Original Research

A novel single-tube multiplex real-time PCR assay for genotyping of thiopurine intolerance-causing variant *NUDT15* c.415C>T

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Impact statement

NUDT15 c.415C>T is a validated pharmacogenetic marker for predicting thiopurine-induced myelosuppression, especially for East Asians. The genetic testing of *NUDT15* c.415C>T is recommended before initiating thiopurine therapy. Using allele-specific TaqMan probes, this study developed a single-tube multiplex real-time PCR assay for *NUDT15* c.415C>T genotyping, which can simultaneously amplify the target gene and the internal control gene in a single reaction. With the advantage of reliability, fastness, and cost-effectiveness, this method can be used as a routine test in clinical settings to guide thiopurine prescription. Moreover, using this method, the distributions of this variant in four Chinese populations were determined, which could provide useful information for personalized medicine of different ethnic groups.

Abstract

Thiopurines are commonly used in the treatment of acute lymphoblastic leukaemia and autoimmune conditions, can be limited by myelosuppression. The *NUDT15* c.415C>T variant is strongly associated with thiopurine-induced myelosuppression, especially in Asians. The purpose of this study was to develop a fast and reliable genotyping method for *NUDT15* c.415C>T and investigate the polymorphic distribution among different races in China. A single-tube multiplex real-time PCR assay for *NUDT15* c.415C>T genotyping was established using allele-specific TaqMan probes. In 229 samples, the genotyping results obtained through the established method were completely concordant with those obtained by Sanger sequencing. The distributions of *NUDT15* c.415C>T among 173 Han Chinese, 48 Miaos, 40 Kazakhs, and 40 Kirghiz were different, with allelic frequencies of 0.06, 0.02, 0.07, and 0, respectively. This method will provide a powerful tool for the implementation of the genotyping-based personalized prescription of thiopurines in clinical settings.

Keywords: *NUDT15*, thiopurine, real-time PCR, genotyping, pharmacogenomics, adverse drug reaction

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Introduction

Thiopurines, including azathioprine, mercaptopurine, and thioguanine, are purine analogs that exert cytotoxic effects when converted to thioguanine nucleotides.[1,2] Clinically, thiopurines are widely used in the treatment of acute lymphoblastic leukaemia (ALL) and autoimmune disease.[3,4] However, thiopurine treatment is limited by myelosuppression, which may lead to the frequent interruption of treatment and cause complications, such as severe neutropenia and sepsis, and even death.[5–7] During medication 25–30% of children with ALL have leukopenia, and thus their treatment is stopped and the disease recurs.[8] Moreover, approximately 5% of European patients with inflammatory bowel diseases (IBDs) using thiopurines develop leucopenia.[9] This condition leads to the discontinuation of therapy in up to 34% of such patients[9] and subsequent disease recurrence.[6,10–12]

A close relationship between thiopurine-induced myelosuppression (TIM) and genetic polymorphisms has been documented. The first validated biomarker is thiopurine S-methyltransferase (TPMT) genetic polymorphism. TPMT can convert thiopurine to methyl thiopurine, which is non-toxic and less active.[13,14] A number of variants that impair TPMT enzymatic activity are associated with

thiopurine-induced toxicity.[5,15,16] Approximately 10% of individuals of European descent carry non-functional *TPMT* variants. By contrast, only 3% of Asians carry *TPMT* genetic variants despite that Asian patients with IBD have higher incidence of TIM (15%).[17–20] In Asia, some patients with normal TPMT activity still have significant toxicity during treatment.[18,19] Thus, variability in TIM is not fully attributed to *TPMT* genetic polymorphism, that is, other factors are implicated in TIM.

Nucleoside diphosphate-linked moiety X-type motif 15 (NUDT15) belongs to the nudix hydrolase enzyme family and can inactivate thiopurine metabolites and decrease their cytotoxicity *in vitro*.[21] Recent genome-wide association studies have revealed that a missense genetic variant in *NUDT15* gene (c.4115C>T, p.Arg139Cys, rs116855232) is strongly associated with TIM in Korean patients with IBD[22] and in East Asian children with ALL.[8] Similar conclusions were replicated in studies performed on Chinese, Japanese, and Indian populations.[8,23,24] The frequency of *NUDT15* c.415C>T is much more common in Asians (around 10%) than in individuals of European origin (up to 2%). Moreover, the tolerable dosage during maintenance therapy with MP in patients suffering from ALL and carrying the *NUDT15* c.415C>T variant is much lower than that in patients carrying the wild-type genotype.[23,25] These findings suggest that *NUDT15* c.415C>T can be used as a promising pharmacogenetic marker for TIM prediction, especially for East Asians. The genetic testing of *NUDT15* c.415C>T is recommended before initiating thiopurine therapy.[26]

Fluorescent real-time PCR, which is carried out in a closed-tube format, has become a very important tool for gene quantification and genetic analysis. With the availability of more sensitive fluorescent dyes, multiple target sequences can be detected in a single reaction, which greatly improves the detection efficiency and reduces experimental cost.[27,28] In this study, using allele-specific TaqMan probes, a single-tube multiplex real-time PCR assay for *NUDT15* c.415C>T genotyping was established, and the distributions of *NUDT15* c.415C>T among four different nationalities in China were determined.

Materials and methods

Samples

Blood samples from 301 unrelated healthy individuals from four different ethnicities in China (173 Han Chinese from Shaanxi Province, 48 Miaos from Yunnan Province, and 40 Kazakhs and 40 Kirghiz from Xinjiang Province). The study was approved by the ethics committees of Northwest University and Shaanxi Provincial Peoples' Hospital. Informed consent was obtained from all participants, in accordance with the Helsinki Protocol.

Genomic DNA extraction

Genomic DNA samples were extracted from 200 μL of ethylenediaminetetraacetic acid anticoagulated peripheral blood with a QIAamp DNA Blood Mini Kit (Qiagen, Shanghai, China). DNA samples were quantified with a NanoDrop spectrophotometer (Thermo Scientific, WI, USA). The extracted genomic DNA was dissolved in an elution buffer TE (10 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid, pH 9.0) and stored at −20°C.

Oligonucleotide primers and probe design

We obtained the information about the rs116855232 from the NCBI database. Based on the sequence data, a forward primer (Fp) and a reverse primer (Rp) were designed for the amplification of a 163-bp DNA fragment encompassing c.451C/T in *NUDT15* gene. For the identification of different genotypes of *NUDT15* c.415C>T, two allele-specific and differently labeled TaqMan probes were designed. One probe (probe-T) labeled with 6-carboxyfluorescein (FAM) was used in the detection of the mutant allele (c.415T), and the other (probe-C) labeled with cyanine dye 5 (CY5) was used in the detection of wild-type allele (c.415C). In addition, a set of primers and probes amplifying the housekeeping gene β-actin (*ACTB*) was designed as the internal control, and the 5′-ends of the probes for *ACTB* were labeled with 6-carboxy-hexachlorofluorescein (HEX; Table 1). Primers and probes were designed following the criteria in designing primers and probes for quantitative real-time PCR methods.[29]

In silico tools, such as primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and uMelt batch (https://www.dna.utah.org/umelt/quartz/umb.php), were used to analyze the melting temperature of primers and probes, check for non-specific hybridization, and assess the formation of secondary structures. Moreover, cross-interactions between primers and probes were evaluated by PCR reactions with one probe (probe-T or probe-C) and one Rp or with two probes (probe-T and probe-C). The reaction system was same as that described in the following real-time PCR

Table 1. Sequences of primers and probes used in this study.

Genes	Primers and Probes	Sequence and modifications (5′–3′)
NUDT15	Fp	GCATCTTTCTTTTCTAGGTTGGGA
	Rp	TCTTCTTTAAATGGATCATAGCCTTG
	Probe-T	FAM-TTCTGGGGACTGTGTTGTTTAAAAGAAC-BHQ2
	Probe-C	Cy5-TTCTGGGGACTGCGTTGTTTAAAAGAAC-BHQ2
ACTB	Fp	CAGCAGATGTGGATCAGCAAG
	Rp	GCATTTGCGGTGGACGAT
	Probe-ACTB	HEX-AGGAGTATGACGAGTCCGGCCCC-BHQ2

Fp: forward primer; Rp: reverse primer; FAM: 6-carboxyfluorescein; BHQ2: Black Hole Quencher-2; CY5: cyanine dye 5; HEX: 6-carboxy-hexachlorofluorescein.

reaction. Gel electrophoresis analysis on the PCR products was used to investigate the non-specific amplification between primers and probes. Under these above measures, high-quality and specific primers and probes were obtained. All the primers and fluorescent probes were synthesized by Sangon Biotech. Co. Ltd (Shanghai, China).

Real-time PCR reaction

The real-time PCR reactions were carried out on Applied Biosystems ViiA™ 7 Real-Time PCR system (Applied Biosystems, CA, USA). All PCR reactions were performed in one tube for the simultaneous amplification of *NUDT15* c.415C>T and *ACTB* gene.

The 20 μL reaction mixture is composed of 250 nM *NUDT15* Fp and Rp, 75 nM *NUDT15* probe-T, 150 nM *NUDT15* probe-C, 100 nM *ACTB* Fp and Rp, 50 nM *ACTB* probe, 20 ng of genomic DNA (20 ng/μL), and 10 μL of 2× Premix Ex Taq (1 U/μL, TaKaRa, Dalian, China). The desired region was amplified by optimizing the PCR reaction at the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 62°C for 40 s. A non-template control was included in each run. Raw data obtained from real-time PCR were analyzed using the amplification-based threshold and the adaptive baseline algorithms provided by the software analysis system of the ViiA™ 7 machine (version 1.1, Applied Biosystems). In all the PCR experiments, at least three biological replicates were performed, and each reaction was run in triplicate.

Performance evaluation of the multiplex real-time PCR assay

The accuracy of the newly established multiplex real-time PCR assay was evaluated by comparing the genotyping results of 229 human DNA samples with those by Sanger sequencing. The detection limits for the established assay were obtained using the following dilutions prepared from *NUDT15* c.415C>T heterozygous positive DNA samples: 10, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 ng/μL. A negative control without DNA template was set up. At least three replicates were performed for each DNA template.

Statistical analysis

Data analysis was performed using SPSS version 20.0 (SPSS, IL, USA). Fisher's exact test was used in determining whether ethnic background influences the distribution of *NUDT15* c.415C>T.

Results

Performance of the multiplex real-time PCR assay

Three standard samples containing different genotypes (wild type: CC, heterozygote: CT, homozygote: TT) of the *NUDT15* c.415C>T variant were used in establishing the multiplex real-time PCR assay. The standard samples were screened from a number of human samples by Sanger sequencing (Supplemental Figure 1).

A simple real-time assay for the simultaneous amplification of *NUDT15* c.415C>T and *ACTB* in one sample was achieved in one single reaction. This method allows the direct visualization of results by three distinct fluorescence signals. The green fluorescence signals of the internal control gene (*ACTB*) were observed in all the samples, indicating that the reactions were successful. When only blue or red fluorescence signal appears in the reaction, the genotype of the detected sample was regarded as wild type (Figure 1(a)) or mutant homozygote (Figure 1(c)), and when both blue and red fluorescence signals occurred, the genotype of the detected sample was regarded as heterozygote (Figure 1(b)).

Limits of detection analysis

A sample with *NUDT15* c.415 CT genotype was tested at different dilutions. At a range of 0.01 − 10 ng DNA templates, the fluorescent signals of the wild-type and mutant templates were detected (Figure 2). Thus, the lowest detection limit of this method is 0.01 ng. However, a higher DNA input is recommended (0.1 − 10 ng) for reliable genotyping.

Accuracy validation of the multiplex real-time PCR assay

We compared the genotyping results of 229 DNA samples with those obtained by Sanger sequencing to verify the accuracy of the established assay. Of these samples, 209 were wild type, 19 were heterozygous, and 1 was homozygous mutant according to the Sanger sequencing results. The genotyping results of the multiplex real-time PCR assay and Sanger sequencing were in complete concordance, and no false positive or false negative results were obtained. The results show that the accuracy of the method developed in this study is 100%.

Distribution of *NUDT15* c.415C>T in different ethnic groups

Using the established method, 301 samples from four ethnic groups in China were screened. The data showed that the allelic frequencies of *NUDT15* c.415C>T in 173 Han Chinese, 48 Miaos, 40 Kazakhs, and 40 Kirghiz were 0.06. 0.02, 0.07, and 0, respectively. The allelic frequencies of *NUDT15* c.415C>T in Han and Kazak were significantly higher than the allelic frequency in Kirgiz ($P < 0.05$). Most of the variant carriers were heterozygotes, and only one was mutant homozygote (Table 2).

Discussion

Thiopurines (e.g. 6-MP) are primarily used in ALL treatment, but can be limited by treatment-related myelosuppression, which may be life-threatening. Recommendations regarding *TMPT* testing to guide thiopurine therapy have already been implemented in clinical practice.[30] A growing body of evidence demonstrated that testing for *NUDT15* polymorphisms may be particularly advantageous in East Asian patients, given the low prevalence of toxicity-associated *TPMT* alleles in this population

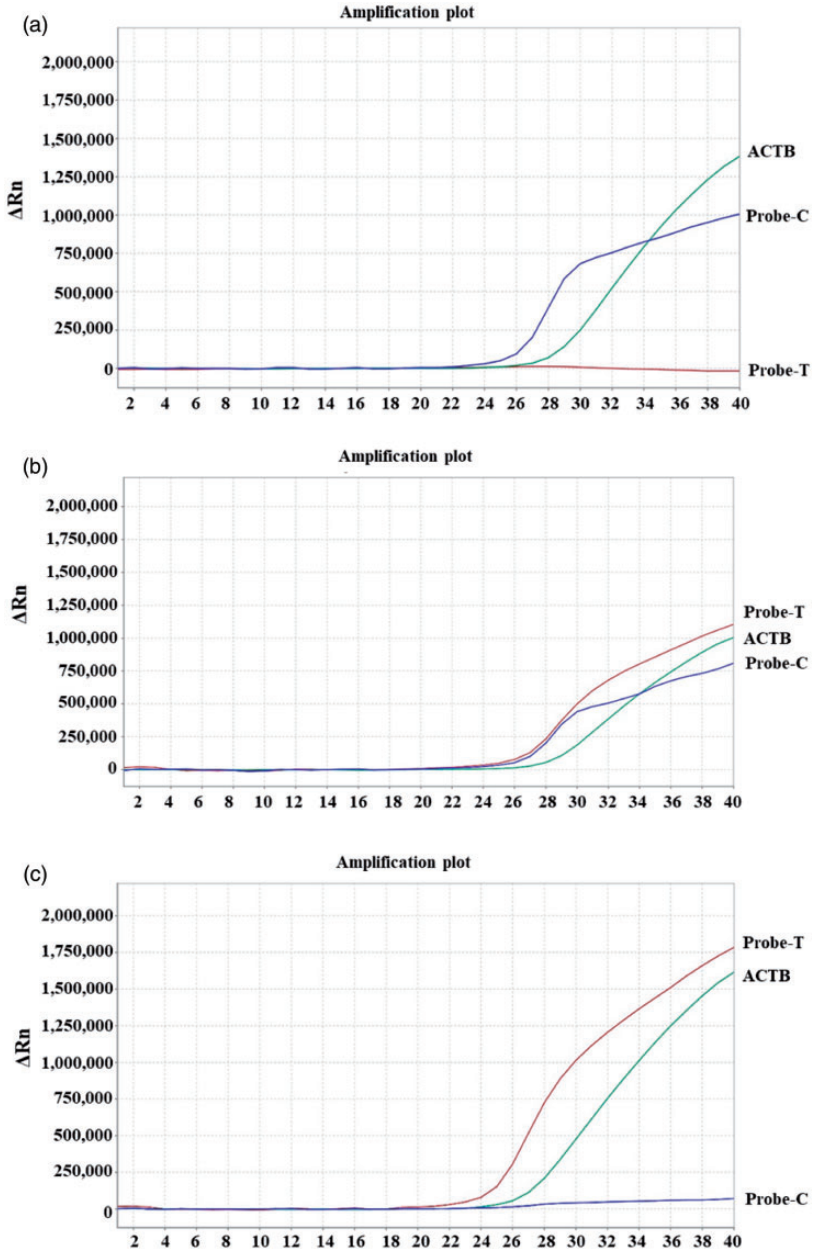

Figure 1. The amplification curves of standard samples with different genotypes of *NUDT15* c.415C>T. (a) Wild-type sample with CC genotype; (b) heterozygous mutant sample with CT genotype; (c) homozygous mutant sample with TT genotype. (A color version of this figure is available in the online journal.)

and the much higher frequency of the *NUDT15* risk allele.[17–20,23,24] Therefore, prospective screening of *NUDT15* variant before initiating thiopurine treatment should also be incorporated into clinical practice to minimize the occurrence of TIM. A convenient genotyping method for *NUDT15* c.415C>T detection is highly useful in the implementation of pharmacogenomic testing for clinical care.

In this study, using multiple allele-specific TaqMan probes, a fast and reliable genotyping method for thiopurine intolerance biomarker *NUDT15* c.415C>T was established. Compared with other available methods, this novel method offers a number of advantages (Supplemental Table 1).

First, Sanger sequencing is regarded as the "gold standard" in determining DNA sequence variations, especially single point mutations.[31] The genotyping results of the established multiplex real-time PCR were completely accordant with those of Sanger sequencing, validating the reliability and accuracy of the established method. In contrast to Sanger sequencing, which is expensive and requires a long processing time, the established method has the advantages of short detection time (1–2 h), low detection cost (about 3 dollars), simple operation, and straightforward result interpretation. Second, although PCR-restriction fragment length polymorphism (PCR-RFLP)[31] and tetra-primer ARMS-PCR assays[32] are low cost and easy to operate, they require the separation of amplified

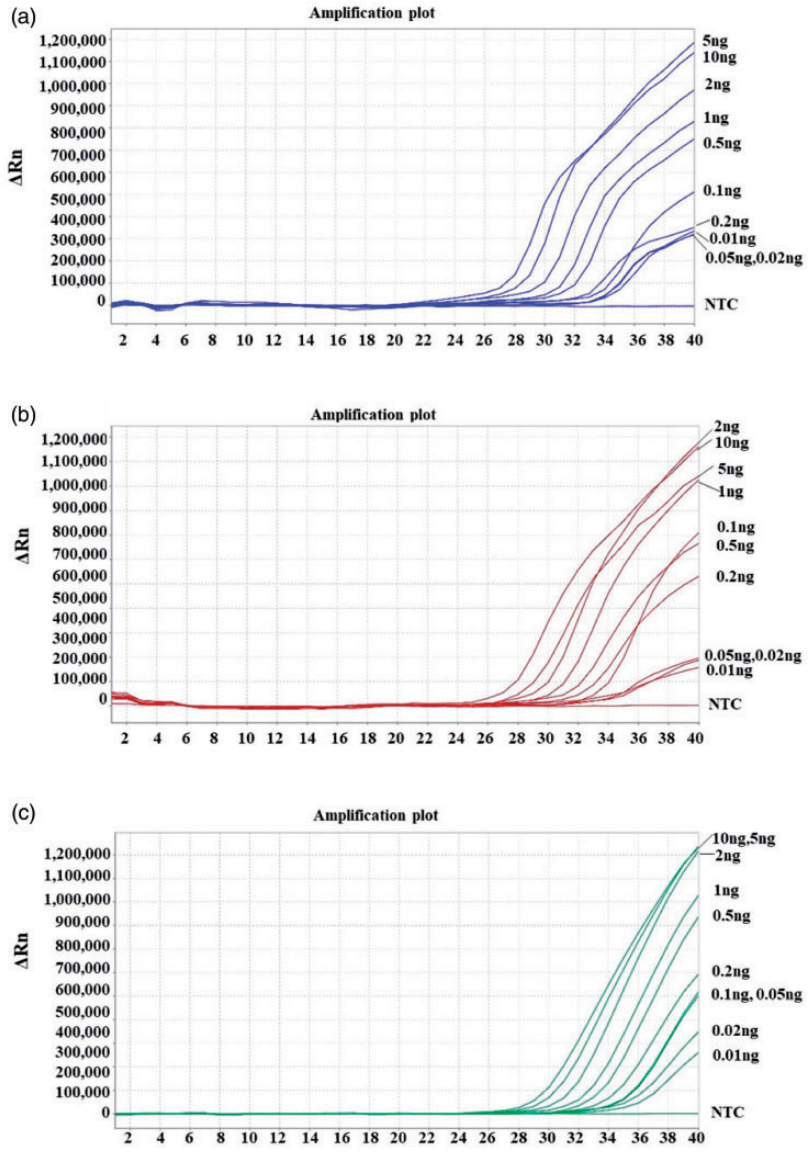

Figure 2. The detection sensitivity of the TaqMan PCR assay for *NUDT15* c.415C>T variant. (a) The amplification curve of wild-type template of *NUDT15* c.415C>T; (b) the amplification curve of mutant template of *NUDT15* c.415C>T; (c) the amplification curves of *ACTB* gene. NTC: non-template control. (A color version of this figure is available in the online journal.)

Table 2. Genotyping results of *NUDT15* c.415C>T in four ethnic groups.

Genotype	Han (*n*)	Miao (*n*)	Kazak (*n*)	Kirgiz (*n*)
CC	154	46	34	40
CT	18	2	6	0
TT	1	0	0	0
Total	173	48	40	40
C allele frequency	0.94	0.98	0.93	1.00
T allele frequency	0.06	0.02	0.07	0.00

products through gel electrophoresis, which cause the frequent occurrence of false positive results and DNA template contamination. Compared with these two methods, the multiplex real-time PCR has a slightly higher cost, but given that the reaction tubes do not need to be opened after amplification, the potential contamination of amplicons is prevented, and thus the risk of false-positive results is considerably reduced. Third, real-time PCR-high resolution melt (PCR-HRM) and TaqMan PCR are both quantitative closed-tube real-time PCR techniques. The difference is that the former uses a high-quality (saturating) double-stranded DNA dye, and the latter uses a TaqMan fluorescent probe, which made the cost of TaqMan PCR higher than HRM. The base of HRM analysis lies in the discrimination of difference in melting temperature between amplification products with mutation alleles and those without. However, other non-targeted mutations in the amplified fragment cause changes in Tm values, and the interpretation of the result is affected or even misinterpreted.[32,33] Furthermore, our established method enables the simultaneous detection of the amplification signals of a targeted gene and internal control in a single reaction by using three TaqMan probes labeled with different fluorophores. In the HRM method, the detection of internal control gene must be performed in

another reaction. The sensitivity of our established assay is quite high, with a limit of detection of 0.01 ng of input DNA, which is much lower than the values obtained through previously reported methods.

Currently, the major genetic factors that can explain the inter-individual variability of TIM vary with ethnicity. *TPMT* genetic polymorphism is strongly associated with TIM in patients with European ancestry, whereas *NUDT15* is strongly associated with TIM in East Asian and Hispanic populations.[22,34–36] This difference among the ethnicities may be partly explained by the varied distributions of *TPMT* and *NUDT15* genetic variants in different populations. *TPMT* non-functional variants are common in European descents but rare in Asians, and *NUDT15* variants are common in Asians but rare in Europeans.[17,19,20] Therefore, investigating the distribution of *NUDT15* c.415C>T among different ethnic populations is helpful for the implementation of *NUDT15* genotyping-based personalized treatments using thiopurines in clinics. China is a multiethnic country with 56 ethnic groups, and significant genetic diversity exists among these ethnic groups. The present study determined the prevalence of *NUDT15* c.415C>T in four different ethnic groups. The results showed that *NUDT15* c.415C>T is common in Han Chinese and Kazak populations but rare or even absent in Miao and Kirgiz populations. The allelic frequency in our studied Han Chinese subjects is consistent with the allelic frequencies reported in other Chinese populations.[37] Kazak and Kirgiz subjects are recruited from Xinjiang Province along the Silk Road, where extensive genetic mixture was observed among East Asians and Western Eurasians.[38] Studies using short tandem repeat analysis demonstrated that the genetic structures of Kazak and Kirgiz are similar to the genetic structure of Uygur.[38] *NUDT15* c.415C>T was not detected in the Kirgiz subjects. This finding is comparable to the data reported in CEU population in the 1000 Genome Project. However, the different distributions of *NUDT15* c.415C>T in Kazak and Kirgiz indicates that the genetic backgrounds of these ethnic groups are not completely the same. Miao, a representative of the southern minorities of China, is genetically related to Southeast Asian populations, such as Dai from Yunnan Province.[35,37–39] The allelic frequency of *NUDT15* c.415C>T in Miao individuals is similar to that reported in Dai (0.048) in the 1000 Genome project. All these data demonstrated genetic diversity in the distribution of *NUDT15* c.415C>T in different Chinese ethnicities, suggesting a different molecular mechanism underlying the variability of TIM in different Chinese races. Further genetic association studies should be conducted in more ethnic groups for the elucidation of genetic factors responsible for TIM in different populations.

Conclusions

In summary, a single-tube multiplex real-time PCR assay for *NUDT15* c.415C>T genotyping was developed. With the advantage of reliability, fastness, sensitivity, and cost-effectiveness, this method can be used as a routine test in clinical settings to implement *NUDT15* genotyping for the prevention of TIM in Asian populations. Our data showed that the distribution of the *NUDT15* c.415C>T variant varies among different Chinese ethnic populations, providing additional information for population pharmacogenomics. Further research should be conducted on patient samples for the evaluation of the analytic performance and pharmacoeconomic value of this method in clinical practice.

AUTHORS' CONTRIBUTIONS

HJW and XYL conceived and designed the research; XYL and YL collected the samples; YWL, KCU, WXW, and SYM performed the experiments; YWL and JYM analyzed the data; HJW and XYL wrote the article. All authors read and approved the final version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study was in compliance with Declaration of Helsinki principle and was approved by the Ethics Committee of Northwest University (China). Informed consent was received before the blood samples were collected.

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SUPPLEMENTAL MATERIAL

Supplementary material for this article is available online.

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