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Application of rapid genotyping of Warfarin individualized pharmacogenetic variants in Warfarin therapy

Defa Huang[1,6], Fangfang Xie[1,6], Shengpeng Xiao[2,6], Minyang Cai[3], Die Hu[1], Baodian Ling[1], Fangsheng Wang[1], Xuan Lin[4], Fangli Song[3], Qi Wang[5] & Tianyu Zhong[1]✉

Warfarin is the most widely used oral anticoagulant in clinical practice. The cytochrome P450 2C9 (*CYP2C9*), vitamin K epoxide reductase complex 1 (*VKORC1*), and cytochrome P450 4F2 (*CYP4F2*) genotypes are associated with warfarin dose requirements in China. Accurate genotyping is vital for obtaining reliable genotype-guided warfarin dosing information. The current method for individualized dosing gene polymorphism detection for warfarin has the disadvantages of being easily contaminated, time-consuming, expensive, and unsuitable for clinical use. Herein, we present a novel application, a multiplex fluorescent melting curve assay of whole-blood direct amplification of nested polymerase chain reaction (PCR), to genotyping single-nucleotide polymorphism (SNPs) rapidly that affect warfarin efficacy. This method requires only 1 µL of whole blood, no DNA extraction, takes less than 2 h, costs less than $1, and is able to accurately distinguish between different SNP sites. Polymorphic loci were detected in whole blood specimens of 181 clinical warfarin-administered patients through nested blood direct PCR fluorescence melting curve analysis and gene sequencing. The results of the nested blood direct PCR multiplex fluorescence melting curve technology were 100% consistent with those of sequencing—characterized by high accuracy and high specificity. The allele frequencies were 94.5% for A and 5.5% for C at *CYP2C9*3* (rs1057910), 7.5% for G and 92.5% for A at *VKORC1* (rs9923231), and 77.1% for G and 22.9% for A at *CYP4F2*3* (rs2108622). For *CYP2C9*2* (rs1799853), only allele C was detected, with a frequency of 100%. Warfarin doses were lower in the *CYP2C9*1*1* genotype population than in the *CYP2C9*1*3* population, lower in the *VKORC1* (AA) population than in the *VKORC1* (GG) population, and higher in individuals with the *CYP4F2*3* mutation (GA/AA) compared with those with wild-type (GG). In summary, the detection and genotyping of four polymorphic SNP sites using a multiplex fluorescent melting curve assay of whole blood direct amplification through nested PCR is highly importance for guiding personalized warfarin anticoagulant therapy.

Warfarin is an oral anticoagulant of the bicoumarin derivative class that works by inhibiting the synthesis of coagulation factors through vitamin K in hepatic cells. It is widely used in the prevention and treatment of thrombotic diseases[1]. Owing to its efficacy and low price, warfarin is the most widely used oral anticoagulant in clinical practice[2]. Despite its widespread use, determining the optimal and safest dosage remains challenging owing to its narrow therapeutic window[3]. Although the introduction of the International Normalized Ratio in the early 1990s allowed for a more consistent and universal management of warfarin therapy, this drug continues to be a key cause of drug-related adverse events, including thromboembolism, ecchymosis, and severe gastrointestinal or intracranial hemorrhage[4]. Hence, it is essential to personalize the warfarin dosage. In recent years, several studies have shown that genetic factors, including polymorphisms in *CYP2C9*, *VKORC1*, and *CYP4F2*, along with 30 other key factors[5], are major contributors to inter-individual differences in warfarin maintenance doses.

The 2017 Clinical Pharmacogenomics Implementation Consortium Guidelines for Genetic Pharmacology to Guide Dosing of Warfarin recommend the three genes with the strongest current scientific evidence for use

[1]Laboratory Medicine, First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, China. [2]Jiangxi Drug Inspector Center, Nanchang 330029, China. [3]Jiangxi Shiningmed Medical Technology Ltd, Ganzhou 341000, China. [4]Department of Trauma Center, First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, China. [5]The First School of Clinical Medicine, Gannan Medical University, Ganzhou 341000, China. [6]These authors contributed equally: Defa Huang, Fangfang Xie and Shengpeng Xiao. ✉email: zhongtianyu@gmail.com

in guiding warfarin dosing, namely *CYP2C9*, *VKORC1*, and *CYP4F2*[6]. The *CYP2C9* gene encodes an enzyme essential for warfarin metabolism, converting it into inactive components. Genetic polymorphisms in the human *CYP2C9* gene, such as *CYP2C9*3* (c.1075 A > C, rs1057910) and CYP2C9*2 (c.430 C > T, rs1799853), which are common in the Chinese population, reduce the activity of the *CYP2C9* enzyme. This results in slower metabolism and clearance of warfarin, making patients more sensitive to the drug and requiring a reduced dose to minimize adverse reactions[7,8]. Genetic polymorphisms in the *VKORC1* gene, which encodes a subunit of the vitamin K epoxide reductase complex, also cause changes in the enzymatic activity of *VKORC*, which in turn affects the anticoagulant effect of warfarin. In particular, *VKORC1* (c.-1639G > A, rs9923231) leads to differences in gene promoter activity, with patients carrying allele A requiring a lower dose of warfarin than GG-pure patients[9–11]. *CYP4F2*, a member of the CYP superfamily mainly found in the liver and kidney, acts as a monooxygenase of vitamin K. The *CYP4F2*3* (c.1297G > A, rs2108622) polymorphism contributes to individual variations in warfarin metabolism in 1-2% of cases. Patients with the AA genotype require a higher warfarin dosage to achieve the same anticoagulant effect[12–14].

Current methods for detecting individualized dose-related polymorphisms in warfarin include restriction endonuclease fragment polymorphism analysis, fluorescence quantitative polymerase chain reaction (PCR), high-resolution solving curves, allele-specific PCR, denaturing high-performance liquid chromatography, gene chips, and gene sequencing[15–18]. However, these methods require purified nucleic acids as templates; this key step increases the testing time and cost. In addition, disadvantages such as expensive instruments and the inability to simultaneously detect multiple gene loci make them unsuitable for clinical testing. In this study, PCR was performed using transgenic DNA polymerase, enabling direct PCR amplification from whole blood samples with just 1 µl of blood, eliminating the need for DNA extraction and minimizing contamination. The use of nested PCR technology enables simultaneous detection of multiple specimens, greatly reducing detection time and meeting the batch processing and speed requirements of clinical testing. In summary, this study aimed to establish the simultaneous detection and genotyping of four polymorphic loci (rs1799853, rs1057910, rs9923231, and rs2108622) in three warfarin-individualized dosing-related genes (*CYP2C9*, *VKORC1*, and *CYP4F2*) in a single reaction system using combined nested PCR and fluorescent probe lysis curve technology.

Materials and methods

Blood sample collection

Patients who received warfarin treatment after cardiac stenting from May to September 2023 at the First Affiliated Hospital of Gannan Medical University were enrolled. In total, 181 whole blood specimens were collected using EDTA anticoagulant or citrate anticoagulant. Informed consent was obtained from all the participants. This study was approved by the Ethics Committee of First Affiliated Hospital of Gannan Medical University. All the studies were conducted in accordance with the principles of the Declaration of Helsinki. Whole blood specimens were collected and stored at 4–6 °C for backup and at -80 °C for long-term storage.

Synthesis of plasmids and design of primer probes

Whole-genome sequencing data for *CYP2C9*, *VKORC1*, and *CYP4F2* were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). Primer premier 5.0 and Oligo 6.0 software were leveraged to design four s elf-quenching probes based on the rs1799853, rs1057910, rs9923231, and rs2108622 polymorphic sites, and the amplification primer pairs were designed according to the corresponding probes. The primers and probes were compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the specificity of amplification and detection. Similarly, the gene sequences of these four polymorphic sites and gene variants were selected for the synthesis of plasmids and homologous sequences. All primers and probes were synthesized by Shanghai Bioengineering Company Limited. The corresponding sequences are listed in Table 1.

Gene polymorphism site	Name (primers, probes)	Sequences (5'-3')
CYP2C9*2 (c.430 C > T, rs1799853)	F1	5'-GGGGAGGATGGAAAACAGAGA-3'
	R1	5'-AGTAAGGTCAGTGATATGGAGTAGGG-3'
	P1	5'-FAM-TCCTCTTGAACACAGTCCT-BHQ1-3'
CYP2C9*3 (c.1075 A > C, rs1057910)	F2	5'-TGTGCCATTTTTCTCCTTTTCC-3'
	R2	5'-GATACTATGAATTTGGGGACTTCGA-3'
	P2	5'-HEX-AGGTCCAGAGATACCTTGAC-BHQ2-3'
VKORC1 (c.-1639G > A, rs9923231)	F3	5'-CTGGGAAGTCAAGCAAGAGAAGA – 3'
	R3	5'-CCAGGGTTCAAGTGGTTCTCG-3'
	P3	5'-ROX-ATTGGCCAGGTGCGGT-BHQ2-3'
CYP4F2 (c.1297G > A, rs2108622)	F4	5'-CCTTCTCCTGACTGCTCCCTT-3'
	R4	5'-CAGCCTTGGAGAGACAGACAGTT-3'
	P4	5'-Cy5-ACAACCCAGCTATGTG-BHQ2-3'

Table 1. Gene polymorphism site primers, probe sequences. F: Forward primer; R: Reverse primer; P: Probes.

Polymerase chain reaction

The PCR system was optimized and tailored to its specific components (final concentrations): 15 μL of 2×SuperEasyTM Mix (UNG)-EDTA, 0.08 μM each of upstream primers for *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, and *CYP4F2*3*, 0.33 μM of downstream primers, 0.08 μM of probes, and 1 μL of whole blood (DNA template), bringing the total volume to 30 μL.

The PCR reaction experiment used SLAN® 96P fluorescent quantitative PCR (Hongshitech, Shanghai, China), with the following reaction conditions: UNG enzyme digestion was set at 37 °C for 5 min. PCR enzyme activation and template degeneration was set in the following order: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 60 s; 50 cycles to obtain amplification products. The fluorescence signal of the corresponding detection channel was collected during the annealing stage, and the melting curve was analyzed after PCR. Melt curve analysis procedure was as follows: denaturation at 94 °C for 2 min and 45 °C constant temperature for 2 min. The melting curve was analyzed utilizing SLAN® 96P fluorescence PCR control software (Hongshitech, Shanghai, China), and 181 PCR products were sent to Anhui General Biology Co., Ltd. for bidirectional sequencing. The Applied Biosystems 3500 genetic analyzer (Carlsbad, California, USA) was used for sequencing.

Statistical analyses

SPSS 18.0 software was used for data processing, with the χ2 test employed to assess whether the allele and genotype frequency distributions of the rs1057910, rs9332127, rs9923231, and rs2108622 polymorphic loci conformed to Hardy–Weinberg equilibrium. The Stata 12.0 software was used to calculate the correlation between genotype and warfarin dose, along with the 95% confidence interval.

Result

Evaluating the effectiveness of the PCR melting curve method

Four pairs of primers and four fluorescent probes were designed to target and detect the four gene polymorphism sites, *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *VKORC1* (rs9923231), and *CYP4F2*3* (rs2108622). Each fluorescent probe was labeled with different fluorescent motifs corresponding to different detection channels (FAM, HEX, CY5, and ROX). Plasmid DNA standards for different genotypes were used to establish standard melting curves for the four gene polymorphism sites (Fig. 1a-d). The melting curves obtained from the wild-type and pure mutant plasmid DNA standards of the four polymorphic loci showed a single melting peak (with different Tm values). Two melting peaks appeared when the specimens tested were heterozygous mutants.

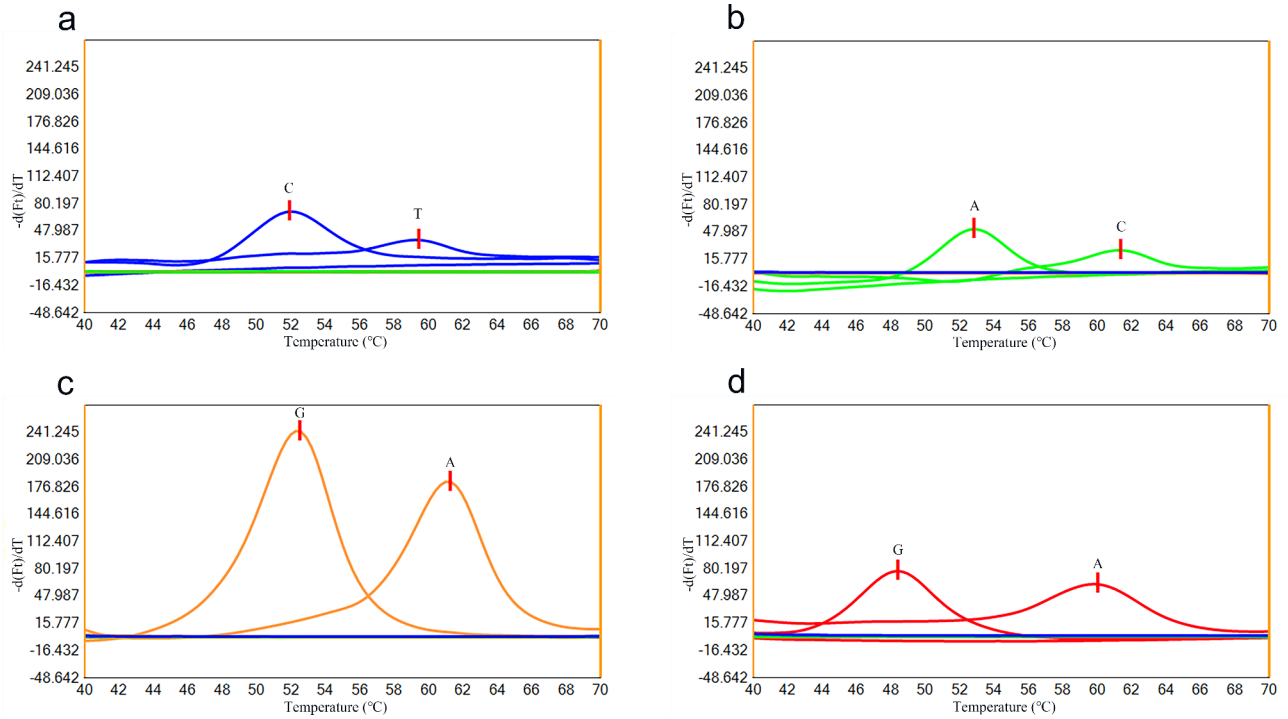

Fig. 1. Plasmid DNA standards to establish standard melting curves for polymorphic sites in four genes. (**a**–**d**) The polymorphic site melting curves graph of *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *VKORC1* (rs9923231) and *CYP4F2*3* (rs2108622), respectively. As *CYP2C9*2* gene for example, a single melting peak appeared and the Tm value was 49.99 ± 1.5 °C, the gene was judged to be wild-type. a single melting peak appeared and the Tm value was 59.08 ± 1.5 °C, the gene was judged to be homozygous variant. two melting peaks appeared, and the Tm values were 49.99 ± 1.5 °C and 59.08 ± 1.5 °C, respectively, the gene was judged to be a heterozygous variant. Genotyping of four polymorphic sites was achieved by the number of melting peaks and Tm values.

Therefore, the number of melting peaks and Tm values could be used to genotype the four polymorphic loci (the Tm values of the melting peaks corresponding to different genotypes are listed in Table 2.)

Next, we tested seven clinical whole blood samples. The assay genotyping results of the seven clinical samples (Fig. 2a-g) showed 100% concordance with the sequencing results (Table 3), and there were no melting peaks in the homologous sequence plasmid standards for polymorphic loci (Fig. 2h). Two clinical whole blood samples were randomly selected for duplicate testing (20 replicate wells per sample) (Fig. 2i-j). These results indicated that the assay had good accuracy, specificity, and reproducibility.

Lower limit of white blood cell number of PCR melting curve method

For clinical testing convenience, we also determined the lower limit of the minimum number of white blood cells required for our method to estimate the minimum blood volume needed for testing. We tested seven clinical whole blood samples corresponding to white blood cell counts of 2,590, 4,080, 7,280, 10,240, 15,050, 20,990, and 40,070. The melting curve peaks of all the assay results distinguished the different genotypes well (Fig. 2a-g). Approximately 1 µL of whole blood of a healthy individual contains 4000–10,000 white blood cells. Thus, we conclude that 1 µL of whole blood was sufficient for our method.

Patients with clinical warfarin anticoagulation usually require monitoring of prothrombin time and international normalized ratio (with a sodium citrate anticoagulant). We tested EDTA-anticoagulated whole blood samples and sodium citrate-anticoagulated whole blood samples, and the results showed that neither anticoagulant affected the test results (Fig. 3a-e). This indicated that the assay was easy to perform in a clinical setting.

Detection result analysis of patient specimens treated with warfarin

Whole blood specimens from 181 patients treated with warfarin were tested for four polymorphic loci, *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *VKORC1* (rs9923231), and *CYP4F2*3* (rs2108622), using whole-blood direct-amplification multiplexed fluorescence lysis curve technology. The results showed the following (Fig. 4a-b) (Table 4): all genotypes at the *CYP2C9*2* (rs1799853) locus were wild-type (CC, genotype frequency 100%); 89.0% of the genotypes at the *CYP2C9*3* (rs1057910) locus were wild-type (AA), 11% were heterozygous mutant (AC), and no purist mutant (CC) was detected; most genotypes at the *VKORC1* (rs9923231) locus genotypes were primarily pure heterozygous mutant (AA), accounting for 85.6%, and wild-type (GG) accounted for only 0.6%; *CYP4F2*3* (rs2108622) locus genotypes GG, GA, and AA were 58.0%, 38.1% and 3.9%, respectively. All samples were sequenced (Supplementary Table 1), and the sequencing results were in complete agreement with the results of the method used, indicating that the reaction system of the whole blood direct amplification multiplex fluorescence melting curve technology had high specificity and accuracy. In addition, we analyzed the relationship between the mutation results of the relevant genes in patients and the warfarin dose. The results revealed that patients with mutations in the *VKORC1* gene had a lower warfarin dose than that of the wild type and a significantly longer warfarin dose adjustment cycle. The intermediate-metabolizing population (*CYP2C9*1*3*) had a lower warfarin dose than the normal metabolizing population (*CYP2C9*1*1*) and a high risk of hemorrhage. Carriers of the A allele in the *CYP4F2*3* gene had a significantly higher warfarin dose than that of the wild type (Fig. 5a-b).

Discussion

Warfarin dose is significantly influenced by the *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, and *CYP4F2* alleles, which is why these genetic variants have been incorporated into algorithms for determining the initial warfarin dose in clinical settings[19]. The frequencies of these alleles and their impact on warfarin dosing have been studied in patients and healthy donors of different ancestries, including African Americans, Caucasians, Japanese, Han Chinese, Indians, and Hispanics[20–22]. The results of these previous studies highlight the differences in the frequency of these alleles and, therefore, in the general warfarin dose requirement among individuals from different geographical regions. Various methods have been developed to detect polymorphisms in individual warfarin dosing-related genes. Although these methods are available and used in clinical practice, their cumbersome and time-consuming procedures and limited number of detectable sites have restricted their broader clinical application. Liu et al. developed a fully integrated and automated microsystem consisting of disposable plastic chips for DNA extraction and PCR amplification combined with a reusable glass capillary array electrophoresis chip in a modular-based format was successfully developed for warfarin pharmacogenetic

Gene polymorphism site	Fluorescence detection channel	Allele	Tm (℃)
CYP2C9*2 (rs1799853)	FAM	C	49.99 ± 1.5℃
		T	59.08 ± 1.5℃
CYP2C9*3 (rs1057910)	HEX	A	51.46 ± 1.5℃
		C	60.80 ± 1.5℃
VKORC1 (rs9923231)	ROX	G	52.96 ± 1.5℃
		A	61.60 ± 1.5℃
CYP4F2*3 (rs2108622)	Cy5	G	49.89 ± 1.5℃
		A	61.40 ± 1.5℃

Table 2. Tm values of wild-type and mutant melting peak in each gene polymorphism site.

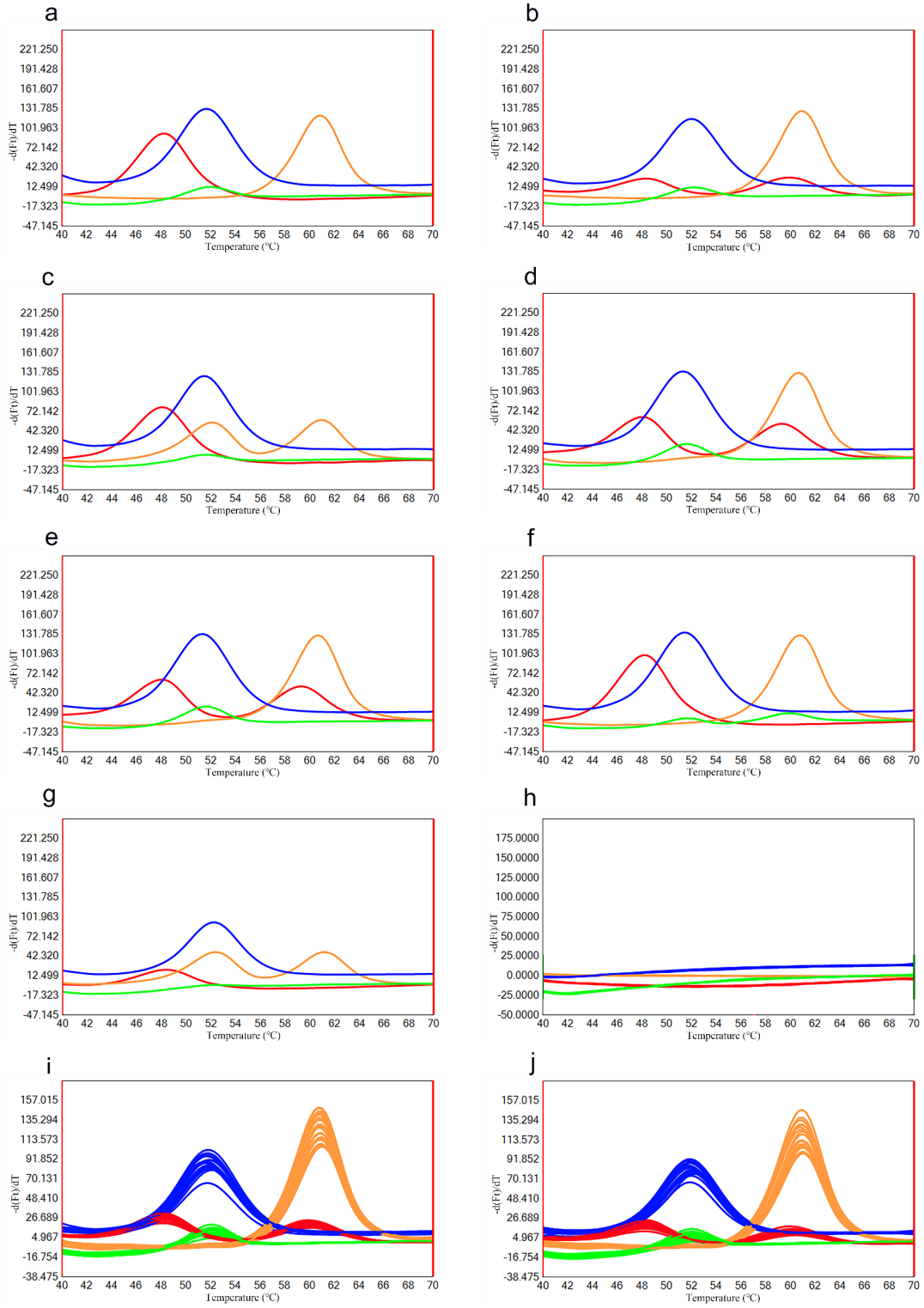

Fig. 2. Evaluating the effectiveness of the PCR melting curve method. (**a**–**g**) The melting curves graph of 4 gene polymorphisms in 7 clinical whole blood samples, and the number of white blood cells in whole blood specimens is 2.59×10^9/L, 4.08×10^9/L, 7.28×10^9/L, 10.24×10^9/L, 15.0510^9/L, 20.99×10^9/L, 40.07×10^9/L respectively; (**h**) The homologous sequence melting curve graph of the *CYP2C9* and *VKORC1* genes, including homologous sequences *CYP2C19*, *CYP2E1*, and *CYP2A13-TYW1*; (**i,j**) The melting curves obtained from 20 repeat trials of two randomly selected clinical specimens.

	CYP2C9*2 (rs1799853)		CYP2C9*3 (rs1057910)		VKORC1 (rs9923231)		CYP4F2*3 (rs2108622)	
Sample number	M1	M2	M1	M2	M1	M2	M1	M2
1	CC	CC	AA	AA	AA	AA	GG	GG
2	CC	CC	AA	AA	AA	AA	GA	GA
3	CC	CC	AA	AA	AA	AA	GG	GG
4	CC	CC	AA	AA	GA	GA	GG	GG
5	CC	CC	AA	AA	AA	AA	GA	GA
6	CC	CC	AC	AC	AA	AA	GG	GG
7	CC	CC	AA	AA	GA	GA	GG	GG

Table 3. Comparison of gene polymorphisms detected by multiplex fluorescence. M1 refers to the gene polymorphism site results detected in this kit, and M2 refers to the sequencing results.

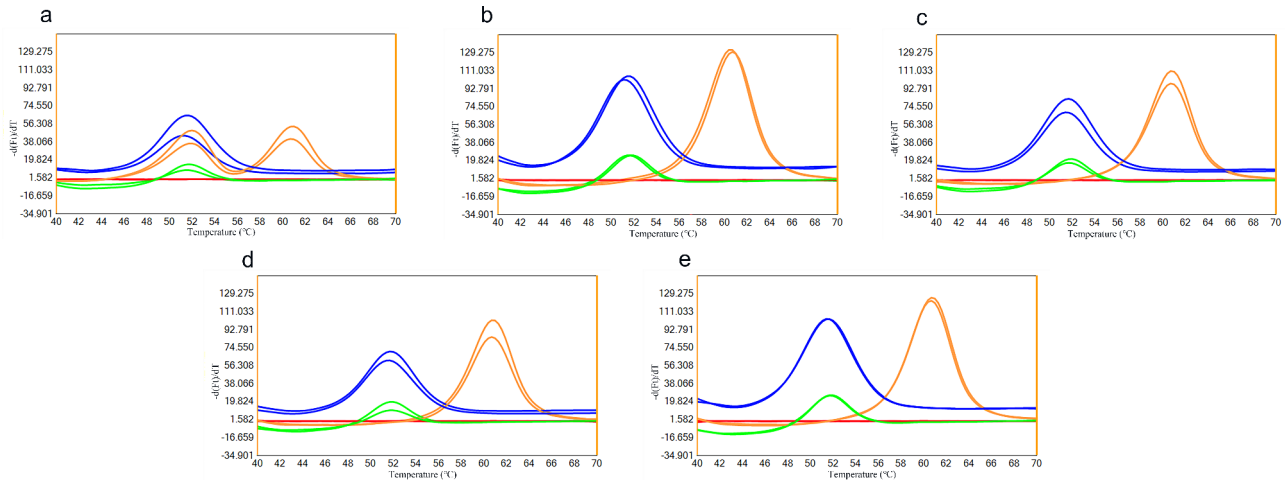

Fig. 3. Analysis of detection results of gene polymorphism by different anticoagulants. (**a**–**e**) The melting curves obtained by multiplex fluorescence melting curve assay of whole blood direct amplification of 5 patients whole blood samples anticoagulated by EDTA and sodium citrate, respectively. The melting curves result of four polymorphisms loci from two samples of whole blood from the same patient were consistent.

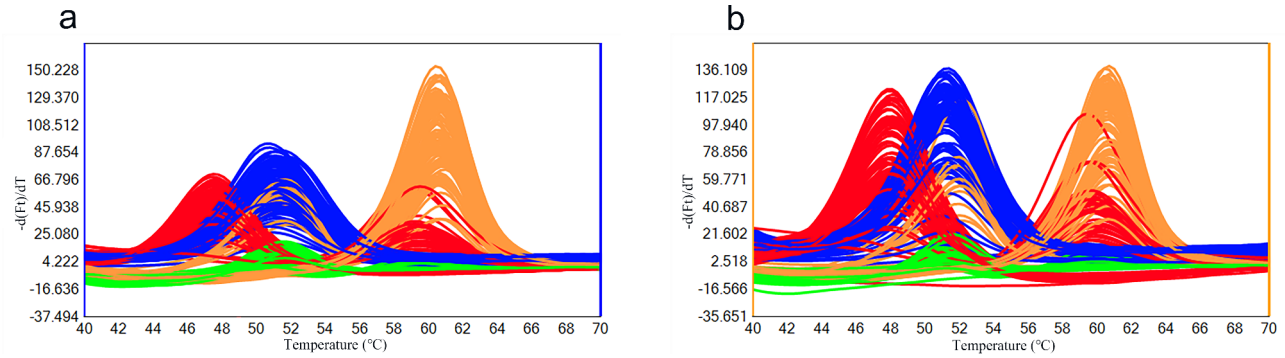

Fig. 4. Gene polymorphism detection melting curve of 181 clinical samples. (**a**) Melting diagram of sample No. 1–95; (**b**) Melting diagram of sample No. 96 – 18.

testing[23]. Although this method is greatly improved compared to some previous methods, it still requires DNA extraction, which is more costly and cumbersome. The whole blood direct amplification multiplex fluorescence melting curve technique established in this study enables genotyping of the warfarin loci at *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *VKORC1* (rs9923231), as well as *CYP4F2*3* (rs2108622) genotyping of the warfarin locus. This method accurately detected four nucleotide polymorphism sites with good reproducibility. Fluorescence intensities of the four channels were determined using fluorescent probes. The melting curves of

Gene polymorphism site	Genotype	Number	Genotype frequency (%)	Allele	Allele frequency (%)
CYP2C9*2 (rs1799853)	CC	181	100	C	100
	CT	0	0	T	0
	TT	0	0		
CYP2C9*3 (rs1057910)	AA	161	89.0	A	94.5
	AC	20	11.0	C	5.5
	CC	0	0		
VKORC1 (rs9923231)	GG	1	0.6	G	7.5
	GA	25	13.8	A	92.5
	AA	155	85.6		
CYP4F2*3 (rs2108622)	GG	105	58.0	G	77.1
	GA	69	38.1	A	22.9
	AA	7	3.9		

Table 4. Genotyping results of 181 samples and frequency of genetic polymorphisms.

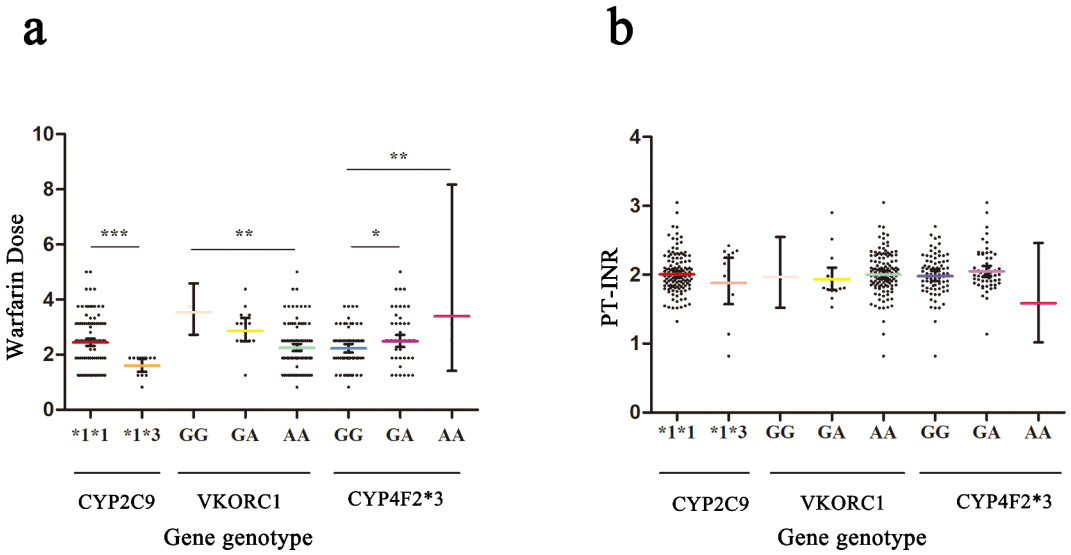

Fig. 5. Correlations between genotyping of 4 polymorphisms and warfarin dose. Divided into 8 groups, *CYP2C9*1*1*, *CYP2C9*1*3*, *VKORC1* (GG), *VKORC1* (GA), *VKORC1* (AA), *CYP4F2*3* (GG), *CYP4F2*3* (GA), *CYP4F2*3* (AA) according to the polymorphism site gene detection results. Among them, *CYP2C9*1*1* represents *CYP2C9*2* and *CYP2C9*3* are both wild types, and *CYP2C9*1*3* represents *CYP2C9*2* wild type and *CYP2C9*3* heterozygous variant type. Comparison of genotyping with clinical warfarin dose (**a**) and corresponding PT-INR values (**b**). The difference in $p < 0.05$ was statistically significant.

each polymorphic site had a △Tm value greater than 5℃, which prevented misinterpretation of genotypic results caused by the close proximity of the melting peaks between single nucleotide mutation sites and unmutated gene sites. The method requires only 1 µL of whole blood, no DNA extraction, takes less than 2 h, costs less than $1, is highly sensitive, can accurately distinguish between different single-nucleotide polymorphism (SNP) sites, and can meet the needs of most clinical whole blood samples for genetic polymorphism detection.

Genotyping of four SNP loci in the *CYP2C9*, *VKORC1*, and *CYP4F2* genes was performed on whole blood samples from 181 patients in the Ganzhou region following warfarin treatment. The aim was to assess the mutation frequency of genes related to individualized warfarin administration and their relationship with warfarin dosing. As shown in Table 4, *VKORC1* (c.-1639G > A, rs9923231) had the highest mutation frequency among the genes related to warfarin individualized dosing, with a frequency of allele A as high as 92.5%; the frequencies of alleles G and A at *CYP4F2*3* (c.1297G > A, rs2108622) were 77.1% and 22.9%, respectively; *CYP2C9*3* (c.1075 A > C, rs1057910) had allele A and C frequencies of 94.5% and 5.5%, respectively; *CYP2C9*2* (c.430 C > T, rs1799853) was detected only with allele C (allele frequency of 100%), and no mutant genes were detected, which is in line with the distribution pattern of the gene in Asian populations. Analysis of the relationship between gene mutations related to individualized warfarin dosing and warfarin dosage in 181 patients revealed that those with *VKORC1* gene mutations required lower doses of warfarin than the wild type group and had significantly longer dosage adjustment cycles. Additionally, patients with intermediate metabolism (*CYP2C9*1*3*) required warfarin doses than those with normal metabolism population (*CYP2C9*1*1*) and had a higher risk of hemorrhage.

Carriers of the A allele in the *CYP4F2*3* gene had significantly higher warfarin doses than the wild type. In summary, the *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, and *CYP4F2*3* genes are the primary factors affecting differences in individual warfarin doses in the Chinese population. Genetic polymorphism testing in patients undergoing warfarin therapy is crucial for shortening the dosage adjustment cycle and reducing the risk of bleeding.

However, this study had certain limitations. Our assay was performed in a controlled environment, and measurements were taken for only a subset of the population. In addition, we were unable to assess certain clinical factors of the patients, such as physical activity and dietary structure, which may affect the daily stable dose of warfarin[24,25]. Furthermore, owing to the limited sample size in this study, expanding the sample size in future phases is essential. Additionally, timely recording of initial doses and dosage adjustment cycles is necessary to provide a theoretical basis for individualized warfarin dosing in clinical practice.

In summary, we established a reliable direct blood PCR method to detect *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, and *CYP4F2*3* SNP loci. This study also demonstrated that the frequencies of *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, and *CYP4F2*3*—the key genetic factors influencing warfarin dose requirements—differ in a sample from Ganzhou (China) compared to other populations of diverse ancestries. This highlights the need to evaluate various Chinese populations to accurately determine the true frequency of these genetic variations.

Data availability

No datasets were generated or analysed during the current study.

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Author contributions

TYZ conceived the original ideas, designed the project. DFH, FFX and SPX wrote the manuscript. DFH per-

formed the majority of the experiments with the help from FFX, SPX, MYC, DH, BDL, FSW, XL, FLS. WQ and TYZ strictly reviewed the manuscript and polished the grammar. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
This project was approved by the Ethical Committee on Scientific Research of First Affiliated Hospital of Gannan Medical University (No. LLSC-2023042401).

Consent for publication
All human blood samples were obtained with written informed consent from all subjects.

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Correspondence and requests for materials should be addressed to T.Z.

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