Regular Article

Single-Nucleotide Polymorphisms, c.415C > T (Arg139Cys) and c.416G > A (Arg139His), in the *NUDT15* Gene Are Associated with Thiopurine-Induced Leukopenia

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While *nucleoside diphosphate-linked moiety X-type motif 15 (NUDT15)* gene polymorphism Arg139Cys (rs116855232) is known to be a risk factor for thiopurine-induced severe leukopenia, association with the *NUDT15* gene polymorphism Arg139His (rs147390019) has not yet been clarified. In addition, the accuracy of TaqMan PCR to assess these two polymorphisms has not been investigated. In this study, we evaluated TaqMan PCR for detection of the *NUDT15* single-nucleotide polymorphisms (SNPs) and examined the clinical impact of Arg139His on thiopurine-induced leukopenia. First, we demonstrated that a TaqMan PCR assay successfully detected the Arg139His polymorphism of *NUDT15* in clinical samples. Next, the *NUDT15* gene polymorphisms (Arg139Cys and Arg139His) were separately analyzed by TaqMan Real-Time PCR in 189 patients from August 2018 to July 2019. The incidences of leukopenia within 2 years were 16.2, 57.9, and 100% for arginine (Arg)/Arg, Arg/cysteine (Cys), and Arg/histidine (His), respectively. The leukopenia was significantly increased in Arg/Cys and Arg/His compared with Arg/Arg. This retrospective clinical study indicated that, in addition to Arg139Cys, Arg139His may be clinically associated with a high risk of leukopenia. Pharmacogenomics will help in selecting drugs and determining the individualized dosage of thiopurine drugs.

Key words *nucleoside diphosphate-linked moiety X-type motif 15 (NUDT15)*, thiopurine, polymorphism, leukopenia

INTRODUCTION

Pharmacogenomics (PGx) is used to predict the therapeutic effects and side effects of drugs based on the gene polymorphisms of their metabolic enzymes and transporters, and supports the dose adjustments and drug selections tailored to individuals.[1)] PGx is an effective tool for the realization of precision medicine, which is performed to prevent and treat diseases by considering individual differences in genetic information, living environment, and lifestyle. A number of basic and clinical studies have already demonstrated the association between gene polymorphisms of pharmacokinetic factors, such as drug metabolizing enzymes and drug efficacies, and the evidence for personalized medicine has been accumulating.[1–4)] It has also been reported that there is a large variation in the frequency of gene polymorphisms among different races.[5)]

Thiopurine drugs, specifically azathioprine and 6-mercaptopurine, are some of the few drugs that can be used for the maintenance of remission in inflammatory bowel disease (IBD),[6–9)] and the number of IBD patients has increased dramatically in recent years. Regarding Crohn's disease (CD), surgery is required in about 60% of cases,[10)] and the gastrointestinal damage caused by the disease and intestinal resection increases and accumulates over time. Therefore, the prevention of postoperative recurrence of CD is an important clinical issue. One solution to this problem is thiopurine therapy, which has been effective in preventing recurrence.[11)] In addition, thiopurine drugs have been used to treat autoimmune diseases such as rheumatoid arthritis and dermatomyositis.[12,13)]

On the other hand, severe leukopenia induced by thiopurine drugs has been a major obstacle to the application of thiopurine therapy.[14)] The Clinical Pharmacogenetics Implementation Consortium (CPIC®) guideline recommend dose adjustment according to *thiopurine methyltransferase (TPMT)* and *nucleoside diphosphate-linked moiety X-type motif 15 (NUDT15)* genotypes.[15)] However, polymorphisms in *TPMT* are rare in Asian populations, and there was no association between *TPMT* genotypes and adverse effects in Japanese patients.[16)] In 2014, an Immunochip-based association study in Korean CD patients revealed that a single nucleotide polymorphism (c.415C > T) in the *NUDT15* gene was strongly associated with acute severe leukopenia induced by thiopurine drugs.[17)] This gene polymorphism causes the 139th amino acid to change from arginine to cysteine (p.Arg139Cys). NUDT15 is an enzyme that hydrolyzes active metabolites, like 6-thioguanine triphosphate and 6-thiodeoxyguanine triphosphate, into inactive metabolites such as 6-thioguanine monophosphate and 6-thiodeoxyguanine monophosphate.[18)] In 2016, it was reported that the *NUDT15* gene polymorphism Arg139Cys was a risk factor for thiopurine-induced severe leukopenia and severe hair loss in Japanese patients with IBD.[19)] A large mul-

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Table 1. Genotypes of *NUDT15* Codon 139

NUDT15 gene	Codon139			Amino acid	Allele
	c.415	c.416	c.417		
Genotype	C	G	T	Arg	Wild type
	T	G	T	Cys	Variant
	C	**A**	T	His	Variant

C, cytosine; G, guanine; A, adenine; T, thymine.

ticenter study in Japan (MENDEL study) reported that all 49 homozygous patients of Arg139Cys discontinued thiopurine drugs early due to adverse effects.[20)]

A polymorphism in Arg139His (c.416G＞A), adjacent to the base of the Arg139Cys polymorphism (c.415C＞T), also occurs rarely, changing the 139th amino acid from arginine to histidine (p.Arg139His)[21)] (Table 1). In previous studies, researchers have mainly focused on the polymorphism of Arg139Cys, and demonstrated that heterozygotes with a single polymorphism of Arg139Cys, as well as homozygotes of Arg139Cys, have significantly increased frequency of leukopenia.[19,20,22)] It has been reported that homozygous patients with the His/His genotype had severe leukopenia.[23)] However, association of the heterozygous Arg/His genotype with adverse effects has not yet been clarified due to the low frequency of Arg139His polymorphism. In the CPIC® guideline for thiopurine, the heterozygous Arg/His genotype is also assigned as "Indeterminate (uncertain function)".[15)]

When the present study began, the ability of TaqMan PCR to accurately detect each polymorphism has not been investigated. Two bases upstream and two bases downstream of the target single-nucleotide polymorphism (SNP) are usually included in the probe for TaqMan PCR. It has not been possible to design a probe avoiding the adjacent SNP. However, it has been necessary to develop the accurate detection assay of adjacent polymorphisms in Arg139His (c.416G＞A) and Arg139Cys (c.415C＞T) with clinical samples.

The present study consists of two parts: first, detection of the *NUDT15* gene polymorphisms by TaqMan PCR assay, and second, a single-center observational clinical study for investigating the impact of these *NUDT15* gene polymorphisms on adverse effects of thiopurine drugs.

MATERIALS AND METHODS

Ethics and Patients The study protocol was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee at Shiga University of Medical Science (Approval No. R2019-147). *NUDT15* gene polymorphism analysis was covered by health insurance from 2019 in Japan and was conducted as a medical service in the Shiga University of Medical Science Hospital.

Japanese adult patients whose *NUDT15* genotypes were determined at Shiga University of Medical Science Hospital between August 2018 and July 2019 were enrolled in the present study. Written informed consent was waived because of the anonymous nature of the data. As an ethical consideration, participants had been provided with the opportunity to opt out from this research based on written information posted on the Shiga University of Medical Science Hospital homepage.

TaqMan PCR Assay for Detection of the *NUDT15* Gene Polymorphisms TaqMan probes and primers for Arg139His polymorphism detection by real-time PCR were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.) by contract synthesis. The sequences of the forward primers, reverse primers, and two allele-specific oligonucleotide probes labeled with a fluorescent reporter dye (VIC or FAM) specific for rs147390019 were 5′-CCCCTGGACCAGCTTTTCTG-3′,5′-CCACCAGATGGTTCAGATCTTCTTTAAA-3′,5′-AAACAACGCAGTCCC-3′ (VIC), and 5′-TTTAAACAACACAGTCCC-3′ (FAM), respectively. For control samples, duplex DNA fragments (470 bases) of c.416G＞A (Arg139His) polymorphism, c.415C＞T (Arg139Cys) polymorphism, and wild type were obtained from Thermo Fisher Scientific by contract synthesis. Heterozygous polymorphisms were mimicked by mixing equal amounts of the two duplex DNA fragments.

Genomic DNA was extracted from peripheral blood hemocytes using the QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, The Netherlands). Arg139Cys (c.415C＞T) genotypes of *NUDT15* codon 139, Arg139His (c.416G＞A) genotypes of *NUDT15* codon 139, and **3C* (c.719A＞G) of *TPMT* genotypes were determined by the genotyping of rs116855232, rs147390019, and rs1142345, respectively, using TaqMan SNP Genotyping Assays and a Step-One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) as previously reported.[19)] The thermal cycler condition for PCR was 40 cycles of 23 s at 95 °C for denaturation and 20 s at 60 °C for annealing and extension.

Direct Sequencing for Detection of the *NUDT15* Gene Polymorphisms To confirm the DNA sequence and presence of c.416G＞A (exon 3 of the *NUDT15*), DNA was extracted and purified by column from the blood samples of Arg/His patients identified by TaqMan PCR assay. The extracted DNA was amplified by GeneAmp® PCR System 9700 (Applied Biosystems) using primers for amplification that were obtained from Thermo Fisher Scientific by contract synthesis. The sequences of the forward and reverse primers were 5′-GTTGGGAGTGGGTTCCTTGGGAAGAACTAC-3′ and 5′-CAAGTACTGGCTGAAAGAGTGGGGGATAC-3′, respectively. DNA products (308 bp) were checked by agarose gel electrophoresis. DNA sequences were determined by direct sequencing. We also investigated the possibility that other polymorphisms are associated with thiopurine drugs-induced leukopenia by sequencing of exon 1–3 of the *NUDT15*. PCR was performed using a KOD -Plus- Neo DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. We used the following primer sequences for amplification as follows: exon 1–2 (3391 bp) forward primer, 5′-AGGCGCGTCCTCCCGCGCGCT-3′; exon 1–2 reverse primer, 5′-TTTCATTTTTTTCAGGCTCTACATTCTTTGGTTCTGAATCATG-3′; exon 2–3 (5048 bp) forward primer, 5′-TGAAACCTGGGAAGAATGTGCTCAAAGG-3′; exon 2–3 reverse primer, 5′-CAAGTACTGGCTGAAAGAGTGGGGGATAC-3′. The sequences of purified amplicons were determined by direct sequencing.

Clinical Study for the *NUDT15* Gene Polymorphisms on Thiopurine-Induced Leukopenia Patients whose *NUDT15* gene polymorphisms were analyzed between August 2018 and July 2019, and who were receiving thiopurine drugs (azathioprine and 6-mercaptopurine) in the Department of Gastroenterology or Dermatology, were enrolled in the present study.

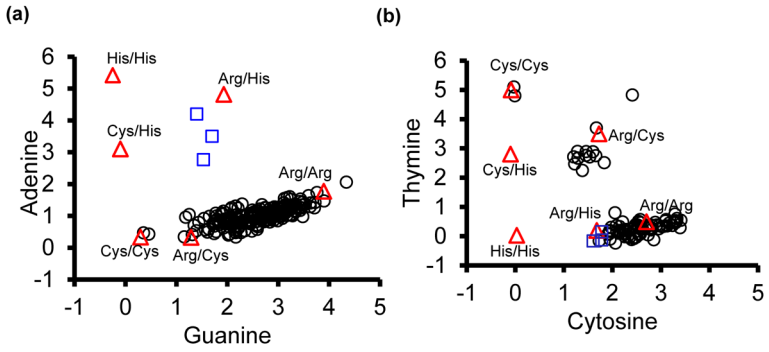

Fig. 1. Allelic Discrimination Plots

(a) Detection of c.416G>A (Arg139His) polymorphisms of *NUDT15*; guanine, VIC; adenine, FAM. (b) Detection of c.415C>T (Arg139Cys) polymorphisms of *NUDT15*; cytosine, VIC; thymine, FAM. Red triangles represent control synthetic duplex DNA fragments for His/His, Cys/His, Cys/Cys, Arg/His, Arg/Arg, and Arg/Cys phenotypes. Black circles represent patient samples. Blue squares represent Arg/His patient samples.

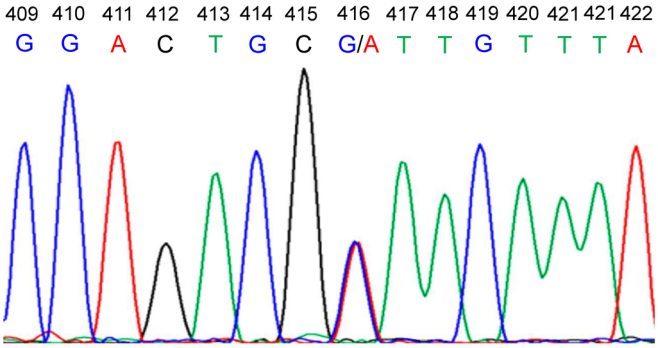

Fig. 2. Direct DNA Sequencing

Direct *NUDT15* sequencing data of Arg/His patients (overlap of A and G at c.416) (C, cytosine; G, guanine; A, adenine; T, thymine).

Patients not receiving thiopurine drugs and patients with an unknown start date of thiopurine drug administration were excluded. Patients in the hematology or pediatric departments were also excluded because the application of thiopurine drugs in these departments was for treating leukemia, and it is difficult to distinguish thiopurine-induced leukopenia from a leukemia-induced blood cell abnormality. The clinical data, including white blood cell (WBC) counts and concomitant drugs, were extracted from the electronic medical records. The observation period was 2 years after the start of thiopurine drug administration. A WBC count $\leq 3000/\mu L$ was defined as the concentration for determining leukopenia (higher than grade 2, CTCAE v5.0). Event-free rate was by the Kaplan–Meier method and log-rank test. Bonferroni's multiple comparison tests were also performed. The baseline characteristics of the patients initially treated with thiopurine drugs were compared using Fisher's exact test. p-Values < 0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics version 27.

RESULTS

Detection of the *NUDT15* Gene Polymorphisms by TaqMan PCR Assay To validate the constructed TaqMan PCR assay for c.416G>A (Arg139His) of the *NUDT15* gene polymorphism, synthetic DNA and clinical samples were analyzed by real-time PCR (Figs. 1a, b). All *NUDT15* phenotypes (His/His, Cys/Cys, Arg/Arg, Arg/His, Arg/Cys, and Cys/His) were separately detected using synthetic duplex DNA fragments by TaqMan Real-Time PCR (Figs. 1a, b). The *NUDT15* gene polymorphisms were analyzed in 189 patients. Among them, three patients with the Arg139His heterozygote variant were detected by real-time PCR (Figs. 1a, b). The amino acid frequencies were 87.5% for Arg, 11.7% for Cys, and 0.8% for His in 189 patient samples. The numbers of Arg/Arg, Arg/Cys, Cys/Cys, and Arg/His patients were 144 (76.2%), 40 (21.2%), 2 (1.1%), and 3 (1.6%) patients, respectively.

Furthermore, to confirm the Arg139His polymorphism detected in the clinical samples by real-time PCR, the direct sequencing of PCR products was conducted. The genomic DNA samples of three Arg/His patients were amplified by PCR, and the PCR amplification products (308 bp) were checked by agarose gel electrophoresis. Direct sequencing of the 3 blood samples revealed the c.416G/A heterozygote genotype (Fig. 2), which corresponded with results obtained from the real-time PCR. The entire DNA sequence of exon 1–3 from the *NUDT15* gene was checked, but no gene polymorphisms were found in all three patient samples, except for c.416G>A.

Clinical Impact of the *NUDT15* Gene Polymorphisms on Thiopurine-Induced Leukopenia To evaluate the clinical impact of the *NUDT15* gene polymorphisms on the frequency of leukopenia, a retrospective observational clinical study was conducted. Ninety patients were selected by patient selection criteria (Fig. 3) out of the patients whose *NUDT15* gene polymorphisms were determined. Fisher's exact test showed that there was no significant difference in patient background between the three genotype groups (Arg/Arg, Arg/Cys, and Arg/His) (Table 2). The patients with

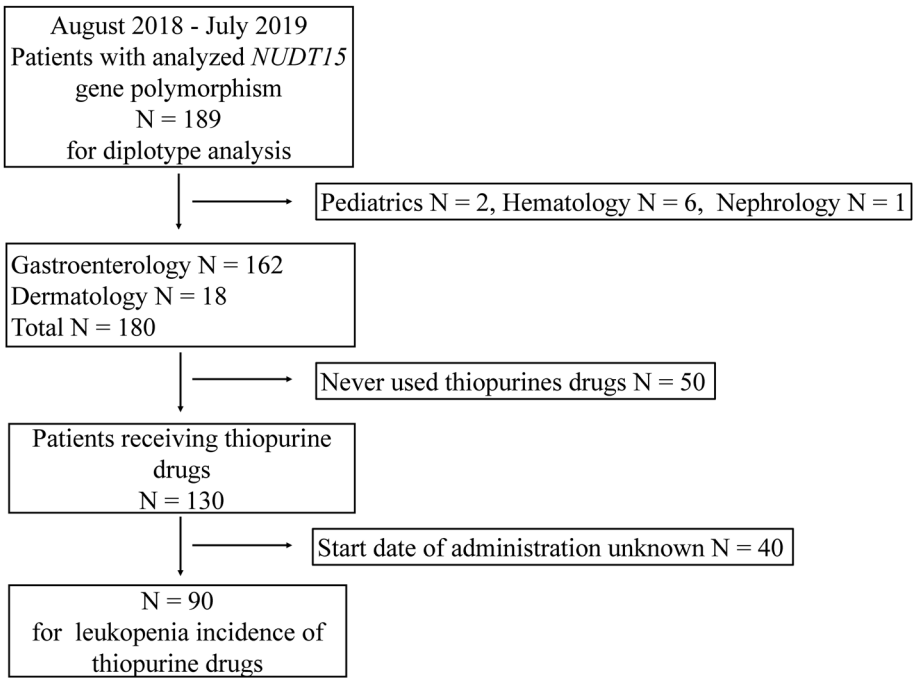

Fig. 3. Patient Selection Criteria

Table 2. Patients Background

NUDT15 genotype	Arg/Arg	Arg/Cys	Arg/His
N	68	19	3
Initial dose of thiopurine drugs (mg/d)	37.5 (20–100)	25 (25–50)	25 (25–100)
Age (years)	37.5 (14–90)	31 (19–68)	45 (38–70)
Gender (F/M)	27/41	10/9	2/1
Body Weight (kg)	54.5 (34.7–107)	53.2 (41.9–85.5)	59 (47.0–70.4)
ALT (U/L)	16 (6–60)	14 (6–82)	16 (9–19)
AST (U/L)	16 (9–43)	14 (9–31)	13 (9–14)
WBC (/μL)	7450 (3000–21000)	7900 (2600–27500)	9900 (4800–10900)
TPMT (**1/*3C*)	1	3	0
5-ASA concomitant	41	12	2
Ulcerative colitis	33	10	1
Crohn's disease	26	8	1
Pemphigoid	3	0	0
Pemphigus	2	0	0
Intestinal Behcet disease	2	0	0
Other diseases	2	1	1

Data are shown as medians (ranges). ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood cells; *TPMT, thiopurine methyltransferase*; 5-ASA, 5-aminosalicylic acid (mesalazine or salazosulfapyridine). There were no homozygous *TPMT*3C/*3C* patients. The 6-merucaptopurine dose was adjusted to azathioprine equivalents by multiplying by 2.

Arg/Arg tended to be administered with thiopurine drugs at a higher initial dose (median 37.5 mg/d) than those for the other genotype groups (median 25 mg/d), but the dose at the time of leukopenia was the same among the three groups (median 50 mg/d). The incidences of leukopenia (Grade 2 or higher, WBC counts $\leq$3000/μL) within 2 years were 16.2% (11/68), 57.9% (11/19), and 100% (3/3) for Arg/Arg, Arg/Cys, and Arg/His groups, respectively. The leukopenia was significantly increased in Arg/Cys and Arg/His compared with Arg/Arg (Fig. 4, $p < 0.01$ by log-rank test). On the other hand, there was no significant difference in leukopenia between Arg/Cys and Arg/His. All three patients with Arg/His heterozygous polymorphism developed leukopenia within 1 year after the start of thiopurine drug administration (77, 210, and 252 d). Because of leukopenia, the azathioprine doses were reduced in two of the three patients with Arg/His. In one of the two patients, the dose was increased from 25 to 50 mg/d on Day 49, and reduced to 37.5 mg/d after the occurrence of leukopenia on Day 211 (WBC count of 2800/μL). One month later (Day 241), because the leukopenia worsened (WBC count of 2500/μL), the dose was reduced from 37.5 to 25 mg/d. After the dose reduction, WBC counts recovered. For another patient, the dose started at 100 mg/d, and reduced to 75 mg/d after the occurrence of leukopenia on Day 78 (WBC count of 2700/μL). After the dose reduction, WBC counts recovered. In the third patient, the dose was increased from 25 to 50 mg/d on Day 21. The dose remained the same after the occurrence of leukopenia on Day 210 (WBC count of 3000/μL),

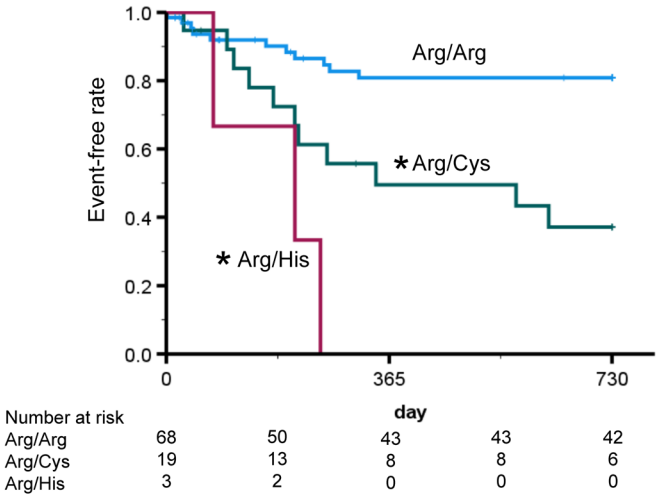

Fig. 4. Effect of *NUDT15* Polymorphism on Incidence of Leukopenia

Event-free rate was assessed by the Kaplan–Meier method and log-rank test. Bonferroni's multiple comparison tests were also performed. The observation period was 2 years after the start of thiopurine drugs administration. A white blood cell count ≤3000/μL was defined as the concentration for determining leukopenia (higher than grade 2, CTCAE v5.0). The whiskers in the figure indicate censored data. Arg/His and Arg/Cys showed significantly increased incidence of leukopenia compared to Arg/Arg (*$p < 0.01$).

and no further leukopenia occurred. Of the 4 patients with *TPMT* *1/*3C, 1 patient had *NUDT15* Arg/Arg and 3 patients had *NUDT15* Arg/Cys. Leukopenia occurred in all of those Arg/Cys patients. Eleven patients (Arg/Arg = 9, Arg/Cys = 2) received allopurinol concomitant with thiopurine drugs, and only one patient (Arg/Arg) developed leukopenia during this combination. The median dosage of thiopurine drug during the azathioprine combination was 30 mg/d. The percentages of patients receiving 5-aminosalicylic acid (5-ASA) (mesalazine or salazosulfapyridine) were 60.3% (41/68), 63.2% (12/19) and 66.7% (2/3) for Arg/Arg, Arg/Cys and Arg/His, respectively. The incidence of leukopenia in the 5-ASA concomitant group (29.1%, 16/55) did not increase compared with that in the group without 5-ASA concomitant (25.7%, 9/35).

DISCUSSION

In the present study, we demonstrated that a TaqMan PCR assay successfully detects the c.416G > A (Arg139His) polymorphism of *NUDT15* in synthetic DNA fragments and clinical samples and distinguishes between c.415C > T (Arg139Cys) and c.416G > A polymorphisms. In addition, the retrospective clinical study indicated that both Arg139Cys and Arg139His are clinically associated with a high risk of leukopenia.

In the previous report,[20] the percentages of Japanese patients with Arg/Arg, Arg/Cys, Cys/Cys, and Arg/His were 74.2, 21.3, 3.8, and 0.5%, respectively. In the present study, the percentages of patients with Arg/Arg, Arg/Cys, Cys/Cys, and Arg/His were 76.2, 21.2, 1.1, and 1.6%, respectively. Thus, only slight differences in the frequencies of polymorphisms were observed between the previous and present reports. The single nucleotide polymorphism (c.415C > T) in Asians is strongly correlated with acute severe leukopenia induced by thiopurine drugs.[19,20,22] Consistent with previous reports, the present study showed that heterozygous patients with the Arg/Cys genotype had significantly increased frequency of leukopenia. It has been reported that 5-ASA inhibits TPMT and may cause myelosuppression with thiopurine drugs,[24,25] while others have reported no association.[26] In the present study, thiopurine-induced leukopenia was not increased by 5-ASA. Allopurinol also inhibits xanthine oxidase, which increases blood levels of 6-mercaptopurine and myelosuppression.[27] The doses of thiopurine drugs were intentionally reduced while allopurinol was used. Thus, thiopurine-induced leukopenia occurred in only one (9.1%) of the 11 patients administered with allopurinol. Therefore, the concomitant drugs are not likely to increase the risk of thiopurine-induced leukopenia.

Two Cys/Cys homozygote patients in the present study received the genotyping tests of *NUDT15* prior to the start of thiopurine dosing. The thiopurine drugs were not administrated to these Cys/Cys patients based on the genotyping results because Cys/Cys homozygotes are known to be at very high risk for leukopenia.[19,20,22] This result indicates that preemptive genotyping tests could be useful in real-world clinical practice. In addition, genotyping for *NUDT15* has reportedly aided women with IBD who are pregnant, or planning, and their partners to avoid adverse infant outcomes.[28] Thus, in some patients, the initial dose was determined according to genotype, which may have resulted in a smaller initial dose for Arg/Cys or Arg/His than for Arg/Arg. On the other hand, many of the patients in the present study had received thiopurine drugs prior to the genotyping tests of *NUDT15*. Recovery of the thiopurine-induced leukopenia in the present study was observed in two of the three Arg/His patients with dose reduction. Therefore, by correcting the dosage of thiopurine drugs according to gene polymorphisms before administration, the frequency of leukopenia may be reduced.

Although the risk of side effects from Arg139His polymorphism was previously unclear due to its low frequency,[15,20] the present study showed for the first time that the heterozygote of Arg139His may have increased risk of thiopurine-induced leukopenia. If Arg139His polymorphism is detected in the preemptive genotyping tests for *NUDT15*, thiopurine dosage should be considered as well as Arg139Cys polymorphism.

A previous report based on *in vitro* analysis has demonstrated that the *NUDT15* p.Arg139His variant leads to reduced electrophilicity and compromised substrate interaction.[29] Arg139Cys has been shown to decrease enzyme activity only slightly more than Arg139His.[29] There was no significant difference in the risk of leukopenia between Arg/Cys and Arg/His in the present study, but both exhibited higher risk than Arg/Arg. Therefore, attention should be paid to the adverse effects of Arg/His as well as Arg/Cys.

Polymorphisms, c.416G > A (Arg139His) and c.415C > T (Arg139Cys), are found in exon 3 of the *NUDT15* gene. The other two variants, p.Val18Ile and p.Val18_Val19insGlyVal in exon 1, have previously been reported[29] In particular, the p.Val18_Val19insGlyVal allele decreased the enzymatic activity of *NUDT15*.[29,30] It has also been reported that the frequency of leukopenia increases in heterozygotes of the p.Val18_Val19insGlyVal variant.[31] In the present study, the entire sequences of exon 1-3 from the *NUDT15* gene were assessed, but no gene polymorphisms were found in all three samples except for c.416G > A. Therefore, the influence of other genetic polymorphisms in *NUDT15* was ruled out, and the leukopenia was most likely due to c.416G > A polymor-

phism.

There were some limitations in the present study, which was conducted at a single institution using a relatively small number of cases, and the frequency of Arg139His polymorphism was very low. The risks associated with Arg139His polymorphism should be examined further in the future. Furthermore, the present study only evaluated the effects of the *NUDT15* gene polymorphisms, but not *TPMT* gene polymorphisms. Gene polymorphisms of inosine triphosphate pyrophosphatase (ITPase) has also been suspected to be partly responsible for adverse effects of thiopurine drugs.[16,32,33] Some studies have suggested a role for *ITPase* variants in thiopurine-induced toxicity.[16,33] A SNP in human *MRP4* (Multidrug resistance protein 4) G2269A also reduced MRP4 function and resulted in the intracellular accumulation of 6-thioguanine nucleotides.[34] Furthermore, *MRP4* G2269A has increased the risk of thiopurine-induced leukopenia in Japanese patients.[35,36] However, the present study did not analyze gene polymorphisms of *ITPase* and *MRP4*. Leukopenia also occurred in some of the Arg/Arg patients, which should not be overlooked.

It is expected that preemptive *NUDT 15* genotyping will help in selecting drugs, determining individualized dosages of thiopurine drugs, and assist the avoidance of adverse effects such as leukopenia.

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Author Contributions All authors had access to the data and a role in writing this manuscript. Tetsuichiro Isono, Daiki Hira, Yoshito Ikeda, Satoshi Noda, Tomohiro Terada, and Shin-ya Morita contributed to the study conception and design. Tetsuichiro Isono was involved in the collection of data. Tetsuichiro Isono and Daiki Hira analyzed the data. Yoshito Ikeda was involved in the sequence analysis. Tetsuichiro Isono and Daiki Hira drafted the manuscript. Yoshito Ikeda, Masahiro Kawahara, Satoshi Noda, Atsushi Nishida, Osamu Inatomi, Noriki Fujimoto, Akira Andoh, Tomohiro Terada, and Shin-ya Morita critically revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest The authors declare no conflict of interest.

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