Research Article

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Pharmacogenomics

Predictive role of *NUDT15* variants on thiopurine-induced myelotoxicity in Asian inflammatory bowel disease patients

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Background: Genetic variants of *TPMT* and *NUDT15* have been reported to predict the inter-patient variability in response and toxicity profiles of patients receiving thiopurine therapy. However, the clinical utility of *TPMT* genotyping in guiding thiopurine doses has been questionable, in part due to underlying differences in the prevalence of *TPMT* variants in both Caucasian and Asian populations. Several *NUDT15* variants have been associated with thiopurine-induced leukopenia, particularly in Asian cohorts. So far, none have been reported in a multiethnic Asian population. **Aim:** To investigate the associations between *TPMT* and *NUDT15* variants with thiopurine-induced myelotoxicity in 129 Asian inflammatory bowel disease patients. **Materials & methods:** Pyrosequencing was performed to screen for *TPMT* and *NUDT15* variants. Intracellular steady-state metabolite concentrations were quantified using liquid chromatography–tandem mass spectrometry. **Results:** Significant declines in nadir white blood cell, absolute neutrophil count and platelet counts were observed with increasing copy numbers of the risk T allele at *NUDT15* c.415C>T locus (overall $p < 0.05$) within 4, 8 and 12 weeks and 6 months after thiopurine initiation. Patients with low and intermediate NUDT15 activity, as inferred from haplotype pairs, had significantly higher risks of leukopenia ($p = 0.000253$) and neutropenia ($p = 0.002$) compared with patients with normal NUDT15 activity. **Conclusion:** These findings highlight the critical relevance of *NUDT15* pharmacogenetics in predicting for thiopurine-induced myelotoxicity and confirm the lack of significance of *TPMT* variants in Asian inflammatory bowel disease patients.

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Keywords: IBD • NUDT15 polymorphisms • thiopurine-induced myelotoxicity • TPMT

Thiopurine drugs, namely azathioprine (AZA) and 6-mercaptopurine (6-MP), have been effectively used as immunomodulators in inducing clinical remission and reducing steroid dependence in patients with active inflammatory bowel disease (IBD) [1–3]. They have also been shown to be effective in maintaining remission in patients with quiescent disease and remain as the cornerstone of pharmacological management of IBD patients due to their effectiveness, safety and affordability; especially when compared with costly biologic therapies [1–3]. However, up to 30–40% of patients discontinue thiopurine therapy or have their treatments interrupted due to adverse effects, particularly myelosuppression [4,5].

Both AZA and 6-MP are pro-drugs that require bioactivation to exert their therapeutic effects. AZA is converted to 6-MP by glutathione *S*-transferase, which then undergoes complex metabolism involving three competing pathways that are catalyzed by TPMT, XO and HGPRT [6,7]. 6-MP metabolism via the multistep pathway of HGPRT results in the production of the pharmacologically active 6-thioguanine nucleotides (6-TGN), which are potentially myelotoxic at supratherapeutic levels exceeding 450 pmole/8 $\times$ 10^8 erythrocytes [6,8,9]. 6-MP methylation via the TPMT pathway leads to the formation of potentially hepatotoxic 6-methylmercaptopurine (6-MMPN) metabolites, while XO catalyzes its metabolism into inactive 6-thiouric acid [6,9].

The observed inter-patient variability in response and toxicity profiles have been postulated to be heavily influenced by polymorphisms in the genes encoding the enzymes involved in thiopurine metabolism. Indeed, pretreatment assessment of *TPMT* genotype status has been recommended in clinical practice guidelines to guide the initial thiopurine doses [10,11]. However, its utility in personalizing thiopurine treatment has remained controversial, particularly in multiethnic Asian population in Singapore where *TPMT* variant alleles have only been shown to occur at a low overall frequency of 2.5% [12]. Such low prevalence of *TPMT* variants does not explain the relatively high rates of thiopurine-induced myelotoxicity in Asian IBD patients.

Recently, Yang *et al.* reported a strong association between a non-synonymous polymorphism in the *NUDT15* gene encoding p.Arg139Cys (rs116855232, c.415C>T) with thiopurine-induced leukopenia in Korean patients with Crohn's disease (CD) [13]. Two other studies by Kakuta *et al.* and Asada *et al.* also reported similar associations in Japanese IBD patients [14,15]. Moriyama *et al.* subsequently reported the presence of three additional variants in *NUDT15* and their associations with thiopurine intolerance in children with acute lymphoblastic leukemia [16]. To date, the frequencies of these variants in Asian IBD patients in Singapore, and their predictive roles for thiopurine-induced myelotoxicity have not been reported. This study therefore sought to evaluate the associations between *TPMT* and *NUDT15* genetic variants with thiopurine-induced myelotoxicity in Asian IBD patients.

Materials & methods

Study population & thiopurine treatment

Patients with confirmed diagnosis of CD and ulcerative colitis (UC) who were treated at the outpatient IBD clinic at the Singapore General Hospital and receiving thiopurine therapy were enrolled (n = 144), of whom 134 provided DNA samples.

Thiopurine doses were typically started at an AZA equivalent dose of 0.5–1.0 mg/kg/day in our institution and escalated up to 2.0–3.0 mg/kg/day as per guidelines at a rate of 0.5 mg/kg/day every 4–8 weeks, unless patients developed severe toxicities which warranted discontinuation of thiopurine therapy. This study was approved by the ethics review committee of the Singapore General Hospital. All recruited patients provided written informed consent to participate in the study.

Clinical data & assessment of myelotoxicity

Demographics and clinical data, including disease characteristics, details of thiopurine treatment and concomitant medications, as well as nadir (lowest) full blood counts within 4, 8 and 12 weeks and 6 months following initiation of thiopurine therapy were reviewed retrospectively from electronic medical records. Patients with missing data were not included in the study. The dose of 6-MP was adjusted to AZA equivalent doses by a factor of 2.08. Myelotoxicity was classified and graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events version 4.0. In addition to leukopenia, incidence of neutropenia and thrombocytopenia, which have not been previously investigated, were also evaluated.

Leukopenia was defined as white blood cell (WBC) count less than 3.0 $\times$ 10^9/l. Neutropenia was defined as absolute neutrophil count (ANC) less than 1.5 $\times$ 10^9/l. Thrombocytopenia was defined as platelet count less than 75 $\times$ 10^9/l. The thiopurine doses at which leukopenia occurred were determined at the time of lowest observed (nadir) WBC count. In addition, we also evaluated the rate of decrease in WBC, ANC and platelet counts from baseline (prior to thiopurine initiation).

Quantification of 6-TGN & 6-MMPN levels in erythrocytes by liquid chromatography–tandem mass spectrometry

Steady-state concentrations of 6-TGN and 6-MMPN metabolites in erythrocytes of patients who had been on stable doses of thiopurine for at least 4 weeks, were quantified using a validated liquid chromatography–tandem mass spectrometry method, which was adapted from Dervieux *et al.* with slight modifications [17]. Briefly, erythrocytes

were harvested from whole blood (6 ml), washed twice with normal saline and stored at -80°C until analysis. Erythrocytes were lysed by extensive vortexing and diluted 1:1 with 0.02 M phosphate buffer. 8-bromoadenine (8-BA; 50 µl) was added to 50 µl of red blood cell (RBC) lysate as internal standard. A total of 375 µl of dithiothreitol (DTT; 25 mg/ml in pure water) was added to protect 6-TGN from oxidation. The metabolites 6-TGN and 6-MMPN were subsequently converted to 6-TG and 6-MMP derivatives by incubation with perchloric acid (70%) for 1 h at 100°C. Appropriately diluted hydrolyzed samples were subsequently injected into the Agilent 1290 UHPLC (Agilent Technologies, CA, USA) tandem QTRAP 5500 MS system (AB Sciex, MA, USA). Mobile phases A and B used were 2.5 mM ammonium acetate containing 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. Chromatographic separation of the metabolites was achieved on an Atlantis T3 analytical column fitted with T3 guard column with an isocratic elution mode. The QTRAP 5500 mass spectrometer was used in positive ionization mode with 5500 V ionization, and the heater set at 450°C for the quantification of 6-TGN and 6-MMPN metabolites. Analyst software version 1.6.2 (AB Sciex) was used for the quantification of peak areas, with linear $1/x^2$-weighted regression.

Pharmacogenetics analyses of *NUDT15* & *TPMT* variants

Peripheral blood samples (6 ml) were obtained from enrolled patients and genomic DNA was extracted using the DNeasy® Blood & Tissue kit according to manufacturer's protocol (Qiagen, Hilden, Germany). Genomic DNA was quantified using NanoDrop ND-1000 Spectrophotometer at 260 nm (DE, USA)

The following variants in the *NUDT15* gene were genotyped: p.Arg139Cys (c.415C>T, rs116855232); p.Arg139His (c.416G>A, rs147390019); p.Val18Ile (c.52G>A, rs186364861); and p.Val18_Val19insGlyVal (c.36_37insGGAGTC, rs554405994). The *TPMT* variants genotyped were as follows: *TPMT*2* (p.Ala80Pro, c.238G>C, rs1800462); *TPMT*3A* (presence of both **3B* and **3C*); *TPMT*3B* (p.Ala154Thr, c.460G>A, rs1800460); *TPMT*3C* (p.Tyr240Cys, c.719A>G, rs1142345); and *TPMT*6* (p.Tyr180Phe, c.539A>T, rs75543815). Genotyping was performed using Pyrosequencing and results were validated against Sanger sequencing. Pyrosequencing and Sanger sequencing primers were designed with PyroMark® Assay Design software 2.0 (Qiagen) and PrimerQuest® Tool (IDT), respectively.

Linkage disequilibrium & statistical analysis

Conformity to Hardy–Weinberg equilibrium was determined using χ^2 tests. Pairwise linkage disequilibrium (LD) analysis was conducted using Haploview software v4.2 (Broad Institute, MA, USA) and described by |D'| and r^2. Haplotype phasing was conducted using PLINK v1.02 to assign haplotype status for each patient [18]. Normality of data was determined by visual inspection of skewness, z-values of kurtosis and D'Agostino–Pearson normality test. Linear mixed effects model was used to assess whether the associations between *NUDT15* c.415C>T (rs116855232) and WBC, ANC and platelet counts were time varying or stable. Likelihood ratio test was used to test models with versus without interaction between *NUDT15* c.415C>T (rs116855232) and time on these three outcome variables.

Logistic regression analyses assuming log-additive and dominant models were performed to determine the odds ratios (OR) of toxicities associated with the variant alleles in *NUDT15* and *TPMT* as well as NUDT15 activity groups. p-values were two sided and considered statistically significant for values lower than 0.05. All plots were generated using GraphPad Prism® version 6.0 (CA, USA), and statistical analyses were performed on SPSS version 14.0 (SPSS, Inc., IL, USA) and STATA version 14.0 (StataCorp LP, TX, USA).

Results

Patient demographics & clinical characteristics

Out of the 134 patients recruited, three patients had received thiopurine treatment for less than 6 months and were excluded from analysis. Two patients were not of Asian ethnicity and were also excluded. None of the enrolled patients had baseline laboratory abnormalities that may have interfered with the interpretation of study results. The demographics and clinical characteristics of recruited patients (n = 129) are listed in Table 1. The majority of the patients were male (n = 87, 67.4%), Chinese (n = 84, 65.1%) and were receiving thiopurine treatment for CD (n = 89, 69.0%). Most patients received AZA (n = 127, 98.4%) and were on concurrent 5-aminosalicylates (n = 83, 64.3%) and steroids (n = 87, 67.4%) at the time of thiopurine initiation.

Table 1. Demographics and clinical characteristics (n = 129).

Characteristics	n	%
Age		
Median (range)	42 (17–18)	
Gender		
Male	87	67.4
Female	42	32.6
Ethnicity		
Chinese	84	65.1
Malay	12	9.3
Indian	31	24.0
Others	2	1.6
Clinical indications of thiopurine therapy		
Crohn's disease	89	69.0
Ulcerative colitis	40	31.0
Thiopurine drug received		
Azathioprine	127	98.4
6-mercaptopurine	2	1.6
Concurrent medications at thiopurine initiation		
5-aminosalicylates	83	64.3
Corticosteroids	87	67.4

Genetic variations in *NUDT15* & *TPMT* in Asian IBD patients

The frequencies of variants in the *NUDT15* and *TPMT* genes are shown in Table 2. The overall variant allele frequency of *NUDT15* c.415C>T (rs116855232) was 11% in this cohort of Asian IBD patients. There were no deviations from Hardy–Weinberg equilibrium ($p = 0.321$) either when individuals of Chinese, Indian and Malay ethnicity were analyzed jointly or separately. The frequencies of *NUDT15* c.52G>A (rs186364861) and c.36_37insGGAGTC (rs554405994) were noticeably lower, with variant allele frequencies of 1 and 4%, respectively when compared with c.415C>T (Table 2). The variant c.416G>A (rs147390019) was nonpolymorphic in this cohort.

As previously reported in patients of east Asian descent, variations in the *NUDT15* gene occur at much higher frequencies than *TPMT* variants in this cohort of Asian IBD patients. None of the patients were carriers of *TPMT*2*, *TPMT*3A* and *TPMT*3B*. Only two patients (1.6%) were heterozygous for *TPMT*3C*, with none being homozygous carrier of the variant allele. Heterozygosity for *TPMT*6* was found only in one patient (0.8%), and the remaining patients were wild-type at this locus. Co-occurrence of variant alleles in both the *NUDT15* and *TPMT* genes was not observed in this study cohort.

Associations of *TPMT* variants with thiopurine-induced myelotoxicity

Myelotoxicity was absent in all three patients who were heterozygous at the *TPMT*3C* and **6* loci, thereby further confirming the lack of predictive role of *TPMT* variants on thiopurine-induced myelosuppression as previously reported in Koreans and Japanese [19–21].

Effects of *NUDT15* variants on nadir WBC, ANC & platelet counts

The pharmacogenetic influence of individual *NUDT15* variants on thiopurine-induced myelotoxicity was first investigated by comparing the differences in nadir WBC, ANC and platelet counts among patients carrying different copy numbers of the *NUDT15* variant alleles within 4, 8 and 12 weeks and 6 months of thiopurine therapy initiation. Statistically significant trends of declining nadir WBC and ANC counts were observed with increasing copy number of the *NUDT15* c.415C>T (rs116855232) risk allele, suggesting strong allele-dose effects (Figure 1A & Table 3). These associations remained significant even after the exclusion of the three patients carrying *TPMT* variants from analysis (data not shown). The distribution in nadir platelet counts between the three genotype groups of *NUDT15* c.415C>T (rs116855232) were significantly different within 8, 12 weeks and 6 months of thiopurine initiation (overall $p < 0.05$).

Table 2. Genotype and allele frequencies of *NUDT15* and *TPMT* variants.

Gene	Variants	n	Genotype frequency (n [%])			Allele frequency	
			CC	CT	TT	C	T
NUDT15	**c.415G>T (rs116855232)**						
	All patients	129	111 (86.0)	16 (12.4)	2 (1.6)	0.89	0.11
	Chinese	84	70 (83.3)	12 (14.3)	2 (2.4)	0.86	0.14
	Malays	12	11 (91.7)	1 (8.3)	0 (0)	0.88	0.12
	Indians	31	29 (93.5)	2 (6.5)	0 (0)	0.92	0.08
	c.416G>A (rs147390019)		**GG**	**GA**	**AA**	**G**	**A**
	All patients	129	129 (100)	0 (0)	0 (0)	1.00	0.00
	Chinese	84	84 (100)	0 (0)	0 (0)	1.00	0.00
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	c.52G>A (rs186364861)		**GG**	**GA**	**AA**	**G**	**A**
	All patients	129	127 (98.4)	2 (1.6)	0 (0)	0.99	0.01
	Chinese	84	83 (98.8)	1 (1.2)	0 (0)	0.99	0.01
	Malays	12	11 (91.7)	1 (8.3)	0 (0)	0.96	0.04
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	c.36_37insGGAGTC (rs554405994)		**WT**	**HET**	**HV**	**WT**	**V**
	All patients	129	118 (91.5)	11 (8.5)	0 (0)	0.96	0.04
	Chinese	84	73 (86.9)	11 (13.1)	0 (0)	0.93	0.07
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
TPMT	****2* (rs1800462)**		**GG**	**GC**	**CC**	**G**	**C**
	All patients	129	129 (100)	0 (0)	0 (0)	1.00	0.00
	Chinese	84	84 (100)	0 (0)	0 (0)	1.00	0.00
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	****3A* (rs1800460 and rs1142345)**		**WT**	**HET**	**HV**	**WT**	**V**
	All patients	129	129 (100)	0 (0)	0 (0)	1.00	0.00
	Chinese	84	84 (100)	0 (0)	0 (0)	1.00	0.00
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	****3B* (rs1800460)**		**GG**	**GA**	**AA**	**G**	**A**
	All patients	129	129 (100)	0 (0)	0 (0)	1.00	0.00
	Chinese	84	84 (100)	0 (0)	0 (0)	1.00	0.00
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	****3C* (rs1142345)**		**AA**	**AG**	**GG**	**A**	**G**
	All patients	129	127 (98.4)	2 (1.6)	0 (0)	0.98	0.02
	Chinese	84	82 (97.6)	2 (2.4)	0 (0)	0.97	0.03
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00
	****6* (rs75543815)**		**AA**	**AT**	**TT**	**A**	**T**
	All patients	129	128 (99.2)	1 (0.8)	0 (0)	0.99	0.01
	Chinese	84	83 (98.8)	1 (1.2)	0 (0)	0.98	0.02
	Malays	12	12 (100)	0 (0)	0 (0)	1.00	0.00
	Indians	31	31 (100)	0 (0)	0 (0)	1.00	0.00

HET: Heterozygous; HV: Homovariant; V: Variant; WT: Wild-type.

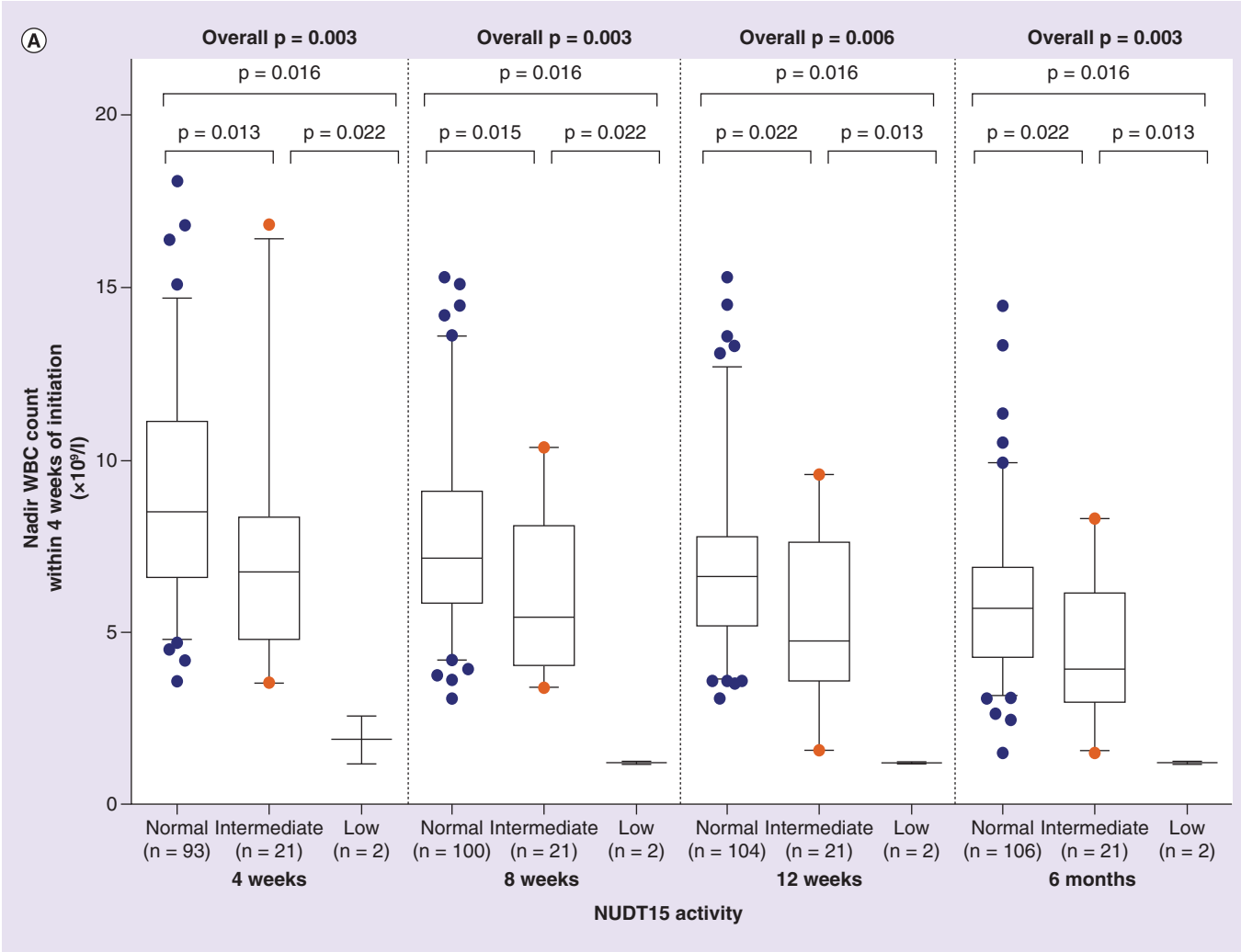


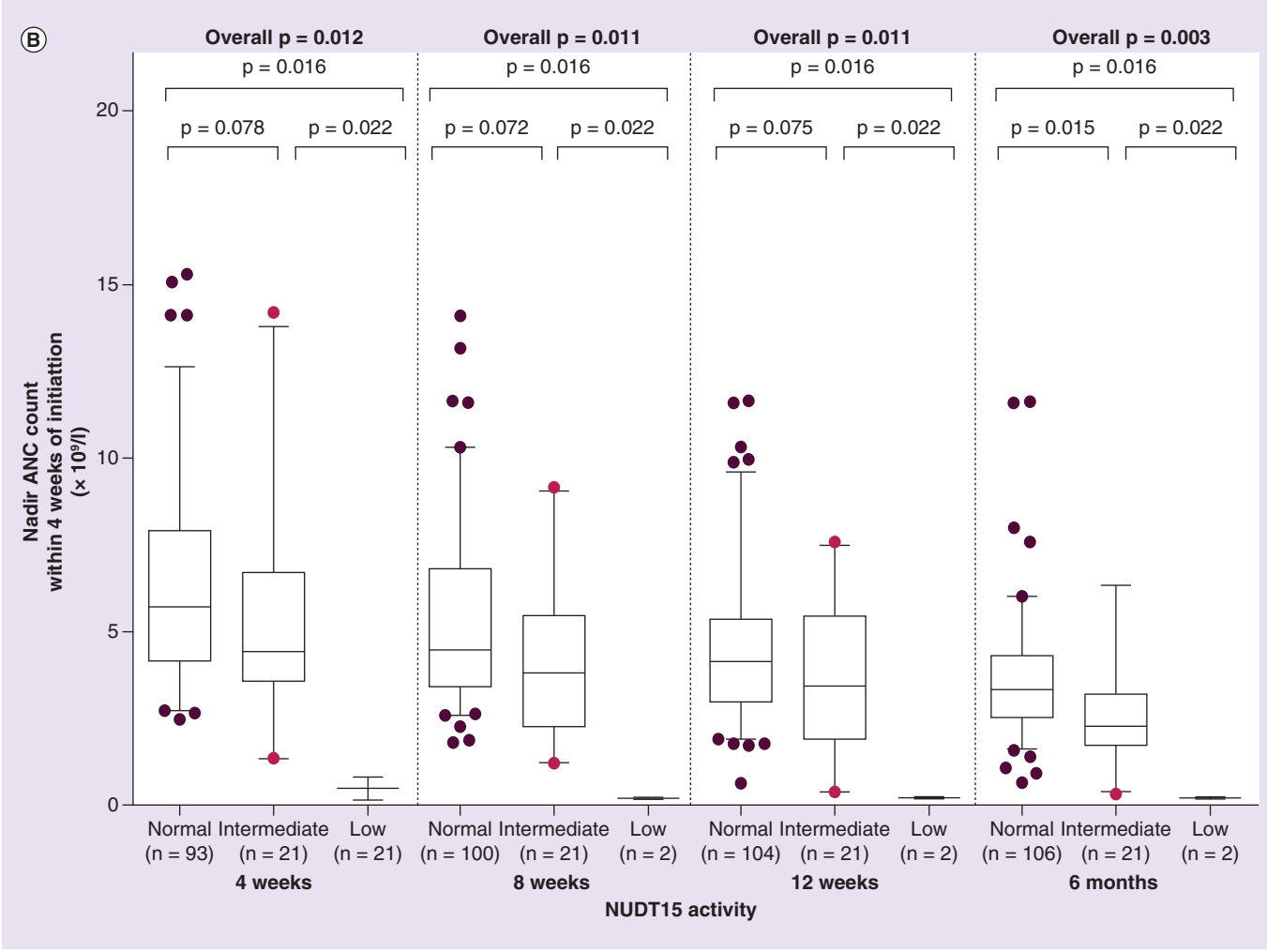
Figure 1. Nadir white blood cell counts within 4, 8 and 12 weeks and 6 months according to NUDT15 activity group. Nadir counts of **(A)** WBC, **(B)** ANC and **(C)** platelet counts within 4, 8, and 12 weeks and 6 months according to NUDT15 activity group.
ANC: Absolute neutrophil count; WBC: White blood cell.

There were no significant trends discernible in the nadir WBC, ANC and platelet counts of patients with regard to c.52G>A (rs186364861) and c.36_37insGGAGTC (rs554405994) within 4, 8 and 12 weeks and 6 months of thiopurine therapy initiation.

Linear mixed effects analyses and likelihood-ratio tests showed no sign of time variation in the associations between *NUDT15* c.415C>T (rs116855232) with WBC count ($p = 0.931$) and ANC ($p = 0.916$). However, the association between *NUDT15* c.415C>T (rs116855232) and platelet counts was time dependent ($p = 0.020$; data not shown). At 4, 8 and 12 weeks and 6 months, patients carrying the T alleles had lower platelet count than those with the wild-type (CC) genotype at this locus; platelet counts: 38.2, 64.2, 74.5 and 71.3 units, respectively. Collectively, these findings highlight the significant role of this *NUDT15* variant in not only predicting for leukopenia, but also neutropenia and thrombocytopenia in thiopurine-treated Asian IBD patients.

Associations of *NUDT15* variants with thiopurine-induced myelotoxicity

NUDT15 c.415C>T (rs116855232) was significantly predictive of thiopurine-induced leukopenia in this cohort (n = 10; 7.8%), with the risk allele being associated with a 22.9-fold increased risk of grade 2 leukopenia and above (OR: 22.9; 95% CI: 5.17–101.4; $p = 3.71 \times 10^{-5}$; Table 3). Similarly, *NUDT15* c.415C>T (rs116855232) was significantly associated with a 13.4-fold higher risk of grade 2 neutropenia and above (n = 10; 7.8%). Trends of faster onset of leukopenia and neutropenia were also observed with increasing copy numbers of the variant *NUDT15*



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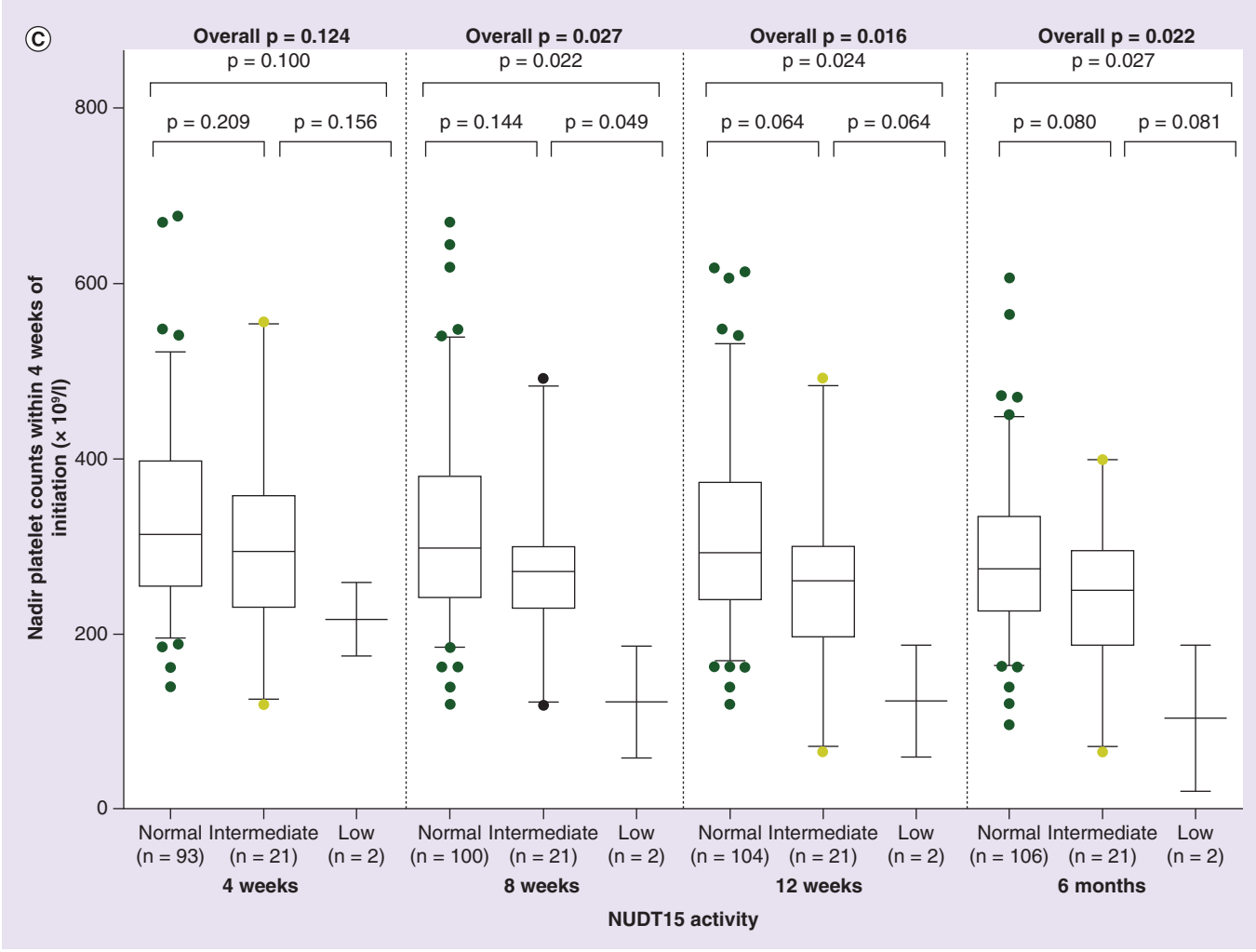
ANC: Absolute neutrophil count; WBC: White blood cell.

c.415C>T (rs116855232) allele (data not shown). Interestingly, *NUDT15* c.36_37insGGAGTC (rs554405994) was also associated with significantly higher risks of leukopenia but not neutropenia. None of the patients carrying variant *NUDT15* c.52G>A (rs186364861) allele had thiopurine-induced leukopenia or neutropenia.

Effects of *NUDT15* variants on steady-state levels of 6-TGN & 6-MMPN

Higher concentrations of thiopurine active metabolites, particularly 6-TGN, have been widely associated with higher risks of myelotoxicity [8]. We therefore investigated the differences in steady-state levels of 6-TGN and 6-MMPN in patients carrying different copy numbers of the *NUDT15* variant alleles in this cohort of Asian IBD patients. Unfortunately, thiopurine therapy was immediately withdrawn from the two patients carrying the homovariant (TT) genotype at the *NUDT15* c.415C>T (rs116855232) locus due to the development of severe myelotoxicity. Since this preceded the monitoring of steady-state thiopurine metabolite levels in these patients, they were excluded from this analysis. No significant differences were observed in 6-TGN and 6-MMPN levels between patients who were wild-type and heterozygous for *NUDT15* c.415C>T (rs116855232) after adjustments for thiopurine doses. Similarly, no significant associations were observed between the other two *NUDT15* variants (c.36_37insGGAGTC [rs554405994] and c.52G>A [rs186364861]) with steady-state 6-TGN and 6-MMPN levels. There was a lack of association observed between WBC and ANC levels with carriers of *TPMT* normal-



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ANC: Absolute neutrophil count; WBC: White blood cell.

NUDT15 variants and *TPMT* intermediate-*NUDT15* variants; most likely due to the low frequency of *TPMT* polymorphic variants in our Asian cohort.

Linkage disequilibrium analysis in Asian IBD patients

Since haplotype- and diplotype-based analyses have been reported to be more robust than genotype-based analyses and *NUDT15* variants investigated in this study were found to be strongly linked [16], we subsequently performed pairwise linkage disequilibrium analysis on the *NUDT15* variants present in this study cohort and identified a moderate linkage between *NUDT15* c.415C>T (rs116855232) and c.36_37insGGAGTC (rs554405994) [$|D'| = 0.69$ and $r^2 = 0.25$]. Haplotype phasing and assessment of the three *NUDT15* variants led to the inference of five haplotypes: reference haplotype *NUDT15*1* (90.3%), *NUDT15*2* (3%), *NUDT15*3* (4.7%), *NUDT15*5* (0.8%) and *NUDT15*6* (1.2%). The *NUDT15*4* haplotype was absent in our cohort of IBD patients. Diplotype analysis revealed a total of six *NUDT15* diplotype groups of which **1/*1* (82.2%), **1/*2* (6.2%) and **1/*3* (6.2%) comprised the most common diplotypes. Rare diplotypes **1/*5*, **1/*6* and **3/*3* occurred at low frequencies of less than 3% each.

Table 3. Associations of *NUDT15* variants with thiopurine-induced leukopenia and neutropenia.

		Genotype groups			Log-additive model	Dominant model	
Variants	Myelotoxicity	CC (n = 111)	CT (n = 16)	TT (n = 2)	p-value	OR (95% CI)	p-value
***NUDT15* c.415C>T (rs116855232)**							
	Leukopenia (WBC $<3.0 \times 10^9$/l)	3 (2.7%)	5 (31.3%)	2 (100%)	0.002*	22.9 (5.17–101.4)	3.71×10^{-5}*
	Grade 2	2	2	0			
	Grade 3	1	3	0			
	Grade 4	0	0	2			
	Neutropenia (ANC $<1.5 \times 10^9$/l)	4 (3.6%)	4 (25.0%)	2 (100%)	0.018*	13.4 (3.30–54.2)	2.79×10–12.79×10^{-4}*
	Grade 2	2	3	0			
	Grade 3	2	0	0			
	Grade 4	0	1	2			
***NUDT15* c.36_37insGGAGTC (rs554405994)**		WT/WT (n = 118)	WT/Ins (n = 11)	Ins/Ins (n = 0)			
	Leukopenia (WBC $<3.0 \times 10^9$/l)	7 (5.9%)	3 (27.3%)	–	–	5.95 (1.29–27.5)	0.022*
	Grade 2	3 (2.5%)	1 (9.1%)				
	Grade 3	4 (3.4%)	2 (18.2%)				
	Grade 4	0 (0%)	0 (0%)				
	Neutropenia (ANC $<1.5 \times 10^9$/l)	8 (6.8%)	2 (18.2%)	–	–	3.06 (0.56–16.6)	0.196
	Grade 2	4 (3.4%)	1 (9.1%)				
	Grade 3	2 (1.7%)	0 (0%)				
	Grade 4	2 (1.7%)	1 (9.1%)				

*$p < 0.05$.
ANC: Absolute neutrophil count; OR: Odds ratio; WBC: White blood cell; WT: Wild-type.

Effects of NUDT15 activity on nadir WBC, ANC & platelet counts

No significant differences were observed in the nadir WBC, ANC and platelet counts of patients with compound-heterozygous diplotypes (*NUDT15 *1/*2*, **1/*3*, **1/*2* and **1/*6*) within 4, 8 and 12 weeks and 6 months of thiopurine initiation (overall $p > 0.05$). The IBD patients in this study were then classified into three NUDT15 activity groups: carriers of the reference diplotype, *NUDT15 *1/*1,* were classified as having normal NUDT15 activity; patients harboring *NUDT15* compound-heterozygous diplotypes (*NUDT15 *1/*2*, **1/*3*, **1/*2* and **1/*6*) were considered to have intermediate NUDT15 activity whereas carriers of compound-homovariant diplotype (*NUDT15 *3/*3*) were classified as having low NUDT15 activity.

Significant differences were observed in the nadir WBC and ANC of patients with different NUDT15 activities within 4, 8 and 12 weeks and 6 months of thiopurine initiation. Specifically, patients with intermediate and low NUDT15 activities had significantly lower nadir WBC and ANC compared with those with normal NUDT15 activity (Figure 1A & B). Significant trends were also observed in the nadir platelet counts of patients with normal NUDT15 activity and those with intermediate and low NUDT15 activity at 8, 12 weeks and 6 months of therapy initiation (Figure 1C). Similar to the findings of the single-variant analysis for *NUDT15* c.415C>T (rs116855232) described earlier, strong gene-dose effects were observed in the diplotype-based association analyses.

Associations of NUDT15 activity with thiopurine-induced myelotoxicity

Logistic regression analyses showed that patients possessing intermediate and low NUDT15 activity were associated with a 15-fold higher risk of leukopenia (95% CI: 3.52–64.1; $p = 0.000253$) compared with patients with normal NUDT15 activity. Similarly, with regard to the risk of developing neutropenia, IBD patients with low and intermediate NUDT15 activities were also significantly associated with nine-fold higher risks compared with patients harboring normal NUDT15 activity (95% CI: 2.30–35.3; $p = 0.002$; Table 4).

Table 4. Associations of *NUDT15* activity with thiopurine-induced leukopenia and neutropenia.

Myelotoxicity	*NUDT15* activity groups			Log-additive model	Dominant model	
	Normal (n = 106)	Intermediate (n = 21)	Low (n = 2)	p-value	OR (95% CI)	p-value
Leukopenia (WBC $< 3.0 \times 10^9$/l):	3 (2.8%)	5 (23.8%)	2 (100%)	0.010*	15.0 (3.52–64.1)	0.000253*
– Grade 2	2	2	0			
– Grade 3	1	3	2			
– Grade 4	0	0	0			
Neutropenia (ANC $< 1.5 \times 10^9$/l):	3 (2.8%)	4 (19.0%)	2 (100%)	0.059	9.0 (2.30–35.3)	0.002*
– Grade 2	2	3	0			
– Grade 3	1	0	0			
– Grade 4	0	1	2			

*$p < 0.05$.
ANC: Absolute neutrophil count; OR: Odds ratio; WBC: White blood cell.

Discussion

Routine screening of *TPMT* genotype status prior to initiation of thiopurine therapy has been recommended in several clinical practice guidelines to reduce the risks of myelosuppression [10,22]. However, the low frequencies of these variants in Asians have greatly diminished its relevance and clinical utility in predicting myelosuppression in this ethnic group. In line with earlier findings in other Asian populations, the results of this study confirm that variations in the *TPMT* gene are rare and do not constitute a significant genetic factor that contribute to the development of thiopurine-induced myelotoxicity in Asian IBD patients.

Importantly, the significant associations between *NUDT15* c.415C<T (rs116855232) with thiopurine-induced leukopenia previously identified in Taiwanese, Koreans and Japanese IBD and acute lymphoblastic leukemia patients [13,14,23] were further confirmed in this cohort of multiethnic Asian IBD patients. Specifically, we observed significant trends of declining nadir WBC counts and more rapid decrease in WBC counts with increasing copy number of the risk T allele (rs116855232), and significantly higher risks of leukopenia in patients carrying the variant *NUDT15* allele at the c.415C>T (rs116855232) locus.

Notably, majority of the infectious complications related to thiopurine therapy have been noted to occur in the absence of leukopenia and risks of thiopurine-related infections have been noted to increase as a consequence of neutropenia [1,24]. Our results also indicate for the first time that the variant T allele at the c.415C<T locus (rs116855232) is predictive of thiopurine-induced neutropenia as well as thrombocytopenia. Taken together, these findings clearly highlight the greater influence of the *NUDT15* c.415C>T variant (rs116855232) on hematological toxicities compared with the low penetrant *TPMT* variants and supports the clinical utility of genotyping for this variant in assessing the risks of thiopurine-induced myelotoxicity prior to treatment initiation in Asian IBD patients.

With regard to the chronological aspects of thiopurine-induced myelosuppression, we performed the analyses with nadir WBC, ANC and platelet counts within 4, 8 and 12 weeks and 6 months of thiopurine initiation as thiopurine-induced myelosuppression has been shown to occur frequently within the first weeks or months of treatment [25]. Indeed, significant trends of lower nadir WBC, ANC and platelet counts with increasing copy of the variant T allele at the c.415C>T (rs116855232) locus were observed across all the four time intervals, further emphasizing the consistency of these observations across the different durations of treatment. The onsets to leukopenia and neutropenia were also observed to be faster with increasing copy number of the variant T allele, where patients who were carriers of two homozygous variant alleles for *NUDT15* c.415C>T (rs116855232) developed thiopurine-induced leukopenia and neutropenia within only 4–8 weeks of treatment initiation (data not shown). Taking into account the strong gene-dose effects observed in the present study and others, optimization of thiopurine dosages based on patients' *NUDT15* genotype status should certainly be considered in future studies aiming to personalize thiopurine therapy.

In addition to the *NUDT15* c.415C>T (rs116855232) variant, which has been more widely investigated, we also investigated the frequencies of other *NUDT15* variants (c.52G>A, c.36_37insGGAGTC and c.416G>A) and their influence on the incidence of thiopurine-induced myelotoxicity in our cohort. Despite the consistently lower trends of nadir WBC, ANC and platelet counts in patients who were heterozygous at the c.52G>A and

c.36_37insGGAGTC loci, statistical significance was not achieved, which may be due in part to the low frequencies of these two functional variants in this study cohort.

It is also plausible that the functional consequences of these two variants on NUDT15 activity may be less pronounced as compared with that of *NUDT15* c.415C>T (rs116855232), as suggested by the functional studies conducted by Moriyama *et al.* [16], which showed markedly lower nucleoside diphosphatase activity for the *NUDT15* c.415C>T gene product compared with the other two variants in *Escherichia coli*. Recently, Carter *et al.* [26] characterized the crystal structure and biochemical activities of the NUDIX family proteins, which includes NUDT15. The findings of Carter *et al.* [26] suggest that the structural change resulting from *NUDT15* c.415C>T (rs116855232) variation would occur at the base of the substrate-binding pocket of the NUDT15 monomer. They also postulated that the arginine to cysteine substitution (p.Arg139Cys) may result in the formation of disulfide bond with an adjacent cysteine residue (Cys140), which may in turn disrupt the conformation of NUDT15 active site. It is possible that the structural disruptions resulting from other variations in the *NUDT15* gene may not occur at sites that are critical to NUDT15 enzymatic activity. However, further studies are essential to fully elucidate the functional consequences of these variants.

Nevertheless, the predictive roles of these less-commonly occurring *NUDT15* variants require further validation in a larger cohort of thiopurine-treated patients. Notably, the results of our diplotype-based association analyses revealed significantly lower nadir WBC, ANC and platelet counts and significantly higher risks of myelotoxicity, particularly leukopenia and neutropenia, in patients with low and intermediate NUDT15 activity as compared with those with normal NUDT15 activity who were wild-type at all loci. Interestingly, comparison of the goodness-of-fit of the regression models showed that the model based on *NUDT15* c.415C>T (rs116855232) alone was better than NUDT15 activity group in predicting thiopurine-induced leukopenia and neutropenia. Although the differences in goodness-of-fit indices between the two models were marginal, the receiver operating characteristic areas were significantly different between the two models, with the model based on *NUDT15* c.415C>T (rs116855232) alone showing consistently better performance in predicting thiopurine-induced leukopenia and neutropenia (data not shown). It is therefore highly plausible that the contributory effect of the *NUDT15* c.415C>T variant is probably greater than the other three variants in the *NUDT15* gene toward development of thiopurine-induced leukopenia and neutropenia. These findings were also supported by the findings of the functional studies done by Moriyama *et al.*, which showed the greatest loss in NUDT15 activity with the *NUDT15* c.415C>T and *NUDT15* c.415C>T plus c.36_37insGGAGTC variants [16]. Given the significant practical advantage of determining *NUDT15* c.415C>T genotype status over NUDT15 activity group, which incorporates the genotype information of three other *NUDT15* variants, it is possible that genotyping for *NUDT15* c.415C>T alone may be sufficient in predicting for thiopurine-induced myelotoxicity.

Moriyama *et al.* also compared the intracellular levels of thioguanine incorporated into DNA in *NUDT15*-knockdown and control human lymphoid cells and demonstrated the accumulation of active metabolites in *NUDT15*-knockdown cells [16], which indicate the role of NUDT15 in inactivating thiopurine metabolites and leading to the postulation that NUDT15 modulates thiopurine-induced myelosuppression via accumulation of 6-TGN. In contrast, however, we found no significant differences in the 6-TGN levels of patients with wild-type genotype and those who were heterozygous at the c.415C>T (rs116855232) locus (data not shown). In accordance with the findings of Asada *et al.* [15], thiopurine-induced leukopenia appears to be mediated by *NUDT15* variants, independent of 6-TGN concentrations. However, further investigations with a larger cohort size and which include the steady-state levels of patients with homovariant (TT) genotype are necessary to confirm these findings.

Conclusion

In conclusion, pharmacogenetic variants in *NUDT15* were found to be significantly associated with the development of thiopurine-induced myelotoxicity in Asian IBD patients. The evidence that *NUDT15* variants confer higher risks of thiopurine-induced leukopenia also supports the compelling need to genotype this pharmacogenetic marker prior to initiation of thiopurine; specifically c.415C>T (rs116855232), which was associated with a 22.9-fold higher risk of leukopenia in our cohort. This study also confirmed the lack of utility of *TPMT* variants as well as the low penetrant *NUDT15* variants (c.52G>A and c.36_37insGGAGTC) compared with *NUDT15* c.415C>T in predicting thiopurine-induced myelosuppression.

Summary points

- Our results revealed a strong dose-allele effect amongst patient carriers of *NUDT15* c.415C>T allele (rs116855232) on decreasing nadir WBC and ANC counts for 4, 8 and 12 weeks and 6 months upon thiopurine initiation.
- Significant associations between *NUDT15* c.415C>T risk alleles and grade 2 leukopenia (OR = 22.9, $p = 3.71 \times 10^{-5}$) and neutropenia (OR = 13.4, $p = 2.79 \times 10^{-4}$) were also observed.
- No significant observations were found between *TPMT* and *NUDT15* variants on steady-state levels of thiopurine-metabolites 6-thioguanine nucleotide (6-TGN) and 6-mercaptomercaptopurine (6-MMPN).
- Patients with intermediate (*NUDT15 *1/*2*, **1/*3*, **1/*2* and **1/*6*) and low NUDT15 (*NUDT15 *3/*3*) activities were found to have lower WBC and ANC counts compared to those with normal NUDT15 activity (*NUDT15 *1/*1*).
- The intermediate and low NUDT15 activity groups were also associated with increased risks in leukopenia (OR = 15.0, p = 0.000253) and neutropenia (OR = 9.0, p = 0.002).
- No significant associations were observed with other *NUDT15* variants (c.52G>A, rs186364861; c.36_37insGGAGTC, rs5544005994), due to the low penetrance of these variants in our Asian population.
- Our study demonstrates the predictive role of *NUDT15* variant c.415C>T on leukopenia risk in a multiethnic Asian population and confirm the lack of clinical utility in *TPMT* genotyping in our cohort.

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