Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2018, Vol. 46(12) 4965–4973

DOI: 10.1177/0300060518787718
journals.sagepub.com/home/imr
SAGE

Clinical application and importance of one-step human *CYP2C19* genotype detection

Ling-Jie Zheng[1], Ning Liu[2], Kun Yang[2], Ai-Feng Wang[1], Zhi-Rong Tan[3] and Xiang Li[2]

Abstract

Background: To directly achieve cytochrome P450 2C19 gene (*CYP2C19*) classification using one-step real-time fluorescent PCR detection and to verify the capabilities of this method with nucleic acid extracted from whole blood samples.

Methods: A human *CYP2C19* genotyping kit based on one-step real-time fluorescent PCR detection was used to analyze whole blood or genomic DNA samples. This method was compared with pyrosequencing and another quantitative (q)PCR kit for its accuracy, repeatability, detection range analysis, sensitivity, specificity, and anti-interference analysis.

Results: The one-step real-time PCR method achieved a 100% accuracy rate compared with pyrosequencing and the other qPCR kit. When detecting different concentrations of known genes, concentrations of each sample ranging from 0.2 to 125 ng/μL could be correctly detected. The genotypes of samples treated with anticoagulants, including EDTA and sodium citrate, and chyle blood samples could be correctly detected.

Conclusion: The one-step detection method demonstrated high accuracy and a wide detection range. It also had high levels of repeatability, sensitivity, and specificity for the assessment of genomic DNA test samples.

Keywords

Real-time fluorescent PCR, CYP2C19, genotype detection, one-step detection

Date received: 25 November 2017; accepted: 18 June 2018

[1]Fuwai Central China Cardiovascular Hospital, Zhengzhou, China
[2]Coyote Bioscience Co., Ltd., Haidian District, Beijing, China
[3]Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, China

Corresponding authors:
Zhi-Rong Tan, Department of Clinical Pharmacology, Xiangya Hospital, Central South University, No. 110 Xiangya Road, Changsha 410008, Hunan, P. R. China.
Email: z2017107@126.com
Xiang Li, Coyote Bioscience Co., Ltd. Haidian District, Beijing, China.
Email: sabrina@coyotebio.com

Introduction

The field of pharmacogenomics has rapidly developed in recent years, and an increasing number of drug genome biomarkers and methods for their detection have emerged.[1] Pharmacogenomics has become a guiding principle in clinical individualized medicine and has been used to assess the risk of serious adverse drug reactions, to guide new drug development, and to evaluate new drugs.[2] The capacity to detect changes in the expression of genes related to drug reactions at the molecular level is a prerequisite for implementing individualized drug therapy.[3] As such, the United States Food and Drug Administration (US FDA) and the Chinese Food and Drug Administration (CFDA) have approved various personalized medicine gene diagnosis kits.[4–6]

Clopidogrel is an antiplatelet agent widely used in acute coronary syndrome in combination with aspirin to reduce the risk of ischemic and thrombotic complications. Clopidogrel is normally prescribed for at least 1 year after percutaneous coronary intervention (PCI) to prevent stent thrombosis.[7,8] It exerts an antiplatelet effect after the metabolic activation of cytochrome P450 2C19 (CYP2C19).[9,10] In March 2010, the US FDA announced a "black box" warning of clopidogrel resistance, stating that it led to adverse cardiovascular events post-PCI which were associated with particular *CYP2C19* alleles. *CYP2C19* mutations can lead to individual differences in enzyme activity, resulting in four different phenotypes: super-fast metabolism (ultrarapid metabolizer, UM), fast metabolism (extensive metabolizer, EM), intermediary metabolism (intermediate metabolizer, IM), and slow metabolism (poor metabolizer, PM). CYP2C19*2 (rs4244285, c.681G>A) and CYP2C19*3 (rs4986893, c.636G>A) are the most important causes of CYP2C19 enzyme defects in Chinese populations,[11] and Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines recommend that patients with *CYP2C19* IM and PM phenotypes should consider changing treatment.[12]

The methods of genetic testing for individualized medicine include PCR direct sequencing, PCR pyrophosphate sequencing,[13] fluorescence quantitative PCR,[14] PCR gene chip,[15] PCR electrophoresis analysis,[16] PCR high resolution melting analysis,[17] allele-specific PCR,[18] PCR restriction fragment length polymorphism,[19] *in situ* hybridization,[20] and others. Among these methods, real-time fluorescent PCR is the most popular because of its high sensitivity, classification accuracy, ease of operation, and quick processing time.

At present, CFDA-approved PCR fluorescence detection kits for *CYP2C19* and vitamin K epoxide reductase complex subunit 1 gene polymorphisms mostly involve whole blood genomic DNA extraction, followed by PCR amplification.[6] As such, they require DNA to be extracted, and the samples added into reaction tubes. Here, we describe a one-step real-time fluorescent PCR detection method in which whole blood samples were used without the need for nucleic acid extraction. This kit adopts the PCR method combined with fluorescence probe *in vitro* amplification technology. The specific probe contains a FAM or ROX fluorescent reporter dye at the 5′ end and a Black Hole Quencher dye at the 3′ end for the direct detection of *CYP2C19*. We also compared our method with other similar kits to provide evidence for its performance in practical applications.

Materials and methods

Subjects

The study included 71 Han Chinese patients (53 males, 18 females), aged 18 years or older (range, 41–78 years), who presented with

acute coronary syndrome. All participants provided their written informed consent before being included in the study, which was approved by the ethics committee of the Institute of Clinical Pharmacology, Central South University (approval number: CTXY - 150001-2).

Genotyping analysis

Genomic DNA was extracted from venous blood of patients with a genomic DNA extraction kit (Promega, Madison, WI, USA). Genotyping of the common loss-of-function CYP2C19*2 variant (rs4244285) and the CYP2C19*3 variant (rs4986893) was performed using TaqMan® single nucleotide polymorphism genotyping assays (Coyote Bioscience, Peking, China). According to the recommendations for the one-step *CYP2C19* genotyping assay kit (Coyote Bioscience), the optimal reaction mixture for real-time fluorescent quantitative PCR (Mini8 Plus, Coyote Bioscience) contained 24 μL CYP2C19*2 PCR reaction mix or 24 μL CYP2C19*3 PCR reaction mix, and 0.2 μL blood sample. Positive and negative controls (1 μL) were included in every reaction tube as recommended by the manufacturer. Hotstar polymerase, dNTP, and FAM-labeled mutant-type probe or ROX-labeled wild-type probe as premix Ex Taq (Takara Bio Inc., Shiga, Japan) were also included. Detection was based on the methods for nucleic acid amplification described in a patent of Xiang Li (Pub. No.: US 2016/0115513 A1). The PCR program involved 40 cycles of 95°C for 2 minutes, 95°C for 5 seconds, and 62°C for 10 seconds.

When an amplification curve was observed at the FAM channel, but not at the ROX channel, the genotype was recorded as AA. When both channels had amplification curves, the genotype was GA, and when only the ROX channel had an amplification curve, the genotype was GG.

The pyrophosphate sequencing method (Xiangya Medical Institute of Central South University) was used as the gold standard for accuracy and specificity analysis. The *CYP2C19* genotyping assay kit (YZY Bio, Wuhan, China), which has been approved by the CFDA, was used as a second comparative method. A total of 20 ng genomic DNA sample was amplified via real-time fluorescent quantitative PCR and pyrophosphate sequencing.

Performance analysis

Accuracy and specificity analysis. A total of 64 clinical cases of unknown genotype were simultaneously used for *CYP2C19* genotyping analysis with the one-step *CYP2C19* genotyping assay kit (Coyote Bioscience), pyrophosphate sequencing, and the *CYP2C19* genotyping assay kit (YZY Bio).

Repetitive analysis. Three known variants, wild-type, heterozygous mutant, and homozygous mutant, were assayed in the blood samples. For genomic DNA, a heterozygous mutant genomic DNA sample was used. These samples were assayed five times with the one-step *CYP2C19* genotyping assay kit.

Detection range analysis. A heterozygous mutant genomic DNA sample was serially diluted to the following concentrations: 125, 25, 5, 1, and 0.2 ng/μL. These samples were then assayed using the one-step *CYP2C19* genotyping assay kit.

Sensitivity analysis. The heterozygous mutation of genomic DNA (1 ng) and whole blood samples were diluted 10 times and used for sensitivity analysis. These samples were then tested seven times with the one-step *CYP2C19* genotyping assay kit, after which statistical analyses were performed.

Anti-interference analysis. Four types of blood samples, including an EDTA anticoagulant

sample, a sodium citrate anti-coagulation sample, a chyle blood sample, and a sample with heme contamination, were assayed with the one-step detection method and the other validated methods.

Statistical analyses. The coefficient of variation (CV) was calculated for the Ct value of each genotype.

Results

Genotyping accuracy and specificity analysis

Clinical blood samples from 64 patients of unknown genotype were used for the genotyping accuracy analysis. Testing was performed in a double-blind manner. The genotyping results showed that the three CYP2C19*2/*3 genotypes were present (Table 1), and that the results of the three methods were consistent with each other. Compared with pyrophosphate sequencing, the accuracy and specificity of the one-step detection method was 100% (Table 2). Partial Ct values and the amplification curve are shown in Table 3 and Figure 1.

Repetitive analysis

Blood samples from three cases with known genotypes (GG, GA, and AA) and one genomic DNA sample of GA type (5 ng)

Table 1. *CYP2C19* genotyping comparison between one-step qPCR genotyping, qPCR genotyping assay kit, and pyrophosphate sequencing

Metabolizer type	CYP2C19*2/*3 genotype	Positive case number			Genotype frequencies (%)
		One-step qPCR genotyping assay kit	qPCR genotyping assay kit	Pyrophosphate sequencing	
EM	GG/GG	18	18	18	28.1
IM	GA/GG	26	26	26	40.6
	GG/GA	8	8	8	12.5
PM	AA/GG	8	8	8	12.5
	AA/GA	0	0	0	0
	GA/GA	2	2	2	3.1
	GG/AA	2	2	2	3.1
	GA/AA	0	0	0	0
	AA/AA	0	0	0	0
Total		64	64	64	100

EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

Table 2. *CYP2C19* genotyping accuracy of the one-step qPCR genotyping assay kit compared with pyrophosphate sequencing

CYP2C19	Genotype	Positive case number		Genotyping accuracy[a]/specificity[b] rate (%)
		One-step qPCR genotyping assay kit	Pyrophosphate sequencing	
*2	GA/AA	36	36	100[a]
*3	GA/AA	12	12	100[a]
*2	GG	28	28	100[b]
*3	GG	52	52	100[b]

Table 3. Partial CT values for *CYP2C19* genotyping

No.	CYP2C19*2	CT value		CYP2C19*3	CT value	
		FAM	ROX		FAM	ROX
1	GG	–	19.23	GG	–	21.40
2	GG	–	19.79	GG	–	20.37
3	GG	–	19.19	GA	23.19	22.97
4	GG	–	19.24	GA	22.64	22.62
5	GA	19.12	19.08	GG	–	21.46
6	GG	–	19.49	GG	–	20.67
7	GA	19.56	19.66	GA	20.94	20.88
8	GG	–	19.75	GA	20.82	20.61

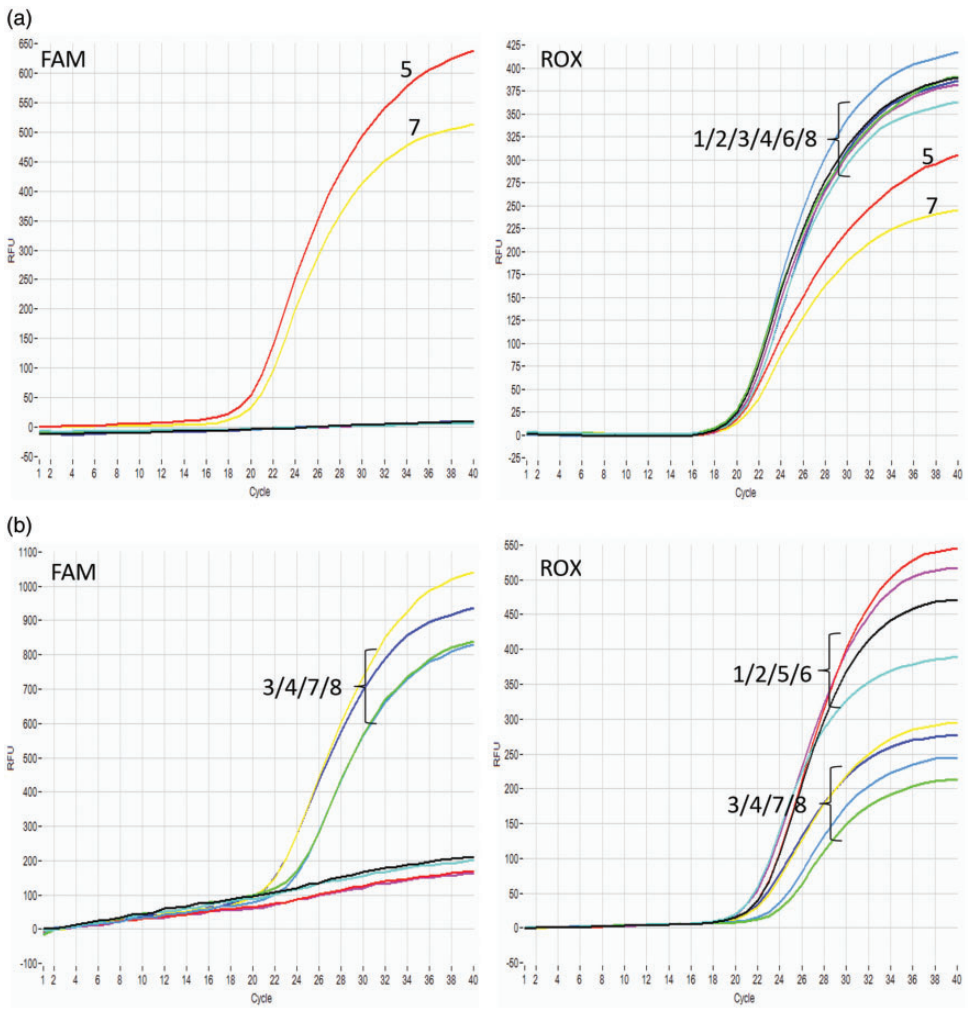

Figure 1. Sample amplification curves for the CYP2C19*2 genotype (a) and CYP2C19*3 genotype (b).

Table 4. One-step detection repeatability analysis

Genotype	SNP	Number of repeats	CV value (%)	Sample type
GG	CYP2C19*2	5	2.56	Blood
AA	CYP2C19*2	5	1.52	Blood
GA	CYP2C19*2	5	2.74/2.01	Blood
GA	CYP2C19*2	5	2.48/2.52	Genomic DNA
GG	CYP2C19*3	5	1.37	Blood
AA	CYP2C19*3	5	1.19	Blood
GA	CYP2C19*3	5	3.50/1.14	Blood
GA	CYP2C19*3	5	0.5/0.24	Genomic DNA

SNP, single nucleotide polymorphism.

were assayed five times each. The CV for each genotype Ct value was less than 5% (Table 4).

Detection range analysis

Heterozygous mutation genotypes of different concentrations from 0.2 to 125 ng/μL could be correctly detected.

Sensitivity analysis

For the genomic DNA sample, the CV values of CYP2C19*2 G and A genotypes were 4.06% and 4.04%, respectively, while those of CYP2C19*3 G and A genotypes were 0.33% and 0.33%, respectively. For whole blood samples, the CV values of CYP2C19*2 G and A genotypes were 2.46% and 2.48%, respectively, and those of CYP2C19*3 G and A genotypes were 3.56% and 3.51%, respectively. All CV values were less than 5%.

Anti-interference analysis

Analysis of the four types of blood samples showed that the sample contaminated with heme was a test failure because the Ct value was more than 43.

Discussion

Individuals with at least one functionally lacking *CYP2C19* allele (CYP2C19*2 or *3 carriers) were previously shown to have lower levels of clopidogrel drug active metabolites, to be prone to clopidogrel resistance, and to have an increased risk of stent thrombosis after PCI.[21] *CYP2C19* detection is therefore necessary for such patients, and CPIC guidelines are recommended to help clinicians understand how available *CYP2C19* genetic testing results can be used to optimize drug therapy. For clopidogrel-resistant patients, *CYP2C19* mutation detection can aid clinicians to switch patients to more effective antiplatelet therapy such as prasugrel or ticagrelor, to overcome their poor response to clopidogrel.[22,23] Indeed, PCI postoperative clopidogrel antiplatelet therapy is one of the most effective ways to reduce the risk of thrombosis.[21,24]

In this study, we used a one-step *CYP2C19* genotyping assay kit based on real-time fluorescent PCR to directly test whole blood samples without the need for a separate nucleic acid extraction step. This technique was shown to be highly accurate and to have a wide detection range. Additionally, its repeatability, sensitivity,

and specificity were similar to those obtained with genomic DNA samples, which satisfies the requirement for clinical *CYP2C19* classification tests.

Compared with other kits, the one-step detection method does not require nucleic acid extraction. This reduces the testing time and cost, which is advantageous because the test report can be issued timely and quickly in a reaction time of approximately 75 minutes. The Mini8 fluorescence quantitative PCR device is also small and portable, does not require calibration, and is easy to operate. Additionally, the number of operative steps required to be performed by clinical inspection personnel is reduced, thereby avoiding the introduction of operator error and contamination. This makes the test more convenient and suitable for clinical applications, and improves the reliability and accuracy of the results. Moreover, because it requires a small sample size (only 0.2 μL of blood), both venous and tip blood are satisfactory means of obtaining samples.

To the best of our knowledge, this is the first study to compare the genotypes of samples treated with anticoagulants, including EDTA and sodium citrate, as well as chyle blood samples, and samples with heme contamination. We showed that the heme-contaminated sample was a test failure, suggesting that this kit cannot be used for samples where there is severe hemolysis or heme contamination.

In conclusion, because one-step molecular diagnosis testing is a rapid and simple operation that does not require highly skilled personnel, it can be used for personalized medicine within hospitals as a point of care test (POCT).[25–27] Indeed, its correct operation and quality control specifications can better guarantee the validity of this method when it is performed as a POCT. Furthermore, this technique may be used to detect other genotypes. Nevertheless, a number of limitations apply when the method is applied directly to the clinic. For example, only certain sample types and instruments can be used for detection. In our future studies, we aim to overcome these limitations and to establish a rapid nucleic acid diagnostic platform to complement the-step real-time fluorescent PCR detection method.

Author contributions

Ling-Jie Zheng conceived the study, generated the first draft, and led the writing of the manuscript. The manuscript was supervised and finalized by Zhi-Rong Tan. All the authors read, edited, and approved the final version of the manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This study was supported by The Research of Major Disease Prevention and Control of Precision Medical Research of Hunan Province Education Department (number 2016JC2069) and The National Development of Key Novel Drugs for Special Projects of China (number 2018ZX09736007-004 and number SQ2017ZX090361 -02).

References

1. Weinshilboum RM and Wang L. Pharmacogenomics: precision medicine and drug response. *Mayo Clin Proc* 2017; 92: 1711–1722.
2. Jones S. Welcome to the 19th volume of Pharmacogenomics. *Pharmacogenomics* 2018; 19: 1–2.
3. Mooney SD. Progress towards the integration of pharmacogenomics in practice. *Hum Genet* 2015; 134: 459–465.
4. Cayre A, Mishellany F, Lagarde N, et al. Comparison of different commercial kits for HER2 testing in breast cancer: looking

for the accurate cutoff for amplification. *Breast Cancer Res* 2007; 9: R64.
5. https://www.fda.gov/MedicalDevices/default.htm
6. http://www.sfda.gov.cn/WS01/CL1026/
7. Desta Z, Zhao X, Shin JG, et al. Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin Pharmacokinet* 2002; 41: 913–958.
8. Pare G, Mehta SR, Yusuf S, et al. Effects of CYP2C19 genotype on outcomes of clopidogrel treatment. *N Engl J Med* 2010; 363: 1704–1714.
9. Han Y, Lv HH, Liu X, et al. Influence of genetic polymorphisms on clopidogrel response and clinical outcomes in patients with acute ischemic stroke CYP2C19 genotype on clopidogrel response. *CNS Neurosci Ther* 2015; 21: 692–697.
10. Saab YB, Zeenny R and Ramadan WH. Optimizing clopidogrel dose response: a new clinical algorithm comprising CYP2C19 pharmacogenetics and drug interactions. *Ther Clin Risk Manag* 2015; 11: 1421–1427.
11. Goldstein JA and de Morais SM. Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* 1994; 4: 285–299.
12. Scott SA, Sangkuhl K, Stein CM, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. *Clin Pharmacol Ther* 2013; 94: 317–323.
13. Alderborn A, Kristofferson A and Hammerling U. Determination of single-nucleotide polymorphisms by real-time pyrophosphate DNA sequencing. *GENOME RES* 2000; 10: 1249–1258.
14. Wang QY, Li Y, Ji L, et al. [Detecting HB-1 Expression Level in Bone Marrow of Acute Leukemia Patients by Real-Time Fluorescence Quantitative RT-PCR]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2018; 26: 8–15 [in Chinese, English Abstract].
15. Kahyo T, Iwaizumi M, Yamada H, et al. Application of digital PCR with chip-in-a-tube format to analyze Adenomatous polyposis coli (APC) somatic mosaicism. *Clin Chim Acta* 2017; 475: 91–96.
16. Sell AM and Visentainer JE. Blood grouping based on PCR methods and agarose gel electrophoresis. *Methods Mol Biol* 2015; 1310: 37–49.
17. Al-Farha AA, Petrovski K, Jozani R, et al. Discrimination between some Mycoplasma spp. and Acholeplasma laidlawii in bovine milk using high resolution melting curve analysis. *BMC Res Notes* 2018; 11: 107.
18. Amr SS, Murphy E, Duffy E, et al. Allele-specific droplet digital PCR combined with a next-generation sequencing-based algorithm for diagnostic copy number analysis in genes with high homology: proof of concept using Stereocilin. *Clin Chem* 2018;
19. Bayraktar S, Duran N, Duran GG, et al. Identification of medically important Candida species by polymerase chain reaction-restriction fragment length polymorphism analysis of the rDNA ITS1 and ITS2 regions. *Indian J Med Microbiol* 2017; 35: 535–542.
20. Yoshimoto M, Ludkovski O, Good J, et al. Use of multicolor fluorescence in situ hybridization to detect deletions in clinical tissue sections. *Lab Invest* 2018; 98: 403–413.
21. Mega JL, Close SL, Wiviott SD, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med* 2009; 360: 354–362.
22. Horenstein RB, Madabushi R, Zineh I, et al. Effectiveness of clopidogrel dose escalation to normalize active metabolite exposure and antiplatelet effects in CYP2C19 poor metabolizers. *J Clin Pharmacol* 2014; 54: 865–873.
23. Lin R, Zhang L, Zhang P, et al. Influence of CYP2C19 loss-of-function variants on the metabolism of clopidogrel in patients from north-western China. *J Clin Pharm Ther* 2015 40: 308–314.
24. Zhang HZ, Kim MH, Guo LZ, et al. CYP2C19 but not CYP2B6, CYP3A4, CYP3A5, ABCB1, PON1 or P2Y12 genetic polymorphism impacts antiplatelet response

after clopidogrel in Koreans. *Blood Coagul Fibrinolysis* 2017; 28: 56–61.
25. St John A and Price CP. Existing and emerging technologies for point-of-care testing. *Clin Biochem Rev* 2014; 35: 155–167.
26. Tiwari AK, Setya D, Aggarwal G, et al. Evaluation of new indigenous "point-of-care" ABO and Rh grouping device. *J Lab Physicians* 2018; 10: 80–84.
27. Huang W, Gaydos CA, Barnes MR, et al. Comparative effectiveness of a rapid point-of-care test for detection of Chlamydia trachomatis among women in a clinical setting. *Sex Transm Infect* 2013; 89: 108–114.