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ORIGINAL ARTICLE—ALIMENTARY TRACT

NUDT15 R139C-related thiopurine leukocytopenia is mediated by 6-thioguanine nucleotide-independent mechanism in Japanese patients with inflammatory bowel disease

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Abstract

Background *NUDT15* R139C (rs116855232) is a recently identified genetic factor responsible for thiopurine-induced leukocytopenia and hair loss. In this study, we investigated the association of *NUDT15* R139C with 6-thioguanine nucleotide (6-TGN) levels and thiopurine-induced leukocytopenia in Japanese patients with inflammatory bowel disease (IBD).

Methods Two hundred and sixty-four subjects (103 healthy volunteers and 161 IBD patients treated with thiopurines) were enrolled. Genotyping for *NUDT15* R139C was performed using Custom TaqMan® SNP genotyping assays.

Results The *NUDT15* C/C, C/T, and T/T genotypes were 80.7, 18.2, and 1.1 %, respectively. The allelic frequency was 10.2 %. Among 161 IBD patients, there was no significant difference in 6-TGN levels among the *NUDT15* genotypes. Forty-five patients (27.9 %) developed leukocytopenia (WBC <3000/μl), and the C/T and T/T genotypes were significantly associated with the development of leukocytopenia ($P = 1.7 \times 10^{-5}$). In these patients, 6-TGN levels were not significantly different between *NUDT15* genotypes. *NUDT15* R139C was significantly associated with early (<8 weeks) ($P = 1.03 \times 10^{-4}$) and late (>8 weeks) leukocytopenia ($P = 4.3 \times 10^{-4}$). The decrease in WBC count at 2 and 4 weeks was significantly higher in patients with the C/T or T/T genotypes as compared to the patients with the C/C genotype. All patients with the T/T genotype ($n = 2$) developed early severe hair loss and severe leukocytopenia (<1000/μl). The logistic regression analysis revealed that *NUDT15* R139C was the sole genetic factor responsible for the thiopurine-induced leukocytopenia ($P = 0.001$).

Conclusions These results suggest that *NUDT15* R139C-related thiopurine-induced leukocytopenia is mediated by a 6-TGN-independent mechanism.

Keywords NUDT15 · IBD · Azathioprine · 6-Mercaptopurine

Introduction

The thiopurine drugs azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used to maintain clinical remission of inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) [1–3]. Despite its efficacy, thiopurine was reported to have drug-induced toxicity, such as myelotoxicity, hepatotoxicity, hair loss, pancreatitis and gastrointestinal intolerance [4, 5]. About 15–30 % of patients discontinue therapy due to side effects [6–9].

The unfortunate side-effect profile of thiopurine despite its clinical efficacy has been explained by individual thiopurine-metabolizing activity, based on the genetic polymorphism of thiopurine-metabolizing enzymes [10, 11]. AZA and 6-MP are metabolized to 6-thioguanine nucleotides (6-TGNs) [10, 11], and 6TGNs mediate the immunosuppressive properties of AZA/6-MP via the inhibition of immune cell proliferation [10]. In this process TPMT (thiopurine *S*-methyltransferase) metabolizes thiopurines to inactive molecules, and variant *TPMT* alleles

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of lower metabolizer are associated with thiopurine-induced leukocytopenia with an elevation of 6-TGN levels [12]. A SNP (single-nucleotide polymorphism) of *TPMT* A719G has been reported to be the most prevalent allele of lower thiopurine metabolize [1, 13].

Genetic polymorphism of inosine triphosphate pyrophosphatase (ITPase) was also suspected as partly responsible for thiopurine intolerance [5, 14–17]. Genetic ITPase deficiency has been reported to result in the cellular accumulation of thioinosine triphosphate (TITP), and some studies have suggested a role for *ITPase* variants in thiopurine-induced toxicity [16, 17].

Multidrug resistance protein 4 (MRP4) functions as a cellular efflux pump for 6-MP and 6-TGNs [18]. A SNP in human *MRP4* G2269A dramatically reduces MRP4 function and results in the intracellular accumulation of 6-TGNs [18]. We have previously reported that *MRP4* G2269A is associated with higher 6-TGN levels and leads to thiopurine-induced leukocytopenia in Japanese patients with IBD [19].

In addition to this genetic predisposition, a considerable number of patients who have not inherited the *TPMT* or *MRP4* variant experience thiopurine-induced leukocytopenia [1], suggesting the contribution of unknown factors responsible for inter-individual variation in thiopurine sensitivity. In 2014, Yang et al. discovered a SNP in *NUDT15* (nucleoside diphosphate–linked moiety *X*-type motif 15) (*NUDT15* R139C) that was strongly associated with thiopurine-induced leukocytopenia [odds ratio (OR) = 35.6, $P = 4.88 \times 10^{-94}$] in Korean CD patients [20]. Sensitivity and specificity of *NUDT15* R139C for thiopurine-induced early (developed within 8 weeks) leukocytopenia were 89.4 and 93.2 %, respectively. Following this study, Kakuta et al. confirmed the association of *NUDT15* R139C with early leukocytopenia in Japanese IBD patients (OR = 28.4, $P = 1.92 \times 10^{-16}$) [21]. These findings suggest that *NUDT15* R139C is a new genetic factor responsible for thiopurine-induced myelotoxicity in East Asian populations. However, these two studies did not evaluate 6TGN levels, which is quite important to understand the functional role of *NUDT15* R139C in promoting thiopurine-induced leukocytopenia.

In this study, we investigated the association of *NUDT15* R139C genotypes with thiopurine-induced leukocytopenia and 6TGN levels. Furthermore, we focused on the association between *NUDT15* R139C and decrease in WBC (white blood cell) count.

Patients and methods

Patients

We enrolled 103 healthy volunteers (female/male 46/57) and 161 IBD patients (UC $n = 89$; CD $n = 72$) attending the gastroenterology outpatient clinic at the Hospital of Shiga University of Medical Science between May 2015 and July 2015. All subjects were Japanese, and their demographic/clinical characteristics are shown in Table 1. All patients were treated with AZA/6MP. The protocol of this study was approved by the Ethics Committee of Shiga University of Medical Science. Written informed consent was obtained from all patients.

Thiopurine treatment and adverse events

The clinical data including 6-TGN levels were reviewed from medical records. The dose of 6MP was converted to an AZA equivalent dose using a conversion factor of 2.08 [22]. Thiopurine therapy was performed according to the treatment guidelines of IBD in Japan. Thiopurines were started at a dose of 0.5–1.0 mg/kg/day (as AZA dose) and adverse events were checked at 7–14 days from the initiation. If patients have no adverse events, we increased to around 1.0 mg/kg/day. The thiopurine dose and 6-TGN levels when the WBC count reached the lowest value (nadir) were used for analysis. Leukocytopenia was graded by common toxicity criteria as follows: Grade 2, 2000–3000/μl; Grade 3, 1000–2000/μl, and Grade 4, <1000/μl [23]. Severe hair loss was defined by the need to wear wigs for long period, according to the criteria described by Kakuta et al. [21].

Table 1 Characteristics of patients and healthy volunteers enrolled in this study

	UC ($n = 89$)	CD ($n = 72$)	Healthy ($n = 103$)	Total ($n = 264$)
Gender (female/male)	44/45	20/52	46/57	110/154
Age, year[a] (mean ± SEM)	37.0 ± 1.4	32.3 ± 1.2	57.7 ± 1.8	44.1 ± 0.89
5-ASA[a], *n* (%)	79 (88.8 %)	66 (91.7 %)		
Prednisolone[a], *n* (%)	67 (75.3 %)	24 (33.3 %)		
Anti-TNF drugs[a], *n* (%)	1 (1.1 %)	29 (40.3 %)		

All of the IBD patients were treated with thiopurine drugs [azathioprine (AZA) or 6-mercaptopurine (6-MP)]. The dose of 6MP was converted to an AZA equivalent dose using a conversion factor of 2.08 [22]

[a] Age, 5-ASA, prednisolone and anti-TNF drugs are the data of thiopurine start

Genotyping of *NUDT15* R139C (rs116855232), *TPMT* A719G (rs1142345), *ITPase* C94A (rs1127354) and *MRP4* G2269A (rs3765534)

Mononuclear cells were isolated from heparinized blood using a Ficoll density gradient. The genomic DNA was isolated by a DNA extraction kit purchased from QIAGEN (Valencia, CA). Genotyping for *NUDT15* R139C (rs116855232), *TPMT* A719G (rs1142345), *ITPase* C94A (rs1127354) and *MRP4* G2269A (rs3765534) was performed using Custom TaqMan® SNP genotyping assays (ID: C_154823200_10, C_19567_20, C_27465000_10 and C_27478235_20; Applied Biosystems, Inc., Foster City, CA) in accordance with information on the Applied Biosystems website (http://www.appliedbiosystems.com).

The PCR was performed according to the manufacturer's instructions provided by Applied Biosystems. The PCR thermal cycling was as follows: initial denaturing at 95 °C for 30 s followed by 40 cycles of 92 °C for 5 s and 60 °C for 20 s. Thermal cycling was performed using a LightCycler 480 system (Roche Diagnostics, Switzerland). Each 96-well plate contained 80 samples of an unknown genotype and 4 reaction mixtures containing the reagents, but no DNA (no-template control). The no-DNA control samples were necessary for the Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide. The genotypes were determined visually based on the dye component fluorescent emission data depicted in the *X*–*Y* scatter-plot of the SDS software.

6-TGN assay

The heparinized blood samples were first washed, and the red blood cells were hydrolyzed by acid, and extracted with phenylmercuric acetate/ethyl acetate. The 6-TGN levels were then measured by high performance liquid chromatography, as previously described [24].

Hardy–Weinberg equilibrium (HWE)

HWE analysis was performed on the research subjects by comparing the detected distribution of allele frequencies with the theoretical distribution estimated on the basis of the SNP allelic frequencies. $P > 0.05$ (Chi-squared statistics) was considered to indicate equilibrium.

Statistical analysis

The AZA dose, 6-TGN levels, and ΔWBC (ΔHb and ΔPlts) were compared using the Mann–Whitney test. The frequencies of leukocytopenia and severe hair loss for each genotype of R139C were compared by 3 × 2 Chi-square tests in univariate analyses. Logistic regression analyses were performed to identify the associations of leukocytopenia with candidate SNPs in multivariate analyses. All analyses were performed using R software version 2.14.1. $P < 0.05$ was regarded as statistically significant.

Results

Genotype frequency

Of the 264 subjects analyzed (161 IBD patients and 103 healthy volunteers), 213 subjects showed a wild type of *NUDT15* R139C (C/C) (80.7 %) (Table 2). Forty-eight subjects showed a heterozygote of *NUDT15* R139C (C/T) (18.2 %) and 3 carried a homozygote (T/T) (1.1 %) (Table 2). The allelic frequency of *NUDT15* R139C was 10.2 % (54/528). No deviation from the Hardy–Weinberg equilibrium (HWE) was detected ($P = 0.66$).

In the same cohort, 7 subjects had a heterozygote of *TPMT* A719G (2.7 %) (Table 2), and the allelic frequency of *TPMT* A719G was 1.3 % (7/528). Sixty-three subjects showed a heterozygote of *MRP4* G2269A (23.9 %) and 5 carried a homozygote (1.9 %) (Table 2). The allelic frequency of *MRP4* G2269A was 13.8 % (73/528). A heterozygote of *ITPase* C94A was detected in 55 subjects (20.8 %) and a homozygote of *ITPase* C94A was detected 6 subjects (2.3 %) (Table 2) [25]. The allelic frequency of *ITPase* C94A was 12.7 % (67/528). These results agreed with previous reports on *TPMT*, *MPR4* and *ITPase* polymorphism in the Japanese population [7, 13, 16, 19, 25].

Association of *NUDT15* genotype with 6-TGN levels and leukocytopenia

The associations of the *NUDT15* variant with 6-TGN levels and the white blood cell (WBC) count were analyzed in 161 IBD patients treated with AZA/6-MP (Table 3). These subjects consisted of 127 patients with the wild genotype (C/C), 32 patients with the C/T genotype, and 2 patients with the T/T genotype. In 161 IBD patients (including those with and without leukocytopenia), there was no significant difference in AZA dose and 6-TGN levels at WBC nadir among the *NUDT15* genotypes (Table 3), suggesting that the *NUDT15* genotype did not affect thiopurine metabolism.

Forty-five of 161 IBD patients (27.9 %) experienced leukocytopenia (Grade 2 or higher; WBC <3000/μl), and *NUDT15* variants (C/T and T/T genotype) were significantly associated with the development of leukocytopenia [$P = 1.70 \times 10^{-5}$, odds ratio (OR) 5.82, 95 % confidence

Table 2 Genotype distribution of *NUDT15, MRP4, TPMT* and *ITPase* gene

Genotype allele	Total ($n = 264$)	UC ($n = 89$)	CD ($n = 72$)	Healthy ($n = 103$)
NUDT15 R139C				
C/C (wild type)	213 (80.7 %)	69	58	86
C/T	48 (18.2 %)	18	14	16
T/T	3 (1.1 %)	2	0	1
Allele frequency	10.2 %	–	–	–
TPMT A719G				
A/A (wild type)	257 (97.3 %)	86	70	101
A/G	7 (2.7 %)	3	2	2
G/G	0	0	0	0
Allele frequency	1.30 %	–	–	–
MRP4 G2269A				
G/G (wild type)	196 (74.2 %)	67	55	74
G/A	63 (23.9 %)	20	16	27
A/A	5 (1.9 %)	2	1	2
Allele frequency	13.8 %	–	–	–
ITPA C94A				
C/C (wild type)	203 (76.9 %)	72	56	75
C/A	55 (20.8 %)	16	16	23
A/A	6 (2.3 %)	1	0	5
Allele frequency	12.7 %	–	–	–

Table 3 Association of *NUDT15* genotype with leukocytopenia and hair loss

NUDT15 genotype	C/C ($n = 127$)	C/T ($n = 32$)	T/T ($n = 2$)	*P* value
AZA dose, mg/kg/day (mean ± SEM)	0.86 ± 0.04	0.80 ± 0.07	0.75	0.27
6TGNs levels, pmol/8 × 10^8 RBC (mean ± SEM)	341.2 ± 17.7	320.5 ± 37.3	341	0.36
Leukopenia (WBC <3000), *n* (%)	25 (19.6 %)	18 (56 %)	2 (100 %)	1.7×10^{-5}
Early leukocytopenia (within 8 weeks), *n* (%)	2 (1.6 %)	2 (6.3 %)	2 (100 %)	1.03×10^{-4}
Grade 4 (WBC <1000)	0	0	2	$6.8 \times 10^{-6\#}$
Grade 3 (WBC <2000)	1	1	0	–
Grade 2 (WBC <3000)	1	1	0	–
No leukopenia	125	30	0	–
Late leukocytopenia (after 8 weeks), *n* (%)	23 (18.4 %)	16 (53.3 %)	–	4.3×10^{-4}
Grade 4 (WBC <1000)	0	0	–	–
Grade 3 (WBC <2000)	3	1	–	–
Grade 2 (WBC <3000)	20	15	–	–
No leukopenia	102	14	–	–
Interval to late leukocytopenia (days) (mean ± SEM)	518.9 ± 98.6	324.8 ± 95.6	–	0.06
ΔWBC at 2 weeks (/μl) (mean ± SEM)	388.6 ± 193.9	−781.1 ± 548.0	−4550	0.021
ΔWBC at 4 weeks (/μl) (mean ± SEM)	575 ± 180.8	−2714.3 ± 532.5	−4430	5.7×10^{-4}
Early severe hair loss, *n* (%)	0	0	2 (100 %)	–

ΔWBC at 2 and/or 4 weeks: Change in WBC count 2 and/or 4 weeks after thiopurine induction. The AZA dose and 6-TGNs levels when the WBC count reached the lowest value were used for analysis

WBC white blood cell, *AZA* azathioprine, *6TGNs* 6-thioguanine nucleotides

P value for early severe leukocytopenia (Grades 3 and 4)

interval (CI) 2.59–13.1] (Table 3). This result was compatible with recent reports on Koreans ($P = 5.49 \times 10^{-5}$) [20] and Japanese ($P = 1.61 \times 10^{-5}$) [21].

Next, we investigated the association of *NUDT15* R139C with early (developed within 8 weeks) and late (developed after 8 weeks) leukocytopenia. As shown in

Table 3, *NUDT15* R139C was significantly associated with the development of early leukocytopenia ($P = 1.03 \times 10^{-4}$, OR 9.33, 95 % CI 2.83–30.8), and a much stronger association was detected with the development of early severe leukocytopenia (Grades 3 and 4) ($P = 6.8 \times 10^{-6}$, OR 22.8, 95 % CI 4.25–122.6). In particular, all patients with the T/T genotype ($n = 2$) developed Grade 4 leukocytopenia (<1000/μl) within 8 weeks.

Recently, Kakuta et al. reported that the presence of *NUDT15* R139C was not related to the development of late leukocytopenia in the Japanese population. However, we detected a significant association between the presence of *NUDT15* R139C and the development of late leukocytopenia ($P = 4.3 \times 10^{-4}$, OR 4.08, 95 % CI 1.9–8.7) (Table 3). Interval to late leukocytopenia tended to be shorter in patients with the C/T genotype than those with the wild (C/C) genotype (Table 3), but statistical significance was not detected ($P = 0.06$).

ΔWBC at 2 and 4 week

To evaluate the association of *NUDT15* R139C with the decrease rate of WBC, we calculated the ΔWBC at 2 and 4 weeks. The ΔWBC at 2 weeks was calculated by the following formula: (the ΔWBC at 2 weeks) = (WBC count at 2 weeks after thiopurine initiation−WBC count at thiopurine initiation). The ΔWBC at 4 weeks was also similarly calculated. As shown in Table 3, ΔWBC at 2 and 4 weeks was significantly higher in patients with the C/T or T/T genotypes as compared to patients with the wild genotype (C/C). In patients with the wild genotype (C/C), ΔWBC values at 2 and 4 weeks were 388.6 and 575, respectively, showing no decrease in these patients. WBC count seemed to decrease much more rapidly in patients with the T/T genotype as compared with patients with the C/T genotype (statistical analysis was not possible due to small sample number). A similar tendency was observed in the rate of decrease of platelets, but the findings were not significant (Supplementary Table 1).

Early severe hair loss

All patients with the *NUDT15* T/T genotype ($n = 2$) developed early severe hair loss, but this phenomenon was not observed in patients with the wild genotype (C/C) or C/T genotype (Table 3).

AZA dose, 6-TGN levels and prednisolone dose in IBD patients with leukocytopenia

In IBD patients with leukocytopenia, AZA dose, 6-TGN levels and prednisolone dose at WBC nadir were not different between *NUDT15* genotypes (Table 4). Furthermore, there were no statistically significant differences in AZA dose and 6-TGN levels among the *NUDT15* genotypes in patients with early and late leukocytopenia (Table 4).We also evaluated the association of other candidate SNPs (*TPMT* A719G, *MRP4* G2269A, and *ITPA* C94A) with the development of leukocytopenia, but no significant association was detected (Supplementary Table 2).

Multivariate analysis

In the logistic regression analysis with *NUDT15*, *TPMT*, *MRP4*, *ITPase* variants and 6TGNs levels, the *NUDT15* variant was only genetic factor significantly contributing to the thiopurine-induced leukocytopenia ($P = 0.001$, OR 5.38, 95 % CI 1.91–15.2) (Table 5). In addition, interaction of *NUDT15* variant with *MRP4* or *ITPase* variants was not

Table 4 AZA dose and 6TGN levels in IBD patients with leukocytopenia

NUDT15 genotype	C/C	C/T	T/T	*P* value
Initial dose of AZA (mg/kg/day)	0.72 ± 0.05 ($n = 25$)	0.73 ± 0.08 ($n = 18$)	0.75 ($n = 2$)	0.37
AZA dose at WBC nadir (mg/kg/day)[a]				
Patients with leukocytopenia	0.96 ± 0.05 ($n = 25$)	0.88 ± 0.09 ($n = 18$)	0.75 ($n = 2$)	0.26
With early leukocytopenia	0.80 ($n = 2$)	0.94 ($n = 2$)	0.75 ($n = 2$)	–
With late leukocytopenia	0.97 ± 0.08 ($n = 23$)	0.88 ± 0.10 ($n = 18$)	–	0.29
6TGN levels at WBC nadir (pmol/8 × 10^8 RBCs)[a]				
Patients with leukocytopenia	370.6 ± 34.4 ($n = 25$)	367.8 ± 55.9 ($n = 16$)	341 ($n = 2$)	0.40
With early leukocytopenia	364.0 ($n = 2$)	292.0 ($n = 2$)	341 ($n = 2$)	–
With late leukocytopenia	371.3 ± 37.5 ($n = 23$)	382.9 ± 64.5 ($n = 16$)	–	0.43
Prednisolone dose at WBC nadir (mg/day)[a]	9.2 ± 1.0 ($n = 3$)	8.3 ± 1.5 ($n = 10$)	12.5 ± 7.5 ($n = 2$)	0.31

AZA dose, 6TGN levels and prednisolone dose were expressed as mean ± SEM

WBC white blood cell, *AZA* azathioprine, *6TGNs* 6-thioguanine nucleotides

[a] The AZA dose, 6-TGNs levels and prednisolone dose when the WBC count reached the lowest value (nadir) were used for analyses

Table 5 Multivariate analysis for thiopurine-induced leukopenia (WBC <3000/μl)

Variables	Odds ratio	95 % CI	*P* value
NUDT15 variant	5.38	1.91–15.2	0.001
TPMT variant	1.31	0.17–9.97	0.79
MRP4 variant	1.71	0.62–4.74	0.30
ITPA variant	0.77	0.24–2.45	0.66
6TGNs	1	1.0–1.00	0.37

CI confidence interval

observed (Supplementary Table 3). Interaction between *NUDT15* and *TPMT* variant could not be evaluated due to small number of patients with *TPMT* variant.

Discussion

In the current study, we demonstrated that *NUDT15* R139C is common in the Japanese population (allelic frequency of 10.2 %), and this finding was compatible with other recent reports of East Asians [20, 21, 26]. The genotype frequency (wild type C/C 80.7 %, heterozygote C/T 18.2 %, homozygote 1.1 %) was similar to that found in recent reports on Koreans [20] and Japanese [21].

Thiopurine-induced leukocytopenia has been mainly explained by *TPMT* variants that induce the intracellular accumulation of 6-TGNs [1, 12]. However, *TPMT* variants can be found in some patients who experience thiopurine-associated leucopenia [19, 27]. Recently, *NUDT15* R139C (rs116855232) has been identified as a novel predictor of thiopurine-induced leukocytopenia in Korean and Japanese patients with IBD [20, 21]. The GWAS (genome-wide association study) in child patients with acute lymphocytic leukemia also proved the association between *NUDT15* R139C and thiopurine sensitivity and showed that *NUDT15* R139C was found frequently in East Asians (9.8 %) and Hispanics (3.9 %) but rarely in Europeans (0.2 %) and Africans (0 %) [26]. However, none of these studies analyzed 6-TGN levels that might aid in explaining the molecular mechanism underlying the association of *NUDT15* variants with thiopurine-induced leukocytopenia. In the present study, we confirmed that *NUDT15* R139C is a strong predictor for thiopurine-induced leukocytopenia and found that *NUDT15* R139C-associated thiopurine leukocytopenia was not accompanied by an elevation of 6-TGN levels, suggesting a thiopurine metabolism-independent mechanism.

Functional changes to NUDT15 by the T to A substitution of rs116855232 remain unclear. 8-xo-7,8-Dihydroguanine (8-oxo-Gua) is the most abundant oxidized purine base [28] generated by reactive oxygen species [29]. NUDT15 hydrolyzes the 8-oxo-Gua-containing DNA precursor (8-oxo-dGTP and 8-oxo-dGDP) to the monophosphate, thereby preventing the incorporation of 8-oxo-Gua-containing nucleotides into DNA [28, 30]. NUDT15 plays an important role in accurate DNA replication as well as in the prevention of abnormal protein synthesis under oxidative stress. Yang et al. explained NUDT15 variant-related thiopurine leukocytopenia from the viewpoint of thiopurine metabolism [26]. Thio-dGTP is one of the active molecules of 6-TGNs, and due to its molecular similarity to 8-oxo-dGDP, thio-dGTP may be hydrolyzed by NUDT15 to the inactive thio-dGMP or thio-dGDP. If *NUDT15* R139C exerts loss-of-function, *NUDT15* R139C will lead to intracellular accumulation of thio-dGTP and result in extensive DNA damage and cytotoxicity. However, this hypothesis does not align with the findings in the present study that 6-TGN levels (including thio-dGTP) were not elevated in patients with *NUDT15* variants.

Rapid development of severe leukocytopenia and severe hair loss was characteristic for patients with the *NUDT15* T/T genotype, and no patients with the C/T genotype had these issues. Severe leukocytopenia and hair loss are also manifestations of strong chemotherapy, and this suggests direct injury to bone marrow- and hair follicle-stem cells. Acute bone marrow suppression is the result of the induction of apoptosis in the proliferating bone marrow stem cells, and several studies have shown that the induction of oxidative stress is primarily responsible for the loss of bone marrow- and hair follicle-stem cells [31–33]. These findings suggest that if *NUDT15* R139C induces loss-of-function, accumulation of oxidative stress and increased apoptosis in bone marrow progenitor cells might be a potential mechanism of *NUDT15* R139C-related thiopurine leukocytopenia. The thiopurine metabolism-independent mechanism as well as the functions of NUDT15 should be extensively investigated in the future.

There have been different observations in recent studies of *NUDT15* variants in East Asians. Yang et al. reported that *NUDT15* R139C was associated with both early (developed within 8 weeks) and late (after 8 weeks) leukocytopenia in the Korean population [20], but Kakuta et al. showed that *NUDT15* R139C was associated with only early leukocytopenia, but not with late leukopenia, in the Japanese population [21]. The current study showed an association of *NUDT15* R139C with early and late leukopenia in the Japanese population, and this was compatible with the findings of Yang et al. The reason for the discrepancy in late leukocytopenia might be due to different adjustments of AZA dosage based on the development of adverse events. Based on these observations, *NUDT15* R139C is considered to be a major genetic factor contributing to the heightened thiopurine sensitivity of East Asians. This is supported by the high frequency of

NUDT15 R139C [26] and low frequency of the *TPMT* variant [13] in East Asians.

In the current study, we evaluated the effects of *NUDT15* R139C not only by evaluating the frequency of leukocytopenia but also by examining the decrease in WBC count. ΔWBC indicates overall alteration of WBC count including patients whose WBC did not decrease below 3000/μl. Therefore, ΔWBC is considered to be a better marker for the evaluation of the rapid effects of *NUDT15* R139C compared to the frequency of leukocytopenia development. ΔWBC was significantly enhanced in patients with the C/T and T/T genotypes as compared to those with the wild C/C genotype. WBC count decreased in patients with the C/T and T/T genotypes after 2 and 4 weeks, but such a decrease was not observed in patients with the wild genotype. These findings clearly indicate a rapid influence of *NUDT15* R139C on hematopoietic activity.

In conclusion, we found that *NUDT15* variant-related thiopurine-induced leukopenia was not associated with 6-TGN levels, and that a decrease in the WBC count was rapidly (within 2 weeks) induced after thiopurine initiation in patients carrying *NUDT15* R139C. Our observations suggest that *NUDT15* R139C is a new genetic factor related to the increased thiopurine sensitivity/toxicity of IBD patients. The utility of routine genotyping for *NUDT15* variants before initiating thiopurine therapy should be considered to reduce the risk of thiopurine-related severe leukocytopenia and hair loss.

Updates This article was updated on December 1, 2015. Two values were corrected in Table 3 (−4.550 should be −4550 and −4.430 should be −4430).

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Compliance with ethical standards

Conflicts of interest The authors, except Akira Andoh, disclose no conflict of interest. Akira Andoh reports speaker fees from AbbVie and Eisai Pharmaceutical.

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