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RESEARCH ARTICLE

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Development and validation of T-ARMS-PCR to detect CYP2C19*17 allele

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Abstract

Background: *CYP2C19*17* (rs12248560) is a functional single nucleotide polymorphism (SNP) in the *CYP2C19* gene. It has been shown that *CYP2C19*17* is associated with the clinical outcome of some drugs metabolized by CYP2C19 and a decreased risk of some diseases. The aim of this study was to develop a reliable and simple method to detect this polymorphism.

Methods: Tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) was used to detect the *CYP2C19*17* polymorphism. A total of 93 samples were screened by this method, and the results of T-ARMS-PCR were validated by DNA sequencing.

Results: There were 91 samples with the CC genotype (97.8%) and two samples with the CT genotype (2.2%). The frequency of the C allele was 98.9%, and the frequency of the T allele was 1.1%. The DNA sequencing results were completely concordant with the T-ARMS-PCR results.

Conclusion: T-ARMS-PCR can detect the *CYP2C19*17* polymorphism with high accuracy, low costs, and a simple process.

KEYWORDS
CYP2C19, *CYP2C19*17*, genotyping, single nucleotide polymorphism, T-ARMS-PCR

1 | INTRODUCTION

P450 cytochromes (CYPs) are a superfamily of enzymes with an absorbance peak of 450 nm. They play critical roles in xenobiotic detoxification, drug metabolism, and natural product biosynthesis.[1] CYP2C19 is a member of P450 family of proteins and is involved in activating/inactivating reactions of a wide spectrum of drugs, such as antidepressants, antiplatelets, antihypertensives, anticancers, antiulcers, and anticoagulant drugs.[2] The human *CYP2C19* gene is located on chromosome 10q24.1-q24.3, and 35 alleles have been identified. CYP2C*19*17* is one of the single nucleotide polymorphisms (SNPs) in the *CYP2C19* gene and the only SNP that possesses increased function of CYP2C19 (https://www.pharmvar.org/gene/CYP2C19). It is characterized by a −806 C > T change (rs12248560) in the promoter region, and its carriers have an ultrarapid metabolization rate of substrates.[3] The mechanism of *CYP2C19*17* affecting metabolization was shown to be an increase in *CYP2C19* expression level. It was shown that the heterozygotes and homozygotes of *CYP2C19*17* had 1.8-fold and 2.9-fold increased *CYP2C19* mRNA levels over *CYP2C19*17* noncarriers in the liver, respectively.[4] A study showed that a specific nuclear protein bound to the element carrying −806T, but not −806C, which consequently caused the increased transcriptional activity of

the *CYP2C19* gene.[5] The impact of *CYP2C19*17* in the clinic is mainly shown in drug pharmacokinetics. One of the most studied drugs is clopidogrel. It is an antithrombus drug that works by preventing platelet aggregation. Clopidogrel is now used as the standard dual antiplatelet treatment with aspirin after coronary operations. Clopidogrel is taken as a prodrug and processed by CYP2C19 to obtain the active thiol metabolite. The active metabolite can inhibit platelet activation and aggregation through binding of the ADP $P2Y_{12}$ receptor on platelet membrane and inhibiting the activation of the downstream glycoprotein IIb/IIIa complex.[6] Studies have shown that T allele carriers are significantly associated with an enhanced response to clopidogrel[7,8] and an increased bleeding risk.[7,9] Omeprazole, the most studied proton pump inhibitor (PPI), is also a substrate of CYP2C19. Studies have shown that patients with the *CYP2C19*17* allele are likely to have therapeutic failure after omeprazole treatment.[5,10] Among children with refractory gastroesophageal reflux disease (GERD) after PPI therapy, the *CYP2C19*17* allele was associated with antireflux surgery (ARS).[11] Voriconazole, a systematic antifungal drug, is also processed by CYP2C19. *CYP2C19*17* has been shown as an important factor for voriconazole therapy failure.[12]

Currently, *CYP2C19*17* can be detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP),[4,10,12] TaqMan assay,[7,8,11] allele-specific PCR (ASP-PCR),[13] high-resolution melting (HRM),[14] pyrosequencing,[10,15] MassARRAY,[15] and DNA direct sequencing.[5] All these methods have their respective merits and shortcomings. For example, ASP-PCR and PCR-RFLP do not require expensive equipment, and they are less expensive than other methods. However, ASP-PCR requires duplex PCRs, and PCR-RFLP involves three steps: amplification, restriction enzyme digestion, and gel electrophoresis. The TaqMan assay can detect SNPs conveniently, but it requires expensive probes and equipment. HRM is a rapid, convenient, high-throughput, and inexpensive method for SNP genotyping, but the accuracy will be affected when another SNP is present in the same amplicon, and the equipment used in HRM is expensive. pyrosequencing, MassARRAY, and DNA sequencing are all accurate methods for detecting SNPs, and DNA sequencing is acknowledged as the gold standard for DNA sequence analysis. But all these methods require expensive equipment. Compared with the abovementioned assays, tetra-primer ARMS-PCR (T-ARMS-PCR) can detect SNPs by four primers using only one PCR and do not need restriction enzymes, probes, and expensive equipment.[16]

Among T-ARMS-PCR, PCR-RFLP, quantitative PCR, and direct sequencing, it was shown that T-ARMS-PCR obtains the most favorable cost-benefit ratio and is reliable, simple, and fast. [17] In this study, we introduced a simple T-ARMS-PCR assay to detect *CYP2C19*17*.

2 | MATERIALS AND METHODS

2.1 | DNA extraction

The samples have been described in our previous study.[18] Oral swab samples were provided by 93 students at Nanchang University, and a salting-out method was used to extract genomic DNA. After DNA quantification by a spectrophotometer (Nanodrop2000, Thermo Fisher Scientific), each sample was diluted to 10 ng/μL and kept at −20°C. The study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Nanchang University, and all the participants signed informed consent.

2.2 | Gene synthesis

A DNA fragment containing *CYP2C19*17* (−1072 to −611, 462 bp) was synthesized and inserted into the SmaI cloning site of the pUC57 plasmid. According to the DNA quantification results, the plasmid sample was diluted to 0.1 ng/μL for PCR amplification.

2.3 | T-ARMS-PCR

Primers FO, RI, and RO in Table 1 were designed by PRIMER1 online software,[15] and primer FI was from Scott et al[13] T-ARMS-PCR was performed in a total volume of 10 μL, including 5 μL of 2 × Taq master mix, 0.4 μM of primer FO, 0.4 μM of primer RI, 0.8 μM of primer FI, 0.4 μM of primer RO, 1 μL of DNA template (including genomic DNA and plasmid DNA), and 2 μL of ddH_2O. The PCR conditions started with the initial denaturation at 94℃ for 3 minutes, 35 cycles of 94℃ for 20 seconds, 56℃ for 20 seconds, and 72℃ for 20 seconds, followed by a final extension at 72℃ for 5 minutes. The PCR products were visualized by 3% agarose gel electrophoresis at 150 V for 30 minutes.

2.4 | DNA sequencing validation

The results of T-ARMS-PCR were validated by DNA sequencing. Six samples showing the CT genotype or CC genotype by T-ARMS-PCR

TABLE 1 Primers used in T-ARMS-PCR of *CYP2C19*17*

Primer	Sequence[a] (5′-3′)	Product size
FO	GAGATCAGCTCTTCCTTCAGTTACAC	Common 462 bp
RO	CACCTTTACCATTTAACCCCCTAAAAA	
FI[b]	TTTTTCAAATTTGTGTCTTCTGTTCTCAAA***T***T	T allele 227 bp
RI	GCGCATTATCTCTTACATCAGAG***C***TG	C allele 292 bp

Abbreviations: FI, forward primer; FO, forward outer primer; RI, reverse inner primer; RO, reverse outer primer.

[a]Specificity-enhancing mismatches are shown in bold italics.

[b]Primer FI was from Scott et al.[13]

were amplified and sent to a company (Sangon) for sequencing. The PCR (10 μL) contained 5 μL of 2 × Taq master mix, 0.4 μM of primer FO, 0.4 μM of primer RO, 0.5 μL of DNA template, and 3.7 μL of ddH_2O. The thermal conditions were the same as T-ARMS-PCR.

3 | RESULTS

A representative T-ARMS-PCR electrophoretogram of *CYP2C19*17* is shown in Figure 1. The CT genotype showed three bands: 227 bp, 292 bp, and 462 bp. The CC genotype showed two bands: 292 bp and 462 bp. The artificially synthesized plasmid containing the TT genotype showed two bands: 227 bp and 462 bp. Among the 93 samples, 91 samples (97.85%) were the CC genotype, and two samples (2.15%) were the CT genotype. The allele frequencies were 98.92% and 1.08% for the C allele and T allele, respectively. The distribution of genotypes was in Hardy-Weinberg equilibrium (χ^2 = 0.0110; P = .9165).

The results of DNA sequencing are shown in Figure 2. The two samples showing three bands by T-ARMS-PCR were the CT genotype, and the other four samples showing 292 bp and 462 bp by T-ARMS-PCR were the CC genotype. The results of T-ARMS-PCR were completely concordant with the DNA sequencing results.

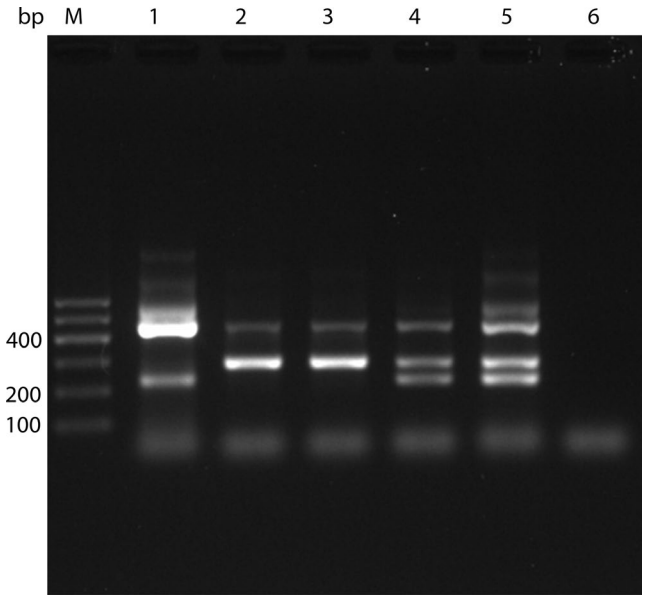

FIGURE 1 Electrophoretogram of T-ARMS-PCR results of CYP2C19*17. Lane M is the DNA size standard marker; lane 1 is the products of the synthesized DNA fragment (TT genotype), with two bands at 462 bp and 227 bp; lanes 2 and 3 are the products of the CC genotype, with two bands of 462 bp and 292 bp; lanes 4 and 5 are the results of CT genotype with three bands of 462 bp, 292 bp, and 227 bp; lane 6 is a negative control

4 | DISCUSSION

In addition to the important role of predicting the clinical outcome of drugs metabolized by CYP2C19, *CYP2C19*17* is also associated with a decreased risk of some diseases, such as breast cancer and Behcet's disease.[19,20] Thus, it is necessary to establish a simple and reliable method to detect this polymorphism.

Because of its reliability, simplicity, and low cost, T-ARMS-PCR has been widely used to detect SNPs and mutations in recent years.[21-27] Using the four T-ARMS-PCR primers designed by PRIMER1 software, we found that the inner primer of the T allele did not have specificity (data not shown) because all the tested samples had the corresponding band of the T allele, but the T allele was rare in Chinese subjects.[28] After searching the literature, we found that Scott et al developed an ASP-PCR method to detect the *CYP2C19*17* allele.[13] We validated the specificity of their T allele primer, and then, the primer was used as our T allele primer (primer FI). We also did not know whether the C allele primer (primer RI) designed by PRIMER1 software was specific because all the tested samples had the corresponding band of the C allele. To prove the specificity of the C allele primer, we synthesized a DNA fragment of the TT genotype. The position of the synthesized DNA fragment in the *CYP2C19* gene was the same as the PCR product of the two outer primers. We did not obtain a PCR product when the synthesized DNA fragment was amplified by primer FO and primer RI, proving that the two primers had the specificity to amplify the C allele. Then, the four primers were added in a single tube at different ratios to obtain the optimal result. As shown in Figure 1, the 227 bp and 292 bp specific fragments of the CT genotype had similar luminance, which suggested that the PCR system and conditions were appropriately optimized. We found that the common band of the CC genotype was absent when we used a specific production lot of 2 × Taq master mix, but the 292 bp specific fragment was not affected. This is consistent with the results of Medrano et al and our previous studies.[28,29] In

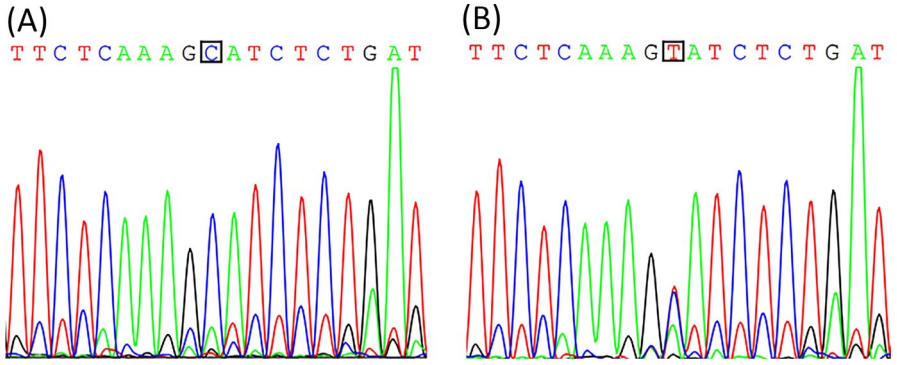

FIGURE 2 The results of DNA sequencing of CYP2C19*17. A, CC genotype; B, CT genotype

this study, no TT genotype sample was found in the 93 samples. The genotype and allele frequencies of *CYP2C19*17* in our study were very similar to those of two Chinese populations.[15,30] The results of T-ARMS-PCR and DNA sequencing were completely consistent, which further confirmed the reliability of the T-ARMS-PCR for *CYP2C19*17* genotyping.

Compared with PCR-RFLP, T-ARMS-PCR does not need a restriction enzyme, so the cost of T-ARMS-PCR is lower than that of PCR-RFLP. T-ARMS-PCR is also cheaper than low-cost ASP-PCR. T-ARMS-PCR runs PCR in a single tube, while ASP-PCR requires two reaction tubes, so ASP-PCR needs more reagents. Compared with the TaqMan assay, HRM, pyrosequencing, MassARRAY, and DNA direct sequencing, T-ARMS-PCR does not require special equipment, so it is particularly suitable for use in common laboratories without expensive equipment.

In conclusion, T-ARMS-PCR is a reliable, simple, and low-cost method for detecting *CYP2C19*17*. It offers an effective screening method for personal medication on *CYP2C19*17*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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