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NUDT15 Polymorphisms Alter Thiopurine Metabolism and Hematopoietic Toxicity

Takaya Moriyama[1,2], **Rina Nishii**[*,1,3], **Virginia Perez-Andreu**[*,1], **Wenjian Yang**[1], **Federico Antillon Klussmann**[4,5], **Xujie Zhao**[1], **Ting-Nien Lin**[1], **Keito Hoshitsuki**[1,6], **Jacob Nersting**[7], **Kentaro Kihira**[2], **Ute Hofmann**[8,9], **Yoshihiro Komada**[2], **Motohiro Kato**[10], **Robert McCorkle**[1], **Lie Li**[1], **Katsuyoshi Koh**[11], **Cesar Rolando Najera**[4], **Shirley Kow-Yin Kham**[12], **Tomoya Isobe**[13], **Zhiwei Chen**[12], **Edwynn Kean-Hui Chiew**[12], **Deepa Bhojwani**[14], **Cynthia Jeffries**[15], **Yan Lu**[15], **Matthias Schwab**[8,9,16,17], **Hiroto Inaba**[18], **Ching-Hon Pui**[18], **Mary V. Relling**[1], **Atsushi Manabe**[19], **Hiroki Hori**[2], **Kjeld Schmiegelow**[7,20], **Allen E. J. Yeoh**[12,21], **William E. Evans**[1], and **Jun J. Yang**[1]

[1]Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

[2]Department of Pediatrics, Mie University Graduate School of Medicine, Mie, Japan

[3]Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University Graduate School of Medicine, Tokyo, Japan

[4]Unidad Nacional de Oncología Pediátrica, Guatemala City, Guatemala

[5]Francisco Marroquin Medical School, Guatemala City, Guatemala

[6]School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[7]Department of Paediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

[8]Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany

[9]Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany

Correspondence: Jun J. Yang, PhD, Department of Pharmaceutical Sciences, Hematologic Malignancies Program, Comprehensive Cancer Center, St. Jude Children's Research Hospital, MS313, 262 Danny Thomas Place Memphis, Tennessee 38105-3678, jun.yang@stjude.org, Phone: (901)595-2517.

*These authors contributed equally

Author Contributions

Supervised research: J.J.Y.; Conceived and designed the experiments: T.M., R.N., H.H., K.S., A.E.J.Y, W.E.E. and J.J.Y.; Performed the experiments: T.M., R.N., V.P.A., X.Z., T.N.L, K.H., J.N., K.K., U.H., R.M., L.L., C.R.N., T.I., Z.C., E.C., C.J., Y.L. and M.S.; Performed statistical analysis: V.P.A., W.Y., ; Analyzed the data: T.M., R.N., V.P.A., W.Y., J.N., U.H., R.M., L.L., T.I., C.J., Y.L. M.S., H.H., K.S. and J.J.Y.; Contributed to reagents/materials/analysis tools: F.A., K.K., Y.K., M.K., K.K., C.R.N., S.K., Z.C., E.C., D.B., H.I., C.H.P., M.V.R., A.M., H.H., K.S. and A.E.J.Y.; Wrote the paper: T.M., W.E.E. and J.J.Y.

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[10]Department of Pediatric Hematology and Oncology Research, National Center for Child Health and Development, Tokyo, Japan

[11]Department of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Japan

[12]National University Cancer Institute, National University Health System, Singapore

[13]Graduate School of Medicine and Faculty of Medicine, the University of Tokyo, Tokyo, Japan

[14]Department of Pediatrics, Children's Hospital of Los Angeles, Los Angeles, California, USA

[15]Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

[16]German Cancer Consortium, German Cancer Research Center (DKFZ), Heidelberg, Germany

[17]Department of Pharmacy and Biochemistry, University of Tübingen, Tübingen, Germany

[18]Department of Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

[19]Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan

[20]The Institute of Clinical Medicine, the University of Copenhagen, Copenhagen, Denmark

[21]Viva-University Children's Cancer Centre, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Abstract

Widely used as anti-cancer and immunosuppressive agents, thiopurines have narrow therapeutic indices due to frequent toxicities, partly explained by *TPMT* genetic polymorphisms. Recent studies identified germline *NUDT15* variation as another critical determinant of thiopurine intolerance, but the underlying molecular mechanisms and its clinical implications remain unknown. In 270 children enrolled in clinical trials for acute lymphoblastic leukemia in Guatemala, Singapore, and Japan, we identified 4 *NUDT15* coding variants (p.Arg139Cys, p.Arg139His, p.Val18Ile, p.Val18_Val19insGlyVal) that resulted in 74.4%–100% loss of nucleotide diphosphatase activity. Loss-of-function *NUDT15* diplotypes were consistently associated with thiopurine intolerance across three cohorts ($P=0.021$, 2.1×10^{-5}, and 0.0054, respectively; meta-analysis $P=4.45\times10^{-8}$, allelic effect size=−11.5). Mechanistically, NUDT15 inactivated thiopurine metabolites and decreased its cytotoxicity *in vitro,* and patients with defective *NUDT15* alleles showed excessive thiopurine active metabolites and toxicity. Taken together, our results indicate that a comprehensive pharmacogenetic model integrating *NUDT15* variants may inform personalized thiopurine therapy.

Introduction

Thiopurines (mercaptopurine [MP], thioguanine [TG], and azathioprine) are widely used anti-cancer and immunosuppressive agents[1–8]. In acute lymphoblastic leukemia (ALL), prolonged daily exposure to MP is a major component of contemporary treatment regimens and indispensable for the cure of this disseminated malignancy[7,9–13]. However, MP can cause severe myelosuppression, resulting in frequent treatment disruptions, necessitating extensive supportive care, and increasing the risk of life-threatening infections[6,13–22].

Thiopurines are also commonly prescribed in patients with inflammatory bowel diseases (IBD, e.g., Crohn's disease and ulcerative colitis), especially for their steroid-sparing potential and efficacy in remission maintenance[4,6,14–17]. Thiopurine treatment for IBD is associated with substantial hematopoietic toxicity, leading to discontinuation of therapy in up to 40% of patients and subsequent disease recurrence[6,18–23]. Therefore, the narrow therapeutic indices of thiopurines point to a strong need for applying evidence-based precision medicine approaches to the use of this class of medications.

As prodrugs, thiopurines are enzymatically converted to thioguanosine triphosphates (TGTP), through multiple sequential anabolic reactions. TGTP is further reduced to deoxy-thioguanosine triphosphate (TdGTP) that is incorporated into double strand DNA (DNA-TG) to trigger futile mismatch repair and eventually apoptosis[1,24–28]. The integration of thioguanine into DNA is widely accepted as one of the principal sites of action for thiopurine drugs and a major process responsible for their cytotoxic effects[8,27–31]. There are multiple pathways that negatively impact thiopurine effects: MP can be directly anabolized by thiopurine methyltransferase (TPMT) to the inactive methyl-mercaptopurine; thiopurine active metabolites can also be catabolized *via* dephosphorylation of thioguanine nucleotides (TGN)[32,33]. Therefore, thiopurine cytotoxicity is determined by the competition between the activation and inactivation pathways, components of which are affected by genetic variations. In particular, single nucleotide polymorphisms (SNPs) in the *TPMT* gene have been shown to cause loss of TPMT enzymatic activity, excessive levels of TGN, and predispose patients to thiopurine-related hematopoietic toxicity across multiple diseases[23,34–38]. In fact, *TPMT*-guided preemptive thiopurine dose adjustment has been shown to substantially reduce the risk of adverse effects without compromising therapeutic efficacy[39–45] and is a prototype of precision medicine approaches to individualize drug therapy based on pharmacogenetics. However, substantial toxicity still occurs in some patients with normal TPMT activity, suggesting that additional variables, including other genetic variants, may contribute to the inter-patient variability in thiopurine metabolism.

Recent genome-wide association studies described a missense variant in the *NUDT15* gene (rs116855232, referred to as the c.415C>T or the p.Arg139Cys variant hereafter) that is strongly associated with thiopurine-related myelosuppression in patients with IBD[46] and in children with ALL[47]. Individuals homozygous for the risk allele at p.Arg139Cys were exquisitely sensitive to MP and tolerated only 8% of the standard dose, and this *NUDT15* variant alone explained 22% of variance in MP tolerance[47]. Encoding a purported purine-specific nucleotide diphosphatase, *NUDT15* is hypothesized to dephosphorylate the thiopurine active metabolites TGTP and TdGTP, thus preventing their incorporation into DNA and negatively affecting the desired cytotoxic effects of thiopurines. However, the exact mechanism by which NUDT15 regulates thiopurine metabolism has not been experimentally examined and the functional consequences of *NUDT15* variants are unknown.

In this study, we systematically identified *NUDT15* variants associated with thiopurine disposition and host toxicity, characterized their enzymatic properties, and comprehensively investigated the molecular pathways linking NUDT15 to thiopurine toxicity. Our results

suggest that a comprehensive pharmacogenetic model integrating *NUDT15* genetic variants may inform strategies to further individualize thiopurine therapy.

Results

NUDT15 variant discovery and function characterization

To systematically characterize *NUDT15* polymorphisms associated with thiopurine toxicity, we sequenced all exonic regions of this gene in 3 cohorts including 270 children treated on frontline ALL clinical trials in Guatemala, Singapore, and Japan (Supplementary Table 1). In total, we identified 4 coding variants located in exons 1 and 3, all of which resulted in changes in amino acid sequence of the NUDT15 protein (Fig. 1A). The c.415C>T and c.416G>A variants were transition substitutions within the codon for amino acid residue Arg139, causing an arginine-to-cysteine and arginine-to-histidine change (p.Arg139Cys and p.Arg139His), respectively. The other 2 variants affected the Val18 residue: the c.52G>A variant resulted in a valine-to-isoleucine conversion (p.Val18Ile), and the c.36_37insGGAGTC led to an in-frame addition of a glycine and a valine residue (p.Val18_Val19insGlyVal). We inferred 5 *NUDT15* haplotypes with distinct combinations of genotype at these 4 variants (referred to as haplotype *1–*5 hereafter, Fig. 1A). In particular, the p.Val18_Val19insGlyVal allele was in high linkage disequilibrium with the p.Arg139Cys allele, and the presence of both defined the common haplotype *2. Analyses of the phased 1,000 Genomes Project data identified similar haplotype patterns, and the prevalence of each haplotype varied substantially by ancestry (Supplementary Fig. 1).

To define the functional consequences of *NUDT15* polymorphisms, we characterized nucleotide diphosphatase activity for each of the 4 variant proteins (Fig. 1B). Wildtype NUDT15 efficiently converted the thiopurine active metabolite TGTP to the monophosphate thioguanosine nucleotide TGMP, with an apparent catalytic efficiency (V_{max}/K_m) of 107.9 ± 0.2. In contrast, 4 variant NUDT15 proteins showed 74.4% to 100% loss of enzymatic activity: V_{max}/K_m of 23.6 ± 0.9, 27.7 ± 1.1, and 14.9 ± 1.1 for the p.Arg139His, p.Val18Ile, p.Val18_Val19insGlyVal variants, respectively; enzymatic activity was not detectable for the p.Arg139Cys or the p.Val18_Val19insGlyVal/p.Arg139Cys variant protein. Similar differences in NUDT15 activity were observed when TdGTP was used as the substrate (Supplementary Fig. 2). While the p.Arg139Cys and p.Val18_Val19insGlyVal individually caused reduction of NUDT15 activity, the protein harboring both variants did not show further loss of function compared to the p.Arg139Cys variant alone (Fig. 1B), suggesting that the *2 haplotype is likely to have similar functional consequence as haplotypes with a single variant (e.g., *3). Thermostability assay showed substantially lower T_m values for all variant NUDT15 proteins (42.6 ± 0.1, 43.6 ± 0.2, 52.5 ± 0.3, 41.5 ± 0.1, and 54.0 ± 0.1 °C, for the p.Arg139Cys, p.Arg139His, p.Val18Ile, p.Val18_Val19insGlyVal/p.Arg139Cys and p.Val18_Val19insGlyVal, respectively), compared to the wildtype protein (56.3 ± 0.3 °C), consistent with perturbation of NUDT15 conformation by these substitutions (Supplementary Fig. 3).

Further, we tested different combinations of variant NUDT15 proteins to determine enzymatic activity for each of the 8 diplotypes observed in our clinical cohorts (Fig. 1C). Equimolar mixtures of the variant and wildtype NUDT15 proteins (equivalent of patients

heterozygous at a single variant, e.g., *1/*3) showed intermediate activity, indicating a gene dosage effect. By comparison, the mixture of two different variant NUDT15 proteins (e.g., *3/*5) showed low activity that was comparable to a single variant with the same total protein concentration (e.g., *3/*3), suggesting that individuals with compound heterozygous genotypes are likely to have similar MP intolerance as those homozygous for a single *NUDT15* variant. Therefore, we classified patients into 3 diplotypic groups (Fig. 1C): normal activity (*1/*1), intermediate activity (*1/*2, *1/*3, *1/*4 and *1/*5), and low activity (*2/*3, *3/*3 and *3/*5). There were no significant differences in enzymatic activity between diplotypes within the intermediate activity group (P = 0.73) or within the low activity group (P = 0.19).

Consistently in the Guatemalan, Singaporean, and Japanese cohorts (N = 159, 79, and 32, respectively), MP tolerance was highest in patients with the normal activity *NUDT15* diplotype followed by those with intermediate activity, and lowest in patients with the diplotypes indicative of low NUDT15 activity (P = 0.021, 2.1×10^{-5}, and 0.0054, respectively, Fig. 2). Of particular note, patients with *1/*2 diplotype (heterozygous at both the p.Arg139Cys and the p.Val18_Val19insGlyVal variants) showed similar degree of MP intolerance as the *1/*3 group (heterozygous at the p.Arg139Cys variant alone, Supplementary Fig. 4), corroborating our observation that single (p.Arg139Cys) and double (p.Val18_Val19insGlyVal/p.Arg139Cys) variant NUDT15 proteins exhibited similar enzymatic activity (Fig. 1B). Additionally, two patients with compound heterozygous *NUDT15* genotype (*3/*5 and *2/*3) were exquisitely sensitive to MP, on the same scale as those homozygous for the variant allele (*3/*3), indicating cumulative effects of *NUDT15* variants (Supplementary Fig. 4). A meta-analysis combining three cohorts indicated strong and consistent effects of *NUDT15* variants on tolerated MP dosage reduction ($P = 4.45 \times 10^{-8}$, allelic effect size of −11.5 [95% confidence interval, −15.6 to −7.4], Fig. 2D) and we did not observe any statistically significant heterogeneity across cohorts (P = 0.34). Including patients with *TPMT* risk variants (rs1800462, rs1800460, and rs1142345) had minimal effects on the association of *NUDT15* diplotype with tolerated MP dosage (meta-analysis $P = 9.96 \times 10^{-10}$ and 9.92×10^{-8} for before and after adjusting for *TPMT* variants respectively, Supplementary Fig. 5), indicating independent contributions of these two genes to MP metabolism and toxicity.

NUDT15 regulates thiopurine activation and cytotoxicity

Because NUDT15 converts TGTP to TGMP (also TdGTP to TdGMP), we hypothesized that NUDT15 prevents the incorporation of these thiopurine metabolites into DNA (DNA-TG) and negatively regulates thiopurine activation and consequently its cytotoxicity. To recapitulate the loss of NUDT15 activity resulting from genetic variation, we first established stable *NUDT15* knockdown in a human lymphoid cell line (Supplementary Fig. 6). In the control cells transduced with scramble shRNA, MP was extensively metabolized and the ratio of TGTP to TGMP was 0.028 (± 0.0052), with TGTP representing only 2.7% (± 0.5%) of total intracellular thioguanine nucleotides (TGN). When *NUDT15* expression was downregulated, the level of TGTP increased significantly with a 7.5-times higher TGTP to TGMP ratio (0.21 [± 0.0067]) and 6.2-times higher %TGTP in TGN (16.8% [± 0.3%]) compared to control cells, indicating a significant shift in equilibrium toward TGTP (Fig.

3A). Consequently, DNA-TG was markedly higher in *NUDT15* knockdown cells compared to control (8,219.9 ± 853.6 fmol/μg DNA and 1,734.7 ± 25.7 fmol/μg DNA, respectively, Fig. 3B), leading to a significant increase in MP-induced apoptosis in these cells (Fig. 3C, Supplementary Fig. 7A). With increasing concentrations of MP, there was concomitant elevation of intracellular DNA-TG, and the rate at which MP was incorporated into DNA (i.e., DNA-TG) was substantially greater in the *NUDT15* knockdown cells compared to control cells (Fig. 3D). Similarly, when we repeated these experiments with TG or azathioprine, *NUDT15* knockdown cells consistently showed increased levels of active metabolites (e.g., TGTP and DNA-TG), and higher susceptibility to TG- or azathioprine-induced apoptosis (Fig. 3E–H, Supplementary Figs. 7B and 8). Together, these results indicate that NUDT15 inactivates thiopurine metabolites and is directly related to their cytotoxic effects, thus providing a clear biological mechanism explaining the susceptibility to thiopurine host toxicity in patients inheriting loss-of-activity variants in *NUDT15*.

NUDT15 variants and thiopurine metabolism *in vivo*

Building upon the *in vitro* results, we next sought to determine how inherited NUDT15 deficiency influences thiopurine activation in patients by directly monitoring DNA-TG levels in white blood cells from children with ALL receiving daily MP treatment. In 32 patients in the Singaporean cohort, DNA-TG levels strongly correlated with the actual MP dosage delivered (P = 0.0024, Fig. 4A). The positive correlation between MP dosage and DNA-TG was also validated in the Japanese cohort (N = 32, $P = 2.2 \times 10^{-4}$, Fig. 4B). The ratio of DNA-TG to MP dosage (i.e., the amount of DNA-TG converted from every unit MP dose) varied significantly by *NUDT15* genotype. In the Singaporean cohort (Fig. 4C and Supplementary Fig. 9A), the average DNA-TG to MP dosage ratio was 6.0 ± 2.7 fmol/μg DNA/mg MP in children with wildtype *NUDT15* (*1/*1). In contrast, those with intermediate activity *NUDT15* diplotypes (*1/*3 and *1/*5) achieved 8.8 (± 5.3 fmol/μg DNA) of DNA-TG for every mg of MP and this ratio was highest in patients with homozygous or compound heterozygous *NUDT15* diplotypes (19.6 ± 6.3 fmol/μg DNA/mg MP, $P = 7.7 \times 10^{-5}$). Similarly in the Japanese cohort (Fig. 4D and Supplementary Fig. 9B), the DNA-TG to MP dosage ratio was 9.6 ± 4.1, 12.3 ± 4.5, and 32.4 fmol/μg DNA/mg MP for children with normal, intermediate, and low activity *NUDT15* diplotypes, respectively ($P = 2.9 \times 10^{-4}$). A meta-analysis combining the Singaporean and Japanese cohorts indicated strong and consistent association of *NUDT15* diplotype with normalized DNA-TG ($P = 2.2 \times 10^{-8}$) and we did not observe significant heterogeneity between cohorts (P = 0.63).

Discussion

The current study has comprehensively characterized the effects of inherited variants of *NUDT15* on the metabolism of thiopurine and on the clinical tolerance of thiopurine toxicity. These novel findings are important for multiple reasons: First, these *NUDT15* variants are highly penetrant and confer exquisite sensitivity to thiopurines (homozygous carriers can tolerate < 10% of standard dosage), with effect sizes comparable to *TPMT* variants that are clinically implemented to guide thiopurine dose reduction[46,47]. In fact, in a multiethnic cohort of children with ALL, the *NUDT15* variant p.Arg139Cys alone explained 22% of variance in MP tolerance[47]. Secondly, *TPMT* variants are generally rare among

Asian populations[48] and thus of limited relevance for guiding thiopurine dosing in these race groups. In contrast, *NUDT15* genetic variation is substantially over-represented in Asians and is their predominant genetic cause for thiopurine toxicity[46,47]. The unequivocal evidence linking *NUDT15* p.Arg139Cys variant to thiopurine toxicity (particularly in Asians[46,47,49,50]) strongly indicates its potential clinical relevance, and also raises the question of whether (or what) clinical action is warranted for these at-risk patients.

A critical barrier to integrating *NUDT15* genotypes into thiopurine dosing algorithm has been the paucity of data to establish the pharmacologic basis for dose reduction for patients with *NUDT15* risk alleles. Addressing this knowledge gap, our results from a variety of laboratory model systems and in patients collectively indicate that NUDT15 deficiency directly resulted in excessive levels of thiopurine active metabolites (TGTP and DNA-TG) and increased host toxicity. Therefore, reducing thiopurine doses for patients who carry the *NUDT15* variants would likely tailor their exposure to a level of TGTP and DNA-TG that is similar to wildtype patients receiving standard thiopurine doses. This is a highly plausible strategy for utilizing *NUDT15* genotype to individualize thiopurine therapy to mitigate toxicity, as it is the same principle used for *TPMT*-based dose adjustments already implemented clinically[51]. In fact, in 285 children with newly diagnosed ALL, leukemic cells with intermediate activity *NUDT15* diplotypes were also significantly more sensitive to TG than those with wildtype diplotype ($P = 0.03$, Supplementary Fig. 10). Therefore, genotype-guided thiopurine dose reductions in ALL patients inheriting *NUDT15* risk alleles would likely minimize side effects without compromising antileukemic efficacy. Future clinical studies will be needed to precisely define the optimal dosage of thiopurines in patients with different *NUDT15* diplotypes. The strength of the association between *NUDT15* variants and tolerated MP dosage also differed slightly among three ALL cohorts (strongest in Singaporeans and weakest in Guatemalans, Fig. 2). Among these three populations, Guatemalans had the lowest tolerated MP dosage even with wildtype *NUDT15* and had smaller differences in MP tolerance across *NUDT15* genotype groups (although still significant). It is possible that other novel genetic variants associated with thiopurine toxicity exist uniquely in Guatemalans, potentially contributing to their overall sensitivity to this class of drugs. These hypotheses should be examined in future studies.

Our targeted sequencing of *NUDT15* identified 4 coding variants, all of which influenced *NUDT15* activity and were linked to thiopurine toxicity (Figs. 1 and 2). Intriguingly, 2 of these variants both affect residue Arg139 that is located in the α helix a2 at the base of the substrate binding pocket of NUDT15[52]. It has been hypothesized that the substitution of arginine with cysteine (p.Arg139Cys) at this position might introduce a disulfide bond and thus structural perturbation that interferes with TGTP binding[52]. In contrast, the arginine-to-histidine change caused by the p.Arg139His variant might lead to a reduced eletrophilicity and compromise substrate interaction. The other 2 *NUDT15* variants locate at residue Val18 within the β sheet at the N-terminus of the protein, and it is unclear how the substitution and insertion at this position structurally hinder NUDT15 function. It is also possible that coding variants can affect NUDT15 protein synthesis and/or degradation and thus indirectly influence enzymatic activity, as we have shown for *TPMT* variants[53]. However, when ectopically expressed in HEK293T cells, the *NUDT15* variants identified herein showed relatively high protein stability over time *in vitro*, comparable to wildtype NUDT15

(Supplementary Fig. 11); it will be important to document similar lack of differences in patients with these *NUDT15* genotypes.

It was originally believed that NUDT15 functions as a sanitizer to remove damaged nucleotides, e.g., oxo-dGTP generated from radical oxygen species[54]. However, this was challenged by more recent data showing a strong preference of NUDT15 for thiopurine metabolites over oxo-dGTP[52]. NUDT15 is also shown to efficiently hydrolyze dGTP with potential importance in purine nucleotide homeostasis[52], but its exact physiological functions remain unknown. It is unclear whether loss-of-function *NUDT15* variants confer susceptibility to any diseases, although there is no evidence in the literature to suggest that this is the case. It is plausible that NUDT15 deficiency has limited effects on endogenous nucleotide metabolism under normal physiological conditions because of compensation by other NUDT proteins with overlapping enzymatic functions[52,55]. In contrast, the prominent role of *NUDT15* in thiopurine inactivation signifies potential importance of the NUDT family of enzymes in the metabolism of other nucleotide analog drugs, which are widely used for diverse human diseases (especially in antiviral therapy)[56]. Therefore, comprehensive identification of pharmacogenetic variants in *NUDT* genes may have broad clinical relevance.

Our results from comprehensive *in vitro* and *in vivo* studies strongly indicate that *NUDT15*-related thiopurine toxicity follows an additive genetic mode of inheritance, with the severity of the phenotype proportional to the cumulative number of risk alleles in *NUDT15*. Patients homozygous or compound heterozygous for *NUDT15* risk alleles experience especially excessive MP toxicity, compared to those with intermediate or normal activity diplotypes (Fig. 2). This is not unexpected because homozygous variant NUDT15 protein is enzymatically inactive (Fig. 1C) and complete loss of this important metabolizing enzyme results in disproportionally high levels of active metabolite and thus toxicity, as also seen in patients with inherited TPMT deficiency[12,35]. The interpretation of *NUDT15* genotype becomes more complicated when a patient carries multiple functional variants. For example, if two variants are on the same haplotype, the patient is likely to have intermediate NUDT15 activity because the remaining wildtype copy is still functional (e.g., *1/*2). In contrast, if each of these two variants affects a different copy of *NUDT15* (compound heterozygous, e.g., *3/*5), enzymatic activity is completely lost in these patients similar to subjects with homozygous variant genotype (e.g., *3/*3). These two scenarios indicate distinct levels of thiopurine tolerance and can be distinguished only through haplotype-based analyses. Using phased sequencing data from the 1,000 Genomes Project, we inferred *NUDT15* diplotypes in major race/ethnic groups worldwide (Supplementary Fig. 1) and experimentally determined the enzymatic activity of each haplotype. *NUDT15* deficiency (carrying low or intermediate activity diplotypes) was most common in East Asians (22.6%) and also prevalent in South Asians (evenly distributed in India, Pakistan, Bangladesh, and Sri Lanka at 13.6%) and Native American populations (e.g., 21.2% in Peruvians and 12.5% in Mexicans). In fact, in our Japanese cohort, approximately 1 in every 3 individuals carried risk variant(s) in the *NUDT15* gene, a frequency that is even higher than that of TPMT deficiency (e.g., 1 in every 12 Europeans or 1 in every 8 Africans)[51]. We have also identified rare *NUDT15* variants in the Broad Institute Exome Aggregation Consortium dataset

including whole exome seq of 60,706 individuals (Supplementary Table 2), although the function of these variants and their effects on thiopurine metabolism are unknown.

As previously described, cases with both variants of *NUDT15* and *TPMT* variants were significantly less tolerant to MP tolerance than those with risk alleles in one of these two genes[47]. Therefore, integration of *NUDT15* in thiopurine dosing algorithm may have a major implication for Asian populations, whereas *TPMT* variants are most informative for thiopurine toxicity in Europeans and Africans. A polygenic dosing algorithm that incorporates both *NUDT15* and *TPMT* variants would potentially provide a robust approach to personalize thiopurine therapy in major racial and ethnic groups represented in diverse populations worldwide.

Online Methods

Patients and thiopurine therapy

A total of 270 children treated on frontline ALL clinical trials in Guatemala, Singapore, and Japan were included in this study: 266 had newly-diagnosed ALL and 4 cases were diagnosed with mixed phenotype acute leukemia or lymphoma (Supplementary Table 1). The Guatemalan cohort comprised 159 patients (self-identified as Native American or Hispanic/mestizo) from the LLAG-0707 ALL protocol[57] at the Unidad Nacional de Oncología Pediátrica, Guatemala City, Guatemala. This protocol included daily MP treatment during the maintenance phase with a planned MP dosage of 50–75 mg/m^2 per day with provisions for dose adjustments based on the degree of myelosuppresion (target white blood cell count between 1.5–3.0 × 10^9/L) or infections. The Singaporean cohort included 79 children treated on the MaSpore ALL 2003 and MaSpore ALL 2010 protocols[58] at National University Hospital, Singapore. Planned daily MP dosage was 50 mg/m^2 and 75 mg/m^2 for the standard/intermediate and high-risk arms respectively, and clinically titrated to a target white blood cell count between 2.0–4.0 × 10^9/L. The Japanese cohort consisted of 32 children treated on the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) ALL-B12 protocol (UMIN000009339) for newly-diagnosed ALL at 13 hospitals and oncology centers in Japan. The standard MP dosage during maintenance phase was 50 mg/m^2 per day and was adjusted to a target white blood cell count between 2.0–3.0 × 10^9/L. In all 3 cohorts, MP dosage was considered to be stable after at least 9 weeks of daily MP dosing during maintenance therapy (with appropriate dose titration), following which the average of daily dosage over at least 14 days was used to define MP tolerance (i.e., tolerated MP dosage).

Patients were selected on the basis of the availability of germline DNA and availability of MP dosing and tolerance history. Germline DNA was extracted from peripheral blood obtained during clinical remission. This study was approved by the respective institutional review boards, and informed consent was obtained from parents, guardians, and/or patients, as appropriate.

NUDT15 sequencing and *TPMT* genotyping

Coding regions (exons 1, 2 and 3) of the *NUDT15* gene were first amplified by polymerase chain reaction (PCR) from germline DNA, followed by Sanger sequencing (primer sequences are provided in Supplementary Table 3). Sequence alignment and comparison were performed using the CLC Genomics Workbench (CLC Bio, Qiagen) to identify genetic variants. *NUDT15* variant genotype was coded as 0, 1, or 2 to indicate the number of variant alleles, and sequencing data were analyzed by using PHASE[59] to infer haplotypes. *TPMT* variants (rs1800462, rs1800460, and rs1142345) were manually genotyped as described previously[60] for all patients, and individuals with variant genotypes (N = 20 and 2 in the Guatemalan and Singaporean cohorts, respectively) were excluded from subsequent analyses unless otherwise indicated.

NUDT15 purification, enzymatic activity measurement, and thermostability assay

Human *NUDT15* cDNA (accession number BC133017) was cloned into the pCold II expression vector (TaKaRa Bio) with an N-terminal His tag, as previously described[54]. Missense and insertion variants (p.Arg139Cys, p.Arg139His, p.Val18Ile, p.Val18_Val19insGlyVal, and p.Val18_Val19insGlyVal/p.Arg139Cys) were introduced by site-directed mutagenesis using the QuikChange II XL kit (Agilent Technologies). Wildtype or variant NUDT15 protein was ectopically expressed in *E. coli* BL21 with IPTG induction (0.5 mM, 24 hours at 15 °C), and purified by affinity chromatography using the TALON metal affinity resin (Clontech). Purified proteins were concentrated with an Amicon Ultra 0.5 ml centrifugal filter (Sigma-Aldrich) in 10 mM pH 8.0 Tris-HCl buffer containing 0.1 mM EDTA, 5 mM DTT and 10% glycerol. Protein purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie Blue staining (Supplementary Fig. 12), and the molecular weight of each NUDT15 protein was confirmed by Time-of-Flight mass spectrometry using the Waters LCT Premier XE MS (Supplementary Table 4).

NUDT15 diphosphatase activity was measured by quantifying the release of pyrophosphate using the PiPer Pyrophosphate Assay Kit (Life Technologies). Each NUDT15 protein (100 ng) was incubated with varying concentrations of TGTP (1.88 μM to 21.4 μM) or TdGTP (0.99 μM to 35.7 μM) at 37 °C for 10 minutes before heat inactivation (99 °C for 20 minutes). Free pyrophosphate was then quantified immediately by fluorescence spectrometry according to manufacturers' instructions, and K_m and V_{max} values were determined by Michaelis-Menten kinetics methods in Prism (GraphPad). Each experiment was done in triplicates and repeated at least three times.

To determine protein thermostability, 1 μg of wildtype or variant NUDT15 protein was incubated with 10× Sypro®Orange (Molecular Probes), and the mixture was heated from 20 °C to 95 °C in increments of 0.2 °C in the Quant Studio 12K Flex real time PCR system (Applied Biosystems). Fluorescence changes were monitored with a charged-couple device camera, with excitation and emission wavelengths at 490 nm and 575 nm, respectively. T_m values, the temperature midpoint for the protein unfolding transition, were calculated based on the Boltzmann model in Prism (GraphPad).

To assess NUDT15 protein stability *in vitro*, variant or wildtype *NUDT15* cDNA with a N-terminal FLAG tag on the pcDNA3.1 backbone (Invitrogen) was transiently expressed in HEK293T cells (American Type Culture Collection, ATCC) using polyethylenimine reagent (Polysciences). Cycloheximide (50 μg/ml) was added 48 hours following transfection, and NUDT15 protein levels were monitored after 0, 24, and 48 hours by Western blot with β-actin as the loading control (anti-FLAG antibody, #2368, Cell Signaling).

NUDT15 knockdown and thiopurine metabolism *in vitro*

Lentiviral vectors containing shRNAs specific for human *NUDT15* (TRCN0000050311) or scramble sequence (SHC016) were purchased from Sigma-Aldrich. Viral particles were prepared using the calcium chloride method in HEK293T cells and stable knockdown was established in the human lymphoid cell line Nalm6 (German Collection of Microorganisms and Cell Cultures, DSMZ) by lentiviral transduction of shRNA and puromycin selection, following procedures described previously[61]. Cell lines were authenticated by karyotyping and cytogenetics and tested negative for mycoplasma contamination. The level of *NUDT15* knockdown was determined by real-time quantitative PCR with human tubulin as the internal control (Supplementary Fig. 6A and primer sequences are provided in Supplementary Table 3). NUDT15 expression was also determined at the protein level by Western blot with β-actin as the loading control (anti-NUDT15 antibody, ab122521, Abcam, Supplementary Fig. 6B).

To determine thiopurine metabolism *in vitro*, 5×10^6 *NUDT15* knockdown or control cells were treated with varying concentrations of MP or TG at 37 °C for 48 hours. Cytosolic thiopurine metabolites (e.g., TGTP, TGDP, and TGMP) were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to a previously reported method[62]. In parallel, aliquots of MP-, TG-, or azathioprine-treated cells were processed to extract genomic DNA, and DNA-TG was measured using LC-MS/MS as described previously[63]. MP, TG, and azathioprine cytotoxicity was determined for *NUDT15* knockdown and control cells by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at the end of the 72-hour drug exposure. In *vitro* experiment were done in triplicates and repeated at least three times.

Thiopurine metabolism in children with ALL

To investigate *in vivo* thiopurine metabolism, DNA incorporation of TdGTP (i.e., the level of DNA-TG) was longitudinally monitored during the maintenance therapy phase in a subset of children enrolled on the MaSpore ALL 2010 and JPLSG ALL-B12 protocols. In the Singaporean cohort (N = 32), peripheral blood was collected every 3 months during maintenance, with a maximum of 3, 4, and 5 time points for those on the high, intermediate, and standard risk arms, respectively. In the Japanese cohort (N = 32), peripheral blood collection was scheduled at the 13^{th} and 41^{st} week of maintenance therapy. For both cohorts, genomic DNA was extracted from leukocytes within 72 hours of blood draw and DNA-TG was quantified using LC-MS/MS as described previously[63], and detailed MP dosing history was recorded for 14 days prior to each sample collection.

To examine the effects of *NUDT15* variants on leukemic cell sensitivity to thiopurine, we also evaluated TG LC50 (drug concentration that is lethal to 50% of cells) in 285 children with newly-diagnosed ALL. Primary ALL cells were exposed to increasing concentrations of TG for 96 hours, after which cell viability was determined using MTT assay to estimate LC50, as described previously[64].

Statistical analyses

Each patient was assigned a *NUDT15* "genetic score" of 0, 1, or 2 indicating low, intermediate, or normal NUDT15 activity, on the basis of his/her *NUDT15* genetic diplotype and experimentally determined enzymatic activity for each diplotype (Fig. 1C). Within each ALL cohort, we first evaluated the association of patient clinical features with tolerated MP dosage, using the Wilcoxon rank test (gender), the Kruskal-Wallis test (ancestry and leukemia type), or the Spearman rank test (age as a continuous variable, Supplementary Table 1). The correlation between *NUDT15* genetic score and tolerated MP dosage (an average value for each patient if there were multiple measurements) was then tested in Guatemalan, Singaporean, and Japanese cohorts separately, using the linear regression model. Age and gender were included as covariates in regression model for the Singaporean cohort because they were related to MP dosage. A similar statistical model was applied to determine the association of *NUDT15* genetic score and the ratio of DNA-TG to MP dosage (DNA-TG/MP dosage) in the Singaporean and Japanese cohorts. Correlation between tolerated MP dosage and DNA-TG was determined by using the Spearman rank test. Kruskal-wallis test was used to evaluate the differences in nucleotide diphosphatase activity, tolerated MP dosage, or normalized DNA-TG among diplotypes within the intermediate activity group and among those within the low activity group. Meta-analyses were also performed combining test statistics of each cohort using random effect model based on inverse variant method[65] with ancestry, age, and gender as covariates, and inter-cohort heterogeneity was tested using the Q-statistic. All statistical tests were two-sided and chosen as appropriate according to data distribution, and the threshold for statistical significance was defined as $P < 0.05$. R (version 3.0) was used for all analyses unless indicated otherwise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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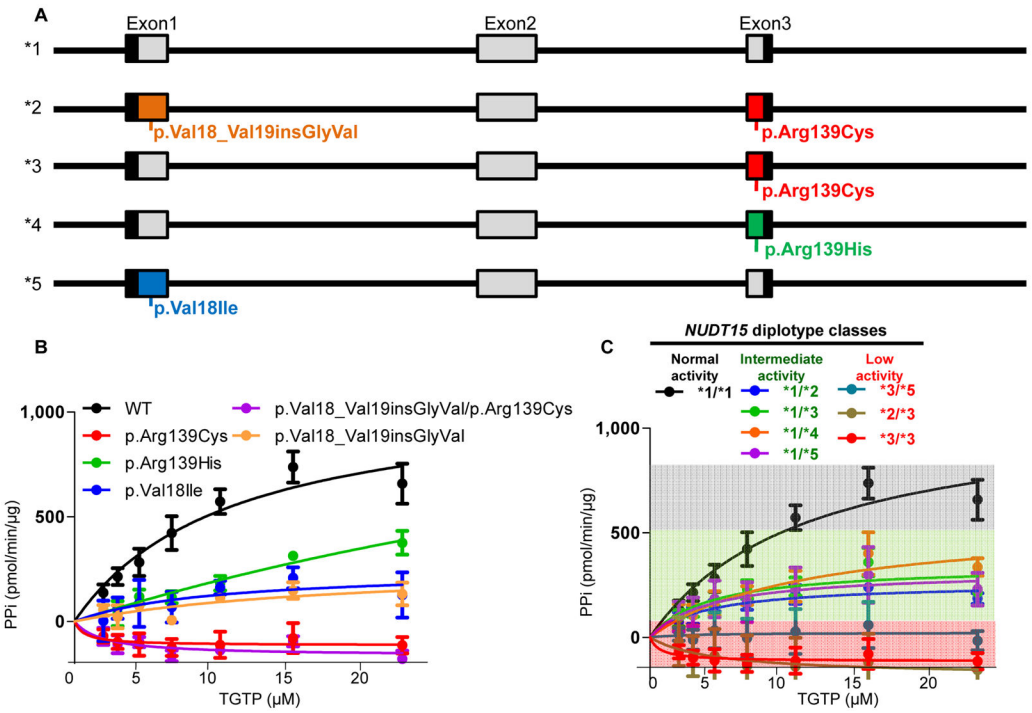

Figure 1. *NUDT15* genetic variants and their effects on nucleotide diphosphatase activity

Four coding variants were identified representing 5 haplotypes (*1 to *5, panel A). Each variant NUDT15 was expressed in *E. Coli* and purified protein was subjected to diphosphatase activity measurement with TGTP as the substrate (panel B). Variant or wildtype proteins were combined to determine the level of NUDT15 activity in patients with different diplotypes (panel C). There were no significant differences in nucleotide diphosphatase activity between diplotypes within the intermediate group (green shade, P = 0.73) or within the low activity group (red shade, P = 0.19), as determined using Kruskal-wallis test. Center values (dots) represent mean of triplicates and error bars indicate standard deviation.

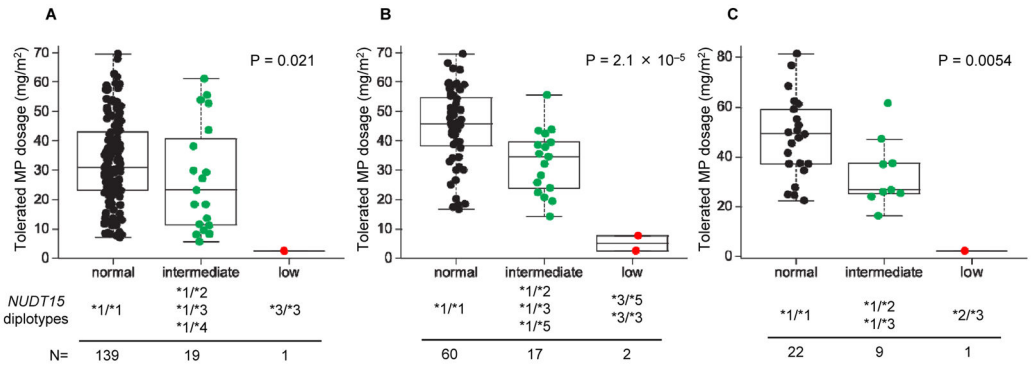

Figure 2. Association of *NUDT15* diplotype with MP tolerance during ALL therapy in Guatemala, Singapore, and Japan

Patients were classified as “normal”, “intermediate”, or “low” NUDT15 activity on the basis of their diplotype at 4 coding variants (panels A, B, and C for the Guatemalan, Singaporean, and Japanese cohorts, respectively). MP dosage was adjusted during maintenance therapy to avoid host toxicities and tolerated MP dosage was defined as the average over at least 14 daily dosages after at least 9 weeks of maintenance therapy. Cases with *TPMT* variants (rs1800462, rs1800460, and rs1142345) were excluded from the analysis. P value was calculated by using linear regression test, after adjusting for co-variates when applicable. In panels A–C, each box includes data between 25th and 75th percentiles, with horizontal line indicating median. Similar association analyses were performed to compare tolerated MP dosage between normal and intermediate *NUDT15* groups (P = 0.04, 0.00049, and 0.0033 for Panels A, B, and C, respectively). Meta-analysis combining test statistics from three cohorts indicated consistent association (Forest plot, Panel D, P = 4.45×10^{-8}), with no significant heterogeneity across cohorts (P = 0.34). Allelic effect size indicates the change in MP dosage for every copy of the *NUDT15* risk allele. The length of each horizontal line represents the range of 95% confidence interval of allelic effect size with the tick indicating median (gray boxes are proportional to weights of each cohort used in meta-analysis). Dashed vertical line denotes allelic effect size in the meta-analysis with the lateral tips of diamond representing 95% confidence interval.

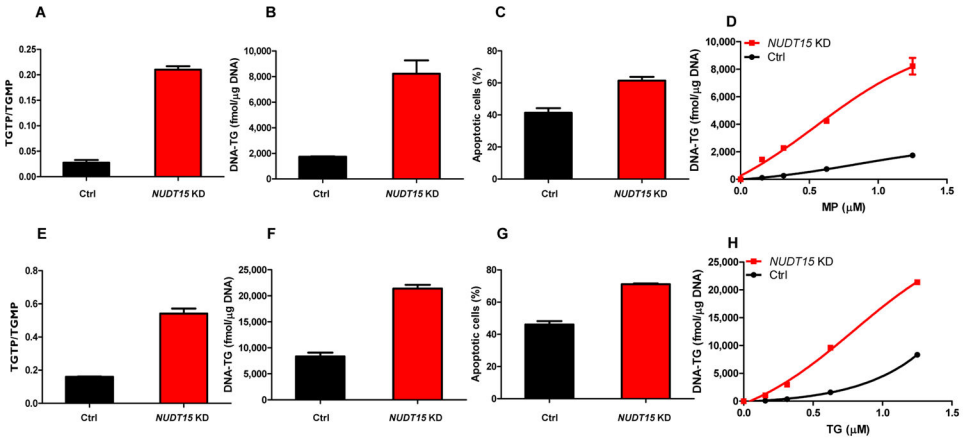

Figure 3. Effects of *NUDT15* on thiopurine metabolism and cytotoxicity

*NUDT*15 knockdown (*NUDT15* KD, red) cells were established by lentiviral transduction of *NUDT15*-specific shRNA, and control cells (black) were transduced with non-targeted vectors. Thiopurine metabolites (cytosolic TGMP and TGTP, and DNA-incorporated thioguanine, [DNA-TG]) were analyzed after treatment with 1.25 μM of MP (A and B) or TG (E and F) for 48 hours. DNA-TG was measured in cells exposed to increasing concentrations of MP (D) and TG (H). Cytotoxicity was determined by MTT assay following 72-hour incubation with MP (C, 1.25 μM) or TG (G, 1.25 μM). Mean values are plotted in each panel with error bars indicating standard deviation from triplicates.

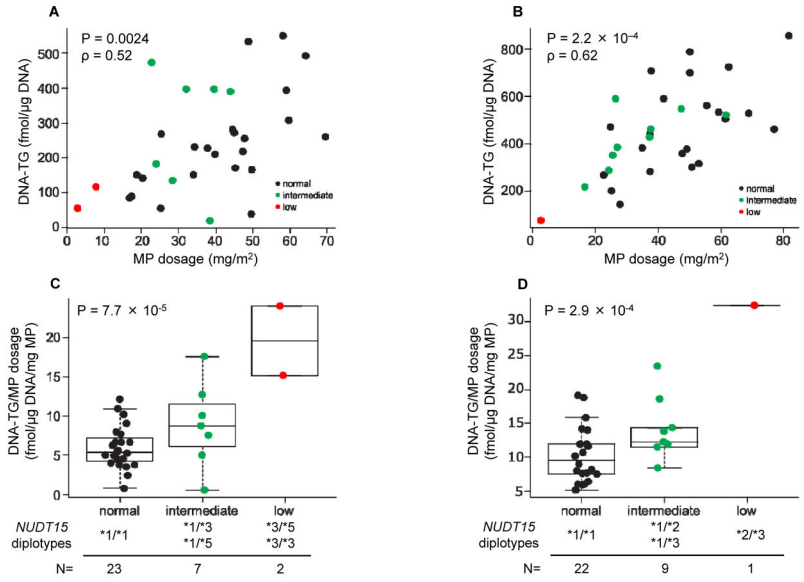

Figure 4. *NUDT15* variants and MP metabolism in children during ALL therapy

DNA-TG levels were analyzed in Singaporean (A and C) and Japanese (B and D) cohorts. Sixty-three and 44 samples were successfully measured from 32 cases with wildtype *TPMT* in Singapore and Japanese cohorts, respectively. An average DNA-TG level was estimated for each patient. The associations between MP dosage and DNA-TG were evaluated by two-sided Spearman rank test (A and B). DNA-TG level was normalized based on actual MP dosage (the average of 14 days) prior to metabolite measurements and then correlated with *NUDT15* diplotype (as normal, intermediate, or low NUDT15 activity) using a linear regression model (two-sided, C and D). Similar association analyses were also performed to evaluate the difference in normalized DNA-TG between normal and intermediate *NUDT15* groups (P = 0.14 and 0.039 for Panels C and D, respectively). Meta-analysis combining test statistics from two cohorts indicated consistent association of *NUDT15* diplotype with normalized DNA-TG ($P = 2.2 \times 10^{-8}$), without significant heterogeneity across cohorts (P = 0.63). Each box includes data between 25^{th} and 75^{th} percentiles, with horizontal line indicating median.