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ORIGINAL ARTICLE

Clinical Trials Study

Early proactive monitoring of DNA-thioguanine in patients with Crohn's disease predicts thiopurine-induced late leucopenia in NUDT15/TPMT normal metabolizers

Ting Yang, Kang Chao, Xia Zhu, Xue-Ding Wang, Sumyuet Chan, Yan-Ping Guan, Jing Mao, Pan Li, Shao-Xing Guan, Wen Xie, Xiang Gao, Min Huang

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Abstract

BACKGROUND
Thiopurine-induced leucopenia significantly hinders the wide application of thiopurines. Dose optimization guided by nudix hydrolase 15 (*NUDT15*) has significantly reduced the early leucopenia rate, but there are no definitive biomarkers for late risk leucopenia prediction.

AIM
To determine the predictive value of early monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides (6TGN) for late leucopenia under a *NUDT15*-guided thiopurine dosing strategy in patients with Crohn's disease (CD).

METHODS
Blood samples were collected within two months after thiopurine initiation for

detection of metabolite concentrations. Late leucopenia was defined as a leukocyte count < 3.5 × 10^9/L over two months.

RESULTS

Of 148 patients studied, late leucopenia was observed in 15.6% (17/109) of *NUDT15*/thiopurine methyltransferase (*TPMT*) normal and 64.1% (25/39) of intermediate metabolizers. In patients suffering late leucopenia, early DNATG levels were significantly higher than in those who did not develop late leucopenia (P = 4.9 × 10^{-13}). The DNATG threshold of 319.43 fmol/µg DNA could predict late leucopenia in the entire sample with an area under the curve (AUC) of 0.855 (sensitivity 83%, specificity 81%), and in *NUDT15/TPMT* normal metabolizers, the predictive performance of a threshold of 315.72 fmol/µg DNA was much more remarkable with an AUC of 0.902 (sensitivity 88%, specificity 85%). 6TGN had a relatively poor correlation with late leucopenia whether in the entire sample (P = 0.021) or *NUDT15*/*TPMT* normal or intermediate metabolizers (P = 0.018, P = 0.55, respectively).

CONCLUSION

Proactive therapeutic drug monitoring of DNATG could be an effective strategy to prevent late leucopenia in both *NUDT15*/*TPMT* normal and intermediate metabolizers with CD, especially the former.

Key Words: Thiopurine-induced late leucopenia; DNA-thioguanine; 6-thioguanine nucleotide; Proactive therapeutic drug monitoring; Crohn’s disease

Core Tip: This study pioneeringly explores if early proactive monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides stable concentration within two months could predict late leucopenia susceptible population under a nudix hydrolase 15 (NUDT15) genotype-guided thiopurines dosing strategy in patients with Crohn’s disease. We first provide evidence indicating that early proactive monitoring of DNATG during the initial stages of thiopurine therapy could be an effective strategy to discern patients susceptible to developing late leukopenia, especially in NUDT15/thiopurine methyltransferase normal metabolizers, with a concentration threshold of 315.72 fmol/µg DNA.

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INTRODUCTION

Thiopurines, mercaptopurine (MP), and azathioprine (AZA) have been widely used in inflammatory bowel disease (IBD) for 70 years[1]. However, up to 10%-30% of patients discontinue therapy primarily due to adverse effects. Thiopurine-induced leucopenia is one of the most common and potentially fatal toxicities, particularly in Asian populations[2].

Thiopurine methyltransferase (TPMT), a key enzyme in thiopurine metabolism, is involved in the inactive conversion of thiopurines. *TPMT* polymorphisms are significantly associated with thiopurine-induced leucopenia and have been a landmark discovery in genomics to guide the clinical application of thiopurines. However, the guiding value of *TPMT* for thiopurine clinical usage is very limited in Asia, because the mutation frequency of *TPMT*3C*, as the most prevalent variant in Asia, is only 1%-3%[3,4]. Until 2014, a genome-wide association study in Korea revealed a common missense variant, nudix hydrolase 15 (*NUDT1*)*3 (rs116855232, R139C, p.Arg139Cys), which strongly increased individual susceptibility to thiopurine-induced leucopenia, bringing the gospel to Asian patients for safe use of thiopurines[5].

Thiopurine-induced leucopenia occurs at any time during treatment, ranging from early (within two months) to late onset (over two months)[6,7]. Thiopurine-induced severe, early-onset leucopenia, we now know, can be attributed largely to *NUDT15* deficiency. Since its introduction, the highly predictive value of *NUDT15*3* for thiopurine-induced leucopenia, mainly for early leucopenia (within two months), has been consistently confirmed, but so far, no definitive conclusion has been reached concerning late leucopenia (over two months)[5,8-10]. In a prospective study, we showed that halved dosage for *NUDT15*3* heterozygous patients reduced the incidence of early leucopenia by approximately 50%, but it did not reduce the risk of late leucopenia[9]. Similar findings were also reported in a Japanese study[10]. As thiopurine-induced late leucopenia is common, occurring in as high as 20%-40% of IBD patients[5,8,10], regular follow-up and therapeutic drug monitoring (TDM) are crucial during long-term thiopurine maintenance therapy.

Thiopurine drugs need to undergo extensive enzymatic conversion into 6-thioguanine nucleotides (6TGN) to exert their pharmacological and cytotoxic action. The role of the 6TGN level as an integral component of thiopurine monitoring to minimize adverse reactions has been investigated for decades[1,11-13]. However, whether 6TGN monitoring can reduce thiopurine-induced leucopenia is controversial and debatable[8,13-17]. Two studies have recently shown that late DNA-thioguanine (DNATG), a *NUDT15*-associated subcellular DNA-incorporated thiopurine metabolite, was strongly related to thiopurine-induced late leucopenia, compared to 6TGN[3,16].

Therefore, it is necessary to verify whether proactive TDM of DNATG or 6TGN in the initial medication phase can predict the occurrence of late leucopenia. After all, early identification of patients at risk of thiopurine-induced late leucopenia is of greater significance than metabolite concentration monitoring after routine blood tests indicating signs of leucopenia.

Herein, we conducted a prospective observational study to explore whether early proactive TDM of DNATG stable concentration within two months could help predict the leucopenia-susceptible population under a *NUDT15* genotype-guided thiopurine dosing strategy in patients with Crohn's disease (CD)[9], compared with 6TGN concentrations over the same period.

MATERIALS AND METHODS

Patient recruitment and study design

Patients diagnosed with CD from June 2019 to December 2022 were recruited in the Sixth Affiliated Hospital, Sun Yat-sen University. After prescribed thiopurines, patients were clinically followed up within two months when blood specimens were collected for TDM (6TGN, DNATG), and every two to three months thereafter. At each follow-up visit, clinical data, and routine blood tests were recorded. Medication adherence was followed up prospectively for a minimum duration of six months to monitor whether leucopenia occurred.

NUDT15 and *TPMT* genotypes were detected before treatment initiation. Patients with *NUDT15* wild-type were prescribed the target dose of 2.0 mg/kg/d AZA or 1.0 mg/kg/d 6-MP, and heterozygous patients were administered the target dose of 1.0 mg/kg/d AZA or 0.5 mg/kg/d 6-MP. Thiopurines were contraindicated in patients with homozygous mutations. The initial dose of AZA was 1.0 mg/kg/d in the wild-type group or 0.5 mg/kg/d in the heterozygous group and then increased to the target dosage in approximately one to two weeks[9]. The dose of 6-MP in mg/kg body weight was obtained by multiplying the dose of AZA by 0.5 as a conversion factor. DNATG and 6TGN levels were determined within the two-month follow up during which patients were medication stable and did not complain of any thiopurine-induced discomfort. The design of this study is depicted in Figure 1.

The clinical information was recorded at every visit until thiopurine discontinuation due to inefficacy, adverse drug reactions, and poor adherence/loss to follow-up. A white blood cell count < 3.5×10^9/L after two or more months of treatment was defined as thiopurine-induced late leucopenia. The exclusion criteria were as follows: Patients with blood transfusion or administration of cyclosporine or methotrexate; patients with insufficient function of the heart, liver, or kidneys; patients with active infection at the sampling time point; and patients suffering from acute adverse reactions within two months. The present study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. This trial is registered with the Chinese Clinical Trials Register (No. ChiCTR2100050295). All patients recruited provided a written informed consent form to participate in the trial.

Genotyping and TDM

DNA was extracted from peripheral leukocytes according to the manufacturer's instructions (TIANGEN DP349-02, Beijing, China). *TPMT**2 (rs1800462, G238C), **3A* (rs100460 and rs1142345), **3B* (rs100460, G460A), **3C* (rs1142345, A719G), and *NUDT15*3* (rs116855232, C415T), **5* (rs186364861, G52A), and **6* (rs746071566) genotypes were determined by Sanger sequencing. Detailed methods of quantitative detection of DNATG concentration in leukocytes and 6TGN concentration in erythrocytes have been published previously[3].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5 (GraphPad, La Jolla, CA, United States) and R software (version 4.2.1; R Foundation). The continuous values are shown as medians (ranges) or median ± interquartile range (IQR) according to the distribution of the values. The nonparametric Spearman test was used for all correlation analyses. χ^2 and Fisher's exact probability tests were used to analyze categorical data, while Mann-Whitney tests were used to analyze measurement data.

The performance and a target threshold of 6TGN or DNATG were evaluated by computing receiver operating characteristic (ROC) curves and the area under the ROC curves (AUC) using the pROC package in R. Cumulative incidence rates of late leucopenia during follow-up were constructed using the Kaplan-Meier method, and the survival curves were compared using the log-rank test with survival and survminer packages in R. Cox proportional hazards regression was used for both univariate and multivariate analysis of thiopurine-induced late leucopenia. Data visualization was performed in Prism 9.5 and the R packages survminer, rms and, ggplot2. Statistical significance was indicated at the 0.05 level.

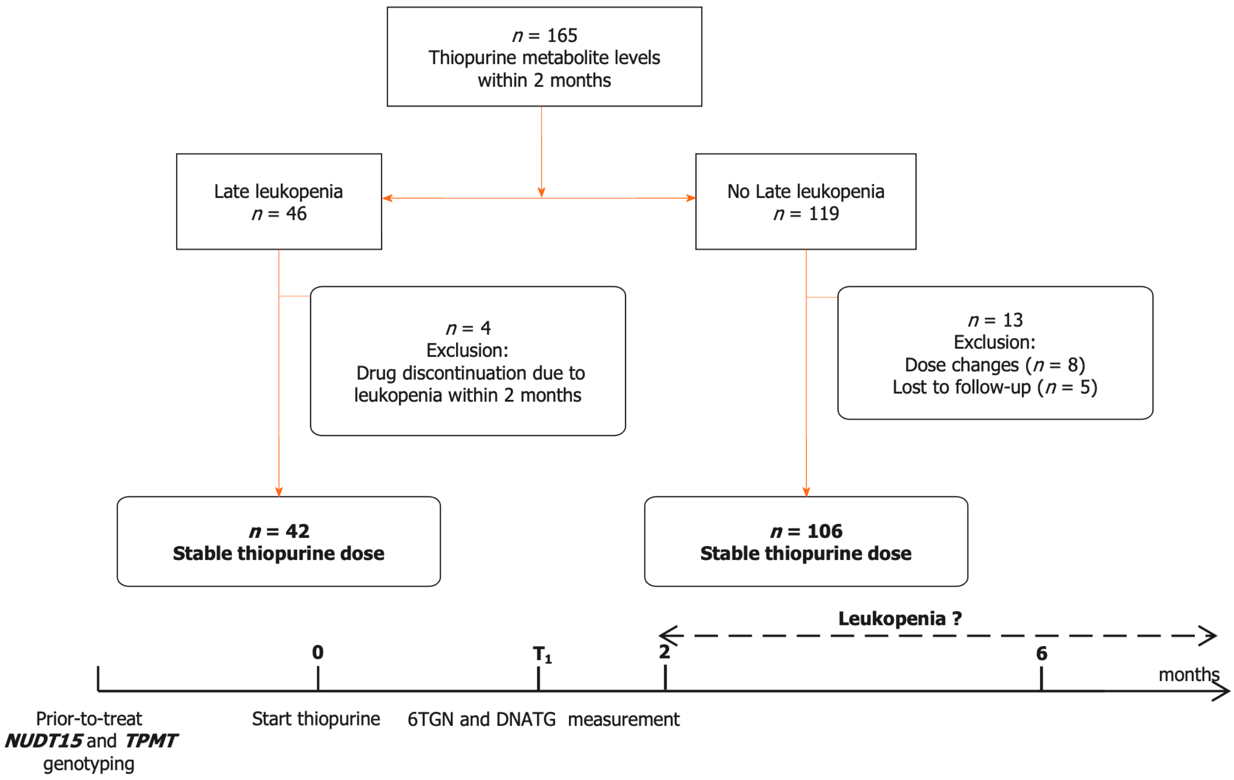


Figure 1 Flow chart of patient enrolment, exclusion, and follow-up. Late leucopenia was defined as a leukocyte count < 3.5 × 10^9/L over two months. The stable dose (2.0 mg/kg/d azathioprine and 1.0 mg/kg/d 6-mercaptopurine, approximately) is reached approximately one to two weeks after the initial administration. NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase; 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine.

RESULTS

Patient characteristics

The flow chart of patient enrollment, exclusion, and follow-up is depicted in Figure 1. A total of 165 patients were recruited in this study, and 17 patients were excluded due to drug discontinuation/dose change/loss of follow-up. A total of 148 patients with DNATG and 6TGN measurements within two months were analyzed in this study. The median patient follow-up time was 13.1 months, with 82.4% of patients followed longitudinally for six months or more. In our study, late leucopenia was observed in 15.6% (17/109) of the *NUDT15/TPMT* normal and 64.1% (25/39) of intermediate metabolizers, consistent with previous observations in similar populations[4,9,15,18]. Of the 148 patients, 3 were heterozygous for *TPMT*3C*, and 37 were heterozygous for low activity *NUDT15* alleles (21 *NUDT15*1/*3*, 14 *NUDT15*1/*2*, 2 *NUDT15*1/*6*). There were no significant differences in sex, age, dose, weight, comedication, or hematologic indices except for platelets (Table 1).

Correlation between early thiopurine metabolite concentrations and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts

There was a significant negative correlation between early DNATG concentration and the minimum peripheral leukocyte, neutrophil, lymphocyte, and, monocyte counts during follow-up (r = -0.3231, P < 0.0001; r = -0.1834, P = 0.0260; r = -0.1793, P = 0.0320; r = -0.2854, P = 0.0004; respectively, Figure 2A-D). Similarly, a marginally significant negative correlation was found between early DNATG concentration and the average peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts during follow-up (r = -0.1806, P = 0.0280; r = -0.1473, P = 0.0740; r = -0.1419, P = 0.0850; r = -0.2392, P = 0.0034; respectively, Figure 2E-H). By contrast, no significant correlation was found between the early 6TGN concentration and the minimum or average peripheral leukocyte, neutrophil, and lymphocyte counts during follow-up, except for monocyte counts (r = -0.2459, P = 0.0026; r = -0.1692, P = 0.0398; respectively, Figure 3).

Association of early thiopurine metabolite concentrations with late leucopenia

Median early DNATG concentrations were much higher in patients who developed late leucopenia than in those who did not [394.9 ± 137.4 fmol/μg DNA (n = 42) *vs* 225.3 ± 139.6 fmol/μg DNA (n = 106), P = 4.9 × 10^{-13}, Figure 4A]. In patients with *TPMT/NUDT15* wild-type (n = 109)], early DNATG concentration [401.1 ± 222.9 fmol/μg DNA (n = 17) *vs* 221.1 ± 140.8 fmol/μg DNA (n = 92), P = 4.9 × 10^{-9}, Figure 5A] was associated with late leucopenia with much higher significance than in heterozygous patients [382.5 ± 143.1 fmol/μg DNA (n = 25) *vs* 240.5 ± 248.3 fmol/μg DNA (n = 14), P = 0.024; 3 for *TPMT*3C*, 21 for *NUDT15*3*, 2 for *NUDT15*6*, 14 for *NUDT15*2*, Figure 5A].

Table 1 Baseline characteristics of the 148 patients included in the study

Characteristics	Patients		*P* value	Odds ratio (95%CI)
	Leucopenia	Nonleucopenia		
Number of subjects (%)	42 (28.4)	106 (71.6)		
Female, *n* (%)	14 (33.3)	22 (20.8)	0.11	1.91 (0.86-4.23)
Age (yr)	29 (12-59)	26 (13-61)	0.03	0.96 (0.92-0.99)
Azathioprine, *n* (%)	37 (26.6)	102 (73.4)	Reference	0.29 (0.07-1.14)
6-mercaptopurine, *n* (%)	5 (55.6)	4 (44.4)	0.08	1.05 (1.01-1.10)
Thiopurine dose (mg)	87.5 (25-100)	75 (50-150)	0.76	0.99 (0.98-1.01)
Azathioprine	75 (25-100)	75 (50-150)		
6-mercaptopurine	50 (37.5-50)	37.5 (25-50)		
Weight (kg)	52 (40-69)	55 (37-79)	0.02	1.05 (1.01-1.10)
Hematologic indices				
Leukocyte count (10^9/L)	6.25 (4.09-16.66)	6.92 (4.09-17.60)	0.83	1.31 (0.11-15.71)
Absolute neutrophil count (10^9/L)	3.98 (1.30-15.55)	4.59 (1.91-14.76)	0.78	0.70 (0.06-8.31)
Absolute lymphocyte count (10^9/L)	1.64 (0.41-4.45)	1.53 (0.27-4.26)	0.79	0.94 (0.57-1.53)
Monocyte count (10^9/L)	0.49 (0.22-1.98)	0.57 (0.10-1.72)	0.66	1.29 (0.42-3.91)
Hemoglobin (g/L)	126 (76-165)	130 (66-166)	0.43	1.01 (0.99-1.02)
Platelets (10^9/L)	287 (122-589)	302 (150-680)	0.10	1.00 (0.99-1.01)
Comedication (%)				
Anti-TNF agents	2 (4.76)	16 (15.09)	0.49	0.46 (0.05-4.09)
Corticosteroids	2 (4.76)	5 (4.72)	0.76	1.41 (0.16-12.47)
Genotypes (phenotypes)				
*NUDT15*1/*1* (NM)	18 (42.86)	93 (87.74)	Reference	
*NUDT15*1/*3* (IM)	13 (30.95)	8 (7.55)	0.95	0.97 (0.34-2.72)
*NUDT15*1/*2* (IM)	9 (21.43)	5 (4.72)	0.61	1.42 (0.37-5.44)
*NUDT15*1/*6* (IM)	2 (4.76)	0	0.99	
*TPMT*1/*1* (NM)	40 (95.24)	105 (99.06)	Reference	
*TPMT*1/*3C* (IM)	2 (4.76)	1 (0.94)	0.19	0.94 (0.47-2.35)

CI: Confidence interval; NM: Normal metabolizer; IM: Intermediate metabolizer; NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase.

By contrast, median early 6TGN concentrations were slightly higher in patients who developed late leucopenia than in those who did not [322.4 ± 210.6 pmol/8 × 10^8 red blood cells (RBCs) (n = 42) *vs* 247.5 ± 182.5 pmol/8 × 10^8 RBCs (n = 106), P = 0.021, Figure 4B]. Notably, the early 6TGN concentration did not exhibit a significant difference in *TPMT*/*NUDT15* heterozygous patients with or without late leucopenia [310.0 ± 180.6 pmol/8 × 10^8 RBCs (n = 25) *vs* 249.9 ± 303.9 pmol/8 × 10^8 RBCs (n = 14), P = 0.55], although it did in *TPMT/NUDT15* wild-type patients with or without late leucopenia [333.7 ± 270.7 pmol/8 × 10^8 RBCs (n = 17) *vs* 247.5 ± 162.9 pmol/8 × 10^8 RBCs (n = 92), P = 0.018] (Figure 5B).

Predictive performance of early thiopurine metabolite concentration for late leucopenia

The AUC of the ROC analysis for early DNATG concentrations was 0.855 (sensitivity 83%, specificity 81%) in the total population (Figure 6A), 0.902 (sensitivity 88%, specificity 85%) in *TPMT*/*NUDT15* wild-type (Figure 6B), and 0.72 (sensitivity 79%, specificity 72%) in heterozygous subgroups (Figure 6C) and the cutoff values of DNATG were 319.43, 315.72, and 354.68 fmol/µg DNA, respectively.

The AUC of the ROC analysis for early 6TGN concentration was 0.622 (sensitivity 66%, specificity 60%) in the total population (Figure 6A), 0.680 (sensitivity 71%, specificity 67%) in *TPMT*/*NUDT15* wild-type (Figure 6B), and 0.560 (sensitivity 50%, specificity 64%) in heterozygous subgroups (Figure 6C). Additionally, discontinuations occurred in 35.7% (15/42) of patients who developed late leucopenia (median follow-up: 5.8 months), with 80% (12/15) of them taking the drug for less than a year. Similarly, early DNATG levels were much more impressive in predicting discon-

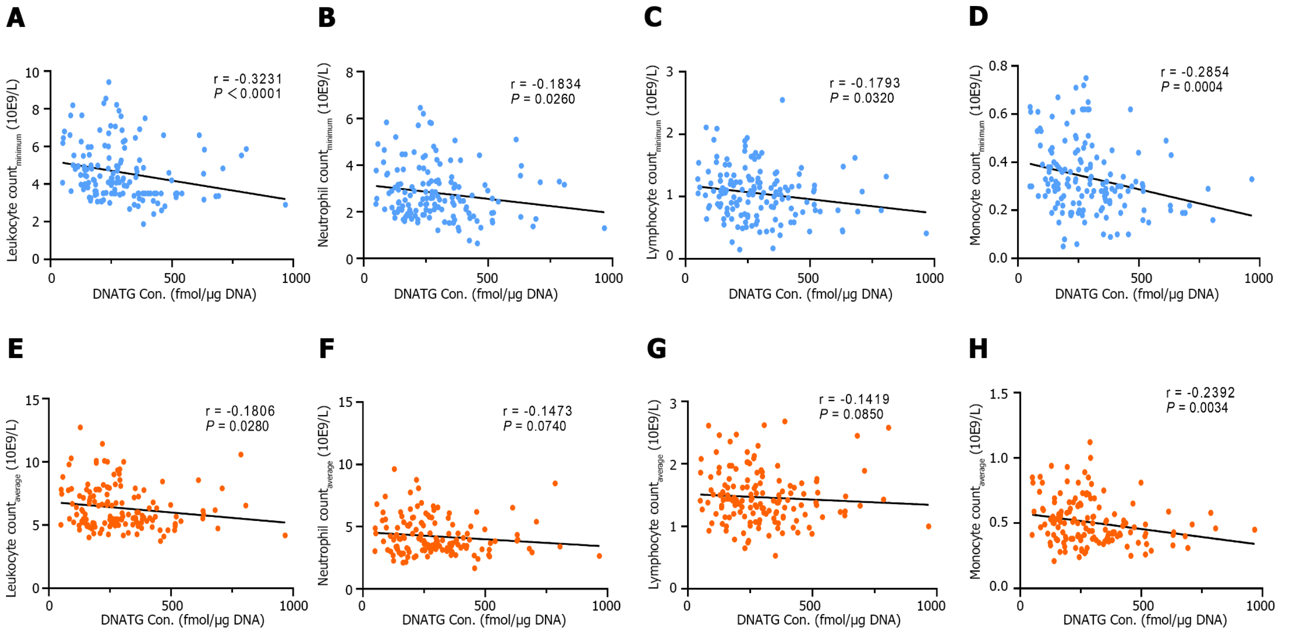


Figure 2 Correlation between early DNA-thioguanine concentration and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. A-D: There was a significant negative correlation between early DNA-thioguanine (DNATG) concentration, and the minimum peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts during follow-up; E-H: A marginally significant negative correlation was found between early DNATG concentration and the average peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. *P* values were determined by the nonparametric Spearman test. DNATG: DNA-thioguanine.

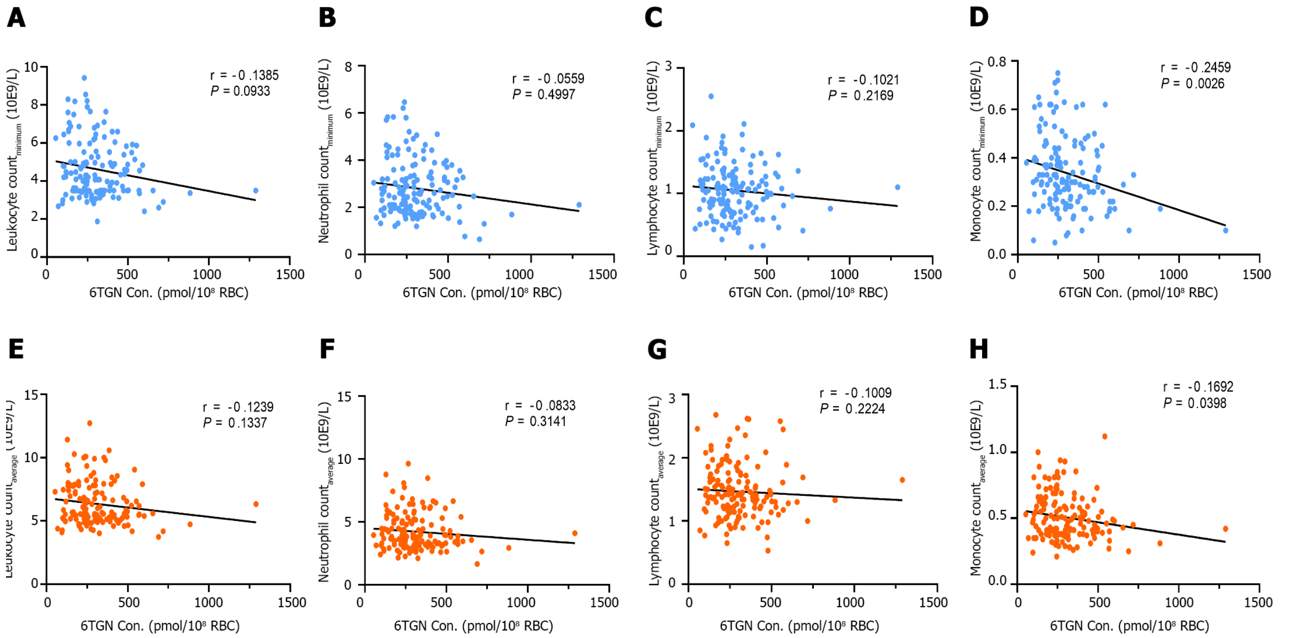


Figure 3 Correlation between early 6-thioguanine nucleotide concentration and peripheral leukocyte, neutrophil, lymphocyte, and monocyte counts. A-H: There was no significant correlation between early 6-thioguanine nucleotides (6TGN) concentration, and the minimum or average peripheral leukocyte, neutrophil, and lymphocyte counts during follow-up ($P > 0.05$) (A-C, E-G). There was a correlation between early 6TGN concentration and the minimum or average peripheral monocyte counts ($P < 0.05$) (D and H). *P* values were determined by the nonparametric Spearman test. 6TGN: 6-thioguanine nucleotides; RBC: Red blood cell.

tinuation events due to recurrent leucopenia, with an AUC of 0.812 (sensitivity 87%, specificity 77%) in the total population, and 0.864 (sensitivity 100%, specificity 67%) in the *TPMT*/*NUDT15* wild-type population (Supplementary Figure 1).

Kaplan-Meier survival analysis showed that the cumulative incidence rates of late leucopenia during follow-up were significantly increased in patients with DNATG concentrations greater than 319.43 fmol/μg DNA in the total population and 315.72 fmol/μg DNA in the *TPMT*/*NUDT15* wild-type population (Figure 7).

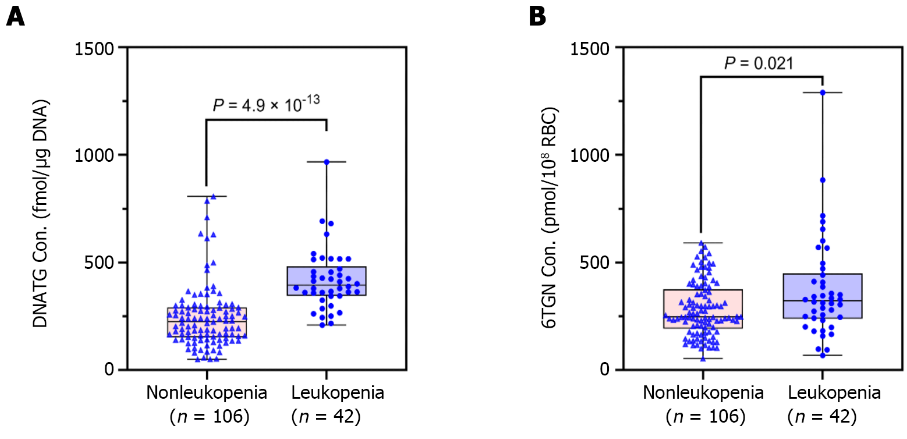


Figure 4 Early DNA-thioguanine concentration was associated with late leucopenia with immense significance compared to 6-thioguanine nucleotides. A: Patients who developed leucopenia had much higher early DNA-thioguanine levels compared with those who did not ($P = 4.9 \times 10^{-13}$); B: Patients who developed leucopenia had slightly higher early 6-thioguanine nucleotides levels compared with those who did not ($P = 0.021$). P values were determined by the Mann-Whitney test. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine.

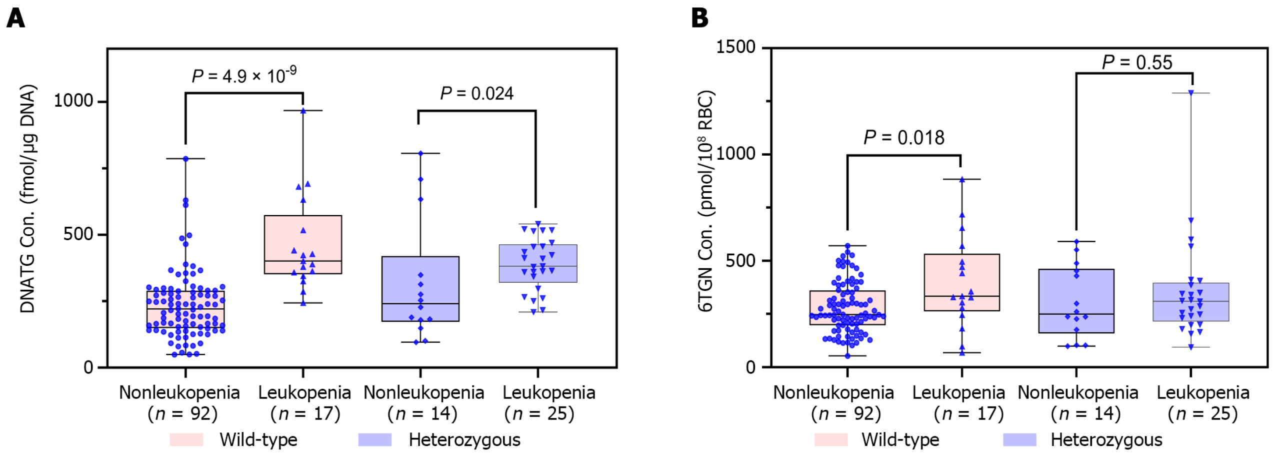


Figure 5 Early DNA-thioguanine concentration was significantly associated with late leucopenia, especially in thiopurine methyl-transferase/nudix hydrolase 15 wild-type patients compared to 6-thioguanine nucleotides. A: Early DNA-thioguanine concentration was associated with late leucopenia, especially in nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) wild-type patients ($P = 4.9 \times 10^{-9}$ *vs* $P = 0.024$); B: 6-thioguanine nucleotides was correlated with late leucopenia in *NUDT15/TPMT* wild-type patients ($P = 0.018$) but not in heterozygous subgroups ($P = 0.55$). P values were determined by the Mann-Whitney test. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; RBC: Red blood cell.

Multivariable prediction model for thiopurine-induced late leucopenia

Univariate Cox regression analysis of 148 patients on stable thiopurine therapy showed that age, weight, early DNATG and 6TGN concentrations, and *NUDT15* genotypes were relevant determinants for the development of late leucopenia ($P < 0.1$). After discarding the nonsignificant factors, 6TGN concentrations, in the multivariate analysis, the final Cox model included age, early DNATG concentration, and *NUDT15* genotypes ($P < 0.05$). Predictive variables and hazard ratios for the development of leucopenia are presented in Table 2. To improve the feasibility of clinical application, a nomogram based on multivariate analysis results is shown in Figure 8.

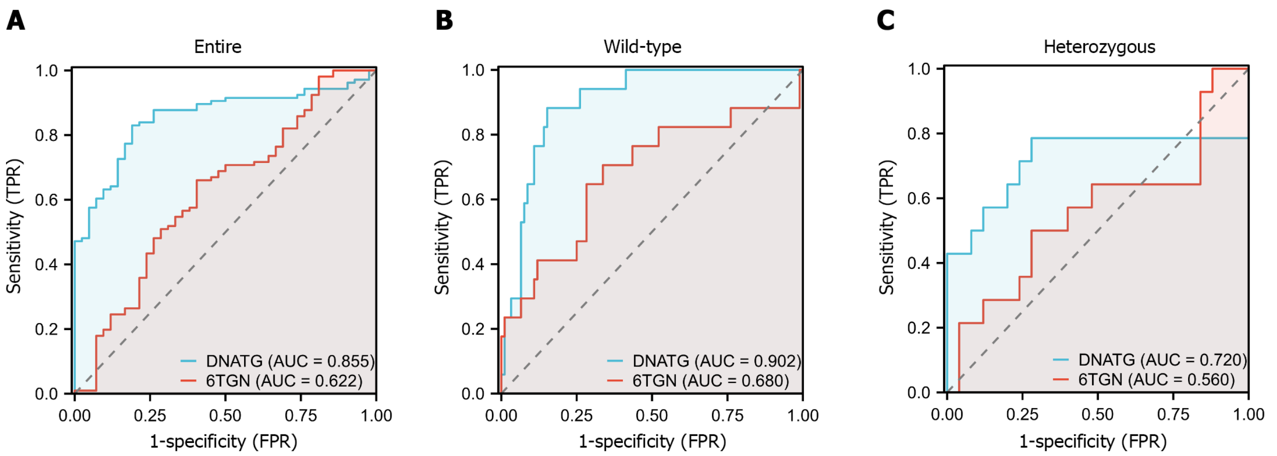
DISCUSSION

This is the first time that the DNATG level has been reported to be applicable in the prediction of thiopurine-induced late leucopenia. To date, few studies have been conducted to determine the worth of DNATG level and its correlation with thiopurine-induced late leucopenia in IBD patients. In our previous study, we found that late DNATG levels were significantly correlated with late leucopenia and were a better independent factor for late leucopenia than 6TGN. Therefore, we proposed that proactive quantification of DNATG may be a good way to predict late leucopenia and this prospective observational study was conducted thereafter. In this study, we also found that early DNATG concentrations were consistent with late concentrations in representative patients with multiple follow-up DNATG measurements (Supplementary Figure 2), which further indicated the reliability and feasibility of early proactive quantification of

Table 2 Univariate and multivariate analysis of independent predictors for leucopenia

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.04 (1.01-1.08)	0.005	1.06 (1.02-1.09)	0.002
Weight (kg)	0.95 (0.91-0.99)	0.013	0.96 (0.92-1.00)	0.076
DNATG (fmol/μg DNA)	1.004 (1.002-1.005)	< 0.001	1.004 (1.002-1.005)	< 0.001
NUDT15 genotypes		< 0.001		
*NUDT15**1/*1	Reference		Reference	
*NUDT15**1/*6	17.99 (3.95-81.90)	< 0.001	11.62 (1.51-89.26)	0.018
*NUDT15**1/*3	4.93 (2.41-10.10)	< 0.001	3.21 (1.54-6.71)	0.002
*NUDT15**1/*2	4.50 (2.01- 10.03)	< 0.001	3.25 (1.41-7.50)	0.006
6TGN (pmol/8 × 10^8 RBC)	1.003 (1.001-1.004)	< 0.001	1.000 (0.998-1.002)	0.98
Sex		0.19		
Female	Reference			
Male	0.64 (0.34-1.22)	0.17		
TPMT genotypes		0.31		
*TPMT**1/*1	Reference			
*TPMT**1/*3C	2.38 (0.57-9.87)	0.25		

HR: Hazard ratio; CI: Confidence interval; NUDT15: Nudix hydrolase 15; TPMT: Thiopurine methyltransferase; 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; RBC: Red blood cell.



Variables	Cut-off value	Sensitivity	Specificity
DNATG	319.43	0.83	0.81
6TGN	303.89	0.66	0.60

Variables	Cut-off value	Sensitivity	Specificity
DNATG	315.72	0.88	0.85
6TGN	303.89	0.71	0.67

Variables	Cut-off value	Sensitivity	Specificity
DNATG	354.68	0.79	0.74
6TGN	239.70	0.50	0.64

Figure 6 Performance of early DNA-thioguanine and 6-thioguanine nucleotides concentrations in the prediction of late leucopenia in the entire population, nudix hydrolase 15/thiopurine methyltransferase wild-type and heterozygous subgroups. A: Performance in the entire population; B: Performance in nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) wild-type subgroups; C: Performance in NUDT15/TPMT heterozygous subgroups. 6TGN: 6-thioguanine nucleotides; DNATG: DNA-thioguanine; AUC: Area under the curve; FPR: False positive rate; TPR: True positive rate.

DNATG.

In the previous decade, investigations on individualized thiopurines were mainly focused on genotypes of *TPMT* and *NUDT15*. The Clinical Pharmacogenetics Implementation Consortium guidelines recommend initial thiopurine dose adjustment based on *TPMT* and *NUDT15* genotypes[19]. Numerous studies have consistently reported that *NUDT15* variants serve as the sole or major factors linked to thiopurine-induced early leucopenia, exhibiting a sensitivity and specificity higher than 90%, while *TPMT* variants demonstrate a sensitivity of 12.1% and a specificity of 97.6%[5]. In addition, some studies have also shown an association between *NUDT15* variants and late leucopenia[8]. In the present

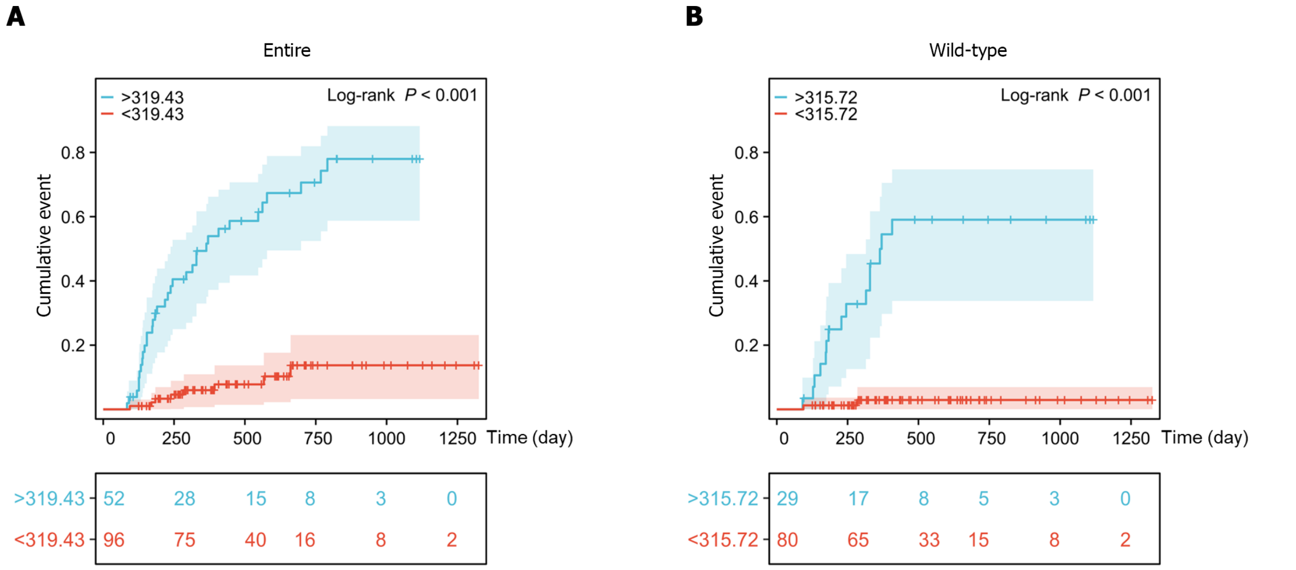


Figure 7 The cumulative events of leucopenia with corresponding DNA-thioguanine thresholds in the entire and thiopurine methyltransferase/nudix hydrolase 15 wild-type populations. A: Cumulative incidence rates were significantly greater in patients with DNA-thioguanine (DNATG) concentrations of more than 319.43 fmol/μg DNA in the entire population; B: Cumulative incidence rates were significantly greater in patients with DNATG concentrations of more than 315.72 fmol/μg DNA in the thiopurine methyltransferase/nudix hydrolase 15 wild-type population. Both log-rank $P < 0.001$. Kaplan-Meier survival analysis followed by the log-rank test was employed to estimate cumulative leucopenia events.

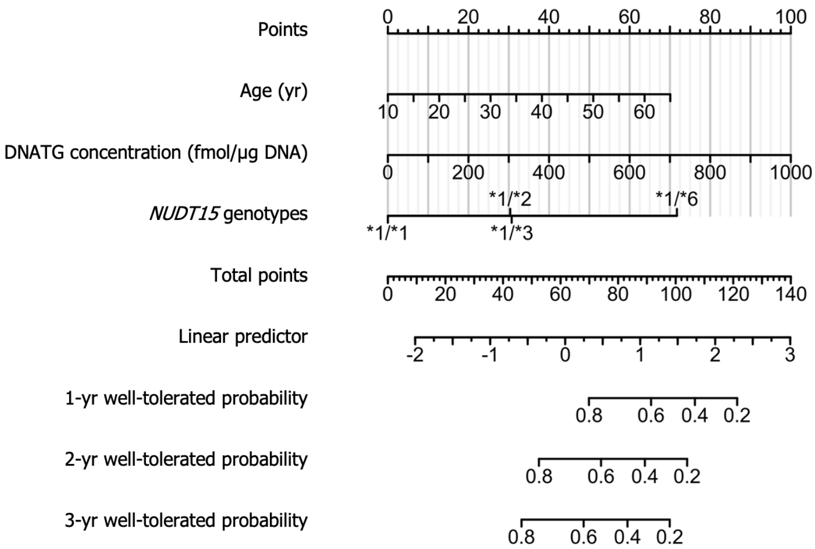


Figure 8 Nomogram based on the multivariate analysis results shown in Table 2. For example, a 65-year-old patient with the wild-type nudix hydrolase 15 (NUDT15)*1/*1, having a DNA-thioguanine (DNATG) concentration of 200 fmol/μg DNA, would score 70 for age, 20 for DNATG concentration, and 0 for the NUDT15 genotype, resulting in a total score of 90. Correspondingly, the estimated 1-year, 2-year, and 3-year well-tolerated (no occurrence of late leukopenia) probabilities are approximately 0.6, 0.4, and 0.3, respectively. DNATG: DNA-thioguanine; NUDT15: Nudix hydrolase 15.

study, we verified that *NUDT15* was a significant independent factor for late risk, and therefore, the importance of pretreatment for the determination of the *NUDT15* genotype cannot be overemphasized.

Nonetheless, a large portion of thiopurine-induced leucopenia cannot be explained by *NUDT15/TPMT* deficiency, which was manifested by the variation in leucopenia in patients receiving optimized thiopurines based on the genotype of *NUDT15/TPMT*[9]. Meanwhile, a high incidence of late leucopenia (10%-20%) can still be seen in the *NUDT15/TPMT* wild-type population[3,4,9,18]. It is very important to identify biomarkers for risk in the populations mentioned above.

TDM of 6TGN has been employed in some clinical settings as a strategy to predict thiopurine-induced toxicity for over two decades although the value of 6TGN TDM is still controversial[20-22]. Kang *et al*[23] reported that the 6-TGN level (goal < 167.1 pmol/8 × 10^8 RBCs) could be used to adjust AZA doses to reduce late thiopurine-induced leucopenia in *NUDT15* intermediate metabolizers.

However, a large number of studies have shown that 6TGN levels are not significantly different between groups with and without leucopenia[8,13-16]. In our previous study[4], late 6TGN levels were significantly associated with late leucopenia in patients with wild-type *NUDT15 R139C* (P = 0.006, median 413.0 *vs* 279.7 pmol/8 × 10^8 RBCs), while no significant association was found between 6TGN levels and leucopenia in patients with *NUDT15 R139C* variants (P = 0.26). Similarly, in the present study, the early concentrations of 6TGN exhibited no significant difference in *TPMT*/*NUDT15* heterozygous patients with or without late leucopenia (P = 0.55), although it did in the entire sample and *TPMT*/*NUDT15* wild-type patients with or without late leucopenia (P = 0.021, 0.018, respectively). This discrepancy may be because in the study performed by Kang *et al*[23], 6TGN concentrations were monitored regularly, and doses were adjusted accordingly so that 6TGN levels would be within the therapeutic range of 235-450 pmol/8 × 10^8 RBCs[24]. In our study, the doses were only adjusted based on genotypes before thiopurine administration.

Furthermore, our current study found that compared to 6TGN, early DNATG levels were more significantly correlated with thiopurine-induced late leucopenia (P = 4.9 × 10^{-13} *vs* P = 0.021). The DNATG threshold of 319.43 fmol/μg DNA predicted late leucopenia in the entire sample with an AUC of 0.855 (sensitivity 83%, specificity 81%), and in the *NUDT15/TPMT* normal metabolizers, the predictability of a threshold of 315.72 fmol/μg DNA was much more impressive with an AUC of 0.902 (sensitivity 88%, specificity 85%). Notably, early DNATG levels were much more impressive in predicting discontinuation events due to recurrent leucopenia with an AUC of 0.864 (sensitivity 100%, specificity 67%) in the *TPMT*/*NUDT15* wild-type population.

In recent years, proactive and reactive TDM strategies in IBD management have been under intense discussion. Reactive TDM in non- or partial responders with active IBD aims to guide treatment changes and routine proactive TDM in patients with quiescent disease aims to reach a target trough concentration during routine clinical care[20]. Many studies have suggested that proactive TDM can provide more clinical benefits than reactive TDM for anti-tumor necrosis factor biologics[25-28]. One Danish study reported that DNATG levels in neutrophils (P < 0.0001) and 6TGN in erythrocytes (P = 0.01) on methotrexate/6-MP maintenance therapy for childhood acute lymphoblastic leukemia, are predictors of neutrophil nadirs within two weeks following high-dose methotrexate infusion[29]. In the present study, it is heartening that early proactive monitoring of DNATG is of very high sensitivity (88%) and specificity (85%) to predict late leucopenia in *NUDT15*/*TPMT* wild-type subgroups that would not benefit from the *NUDT15* genotype-guided dosing strategy.

In general, pretreatment determination of the *NUDT15* genotype is necessary to identify Asian patients with IBD who are susceptible to thiopurine-induced leucopenia. Proactive DNATG therapeutic monitoring is very important, especially for many patients with wild-type *NUDT15*/*TPMT*, to avoid late risk.

There are several shortcomings in our study. First, this was a prospective observational study with relatively small sample sizes. In our study, once leucopenia occurred, patients discontinued the thiopurines, switched therapies, or followed a strategy of empiric dose adaptation due to the patient's wishes and physician discretion. We did not fully determine the benefits of reactive DNATG-TDM-guided dose adaptation decisions with *NUDT15*-guided initial medication. Further randomized clinical trials are warranted to probe whether routine reactive DNATG monitoring, compared with empiric treatment changes, helps to reduce the incidence of drug withdrawal due to late leucopenia in IBD patients.

CONCLUSION

In this prospective study of CD patients receiving thiopurines under *NUDT15* genotype-guided dosing strategy, we demonstrated that in addition to the *NUDT15* genotype, early proactive monitoring of DNATG was highly sensitive in predicting thiopurine-induced late leucopenia. With the cutoff value of 319.43 fmol/μg DNA, late leucopenia cases could be predicted with 83% sensitivity and 81% specificity, and with the cutoff value of 357.05 fmol/μg DNA, the discontinuation of thiopurines could be predicted with 87% sensitivity and 77% specificity. DNATG levels were more significantly associated with late leucopenia than 6TGN levels, which was manifested especially in patients with *NUDT15*/*TPMT* wild-type, with a cutoff value of 315.72 fmol/μg DNA (sensitivity 88%, specificity 85%). This study provides empirical evidence indicating that early monitoring of DNATG levels, offers a predictive capability to discern patients susceptible to developing late leucopenia during the initial stages of thiopurine therapy, especially for many patients with wild-type *NUDT15*/*TPMT*.

ARTICLE HIGHLIGHTS

Research background

Thiopurine-induced leucopenia significantly hinders the use of thiopurines. Genotype-guided dose optimization has significantly reduced the early leucopenia rate, but there are no definitive biomarkers for late risk prediction.

Research motivation

Can we identify definitive predictors for late risk as early as possible under current genotype-guided thiopurine dosing strategy?

Research objectives

To determine the predictive value of early monitoring of DNA-thioguanine (DNATG) or 6-thioguanine nucleotides (6TGN) for late leucopenia in patients with Crohn's disease (CD).

Research methods

Blood samples were collected within two months after thiopurine initiation for detection of metabolite concentrations. Late leucopenia was defined as a leukocyte count < 3.5×10^9/L over two months.

Research results

In patients suffering late leucopenia, early DNATG levels were significantly higher than those who did not ($P = 4.9 \times 10^{-13}$) . DNATG threshold of 319.43 fmol/μg DNA could predict late leucopenia in the entire sample collection with an area under the curve (AUC) of 0.855 (sensitivity 83%, specificity 81%), and in the nudix hydrolase 15 (NUDT15)/thiopurine methyltransferase (TPMT) normal metabolizers, the prediction performance of a threshold of 315.72 fmol/μg DNA was much more remarkable with an AUC of 0.902 (sensitivity 88%, specificity 85%). 6TGN had a relatively poor correlation with late leucopenia whether in the entire sample collection ($P = 0.021$) or NUDT15/TPMT normal or intermediate metabolizers ($P = 0.018$, $P = 0.55$, respectively).

Research conclusions

Early DNATG concentration was significantly correlated with thiopurine-induced late leucopenia in CD patients, especially in NUDT15/TPMT normal metabolizers.

Research perspectives

Proactive therapeutic drug monitoring to keep DNATG concentration below 320 fmol/μg DNA could be an effective strategy to prevent thiopurine-induced late leucopenia in CD patients.

FOOTNOTES

Author contributions: Yang T, Chao K, and Zhu X contributed equally to this work. Huang M and Gao X contributed to the study conceptualization and supervision and funding acquisition; Chao K and Gao X contributed to the acquisition and curation of data; Mao J and Li P helped to collect and process the blood samples; Guan SX and Xie W monitored the project progress and contributed to the writing and revision of the manuscript; Zhu X, Wang XD, and Guan YP helped to detect the genotypes and revise the manuscript; Yang T and Chan S detected drug concentrations; Yang T administered the project, analyzed the data, and wrote the manuscript; and all authors have read and approved the final manuscript.

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