Dear Science Editor Jin-Zhou Tang,

We deeply appreciate your wonderful jobs and the reviewers' comments on our manuscript (50351). The comments are valuable and very helpful for improving our paper. We have revised and submitted back the revised manuscript according to the reviewers' comments. Our response to the comments point-by-point is appended at the letter.

Hope we have addressed all the reviewers' concerns and our revised manuscript will meet all the requirements of the journal, *World Journal of Gastroenterology.*

Thank you very much for your consideration!

Sincerely yours,

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Responses to Comments of Reviewer

Reviewer 1

I think this topic is of utmost importance and relevance for the utilization of thiopurines in IBD care. We are always searching for biomarkers we can use to assist

us with the appropriate dosing. Having two markers work hand in hand to attain a great positive and negative predictive value is very beneficial. The authors did a great job demonstrating this in their study design and methods. The methods can be slightly more clear and maybe add something about the practicality of utilizing these tests in real world practices.

[Answer] Thank you very much for your professional comments.

1. We have elaborated on the details of *NUDT15* R139C/*TPMT**3C and 6TGN/6MMPR detections in the part of methods as follows:

NUDT15 R139C and TPMT*3C genotyping

We used allele-specific polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) to test the genotypes of *NUDT15* R139C (rs116855232) and *TPMT*3C* (rs1142345). The sequences of the primers for *NUDT15* R139C were 5-AGCTTACCCAAATAAACACCCT-3, 5-TGGGGGATACATTAAGAGACTGC-3, and for *TPMT*3C* were 5- AAGTGTTGGGATTACAGGTG-3, 5-TCCTCAAAAACATGTCAGTGTG-3, respectively. PCR amplification started at 94°C for 5 min, continued with 30 cycles of 94°C for 60 s, 59°C for 30s and 72°C for 30 s. Final extension was performed at 72°C for 10 min. The PCR product was digested with restriction enzyme HpyCH4III (New England Biolabs, Hertfordshire, United Kingdom) for *NUDT15* R139C testing and the enzyme AccI (New England Biolabs, Hertfordshire, United Kingdom) for *TPMT*3C* testing.

6TGN and 6MMPR levels detection

6TGN and 6MMPR concentrations in patients' erythrocyte lysates were determined using the Dervieux et al's method[1] by the high performance liquid chromatography. Firstly, 6TGN were hydrolyzed (100 °C for 45 min) into their bases (6-thioguanie) and 6MMPR were converted into their derivatives by perchloric acid. Then they

were separated by a C18 column with the mobile phase consisting of methanol - 20 mmol/L potassium dihydrogenphosphate - triethylamine (pH adjusted to 3.2 using phosphoric acid) (5:95:0.1) with the flow rate of 1.0 mL/min. Finally, the wavelength for detection was set at 345 nm for 6TGN and at 303 nm for 6MMPR.

2. We have added some suggestions about the practicality of utilizing these tests in the part of the discussion as follows “Based on this model, we can distinguish the thiopurine refractoriness if the patient has the steady-state 6TGN levels above the cut-off values without efficacy, which suggests the thiopurine dose should not be increased and the alternative therapeutic agent should be chose to avoid the incidence of TIL.”

Reviewer 2

In this research manuscript, the authors explored the possibility of incorporating the levels of 6TGN and *NUDT15* R139C polymorphisms to predict the occurrence of leukopenia in Crohn's disease patients. The study poses substantial novelty and the finding is helpful to delineate practical cut-off 6TGN levels of CD patients with different *NUDT15* R139C polymorphisms. The manuscript title is concise to reflect the finding of the study. The research objectives are realistic and measurable. The study is soundly designed. Research data is analyzed sufficiently with appropriate statistical methods. The conclusion is suitable to summarize the study findings, with reasonable study limitations.

Below are some comments for consideration to the authors:

1. In context of leukopenia in this manuscript, it should be specified as thiopurine-induced leukopenia, instead of leukopenia in general. On the other hand, *NUDT15* mutation should be stated specifically as *NUDT15* R139C.

[Answer] Thank you very much for your professional comments. We have changed the word "leukopenia" into "thiopurine-induced leukopenia" in the title and also we have used "TIL" as the abbreviation for "thiopurine-induced leukopenia" in our whole manuscript according to the study of van Gennep. S et al[2]. On the other hand, we have replaced "*NUDT15*" into "*NUDT15* R139C" in our title and our manuscript.

2. Methods: Elaborate on the details of the methods/assays used for the determination of 6TGN/6MMPR and *NUDT15* genotyping.

[Answer] Thank you very much for your professional suggestions. We have revised the methods in our manuscript as follows:

NUDT15 R139C and TPMT*3C genotyping

We used allele-specific polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) to test the genotypes of *NUDT15* R139C (rs116855232) and *TPMT*3C* (rs1142345). The sequences of the primers for *NUDT15* R139C were 5-AGCTTACCCAAATAAACACCCT-3, 5-TGGGGGATACATTAAGAGACTGC-3, and for *TPMT*3C* were 5- AAGTGTTGGGATTACAGGTG-3, 5-TCCTCAAAAACATGTCAGTGTG-3, respectively. PCR amplification started at 94°C for 5 min, continued with 30 cycles of 94°C for 60 s, 59°C for 30s and 72°C for 30 s. Final extension was performed at 72°C for 10 min. The PCR product was digested with restriction enzyme HpyCH4III (New England Biolabs, Hertfordshire, United Kingdom) for *NUDT15* R139C testing and the enzyme AccI (New England Biolabs, Hertfordshire, United Kingdom) for *TPMT*3C* testing.

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3. Methods: *TPMT*3C* genotyping is not mentioned in method and abstract.

[Answer] Thank you very much for your professional comments. We have added the method of *TPMT**3C genotype detection in the part of the methods and abstract.

4. Results: Only CD patients were involved in this study; therefore the patients should be referred as CD patients, instead of IBD patients.

[Answer] Thank you very much for your professional comments. We have revised "Clinical characteristics of IBD patients" into "Clinical characteristics of CD patients" in the results.

5. Results: Diplotype of *NUDT15* R139C and *TPMT*3C* is mentioned in results, however diplotype analysis is not stated in methods. In addition, results on diplotype analysis is not shown.

[Answer] Thank you very much for your professional comments. We have added the method of *NUDT15* R139C and *TPMT**3C diplotypes analysis in the part of statistic analysis. "The X^2 method or Fisher's test were used to analyze the associations of *NUDT15* R139C/*TPMT**3C diplotypes with thiopurine-induced leukopenia". Meanwhile these results of *NUDT15* R139C/*TPMT**3C on diplotypes analysis were shown in Table 1.

Table 1. Characteristics of the 411 CD patients included in this study. Patients carrying the *NUDT15* CT and TT genotypes were more likely to develop TIL ([a]*P*<*0.01*).

Characteristics	All patients	With leukopenia	Without leukopenia	*P*-values
No. of subjects (%)	411	72 (17.5)	339 (82.5)	-
Gender				
Male, n (%)	305 (74.0)	52 (17.0)	253 (83.0)	0.180
Female, n (%)	106 (26.0)	20 (18.9)	86 (81.1)	
NUDT15 R139C				
CC, n (%)	342 (80.4)	35 (10.2)	307 (89.8)	2.40×10^{-15}
CT, n (%)	65 (17.9)	33 (50.2)	32 (49.8)	
TT, n (%)	4 (1.7)	4 (100)	0	
*TPMT**3C				
AA, n (%)	398 (96.8)	68 (17.1)	330 (82.9)	0.26
AG, n (%)	13 (3.2)	4 (30.8)	9 (69.2)	
Age in years, median (range)	28 (12~70)	26(12~70)	27(12~60)	0.734
Medication				
Azathiopurine	337 (82.0)	57 (17.3)	273 (82.7)	0.113
Mercaptopurine	74 (18.0)	14 (18.9)	60 (81.1)	

Thiopurines dose, mg/kg/d, median (range)	1.7 (0.2~3.1)	1.5 (0.5~2.6)	1.7 (0.2~3.1)	0.030
CD				
Ileal L1	34	6	28	
Colorectal L2	21	6	15	0.495
Ileocolonic L3	292	48	244	
Upper gastrointestinal L4	61	12	49	
Co-medication	211	45	166	0.74
Corticosteroids	79	15	64	
Thalidomide	51	13	38	
Anti-TNF agents	64	13	51	
5-ASA	4	0	4	
Allopurinol	13	4	9	

6. Gene names should be in italic.

[Answer] Thank you very much for your professional suggestions. We have already modified all of the gene names into italic.

7. Some data was not stated clearly. Example: Pg11, "…leukopenia were found in the subgroups (P=0.55, P=0.30)." – the subgroups should be specified as CC and CT respectively.

[Answer] Thank you very much for your professional suggestions. We have revised the sentence as "No correlations between the 6MMPR concentrations and TIL were found in the subgroups of CC (P=0.55) and CT (P=0.30), respectively."

8. Pg13, "…since half of patients who developed leukopenia (37/72) carried the T allele (CT/TT)." – May consider to rephrase or remove this statement as it seems vague. It could be stated the other way round as 'half of the leukopenia patients are C allele carries' and the statement is still true.

[Answer] Thank you very much for your professional comments. We have removed this statement.

9. There are some inconsistencies of terms used throughout of the manuscript. Eg, deoxy-TGTP or dTGTP; deoxy-TGMP or dTGMP; VS or vs; tiopurine or thiopurine.

[Answer] Thank you very much for your professional suggestions. We have corrected these words and checked the consistence of these terms in our manuscript."

10. Pg16, "…(<319.2 pmol/8×108 RBC) or the TT genotype (>411.5 pmol/8×108 RBC)." – Please check, it should be "…(<319.2 pmol/8×108 RBC) and the CC genotype (>411.5 pmol/8×108 RBC)."?

[Answer] Thank you very much for your professional suggestions. We are sorry for this mistake and we have corrected as follows "the CT genotype (<319.2 pmol/8×10^8 RBC) or the CC genotype (>411.5 pmol/8×10^8 RBC)."

11. Supplement figure 1 is not cited in the manuscript.

[Answer] Thank you very much for your professional comments. Supplement figure 1 was mentioned in the second graph of the results "For *TPMT**3C, the patients carrying *TPMT**3C variants had excessively higher 6TGN (P=2.0×10^{-6}) and lower 6MMPR levels (P=3.7×10^{-4}), which could not be offset by the thiopurine dose (P=4.8×10^{-5}, P=7.7×10^{-5}) (Supplement figure 1)". And also we cited it in the third graph of the discussion."

12. Table 2 (odds ratios and 95% CI): Odds ratios and 95% CI are not correctly included in the table? The ranges given do not seem like a valid 95% CI neither.

[Answer] Thank you very much for your professional suggestions. We are sorry for this mistake in Table 2. Actually the values in this column were the asymptotic 95% confidence interval in ROC curves. We have revised Table 2.

Table 2. Relationship between the concentrations of 6TGN and thiopurine-induced TIL in different *NUDT15* R139C genotype groups among 411 patients according to the ROC curves. In subgroups, the area under curve (AUC) was increased from 0.57 to 0.65 and 0.70. Moreover, in the CT group, the specificity was as high as 96.9%, with a sensitivity of 42.4%, and in the CC group, the specificity was 73.3%, with a high sensitivity of 60.0%, compared with the values in the total samples.

NUDT15 Genotype (no. of patients)	6TGNs (pmol/8*10^8 RBC)	*P* value	AUC	95% CI	Sensitivity (%)	Specificity (%)
CT+CC	≥474.7	0.071	0.57	(0.49~0.65)	34.7	82.6
	<474.7					
CC (342)	≥411.5	9.4×10^{-5}	0.70	(0.61~0.79)	60.0	73.3
	<411.5					
CT (65)	≥319.2	0.039	0.65	(0.51~0.79)	42.4	96.9
	<319.2					

13. Table 3 (categories 2 and 3): "…6TGN >301. 1" – the cut-off range should be 319.2 as stated in the manuscript instead.

[Answer] Thank you very much for your professional suggestions. We have corrected this mistake in Table 3.

Reference

[1]. Dervieux T, Boulieu R. Simultaneous determination of 6-thioguanine and methyl 6-mercaptopurine nucleotides of azathioprine in red blood cells by HPLC. *Clin Chem* **1998**, 44(3):551-555.

[2]. van Gennep S, Konte K, Meijer B, Heymans MW, D'Haens GR, Lowenberg M, de Boer NKH. Systematic review with meta-analysis: risk factors for thiopurine-induced leukopenia in IBD. *Aliment Pharmacol Ther* **2019**, 50(5):484-506.