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ORIGINAL ARTICLE

Whole-Blood Thiopurine S-Methyltransferase Genotype and Phenotype Concordance in Iranian Kurdish Ulcerative Colitis (UC) Patients

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SUMMARY

Background: **Thiopurine methyl transferase (TPMT), a drug-metabolizing enzyme, catalyzes methylation and consequently, the metabolism of thiopurine compounds used for treatment of inflammatory bowel disease (IBD). Individuals who are homozygous recessive or have extremely low TPMT activity need to avoid thiopurines because of concern for significant leukopenia. The aim of this research was to determine TPMT phenotypes and genotypes in IBD patients to predict the risk of thiopurine toxicity before treatment.**
Methods: **The present case-control study consisted of 210 ulcerative colitis patients and 212 unrelated healthy controls from the population of western Iran. TPMT phenotype and genotype were determined by HPLC and allele specific PCR and PCR-RFLP, respectively.**
Results: **TPMT phenotyping and genotyping were compatible and demonstrated no frequency for deficient, 2.2% for low, and 97.8% for normal-activity which is different compared with the results of other studies. There was a significant negative correlation between TPMT activities as calculated based on nmol6MTG/gHb/h and the Hb levels in both UC ($r = -0.54$, $p < 0.001$) and control groups ($r = -0.27$, $p < 0.001$). Interestingly, a significant positive correlation between Hb levels and TPMT activities was seen when the enzyme activity was calculated in mU/L in both UC patients ($r = 0.14$, $p = 0.05$) and in control subjects ($r = 0.43$, $p < 0.001$). The overall concordance rate between TPMT phenotypes and genotypes of mutants to alleles (9 out of 422), based on receiver-operating characteristic (ROC) curve, yielded a sensitivity of 94.7% and specificity of 90% for mU/L and a sensitivity of 85.6% and specificity of 90% for nmol6MTG/gHb/h.**
Conclusions: **The use of mU/L is more appropriate than nmol6MTG/gHb/h for expressing TPMT activity, and there is better correlation between genotypes and phenotypes of TPMT based on mU/L. The frequency of known mutant TPMT alleles in western Iran (Kurd population) is low suggesting low risk of thiopurine drug toxicity in IBD patients from this region.**
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KEY WORDS

thiopurine methyl transferase (TPMT), phenotype and genotype, thiopurine toxicity, inflammatory bowel disease (IBD), SNP

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) (collectively termed inflammatory bowel disease (IBD) are complex disorders distinguished by inflammation of the large intestine and ulcers may form on the surface (UC) or anywhere in the GI tract from the mouth to the anus (CD) [1]. These diseases, however, were low or non-existent in Iran 50 years ago [2,3] but are now increasing [4,5]. The main initial goal of treatment in IBD is to induce and maintain remission of disease, and eventually, to achieve mucosal healing and a reduction in the need for hospital admission, achieve mucosal healing, and a reduction in the need for hospital admission and surgery [6,7]. Thiopurine compounds 6-mercaptopurine (6-MP) and azathioprine (AZA) are the most frequently used immunosuppressive drugs in IBD. They are proven to be effective in active disease, to induce remission especially in steroid dependent and steroid refractory CD and UC patients [8,9], and maintain clinical remission in both UC and CD. Although thiopurine drugs are widely used, gastrointestinal intolerance, pancreatitis, hypersensitivity, and myelosuppression are observed in up to 30% of patients. These adverse drug responses (ADR) often result in the withdrawal of treatment [10-12]. In some instances the unexpected reaction to therapy can be fatal, for example in cases of severe myelosuppression [13]. Although the field of pharmacogenetics dates back to the 1950s, when it was first proposed that inherited traits may account for individual variability in drug response [14] but pharmacogenetics in IBD seems currently to be of immediate scientific as well as of capital clinical importance in view of thiopurine therapies. The link of differences in gene expression (genotype) to drug response (phenotype) and advances in this field are helping to better predict drug efficacy and toxicity [15,16]. Indeed, in the USA the Federal Drug Administration has discussed adding advice to thiopurine drug packaging recommending that the status of drug metabolizing enzyme thiopurine methyltransferase (TPMT) should be checked [17].

Thiopurine methyltransferase (TPMT) (EC 2.1.1.67), a ubiquitous cytosolic enzyme catalyzes S-methylation of aromatic and heterocyclic sulfhydryl compounds such as thiopurine compounds, 6-MP and AZA [18]. Individuals who are homozygous recessive or have extremely low TPMT activity need to avoid thiopurines because of concern for significant leukopenia. The TPMT genetic polymorphism is a significant factor responsible for serious adverse drug reactions in patients treated with thiopurines and may also contribute to individual variation in therapeutic efficacy. At least twenty five TPMT genetic polymorphisms have been identified [19], the majority of which are associated with decreased levels of TPMT activity. The predominant variant alleles are TPMT*3A, TPMT*3B, TPMT*3C and to a lesser extent TPMT*2. Approximately 0.3% of the general population has low to absent TPMT enzyme activity, 11% has intermediate levels, and 89% has normal to high levels of the enzyme activity [10]. However, differences in specific mutant allele frequency have been observed between population groups [20].

The aim of the present study was to determine the frequency of TPMT polymorphisms and their activities in the prediction of adverse events, before thiopurine therapy in IBD patients in Iran´s Kurdish western population.

MATERIALS AND METHODS

Patients

The study protocol was approved by the Research Ethics Committee of the Iranian ministry of Health and was in accordance with the World Medical Association Declaration of Helsinki [21]. This case-control study consisted of 210 unrelated UC patients and 212 unrelated healthy individuals recruited from Mahdieh Clinic of Kermanshah Medical University. Peripheral blood was collected from each individual after obtaining a written consent.

TPMT genotyping

Genomic DNA was extracted from 5 mL peripheral blood using the phenol chloroform extraction method as described previously [22]. Primer design and restriction enzyme analysis were performed according to previous study with minor modifications [23]. Allele-specific polymerase chain reaction (PCR) was used to screen for the TPMT*2 G238C mutation. P2C (5' -TAA ATA GGA ACC ATC GGA CAC-3') (reverse) with either P2W (5' - GTA TGA TTT TAT GCA GGT TTG-3') or P2M (5' -GTA TGA TTT TAT GCA GGT TTC-3') (forward) primers were used to amplify and detect wild type or TPMT*2 G238C mutant polymorphism, respectively. The vaspin gene amplification was used as a control (Forward primer; 5'-GGA GGC AGA CCA GGC ACT AGA AA-3', reverse primer; 5'-ACC ATC TCT CTG GCT TCA GGC TTC-3'). The 254 bp PCR products were separated on a 2.5% agarose gel (Figure 1). PCR-RFLP was set up to analyze TPMT G460A (TPMT*3B) and A719G (TPMT*3C) a point mutations. The 694-bp fragment containing nucleotide G460A representing TPMT*3B polymorphism was amplified with P460F (5' -AGG CAG CTA GGG AAA AAG AAA GGT G-3') and P460R (5' -CAA GCC TTA TAG CCT TAC ACC CAG G-3') primers. PCR amplification consisted of an initial denaturing step at 94°C for 5 minutes followed by 33 cycles of denaturing at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension step

was performed at 72°C for 5 minutes. In wild type alleles, *MwoI* digestion of the PCR product yielded 2 fragments of 443bp and 251 bp long (Figure 1).
To analyze TPMT A719G polymorphism, a 373-bp DNA fragment containing nucleotide 719 was amplified with P719F (5' -GAG ACA GAG TTT CAC CAT CTT GG-3') and P719R (5' - CAG GCT TTA GCA TAA TTT TCA ATT CCT C-3') primers. PCR reaction consisted of an initial denaturing step at 94°C for 5 minutes followed by 33 cycles of denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The final extension step was performed at 72°C for 5 minutes. In the presence of mutation, *AccI* digestion of PCR product yielded two fragments of 283 and 90 bp (Figure 1). Samples with one deficient allele (TPMT*1/*2, *1/*3C, *1/*3B, *1/*3A) were genotyped as heterozygous, and samples with two deficient alleles (TPMT*2/*3C, *2/*3B, *3C/*3B, *2/*3A etc.) were genotyped as homozygous. The samples that carried both G460A and A719G mutations were named TPMT *3A (Figure 1).

TPMT phenotyping
The TPMT activity in erythrocytes was determined by High Performance Liquid Chromatography (HPLC) (Agilent Technologies 1200 Series, Germany) using 6-thioguanine as a substrate. The MZ analytical 5 µ reverse-phase HPLC column (C 18, 150 X 4.6 mm Germany) was used as a stationary phase at ambient temperature as previously described by Ford & Berg with minor modification [17].

Sample collection and storage
Blood samples were collected in EDTA containing vials, aliquoted and stored for a maximum of 2 days at 4°C prior to analysis or at -80°C until use.

Statistical analysis
The TPMT genotype frequencies in UC patients were compared to controls using the χ^2 test. Data was analyzed first for normality of distribution by using the Kolmogorov-Smirnov test. Results were expressed as mean ± SD for normally distributed data, median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data. Comparison of groups was carried out with Student's *t*-test, Mann-Whitney U test, and one-way ANOVA as appropriate. The correlation values of TPMT activities in mU/L and in nmol6MTG/gHb/h, Hb levels, BMI, and age of IBD and control groups were calculated using Pearson's correlation. Receiver-Operating Characteristic (ROC) Curve analysis was generated using genotype and phenotype information for the overall concordance rate when using mU/L or nmol6MTG/gHb/h for expressing TPMT activities unit.
Comparison of TPMT activities in mU/L and in nmol6MTG/gHb/h and Hb level between TPMT wild type and mutant genotypes in all samples were calculated using two-tailed Student's *t*-test. Statistical significance was assumed at $p < 0.05$. The SPSS statistical software (SPSS for Windows 16; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

RESULTS

The clinical, laboratory, and demographic characteristics of participants are summarized in Table 1. There was no significant difference between the mean of TPMT activity in mU/L, age, BMI, Hb and gender of the two groups. However, when TPMT was calculated in nmol6MTG/gHb/h, the concentration of TPMT in UC patients was significantly higher than the control group (46.5 (40.9 - 53.8) vs. 45 (41.2 - 50.6), p = 0.026, respectively).
Correlations between TPMT activities in mU/L and in nmol6MTG/gHb/h and Hb level, BMI, and age in UC patients and control group are shown in Table 2. There was a significant negative correlation between TPMT activities calculated based on nmol6MTG/gHb/h and the Hb levels in both UC (r = -0.54, p < 0.001) and control groups (r = -0.27, p < 0.001). Interestingly, a significant positive correlation between Hb levels and TPMT activities were seen when the enzyme activity was calculated in mU/L in both UC patients (r = 0.14, p = 0.05) and in control subjects (r = 0.43, p < 0.001). These data suggested that the level of TPMT activity when calculated in nmol6MTG/gHb/h may be misleadingly high in individuals with low Hb concentration. To confirm this idea, we divided the UC patients and control group into two subgroups based on Hb level less than 12 and ≥ 12 g/dL and calculated their TPMT activity in mU/L and in nmol6MTG/gHb/h (Table 3). As predicted, the analyses showed that in both UC patients and the control group, individuals with Hb < 12 g/dL had higher TPMT activities when measured in nmol6MTG/gHb/h than individuals having Hb level ≥ 12 g/dL (57.2 (54.7 - 68) vs. 45.4 (40.5 - 50.1), p < 0.001, and 55.6 (48.8 - 60) vs. 44.6 (40 - 49.3), p < 0.001). Thus, test results may show falsely high TPMT activity when it is measured in nmol6MTG/gHb/h.
The overall concordance rate between TPMT phenotypes and genotypes of mutant alleles based on receiver-operating characteristic (ROC) curve was demonstrated in Figure 2. We found a sensitivity of 94.7% and specificity of 90% for mU/L and a sensitivity of 85.6% and specificity of 90% for nmol6MTG/gHb/h.
Frequencies of TPMT genotypes in UC patients, control group, and total samples are shown in Table 4. We detected only 9 TPMT mutant genotypes (9/422; 2.2%) in all samples studied. The frequency of the TPMT genotypes in UC patients were: 1% TPMT*1/*3C (2/210), 1% TPMT*1/*3B (2/210), and 1% TPMT*1/*3A (2/210) and in control group was 1.4% TPMT*1/*3C (3/212). TPMT*1*3A, and TPMT*1*3B alleles were also detected in UC patients, but no TPMT*1*2 allele was observed in either group.

Table 1. The demographic characteristics and distribution of the thiopurine methyltransferase (TPMT) activities and other risk factors in inflammatory bowel disease (IBD) patients and control group in a population from Kermanshah province.

	IBD patient (n = 210)	Control subjects (n = 212)	p-values
Age (years)	35.9 ± 13.2	34 ± 14.2	0.58
Gender (M/F)	86/124	96/116	0.27
TPMT activity (nmol6MTG/gHb/h)	46.5 (40.9 - 53.8) *	45 (41.2 - 50.6)	0.026
TPMT activity (mU/L)	108.9 ± 20.1	111.7 ± 19.8	0.15
Hb (g/dL)	13.8 ± 1.85	14.6 ± 1.73	< 0.001
BMI (Kg/m^2)	24 ± 3.97	24.1 ± 4.51	0.87

6MTG - 6methylthioguanine.
* Median and interquartile range (IQR) for non-normally distributed data and percentages for categorical data.

Table 2. Correlation of TPMT activities in mU/L and nmol6MTG/gHb/h with Hb concentration, BMI, and age in IBD patients and control group.

	IBD patients					Control group				
	TPMT activity (mU/L)	TPMT activity (nmol6MTG/ gHb/h)	Hb g/dL	BMI	Age	TPMT activity (mU/L)	TPMT activity (nmol6MTG/ gHb/h)	Hb g/dL	BMI	Age
TPMT activity (mU/L)	r = 1	r = 0.72 p < 0.001	r = 0.14 p = 0.05	r = 0.03 p = 0.7	r = 0.05 p = 0.5	r = 1	r = 0.73 p < 0.001	r = 0.43 p < 0.001	r = -0.07 p = 0.9	r = -0.15 p = 0.029
TPMT activity (nmol6MTG/ gHb/h)	r = 0.72 p < 0.001	r = 1	r = -0.54 p < 0.001	r = -0.08 p = 0.3	r = -0.07 p = 0.34	r = 0.72 p < 0.001	r = 1	r = -0.27 p < 0.001	r = -0.02 p = 0.77	r = -0.08 p = 0.26
Hb g/dL	r = 0.14 p = 0.05	r = -0.54 p < 0.001	r = 1	r = 0.12 p = 0.1	r = -0.04 p = 0.54	r = 0.43 p < 0.001	r = -0.27 p < 0.001	r = 1	r = 0.03 p = 0.7	r = -0.07 p = 0.3
BMI (Kg/m^2)	r = 0.03 p = 0.68	r = -0.08 p = 0.3	r = 0.12 p = 0.1	r = 1	r = 0.3 p < 0.001	r = -0.01 p = 0.9	r = -0.02 p = 0.77	r = 0.03 p = 0.7	r = 1	r = 0.4 p < 001
Age (years)	r = 0.05 p = 0.5	r = 0.07 p = 0.34	r = -0.04 p = 0.6	r = 0.3 p < 0.001	r = 1	r = -0.15 p = 0.029	r = -0.08 p = 0.26	r = -0.07 p = 0.3	r = 0.4 p < 001	r = 1

Table 3. Comparison of TPMT activities in mU/L and nmol6MTG/gHb/h, Hb concentration, and BMI between subjects with Hb concentration less than and more than 12 g/dL in IBD patients and control group separately.

	Control group		IBD patients	
	Hb < 12 g/dL n = 13	Hb ≥ 12 g/dL n = 199	Hb < 12 g/dL n = 31	Hb ≥ 12 g/dL n = 179
TPMT activity (mU/L)	101 ± 12.4	112.3 ± 19.9	101.9 ± 20.2	110 ± 19.6
	p = 0.036		p = 0.041	
TPMT activity (nmol6MTG/gHb/h)	55.6 (48.8 - 60) *	44.6 (40 - 49.3) *	57.2 (54.7 - 68) *	45.4 (40.5 - 50.1) *
	p < 0.001		p < 0.001	
Hb g/dL	12.4 (11.6 - 12.75) *	15 (14.2 - 16 *)	12.1 (11.2 - 12.5) *	14.4 (13.7 - 15.6) *
	p < 0.001		p < 0.001	
BMI (Kg/m^2)	23.7 ± 3.4	24.1 ± 4.6	23.1 ± 3.8	24.3 ± 4
	p = 0.78		p = 0.15	

* Median and interquartile range (IQR) for non-normally distributed data and percentages for categorical data.

Table 4. Frequencies of thiopurinemethyltransferase (TPMT) genotypes in patients with IBD and control group.

TPMT Genotype	SNP position & amino acid substitution	Number of IBD patients (n = 210)	Number of control group (n = 212)	% of samples study (n = 422)
TPMT*1/*2	238 G>C Ala80Pro	0%	0%	0%
TPMT*1/*3C	719 A>G Tyr240Cys	2/210 (1%)	3/212 (1.4%)	5/422 (1.2%)
TPMT*1*/3B	460 G>A Ala154Thr	2/210 (1%)	0%	2/422 (0.5%)
TPMT*1/*3A	460 G>A and 719A>G Ala154Thr and Tyr240Cys	2/210 (1%)	0%	2/422 (0.5%)
TPMT*1/*1	wild type	206/210 (98%)	207/212 (98.2%)	413/422 (97.8%)

Table 5. Comparison of TPMT activities in mU/L and nmol6MTG/gHb/h and Hb concentration between TPMT wild and mutant genotypes in all samples of the study.

	TPMT wild genotypes	TPMT mutant genotypes	p-value
TPMT activity (mU/L)	110.8 ± 19.4	63.4 ± 14.9	< 0.001
TPMT (nmol6MTG/gHb/h)	46.7 ± 9.02	25.5 ± 5.6	< 0.001
Hb (g/dL)	14.2 ± 1.8	14.1 ± 1.5	0.93

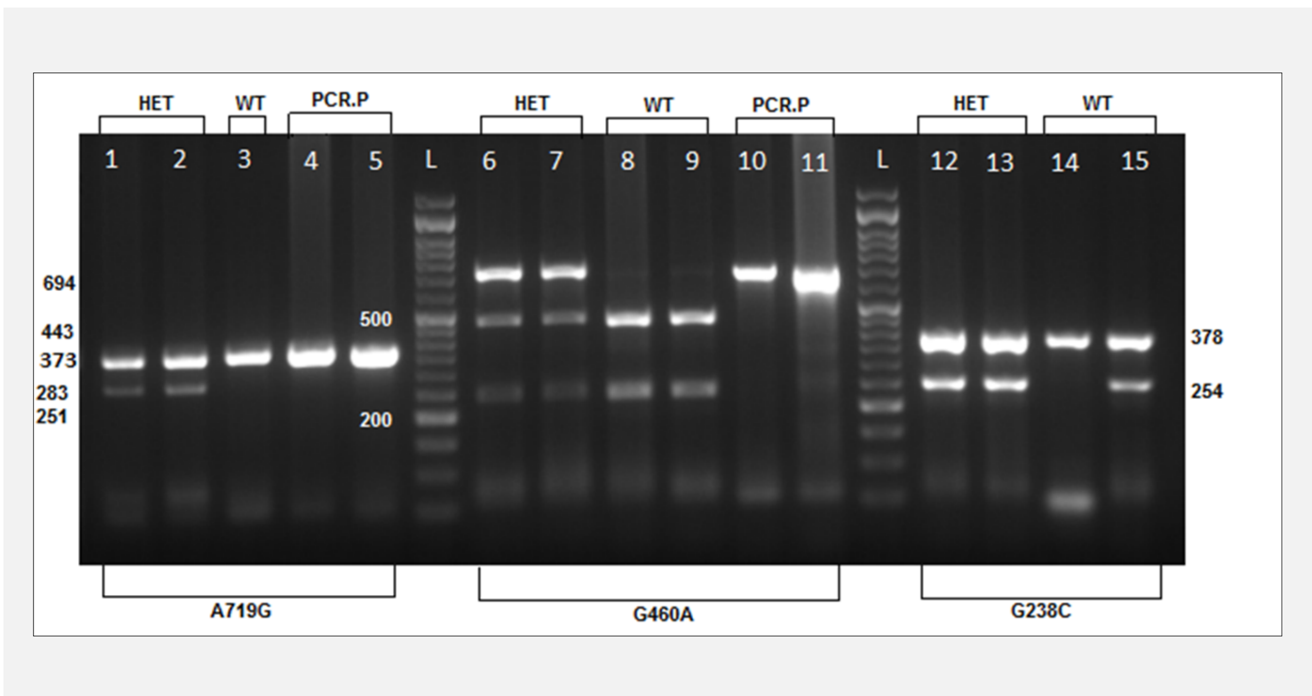
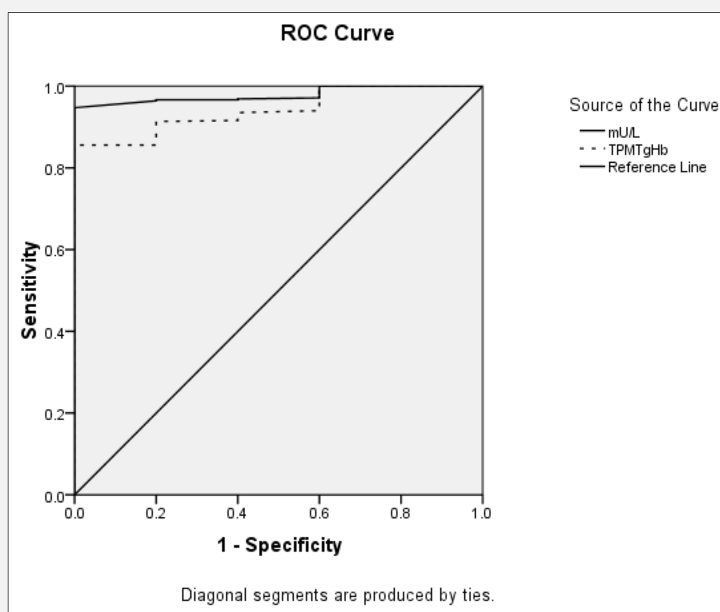


Figure 1. Agarose gel electrophoresis of TPMT alleles as analyzed by PCR-RFLP and allele-specific PCR.

L - 50 bp DNA ladder, HET - heterozygous mutant, WT - homozygous wild type, PCR.P - PCR product. The PCR fragments were separated on a 2.5% agarose gel electrophoresis.



Area under the curve					
Test Result Variable(s)	Area	Std. error [a]	Asymptotic sig. [b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
TPMT mU/L	0.978	0.010	0.000	0.959	0.998
TPMT nmol6MTG/gHb/h	0.942	0.026	0.001	0.891	0.992

[a.] Under the nonparametric assumption.
[b.] Null hypothesis: true area = 0.5.

Figure 2. Receiver-Operating Characteristic (ROC) Curve analysis was generated using genotype and phenotype information for the overall concordance rate when using mU/L or nmol6MTG/gHb/h for expressing TPMT activities unit.

The Hb level and TPMT activities in both mU/L and in nmol6MTG/gHb/h in individuals carrying the wild type TPMT gene were compared with those carrying mutant genotypes of TPMT in Table 5. Individuals with mutant genotypes of TPMT had significantly lower TPMT activities in mU/L and in nmol6MTG/gHb/h than those with wild type genotype of TPMT.

DISCUSSION

This study was designed to evaluate TPMT enzyme activity and its correlation with TPMT genotypes in patients with UC in Iran's western population. To the best of our knowledge, this is the first analysis of phenotype-genotype correlation of TPMT performed in UC patients and healthy individuals with the same ethnic background. It is commonly accepted that 1 in 300 (0.3%) individuals has TPMT deficiency, 11% of the population have low TPMT activity, and TPMT activity is normal in approximately 89% of individuals. In our study, this clearly defined trimodal frequency distribution of TPMT was not demonstrated. We found that 97.8% of the population in western Iran had normal TPMT activity, 2.2% of the population had intermediate activity and no one had low TPMT activity. The frequency of the known mutant of TPMT alleles in western Iran is low and TPMT*3C appears to be the most prevalent among the mutant TPMT alleles tested in this population. These data suggest that the risk of toxicity of thiopurine drugs is low to non-existence in patients with UC from this region.
To determine the toxicity of thiopurine drugs, the majority of the centers around the world are using the TPMT activity in relation to hemoglobin level or RBC content of the blood cell lysate. Here, we found a signif-

icant negative correlation between TPMT activity in nmol6MTG/gHb/h and the Hb levels in both healthy and UC groups. In addition, we found that correlation of TPMT activity with toxicity of thiopurine drugs in IBD patients is significantly affected by hemoglobin level. TPMT activity is significantly greater in UC patients than in the control group when it is calculated in nmol6MTG/gHb/h, whereas, when TPMT activity is calculated in mU/L, no statistically significant difference was seen between the two study groups. The observed difference is due to a lower hemoglobin level in UC patients than control group. In support of this theory we divided IBD patients and control group into two subgroups based on Hb level, less than 12 and ≥ 12 g/dL. We noticed that patients with low hemoglobin level had misleadingly high TPMT when it was measured in nmol6MTG/gHb/h; however, this was not seen when results were expressed as mU/L TPMT. This data highly recommends that TPMT activity be calculated and reported in mU/L instead of nmol6MTG/gHb/h to avoid the influence of Hb level on the results.
In addition, ROC curves allow the establishment of cut points that maximize sensitivity and specificity [24], so the overall concordance rate between TPMT phenotypes and genotypes of mutants alleles (9 out of 422) showed a sensitivity of 94.7% and specificity of 90% with mU/L and a sensitivity of 85.6% and specificity of 90% with nmol6MTG/gHb/h units.

CONCLUSION

Results of this study suggest that expressing TPMT activity in mU/L is more appropriate than in nmol6MTG/gHb/h. There is a better correlation between genotypes and phenotypes of TPMT based on mU/L. The frequency of the known mutant of TPMT alleles in western Iran is low suggesting a low risk of thiopurine drugs toxicity in IBD patients from this region. This information might be of value to gastroenterologists, oncologists, dermatologists, rheumatologists, and other clinicians who prescribed thiopurine derivatives to patients from this area of Iran.

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Declaration of Interest:
No potential conflicts of interest relevant to this article were reported.

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