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Polymorphism in CYP2C9 as a Non-Critical Factor of Warfarin Dosage Adjustment in Korean Patients

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Cytochrome P4502C9(CYP2C9) is largely responsible for terminating anticoagulant effect by hydroxylation of S-warfarin to inactive metabolites. Mutations in the CYP2C9 gene result in the expression of allelic variants, CYP2C9*2 and CYP2C9*3 with reduced enzyme activity compared to wild type CYP2C9*1. The aim of this study was to assess relationship between requirement of warfarin dose and polymorphism in CYP2C9 in Korean population. Patients on warfarin therapy for longer than 1 year were included from July 1999 to December 2000 and categorized as one of four groups; regular dose non-bleeding, regular dose bleeding, low dose non-bleeding and low dose bleeding. Low dose was defined as less than 10 mg/week for 3 consecutive monthly follow-ups. Bleeding complications included minor and major bleedings. Blood samples were processed for DNA extraction, genotyping and sequencing to detect polymorphism in CYP2C9. Demographic data, warfarin dose per week, prothrombin time (INR), indications and co-morbid diseases were assessed for each group. Total 90 patients on warfarin were evaluated; The low dose group has taken warfarin 7.6±1.7 mg/week, which was significantly lower than 31.4±0.9 mg/week in the regular dose group ($p<0.0001$). The measured INR in the low dose group was similar to that of the regular dose group (2.3±0.7 vs. 2.3±0.6, $p=0.9$). Even though there was a higher possibility of CYP2C9 variation in the low dose group, no polymorphism in CYP2C9 was detected. All patients were homozygous C416 in exon 3 for CYP2C9*2 and A1061 in exon 7 for CYP2C9*3. The DNA sequencing data confirmed the homozygous C416 and A1061 alleles. In conclusion, polymorphism in CYP2C9 is not a critical factor for assessing warfarin dose requirement and risk of bleeding complications in a Korean population.

Key words: CYP2C9, Warfarin, Polymorphism

INTRODUCTION

Warfarin is the most widely administered oral anticoagulant to prevent thromboembolic complications in cardiovascular diseases such as atrial fibrillation, heart valve replacement, venous thrombosis and pulmonary embolism (Hirsh and Dalen *et al.*, 1998). Bleeding is a major complication of warfarin that can result in fatal hemorrhagic strokes. The estimated average annual frequencies of fatal, major, and minor bleedings are 0.6, 3.0, and 9.6%, respectively (Landefeld and Beyth, 1993). The risk factors of bleeding include the intensity of anticoagulation, age, the length of therapy, co-morbid diseases and concurrent medications. Dosage for maintaining target range of the international normalized ratio (INR) is highly variable and influenced by various patient or treatment factors including dietary intake, compliance, interacting drugs and genetic polymorphism of metabolic enzyme CYP2C9 (James *et al.*, 1992; Furuya *et al.*, 1995; Aithal *et al.*, 1999).

Warfarin is administered as a racemic mixture of roughly equal amount of R and S enantiomers. Between the two enatiomers, the *S*-form is 5 times more potent as a vitamin K antagonist than the *R*-form. Metabolism of the active enantiomer by CYP2C9 leads to a formation of 6-

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and 7-hydroxywarfarin, pharmacologically inactive forms (Kaminsky and Zhang, 1997; Miners and Birkett, 1998). The CYP2C9 has three variants that differ in their metabolic capabilities with 16-20% of 2C9*2 homozygotes and 5% of 2C9*3 homozygotes compared to 2C9*1 wild type (Rettie and Wienkers *et al.*, 1994; Takahashi and Kashima *et al.*, 1998). The frequency of CYP2C9*2 and 2C9*3 is about 20% and 8.5% in Caucacians, respectively. The CYP2C9 variants have been reported to be very rare in Chinese, Japanese and Korean populations (Redman AR, 2001; Yoon and Shon *et al.*, 2001). Unfortunately, however, the relationship between the racial difference of CYP2C9 metabolism and the alteration in the variability of warfarin pharmacokinetics has not been directed studied. Therefore, we investigated the CYP2C9 polymorphism in Korean patients under warfarin therapy and attempt to study the relationship between CYP2C9 polymorphism and the dose requirement of warfarin or the experience of bleeding complications.

MATERIALS AND METHODS

Subjects

Subjects were recruited from patients visiting the anticoagulation clinic to monitor INR regularly and to adjust warfarin dose from July 1999 to December 2000 at Samsung Medical Center, Seoul, Korea. The institutional review board reviewed the study and each patient has given informed consent. The volunteers were divided into 4 groups: 1) regular dose, no bleeding; 2) regular dose, bleeding; 3) low dose, no bleeding; and 4) low dose, bleeding. The low dose group was on warfarin less than 10 mg/week for longer than 3 months without apparent causes for low-dose requirement such as drug interactions or hepatic insufficiency. The patients did not have any major organ failures and bleeding tendency. The threshold of low dose was defined by approximately 2 standard deviations below the mean weekly dose of warfarin (28.26± 10.26 mg/week) in greater than 1,000 patients under warfarin therapy in our anticoagulation clinic. Patients on warfarin dose greater than 10 mg/week were included in the regular dose group. Bleeding complications included experiences of minor or major bleeding events.

Clinical data collection and assessment

Database of the anticoagulation clinic was reviewed for demographic characteristics, the weekly warfarin dose, the measured INR, indications, bleeding events and comorbid diseases. Bleeding complications were classified as minor bleeding (mild nose bleeds, bruise, microscopic hematuria, and mild gingival, conjunctival or anal bleeding) and major bleeding (gross hematuria, gastrointestinal bleeding requiring medical evaluation or blood transfusion, and cerebral hemorrhage).

DNA isolation

Chromosomal DNA was purified from 2 mL of blood samples using Genome whole blood kit (Bio101, Carlsbad, CA, USA) according to manufactures protocol. The kit consisted of cell lysis/denaturation solution, Rnase Mix, salt-out mixture, cell suspension solution and blood washing solution. A crude extract of genomic DNA was obtained from 2 mL whole blood for polymerase chain reaction (PCR) analysis. Whole blood packed cells were mixed with blood washing solution and centrifuged at 1,500×g for 15 minutes. The supernatant was aspired and the cell pellet was washed twice. The final cell pellet was resuspended in cell suspension solution. Rnase Mix, denaturing solution, and protease solution were added. After incubation at 55°C for 2 h, salt-out mixture was added and centrifuged at 12,000×g for 10 min. To the supernatant, 2 mL TE (i.e., 10 mM Tris buffer of pH 7.5 and 1 mM EDTA) buffer and 8 mL of 100% ethanol were added and mixed. The ethanol phase was eliminated and DNA was dried in the air and dissolved in sterile TE buffer. Subsequently, the DNA preparation was subjected to the PCR reaction for CYP2C9 (see below).

Amplification of DNA by polymerase chain reaction

The exon 3 and exon 7 of CYP2C9 were amplified using polymerase chain reaction (PCR). The primers (Bioneer, Taejeon, Korea) used for PCR are listed in Table V. The PCR reaction was carried out in a 50 μL solution consisting of 1×*Taq* buffer (10 mM Tris-Cl, 50 mM KCl, and 15 mM $MgCl_2$, pH 8.3), 0.2 mM of 4 dNTPs, 80 nmol of each primer, 125 ng of chromosomal DNA as a template and 1.25 U of *Taq* polymerase (Takara-Korea, Seoul, Korea). Thirty-five cycles of PCR (94°C for 45 sec, 61°C for 30 sec, and 72°C for 3 min) were followed by a 10 min incubation at 72°C. To minimize error in the reverse transcription and PCR procedures, all reactions were carried out in duplicate with each primer set. The amplified cDNA fragments were seperated by agarose gel electrophoresis and were visualized by ethidium bromide staining.

Genotyping test and DNA sequencing

For genotyping of exon 3 and exon 7, PCR products were digested with 5 U of *AvaII* and *KpnI* at 37°C for 1 h, respectively. The digested PCR products were analyzed on 4% low melting point agarose gel (NuSeive, USA) and visualized by ethidium bromide staining. After purification of PCR products using PCR purification kit (Nuceogen, Seoul, Korea) according to manufacturer, the DNA fragments were sequenced using a DNA sequencer (MegaBace 1000, Amersham, USA). The comparison of sequence

was performed using MegaBACE software version 1.0.

Statistical analysis

SPSS 10.0 statistical program was used for data analysis. Demographic and clinical characteristics were compared with ANOVA, *t*-test and χ^2-test. $P<0.05$ was accepted as denoting statistical significance.

RESULTS AND DISCUSSION

Subjects

Total 90 patients were screened for genotyping in each group; 71 in the regular dose group (35 no bleeding and 36 bleeding group) and 19 in the low dose group (12 no bleeding and 7 bleeding group) (Table I). The measured INR was similar for both dose groups (i.e., 2.3±0.7 in the regular dose and 2.3±0.6 in the low dose, respectively). The required dosage of warfarin, 7.6±1.7 mg/week, for maintaining therapeutic range of INR in the low dose group was found to be significantly low for the regular dose group (i.e., 31.4±10.9 mg/week, $p<0.0001$). In the low dose group, mean older age (63.5±12.3 vs. 74.7±8.9 years) and mean lower weight (62.0±10.6 vs. 53.2±10.7 kg) might contribute to the reduced dosage of wafarin while maintaining the comparable pharmacological effect. Minor bleeding events were 41% (29/71) in the regular dose group and 37% (7/19) in the low dose group. Approximately 10% (7/71) of the regular dose group was associated with major bleeding events (Table II). Indications and comorbid conditions were also different among groups (Table III and IV).

Genotyping of PCR products in exon 3 for CYP2C9*2

In this study, genotyping of PCR product in exon3 for CYP2C9*2 was carried out by the identification of the absence of a restriction site in the variant. Specifically, the $C_{416}\rightarrow T$ change in the sequence of GGAC**T** in exon 3 leads to the recognition site of *Ava*II (GGAC**C**). PCR products containing C_{416} (wild type) were digested with *Ava*II and resulted in two bands with 363bp and 57 bp while PCR products containing T_{416} (variant) should not be

Table I. Characteristics of patients

Characteristics	Regular dose			Low dose			
	No bleeding N=35	Bleeding N=36	N=71	No bleeding N=12	Bleeding N=7	N=19	p-value Regular vs. Low
Sex (M/F)	10/25	21/15	31/40	8/4	3/4	11/8	0.27
Age (year)	61.9± 3.1	65.3±11.5	63.5±12.3	75.2±9.2	71.7±2.3	74.7± 8.9	0.0003
Weight (kg)	58.7±10.3	65.2±10.1	62.0±10.6	52.2±0.7	54.9±1.2	53.2±10.7	0.002
Warfarin dose (mg/week)	32.8±12.5	30.1± 9.0	31.4±10.9	7.4±1.9	8.0±1.2	7.6± 1.7	<0.0001
Measured INR	2.2± 0.4	2.4± 0.9	2.3± 0.7	2.4±0.4	2.1±0.9	2.3± 0.6	0.90

Table II. Bleeding complications

Bleeding Complications	Regular dose			Low dose		
	No bleeding N=35	Bleeding N=36	N=71 (%)	No bleeding N=12	Bleeding N=7	N=19 (%)
Major	0	7	7(10)	0	0	0
Minor	0	29	29(41)	0	7	7(37)
None	35	0	35(49)	12	0	12(63)

Table III. Indications for Warfarin Therapy

Indications	Regular dose			Low dose		
	No bleeding N=35 (%)	Bleeding N=36 (%)	N=71(%)	No bleeding N=12 (%)	Bleeding N=7 (%)	N=19(%)
Atrial fibrillation	12 (34)	17 (47)	29(41)	6 (50)	3 (43)	9 (47)
Valve replacement	6 (17)	6 (17)	12(17)	0 (0)	2 (29)	2 (3)
Heart/Valve disease	6 (17)	9 (25)	15(21)	5 (42)	1 (14)	6 (8)
Cerebral Infarction /TIA	17 (49)	14 (39)	31(44)	3 (25)	5 (71)	8 (11)
DVT/PE	4 (11)	1 (3)	5(7)	3 (25)	0 (0)	3 (4)

Table IV. Co-morbid conditions with warfarin therapy

Co-morbid conditions	Regular dose			Low dose		
	No bleeding N=35 (%)	Bleeding N=36 (%)	N=71 (%)	No bleeding N=12 (%)	Bleeding N=7 (%)	N=19 (%)
Hypertension	16 (46)	12 (33)	28 (39)	2 (17)	3 (43)	5 (26)
Diabetes	6 (17)	6 (17)	12 (17)	2 (17)	0 (0)	2 (11)
Stroke history	6 (17)	3 (8)	9 (13)	3 (25)	2 (29)	5 (26)
Hyperlipidemia	2 (6)	0 (0)	2 (3)	1 (8)	0 (0)	1 (5)
Alcohol	2 (6)	5 (14)	7 (10)	0 (0)	0 (0)	0
Smoking	2 (6)	4 (11)	6 (8)	0 (0)	1 (14)	1 (5)

digested with *Ava*II to give a 420 bp single band. The heterozygotes would give 3 bands of 420, 363, and 57 bp. In 90 subjects tested, all samples showed two bands by *Ava*II complete digestion, indicating that all samples were the homozygous C_{416} and the DNA sequencing data confirmed the homozygous C_{416} alleles (Fig. 1a, Fig.10a, Table V and VI).

Genotyping of PCR products in exon 7 for CYP2C9*3

To determine the presence of Ile_{359}→Leu change in exon 7, *Kpn*I digestion of PCR products was carried out. A mismatched primer TGCACGAGGTCCAGAGGTAC$_{1060}$ was used to detect codon Leu_{359}. A C_{1061} in the sequence together with a mismatched codon G_{1057} creates the recognition site of KpnI (GGTA**C**C). PCR products containing A_{1061} (wild type) would not be digested and resulted in an 131 bp single band while samples with C_{1061}(variant) would be readily digested with *Kpn*I to give two bands with 110 bp and 21 bp. All 90 subjects in this study showed a single 131 bp band. As a result, all samples are the homozygous A_{1061} and the results were confirmed by DNA sequencing (Fig. 1b, Fig. 2b, Table V and VI).

In this study, all patients under warfarin therapy showed

Table VI. CYP2C9 genotype distribution in patients with warfarin therapy

Genotype	Regular dose, no bleeding N=35	Regular dose, bleeding N=36	Low dose, no bleeding N=12	Low dose, bleeding N=7
CYP2C9*1/*1	35/35	36/36	19/19	7/7
CYP2C9*2/*1	0	0	0	0
CYP2C9*3/*1	0	0	0	0
CYP2C9*2/*2	0	0	0	0
CYP2C9*/*3	0	0	0	0
CYP2C9*3/*3	0	0	0	0

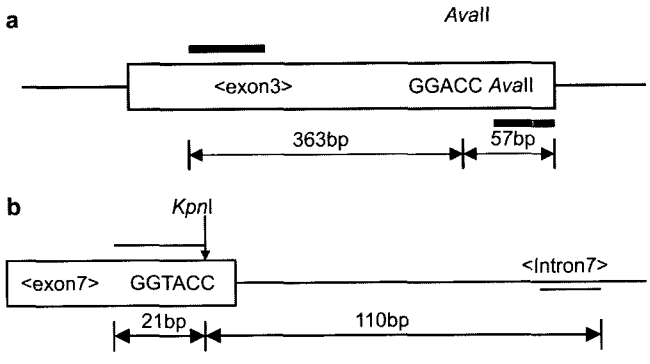

Fig. 1. Scheme for detection of CYP2C9 Arg_{144} (a) and Ile_{359} (b) allele.

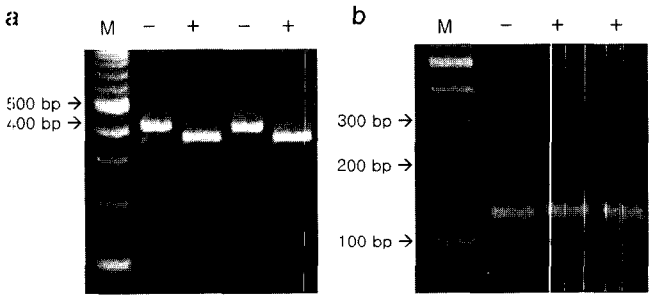

Fig. 2. Analysis of PCR products containing T_{416} or C_{416} (a) and A_{1061} or C_{1061} (b). Digestion of PCR products were performed with (+) or without (-) restriction enzyme *Ava*II(a) and *Kpn*I(b), respectively.

Table V. Sequences and locations of primers used in PCR. The mismatched nucleotide sites are boxed (Wang *et al.*, 1995)

Primer	Sequence	Location
Exon3 forward	GGATATGAAGCAGTGAAGGAA	221-241
Exon3 reward	GGCCTTGGTTTTTCTCAACTC	469-449
Exon7 forward	AAACATGGAGTTGCAGTGTAG	(+36)-(+16) downstream from exon7
Exon7 reward	TGCACGAGGTCCAGAGGTAC	1041-1060

wild type CYP2C9 with full enzyme activity for metabolism of S-warfarin. Even the low dose group with mean 10 mg/week did not have the variants associated with impaired metabolism (CYP2C9*2 and CYP2C9*3). The number of subjects in the low dose group was rather limited (i.e., 19 patients in the group), which may have contributed to the absence of variants in this study. However, in a study with 574 Korean subjects, CYP2C9*2 variant was not found while CYP2C9*3 variants were only rarely found (i.e., heterozygotes, 2.3%; homozygotous variant, one) (Yoon *et al*, 2001). Therefore, the lack of variant in our patient population was somewhat expected from the previous study. In British study, however, there was a strong association between CYP2C9 genotype and warfarin sensitivity and, as a result, low warfarin dose group was six times more likely to be positive for one or more of the variant alleles with low capacity of S-warfarin metabolism (Aithal *et al*, 1999). Indeed, the genetic variation in Chinese, Japanese and Korean (Table VII, Kimura *et al*., 1998; Nasu *et al*., 1997; Wang *et al*., 1997; Stubbins *et al*., 1995; Sullivan-Klose *et al*., 1996; Yoon *et al*., 2001) were found to be quite comparable. Taken together, a racial difference between the Caucasians and the Asians on the CYP2C9 metabolism may be taken into consideration for the warfarin sensitivity.

In this study, we could not identify CYP2C9 variants in patients of low dose group, indicating that CYP2C9 polymorphism is not the primary determinant for the dosing requirement. Other risk factors, such as the advanced age, weight, co-morbid conditions and interactions with drug or food, have to be taken into consideration for the determination of therapeutic warfarin dosage (Landefeld and Beyth, 1993) for Korean patients. In this study, we found that an extremely low dose of warfarin is necessary in significant number of patients. Underlying mechanism for the ethnic difference is unclear. However, since both enantiomers of warfarin may be metabolized by other CYP enzymes such as CYP2C19, CYP3A4, and 1A2 (Kaminsky and Zhang, 1997), it is possible that variants of these enzyme systems may be responsible for the lower dose requirement of warfarin in these Korean patients.

In summary, Korean patients who required very low dose of warfarin for maintaining therapeutic range of INR did not have CYP2C9 genetic variants with less enzyme activity. Therefore, these observations indicated that CYP2C9 polymorphism does not appear to be a critical factor for Korean patients under warfarin therapy in the assessment of therapeutic effects and complications.

Table VII. Frequency of the amino acid variants in the CYP2C9 gene among different ethnic groups

Racial group	Exon 3 (no, frequency) Arg144	Cys144	Exon 7 (no, frequency) Ile359	Leu359
Korean (This study)	90/90	0/90	90/90	0/90
	1	0	1	0
Korean	574/574	0/574	561/574	13/574
(Yoon *et al*., 2001)	1	0	0.987	0.011
Japanese	140/140	0/140	137/140	3/140
(Kimura *et al*., 1998)	1	0	0.979	0.021
Chinese	103/103	0/103	111/115	4/115
(Wang *et al*., 1995)	1	0	0.983	0.017
English	78/100	22/100	84/100	16/100
(Stubbins *et al*., 1995)	0.875	0.125	0.915	0.085
American	92/100	14/100	94/100	10/100
(Sullivan-Klose *et al*., 1996)	0.92	0.080	0.94	0.06

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