East Asians). This is not surprising considering the association of rs761191455 with thiopurine-induced leucopenia, as the variant allele of this SNP results in a frameshift of *NUDT15* at the amino acid residue 115 (p.E115G fsTer4), inducing the loss of the enzymatic domain of *NUDT15*. Although the patient carrying the variant allele of rs751671087 (inducing p.G161R) developed leucopenia, he also has the heterozygous genotype of rs116855232, and it is thus unclear if the novel variant conferred any additional risk of toxicity. Patients with a variant allele of rs149436418 (inducing p.F52L) can be tolerant to a relatively higher

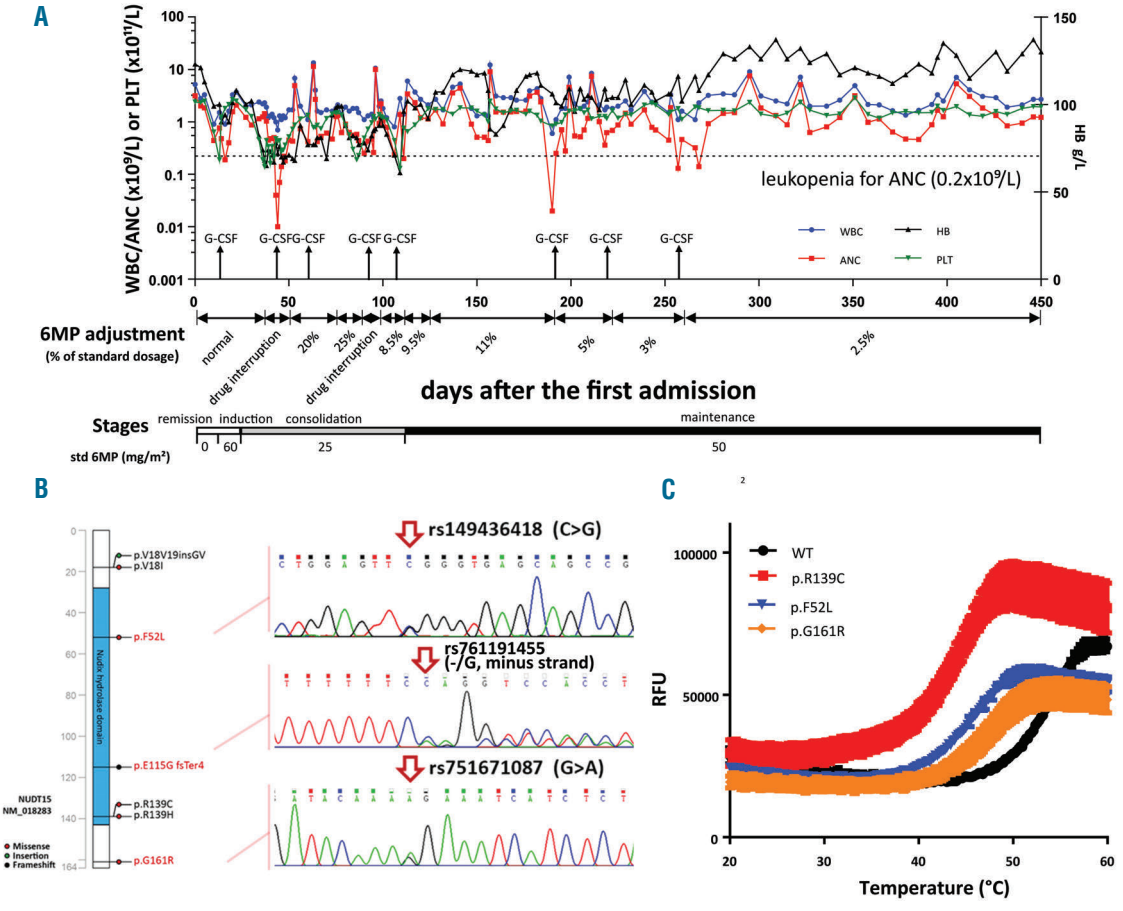


**Figure 1. Characterization of *NUDT15* variants in patients and protein function.** (A) A hemogram record of the rare case with *NUDT15*$^{risk/risk}$*TPMT*$^{wt/risk}$ genotype. Therapy phases, 6-MP dosage adjustment and G-CSF introduction is indicated in the time frame. The dashed line indicates the threshold of the third degree of leukopenia (ANC <0.2x$10^9$/L). (B) Position of known (black letters) and novel (red letters) SNPs in the *NUDT15* coding region, with sequence information exhibited for the novel variants. (C) Thermal stability of the NUDT15 protein, with p.R139C and wild-type included as controls. The inflection point of each curve indicates the temperature for protein unfolding, which is considered as the measurement of protein stability. RFU: relative fluorescence unit; WT: wild-type; ANC: absolute neutrophil count; 6-MP: 6-mercaptopurine; WBC: white blood cell; PLT: platelet.

dose of 6-MP than most rs116855232 carriers with a short-term 6-MP withdrawal and prescription of G-CSF after the development of leukopenia, suggesting the modest effect of this variant on 6-MP-induced leukopenia. Protein stability rather than enzymatic activity is considered to be crucial for *NUDT15* function,[15] we therefore conducted a thermal stability assay, and found reduced Tm values for all three mutant *NUDT15* proteins (41.83 ± 0.23°C, 44.34 ± 0.09°C, and 45.91 ± 0.21°C for p.R139C, p.F52L, and p.G161R, respectively) compared to wild-type (wt) *NUDT15* (52.86 ± 0.07°C) (Figure 1C), suggesting the role of rs751671087 and rs149436418 on thiopurine response is that of impacting protein stability. Therefore, the sensitivity to predict thiopurine-induced leukopenia will further increase (68.8% *vs.* 81.3%) after combining the functional common and novel rare SNPs in *NUDT15* (excluding rs554405994 due to redundancy) and *TPMT* (Table 1), without greatly impacting the specificity (97.1% *vs.* 95%).

Additionally, we observed another common thiopurine-induced ADR, hepatotoxicity, in our patients (N = 37), and conducted an association analyses of such an event with *NUDT15* and *TPMT* SNPs. However, no significant association could be detected between thiopurine-induced hepatotoxicity with *NUDT15* (*P*=0.56) and *TPMT* variants (*P*=0.17) (Table 1). Due to the potential clinical harm hepatotoxicity poses to patients, adjustment could be required for the initial 6-MP treatment. Therefore, further genetic screening is warranted in order to find the pharmacogenetic markers for thiopurine-induced hepatotoxicity.

Collectively, we found a significant association between the reported/novel *NUDT15* and *TPMT* SNPs with thiopurine-induced leukopenia, but not hepatotoxicity. Patients with the *NUDT15*$^{risk/risk}$*TPMT*$^{wt/risk}$ genotype will suffer more severe leukopenia, and should be initially treated with a much lower dosage of 6-MP at a clinical level. Taken together, our results suggest that the detection of all potential functional variants in these two genes is highly recommended in the individualized usage of 6-MP in ALL treatment.

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*Funding: this work was supported by grants from the National Key Research and Development Program of China (No. 2016YFC0905000 [2016YFC0905002] and No. 2017YFC0909300), the National Natural Science Foundation of China (No. 81522028, and No. 81728003) and the US National Institutes of Health (R01GM118578).*

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*doi:10.3324/haematol.2018.187658*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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