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Multiplex pyrosequencing method to determine *CYP2C9*3*, *VKORC1*2,* and *CYP4F2*3* polymorphisms simultaneously: its application to a Korean population and comparisons with other ethnic groups

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Abstract Warfarin is an anticoagulant that is difficult to administer because of the wide variation in dose requirements to achieve a therapeutic effect. CYP2C9, VKROC1, and CYP4F2 play important roles in warfarin metabolism, and their genetic polymorphisms are related to the variability in dose determination. In this study we describe a new multiplex pyrosequencing method to identify *CYP2C9*3* (rs1057910), *VKORC1*2* (rs9923231), and *CYP4F2*3* (rs2108661) simultaneously. A multiplex pyrosequencing method to simultaneously detect *CYP2C9*3, VKORC1*2,* and *CYP4F2*3* alleles was designed. We assessed the allele frequencies of the polymorphisms in 250 Korean subjects using the multiplex pyrosequencing method. The results showed 100 % concordance between single and multiplex pyrosequencing methods, and the polymorphisms identified by pyrosequencing were also validated with the direct sequencing method. The allele frequencies of these polymorphisms in this population were as follows: 0.040 for *CYP2C9*3*, 0.918 for *VKORC1*2*, and 0.416 for *CYP4F2*3*. Although the allele frequencies of the *CYP2C9*3* and *VKROC1*2* were comparable to those in Japanese and Chinese populations, their frequencies in this Korean population differed from those in other ethnic groups; the *CYP4F2*3* frequency was the highest among other ethnic populations including Chinese and Japanese populations. The pyrosequencing methods developed were rapid and reliable for detecting *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3*. Large ethnic differences in the frequency of these genetic polymorphisms were noted among ethnic groups. *CYP4F2*3* exhibited its highest allele frequency among other ethnic populations compared to that in a Korean population.

Keywords *CYP2C9* · *VKORC1* · *CYP4F2* · Pyrosequencing · Pharmacogenetics · Ethnic difference · Koreans

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Introduction

Warfarin is the most widely used anticoagulant drug for preventing cardiovascular diseases after ischemic stroke and thromboembolism related to atrial fibrillation, deep vein thrombosis, and pulmonary embolism [1].

However, determining the warfarin dosage is a challenge to clinicians because of its narrow therapeutic range and intersubject variability in the internationalized normal ratios obtained [2, 3].

An increasing number of genetic variations affecting warfarin pharmacokinetics and/or pharmacodynamics have a major impact on dosage requirements such as polymorphisms in the *CYP2C9*, *VKORC1*, and *CYP4F2* genes [3, 4].

Warfarin is metabolized by CYP2C9, a drug metabolizing enzyme, and the warfarin response is related to CYP2C9 genetic polymorphisms. Several variants in the CYP2C9 gene have been reported, but the most prevalent and most studied are the *CYP2C9*2* and *CYP2C9*3* polymorphisms. The *CYP2C9*2* allele is the result of a C>T transition at position 430 of the CYP2C9 gene, leading to an Arg-to-Cys substitution at residue 144 in the CYP2C9 molecule. The *CYP2C9*3* allele is the result of an A>T transition at position 1075 in the CYP2C9 gene, leading to an Ile-to-Leu substitution at residue 359 in the

CYP2C9 gene. Both alleles lead to a significant reduction in CYP2C9 enzymatic activity, representing the major cause of decreased CYP2C9 enzymatic activity, but *CYP2C9*2* is not found in the Asian population including Koreans [1, 5–8].

Vitamin K epoxide reductase complex unit 1 (VKORC1) is an enzyme that recycles vitamin K 2,3-epoxide, reducing the amount of vitamin K-dependent clotting factors (factor II, VII, IX and X). VKORC1 is one of the most important genetic determinants for warfarin dosing [9–11], and polymorphisms in this gene may explain 20–30 % of the variation in warfarin dosing [1, 12, 13]. Among its polymorphisms, the functional promoter polymorphism G>A at position –1639 (in complete disequilibrium linkage with the intronic polymorphism 1173C>T) influences the warfarin dose requirement [9, 10].

CYP4F2 is primarily responsible for metabolizing arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE). Because 20-HETE is a potent cerebral artery vasoconstrictor, many studies have revealed that *CYP4F2* gene polymorphisms are associated with ischemic stroke [14, 15]. CYP4F2 is involved in the metabolism of vitamin K in addition to vitamin K reductase [16]. Additionally, a V433 M polymorphism (*CYP4F2*3*, rs2108622) is associated with variations in vitamin K metabolic activity in vitro.

The distribution of the *CYP2C9*3, VKORC*3*, and *CYP4F2*3* polymorphisms has been extensively assessed in many populations alone and together. Recent studies have revealed that pharmacogenetic models using these polymorphisms and clinical factors facilitate more accurate predictions of warfarin dose in various populations [1, 12, 17–19]. However, literature reviews showed that previous genetic analyses of these polymorphisms were conducted either by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP), real-time PCR, or direct sequencing analyses. However, these methods are time-consuming or cost-ineffective [20, 21]. Therefore, we developed a method to identify *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* using a pyrosequencing method. The relatively low cost and rapid results of a pyrosequencing analysis are an advantage when genotyping population data.

Pyrosequencing is a non-electrophoretic, real-time DNA sequencing technology. It involves hybridization of a primer to a single-stranded PCR template and initiating the sequencing analysis by adding nucleotides [22]. It is consistent, easy to use, economically viable, and generates a high throughput analysis with a very high success rate [22]. Generally, one PCR fragment is produced for each sequencing reaction in the genotyping assay. Recently, a new single-tube multiplex pyrosequencing method for several polymorphisms in a single pyrosequencing reaction was developed [21, 23].

In this study, we developed a multiplex pyrosequencing method that can clinically detect the *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* polymorphisms simultaneously. To validate this method and to compare these allele frequencies among ethnic populations, we investigated the allelic frequencies of *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* in a Korean population, and compared them to those in other ethnic groups.

Materials and methods

Subjects and methods

Genomic DNA samples were obtained from 250 unrelated Korean subjects, and written and informed consent was obtained. This study protocol was approved by the ethics committee of Anam Hospital, Seoul, Korea.

Pyrosequencing method for detecting the *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* polymorphisms

Genomic DNA was isolated from peripheral leukocytes, as described previously [21]. We developed a pyrosequencing method to identify the following single nucleotide polymorphisms (SNPs): *CYP2C9*3* c.1075A>T (rs1057910), *VKORC1*2* (i-1639G>A) (rs9923231), and *CYP4F2*3* c.1347C>T (rs2108622). The primers used for the PCR reaction and pyrosequencing are described in Table 1. PCR reactions were carried out to amplify sequences and identify each SNP using newly developed primer sets after attaching biotin to the 5′ end of each forward (or reverse) primer using PSQ Assay Design software (Pyrosequencing AB, Uppsala, Sweden). The DNA fragments containing polymorphic sites were amplified using newly developed primer sets after attaching biotin to the 5′ end of each forward (or reverse) primer using PSQ Assay Design software (Pyrosequencing AB). PCR was performed in a reaction volume of 30 μl containing genomic DNA (30 ng), 10 × PCR buffer, dNTPs (2.5 mM), 10 pmol primers (1 μl each) and 5 U Taq polymerase (iNtRON, Seongnam, Korea). PCR reactions were carried out with an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. A final termination step was performed at 72 °C for 5 min.

For the pyrosequencing reactions, 60 μl of the PCR template in a single well was immobilized by incubation (with shaking at 1400 rpm, 10 min, room temperature) with a mixture of 5 μl streptavidin beads (Streptavidin Sepharose High Performance, GE Healthcare Bio-Science

Table 1 Oligonucleotide primers used for polymerase chain reaction and pyrosequencing to detect the *CYP2C9*3, VKORC1*2*, and *CYP4F2*3* polymorphisms

SNP		Sequence	size
*CYP2C9*3*	Forward	5′-B-CAC GAG GTC CAG AGA TAC-3′	179
	Reverse	5′-CGG TGA TGG TAG AGG TTT A-3′	
	Sequencing	5′-TGG GGA GAA GGT CAA-3′	
*CYP4F2*3*	Forward	5′-TTATCTGCCTCATCAGTGT-3′	185
	Reverse	5′-B-CTTGGAGAGACAGACAGTT-3′	
	Sequencing	5′-CCCATCACAACCCAG-3′	
*VKORC1*2*	Forward	5′-ACCTGGGCTATCCTCTGTT-3′	166
	Reverse	5′-B-ATGAAAAGCAGGGCCTAC-3′	
	Sequencing	5′-TCCCATCCTAGTCCA-3′	

B, biotinylated at the 5′-end of the primer; SNP, single nucleotide polymorphism

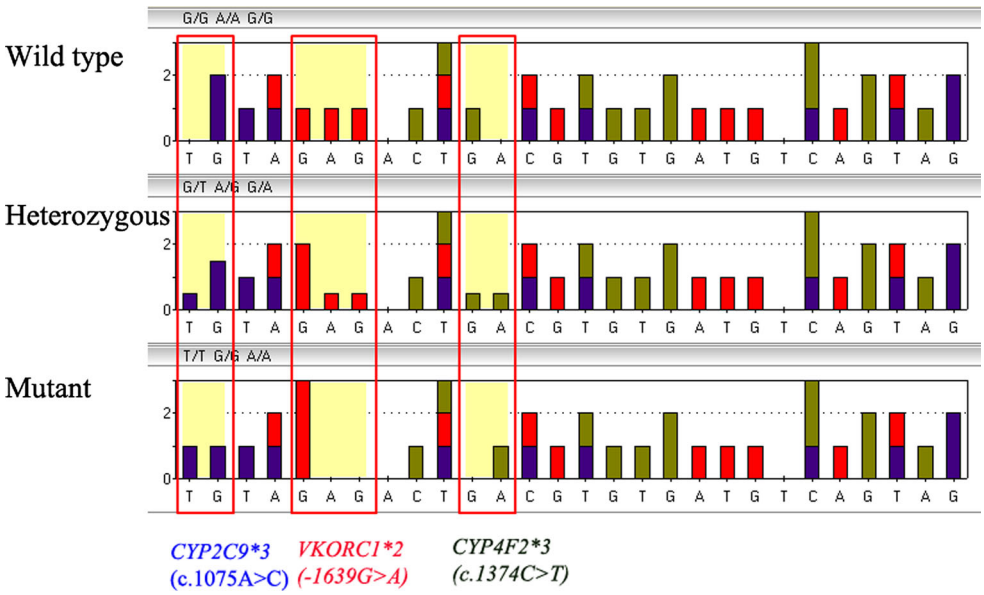

Fig. 1 Multiplex pyrosequencing histogram for *CYP2C9*3, VKORC1*2,* and *CYP4F2*3* as predicted by pyrosequencing software. *CYP2C9*3* (c.1075A > C), *VKORC1*2* (–1639G > A), and *CYP4F2*3* (c.1374C > T) are shown as blue, red, and green bars, respectively. (Color figure online)

AB, Uppsala, Sweden) and 70 µl binding buffer. A 50 µl aliquot of annealing buffer containing each 0.1 µM sequencing primer was incorporated into each well for primer annealing. All liquid was removed by a Vacuum Prep Workstation (Pyrosequencing AB) for strand separation. The beads captured on probes were incubated in 70 % ethanol, and the solution was flushed through filters for 5 s. The beads were then treated with a denaturing solution (0.2 M NaOH) that was flushed through filters for 5 s. A wash buffer (10 mM Tris–acetate, pH 7.6) was used to rinse the beads for 5 s. All liquid was completely drained from the probes, and the beads were released into a PSQ 96 Plate Low (Pyrosequencing AB) containing the sequencing primer. The PSQ 96 Plate Low was heated at 85 °C for 2 min, and the reactions were allowed to cool to room temperature. The resulting mixture was analyzed on a PSQ 96MA Pyrosequencer (Pyrosequencing AB). The accuracy of pyrosequencing was validated by direct DNA sequencing for the randomly selected samples using the same genomic DNA.

Statistical analysis

Genetic equilibrium and linkage disequilibrium were assessed according to the Hardy–Weinberg equilibrium using SNPalyzer ver 7.0.1 (Dynacom Co., Ltd, Yokohama, Japan).

Results

We developed a multiplex pyrosequencing method to identify each SNP for *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* simultaneously. Representative predicted histogram patterns for each genotype are presented in Fig. 1. The assay was designed to generate a specific sequence for each SNP by setting a suitable nucleotide addition order. Nucleotide sequences and pyrograms obtained to identify respective SNPs were consistent with the predicted histograms (Fig. 2). The SNPs and their sequencing data obtained from the pyrosequencing method were validated

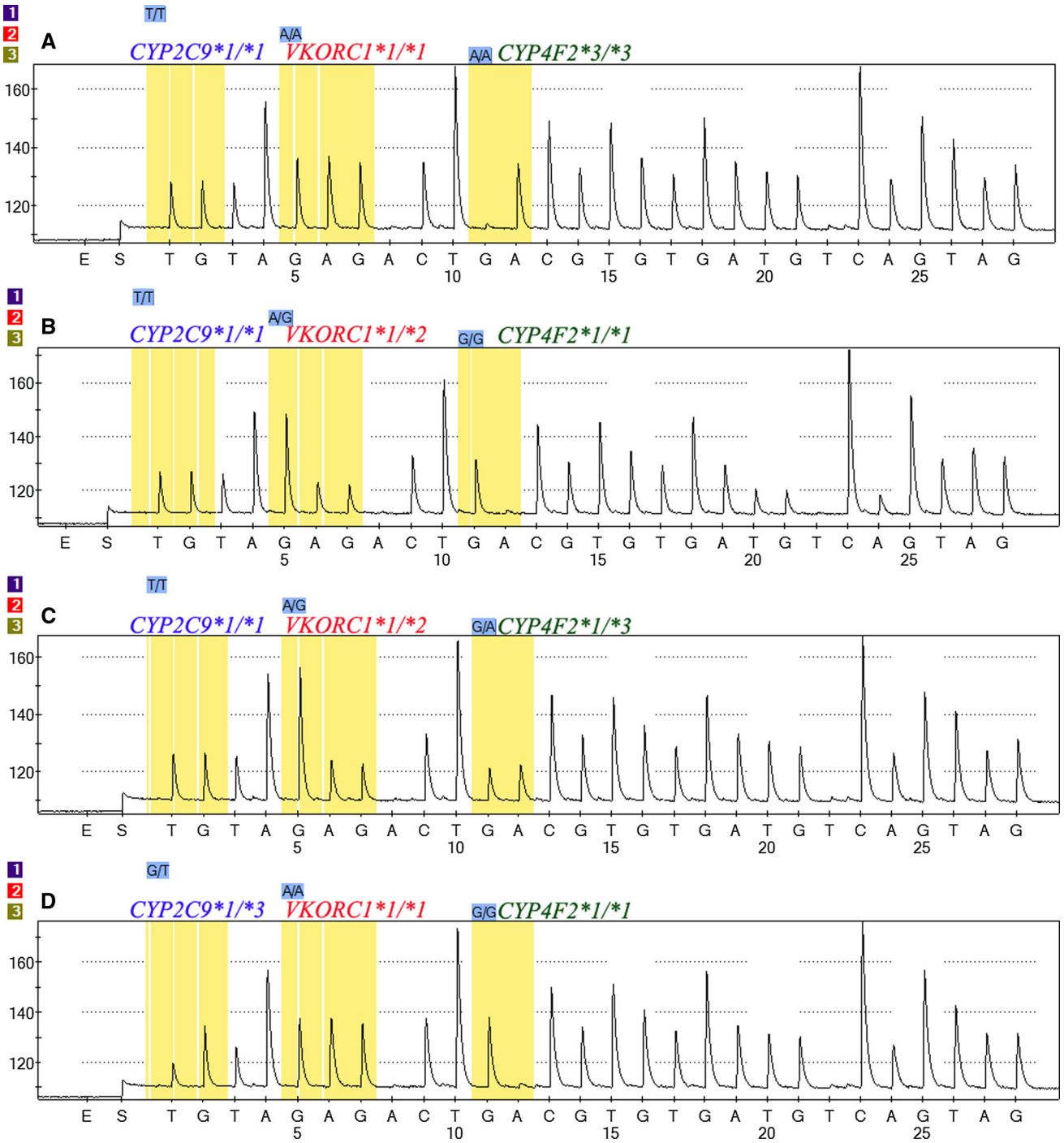

Fig. 2 Representative pyrosequencing pyrograms for the *CYP2C9*3, VKORC1*2,* and *CYP4F2*3* genotypes

by comparisons with the direct DNA sequencing of SNPs for randomly selected samples, and the results showed 100 % concordance with the present pyrosequencing results, indicating 100 % specificity and sensitivity for the method.

When we analyzed the SNPs for *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* with the newly developed method in 250 unrelated Korean subjects, the observed allele frequencies of *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* polymorphisms were as follows: 0.040 for *CYP2C9*3*, 0.918 for *VKORC1*2*, and 0.416 for *CYP4F2*3* (Table 2). All allele frequencies of *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* met Hardy–Weinberg equilibrium ($\chi^2 = 0.0271$, $P = 0.870$ for *CYP2C9*3*; $\chi^2 = 0.0231$, $P = 0.879$ for *VKORC1*2*; $\chi^2 = 0.1035$, $P = 0.748$ for *CYP4F2*3*).

Table 2 Genotyping and allele frequencies of the *CYP2C9*3, VKORC1*2*, and *CYP4F2*3* polymorphisms in this study

SNP	Genotype	No.	Frequencies	Allele	Frequencies
*CYP2C9*3*	T/T	230	0.920	T	0.960
	T/G	20	0.080	G	0.040
	G/G	0	0.000		
*VKORC1*2*	G/G	2	0.008	G	0.082
	G/A	38	0.148	A	0.918
	A/A	210	0.844		
*CYP4F2*3*	C/C	86	0.348	C	0.584
	C/T	119	0.472	T	0.416
	T/T	45	0.180		

When we compared the allele frequencies of these polymorphisms against data reported previously in other ethnic groups, the frequencies of the *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* showed large differences between the various ethnic groups (Table 3).

Table 3 Comparisons of *CYP2C9*3, VKORC1*2*, and *CYP4F2*3* allele frequencies with those in other ethnic groups

SNP	Population	Frequency	Reference
*CYP2C9*3*	Korean (present study)	0.040	Present study
(rs1057910)	Han-Chinese	0.028	Lee et al. [8]
	Japanese	0.021	Nasu et al. [6]
	Taiwanese	0.027	Chern et al. [7]
	Indonesian	0.037	Suriapranta et al. [31]
	Turkish	0.100	Aynacioglu et al. [26]
	British	0.085	Stubbins et al. [27]
	India	0.122	Shalia et al. [41]
	European American	0.060	Sullivan-Klose et al. [42]
	African American	0.005	Sullivan-Klose et al. [42]
*VKORC1*2*	Korean (present study)	0.918	Present study
(rs9923231)	Han-Chinese	0.878	Lee et al. [8]
	Japanese	0.900	Yoshizawa et al. [29]
	Canadian	0.405	Wells et al. [32]
	British	0.413	Biss et al. [43]
	Indonesian	0.230	Suriapranta et al. [31]
	French	0.445	Pautas et al. [19]
	India	0.120	Kumar et al. [44]
	European American	0.398	Kumar et al. [44]
	African American	0.102	Kumar et al. [44]
*CYP4F2*3*	Korean (present study)	0.416	Present study
(rs21086622)	Han-Chinese	0.236	Lee et al. [8]
	Japan	0.277	Fu et al. [45]
	Canadian	0.297	Wells et al. [32]
	British	0.288	Biss et al. [30]
	Indonesian	0.189	Suriapranta et al. [31]
	French	0.294	Pautas et al. [19]
	India	0.418	Kumar et al. [44]
	European American	0.232	Kumar et al. [44]
	African American	0.092	Kumar et al. [44]

Discussion

We developed a rapid and robust pyrosequencing method to detect the *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* polymorphisms simultaneously and applied this method to identify these SNPs in a Korean population. The results showed substantial differences in allele frequencies of the *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* genotypes between our Korean sample and other ethnic groups.

A literature review revealed that *CYP2C9*3*, *VKORC1*2*, and *CYP4F2*3* polymorphisms have been detected individually using PCR–RFLP, real-time PCR, or direct sequencing. However, this is the first study to identify these polymorphisms simultaneously using a pyrosequencing method.

S-Warfarin, the more potent enantiomer of racemic warfarin, is almost exclusively metabolized to 7-hydroxywarfarin by CYP2C9 [2]. Most warfarin studies have focused on *CYP2C9*2* and *CYP2C9*3* polymorphisms. Compared with subjects homozygous for *CYP2C9*1*, homozygous *CYP2C9*2* reduces CYP2C9 enzyme activity to 12 %, whereas homozygous *CYP2C9*3* reduces enzyme activity to 5 % [2, 24]. In accordance with this, a systematic review established that *CYP2C9*2* and *CYP2C9*3* alleles lead to 17 and 37 % reduction in the daily warfarin dose, respectively [25]. In the present study, the allele frequency of *CYP2C9*3* was 0.040. This finding was comparable to that of other Asian populations including Japanese and Chinese [6–8]. However, we did not assess the *CYP2C9*2* polymorphism because it is not found in Asian populations including Koreans [6–8]. Unlike East Asian populations, a higher frequency of *CYP2C9*3* is found in Romanian, Turkish, and British populations [26, 27].

The *VKORC1* genotype predicts 20–30 % of interpatient variability in warfarin dose in white and Asian populations [1, 28]. In particular, a functional promoter polymorphism G>A at position -1639 is in complete linkage disequilibrium with the intronic i-1173C>T and influences warfarin dose requirement [9, 10]. The allele frequency of *VKORC1*2* was 0.918 in this population, suggesting that the variant for the *VKORC1*2* genotype is a dominant allele in Koreans. This finding was similar to East Asian populations including Chinese and Japanese [29]. However, we also found that it is a far higher

frequency than those observed in British, Canadian, French, and Indonesian populations [19, 30–32]. Many studies have shown that patients with the *VKORC1*2* polymorphism have lower dose requirements for warfarin gene-dose dependently [8, 29].

CYP4F2 is a vitamin K1 oxidase involved in the metabolism of vitamin K1 to vitamin K1 dihydroquinone [16]. An in vitro study showed that the *CYP4F2*3* polymorphism, encoding a V433 M amino acid change, causes reduced CYP4F2 protein content and enzyme activity [16]. Interestingly, CYP4F2 is the enzyme responsible for most 20-HETE in the kidney, and the *CYP4F2*3* polymorphism is associated with the development of hypertension and other cardiovascular outcomes in different studies [33–35]. In this study, we found that the *CYP4F2*3* allele frequency was 0.416. When we reviewed the allele frequency in other ethnic groups, we observed that the allele frequency of most ethnic groups was < 0.300. Intriguingly, although the frequencies of *CYP2C9*3* and *VKORC1*2* in our study were similar to the East Asian populations, the Japanese (0.277) and Chinese (0.236) data are rather more comparable to French (0.294), British (0.0.288), or Canadian (0.297) populations [19, 31, 32, 36]. It suggests that the minor allele frequency (MAF) of the Korean population was relatively higher in *CYP4F2*3* than those of other populations. Consistently, other studies done with other Korean populations also exhibited similar allele frequencies with the present findings ($MAF = 0.34 \sim 0.35$; $P > 0.05$) [37, 38]. It would appear that the contribution of this polymorphism to the stable warfarin doses could be greater in the Korean population compared with other ethnic populations [37].

In this study, we developed a new pyrosequencing method to analyze three genetic polymorphisms, which play a crucial role in warfarin dosing determination. These polymorphisms are also associated with the dosing of other anticoagulants, such as phenprocoumon and acenocoumarol [4, 39, 40]. The main advantages of a pyrosequencing assay are the relatively lower cost and time consumption [22, 23]. Usually, only one PCR product is used to analyze the genotype in these polymorphisms. However, we developed a multiplex pyrosequencing method to detect these polymorphisms to reduce laboratory steps and to make it more cost-effective [22, 23]. Furthermore, we reduced the time to analyze these polymorphisms simultaneously using the multiplex tool. Thus, we believe this method should be easier to apply to large population studies and routine clinical use [21]. One limitation of present study is that we analyzed *CYP2C9*3*, *VKORC1*2*, and *CYP4F2**3 polymorphisms only in a Korean population. We could compare their frequencies with other populations based on the literature but it should be better to extend the analyses in other ethnic groups to make the method we developed more convincing and gain a better comparison with other previous methods.

Conclusion

The multiplex pyrosequencing method developed is a rapid and reliable genotyping method to identify *CYP2C9*3*, *VKORC1*2*, and *CYP4F2**3 polymorphisms simultaneously. A large difference in *CYP2C9*3*, *VKORC1*2*, and *CYP4F2**3 polymorphisms was noted when comparing this Korean population with other ethnic groups. In particular, *CYP4F2*3* in this Korean population showed a higher frequency than that in the other ethnic populations.

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