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A haplotype of *CYP2C9* associated with warfarin sensitivity in mechanical heart valve replacement patients

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- *CYP2C9* single nucleotide polymorphisms (SNPs) are important in safe and effective oral anticoagulation with warfarin use.
- Although *CYP2C9*2* and **3* are important genetic factors for the warfarin dose, one of the *CYP2C9* SNPs, IVS-65G>C, has been suggested to be associated with warfarin sensitivity. However, as of yet, there has been no explanation about the possible mechanism and linkage analysis.

WHAT THIS PAPER ADDS

- New information on *CYP2C9* SNPs and their occurrences in common haplotype structures in healthy unrelated Koreans and in individuals who require low warfarin dose after mechanical heart valve replacements (MHVRs) were studied.
- Additional evidence showed that an Asian dominant haplotype consisting of -1565C>T, -1188T>C, IVS3+197G>A, IVS3-334C>T, IVS3-65G>C, IVS4-115A>G and IVS5-73A>G could be associated with a low warfarin maintenance dose in mechanical heart valve replacement (MHVR) patients.

AIMS

The objectives of this study were to determine the distribution of *CYP2C9* variants in Koreans and investigate their association with warfarin dose requirements in patients who received MHVRs.

METHODS

All nine exons, intron–exon junction, and promoter region of *CYP2C9* were amplified and directly sequenced in 50 healthy normal Koreans. Additional direct DNA sequencing of the *CYP2C9* gene was conducted in 36 of the 267 MHVR patients who required low maintenance warfarin doses without carrying *CYP2C9*3* and *VKORC1 1173T* mutations. The effects of *CYP2C9* genetics on warfarin maintenance dose were assessed in 267 MHVR patients.

RESULTS

Thirty-nine single nucleotide polymorphisms (SNPs) including seven previously unidentified SNPs were identified in 50 Koreans by direct DNA sequencing. One of the *CYP2C9* haplotypes exhibited an association with warfarin low dose requirement. The adjusted odds ratio for the haplotype between the low dose group and the normal subjects was 2.5 (95% confidence interval 1.05, 6.16). This haplotype consisting of -1565C>T, -1188T>C, IVS3+197G>A, IVS3-334C>T, IVS3-65G>C, IVS4-115A>G, and IVS5-73A>G was found in 15% of 36 MHVR patients who required low warfarin doses, while 4% of 50 normal healthy subjects exhibited this haplotype. One of the SNPs comprising this haplotype, -1565C>T, apparently changed a protein binding pattern as observed in electrophoretic mobility shift assay.

CONCLUSION

The haplotype including -1565C>T, -1188T>C, IVS3+197G>A, IVS3-334C>T, IVS3-65G>C, IVS4-115A>G, and IVS5-73A>G seems to be associated with low warfarin dose requirement and this haplotype could be considered in the development of a warfarin dose prediction model for Asian populations.

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Introduction

Among the four *CYP2C* genes, *CYP2C9* is the most abundantly expressed in the human liver [1]. The enzyme CYP2C9 is reported to metabolize approximately 16% of human drugs, including the antidiabetic drugs tolbutamide [2] and glipizide [3], the anticonvulsant phenytoin [4], the anticoagulant warfarin [5], the antihypertensive drug losartan [6], the diuretic torasemide [7], and numerous nonsteroidal anti-inflammatory drugs (NSAIDs) [8]. CYP2C9 also metabolizes endogenous substrates, such as arachidonic acid and linoleic acid [9]. CYP2C9 is a genetically polymorphic enzyme responsible for interindividual variation in the metabolism and disposition of many widely used drugs. In particular, genetic polymorphisms in *CYP2C9* have caused variable warfarin dose requirements and risk for adverse events, such as thromboembolic or hemorrhagic complications.

At present, more than 30 single nucleotide polymorphisms (SNPs) of *CYP2C9* have been reported to the Human P450 Allele Nomenclature Committee. The two most important allele variants, *CYP2C9*2* and *CYP2C9*3*, occur at frequencies of 10.5 and 8.4%, respectively, in Caucasians [10], while these are low in Africans at 2–4 and 1–2%, respectively [11, 12]. In Asians, *CYP2C9*2* has not been detected, and *CYP2C9*3* has been reported at frequencies of 3–6% [13–15]. *CYP2C9*8*, **9*, and **11* were found in Africans with relatively high frequencies at 3.6, 13, and 5.6%, respectively. However, these SNPs are low or undetected in other racial groups, indicating apparent ethnic differences in the frequencies of *CYP2C9* variants [16, 17].

Warfarin is an anticoagulant drug, which is administered as an equal mixture of (S)- and (R)-enantiomers for the treatment of thrombosis and embolism in many disorders. Most of the anticoagulation effect is attributable to the (S)-enantiomer, which is deactivated predominantly by *CYP2C9* [5]. Frequent blood tests for a stable international normalized ratio (INR) are performed to reduce the unwanted side effects of warfarin. Although *CYP2C9*2* and **3* are important genetic factors for the determination of warfarin dose in Caucasians, these alleles are not significant contributing factors in Asians due to the very low allele frequencies. More extensive searches would be required in other ethnic groups in order to obtain a complement of *CYP2C9*-defective alleles. Ethnic variation in *CYP2C9* polymorphisms is apparent, but no discovery studies on *CYP2C9* genetic polymorphisms have been carried out in Koreans via direct DNA sequencing. Therefore, we sequenced the *CYP2C9* gene in 50 normal healthy subjects and evaluated the allele distributions and frequencies for the first time in a Korean population. An additional purpose of this study was to investigate the influence of *CYP2C9* variants on warfarin maintenance dose in a well-characterized Korean cohort of MHVR patients [18] through direct DNA sequencing of 36 out of 267 MHVR patients who required low maintenance warfarin doses.

Methods

DNA samples and subjects

Genomic DNA was prepared from peripheral whole blood using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) and stored at −20°C until use. Fifty healthy subjects were selected randomly from the south eastern part of the Korean peninsula to investigate genetic polymorphisms of *CYP2C9* in Koreans. Genomic DNA samples of 50 Korean subjects were obtained from the DNA Repository of the Pharmacogenomics Research Center, Inje University [19]. All the participants were healthy according to their medical histories, physical examinations and routine laboratory tests. MHVR patients were recruited from the Outpatient Clinic of Busan Paik Hospital between September 2005 and March 2007. Data during visits such as warfarin dose, INRs, bleeding events, co-medications, foods, gender, weight and age were collected. Warfarin dose was adjusted every day based on daily INR for the first 2 weeks in the hospital. Once a stable dose of warfarin was reached, the INR was monitored during intervals of 4–8 weeks. A stable dose was defined as the warfarin dose when INR values were within a therapeutic range (1.7–2.8) three times consecutively. Stable warfarin doses ranged from 1.5 mg day^{-1} to 9.5 mg day^{-1}. Of the 267 MHVR patients, 36 individuals who required low maintenance doses of warfarin (3 mg day^{-1}) at Paik Hospital, Busan, Korea, were selected as the low dose group and used for full DNA sequencing of the *CYP2C9* gene. The low and high dose (5.5 mg day^{-1}) groups were selected from ~15% of individuals at each end of a normal distribution curve describing data that cluster around the mean.

All participants provided written informed consent prior to the study. The institutional review board (IRB) of Busan Paik Hospital, Inje University College of Medicine, Busan, Korea, approved the protocol. Details on MHVR patients in the present study were described in the previous report [18].

DNA sequencing and variant identification

For direct DNA sequencing of the *CYP2C9* gene, primers for the amplification of all exons, exon/intron junctions, 3377 bp from the translation start site, and up to 300 bp after the stop codon at the 3′-untranslated region (3′-UTR) were designed according to a previously reported method with slight modifications [20]. The amplified PCR products were directly sequenced after gel electrophoresis to ensure proper amplification, as described previously [19].

Genotyping

A new pyrosequencing method was developed to detect the presence of IVS4-115A>G. The 646-bp fragment

containing an *IVS4-115A>G* variant was amplified using the following primer sequences: forward, 5′-CTGGTTAGAA TTGATCCTCTG-3′, and reverse, 5′-Biotin_GGTAGTTTATATTT CTGTGGGCTC-3′. After amplification of the DNA fragment, a sequencing primer, 5′-AAATTTCCCCATCAAG-3′ was used to detect the *IVS4-115A>G* variant. PCR was performed using a GeneAmp PCR 9700 instrument (Applied Biosystems, Foster City, CA, USA). Briefly, the PCR reaction included 0.25 μM of each primer and 100 ng of genomic DNA in a 20 μl reaction. PCR was performed using one cycle of initial denaturation (5 min, 94°C); 35 cycles comprising of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; followed by a final extension at 72°C for 7 min. The reaction mixture was analyzed on a PSQ 96MA Pyrosequencer (Pyrosequencing AB, Uppsala, Sweden). The accuracy of pyrosequencing was verified by direct DNA sequencing using the same genomic DNA. Details of the genotyping method for *VKORC1* 1173 C>T employing the use of pyrosequencing were described in a previous report [18]. Except for the primer set and sequencing primer, the procedure for pyrosequencing was the same as described previously [21].

Electrophoretic mobility shift assays (EMSA)

Nuclear proteins from human liver tissues were isolated by a NE-PER Nuclear and Cytoplasmic Extraction Reagent (PIERCE, Rockford, USA) system according to the manufacturer's protocol. Liver tissues from Korean donors were obtained from the Busan Paik Hospital (Busan, Korea) affiliated with the tissue repository bank at INJE Pharmacogenomics Research Center (Inje University College of Medicine, Busan, Korea). The approval for the usage of human liver tissues was obtained according to the institutional guideline. [^{32}P]-dATP was incorporated into oligonucleotide primers using T4-polynucleotide kinase (USB, Cleveland, Ohio, USA). The labelled probe (approximately 150 000 counts min^{-1}) was incubated in the presence or absence of nuclear proteins in a 25 mM Hepes buffer (pH 7.8) containing 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM EDTA, 0.5 mM dithiothreitol, 2.5% glycerol and 2% Ficoll 400. Various cold competitors were added to the reaction before the addition of isolated nuclear proteins. After 30 min of incubation at 4°C, the reaction mixture (20 μl) was loaded onto a 5% nondenaturing polyacrylamide gel for electrophoresis in a TAE buffer for 3 h at 100 V. After electrophoresis, the gel was dried and exposed to XAR film (KODAK BioMax, MO, USA). The oligonucleotide probe sequences used were as follows (mutated sequence is underlined): wild-type probe, 5′-CCTCATTCCGGAAATGG GT-3′; variant probe M1 (1565C>T), 5′-CCTCATTC<u>T</u>GGAAA TGGGT-3′; and multiple base mutation probe M2, 5′-CCTCA<u>AGAATTCCC</u>TGGGT-3′.

Statistics

The allele frequency, the Hardy-Weinberg equilibrium, 95% confidence interval (CI) and linkage disequilibrium (LD) were analyzed using a SNPAlyse software (Dynacom Co., Yokohama, Japan). Pairwise LD analysis was performed by |D′| and r^2 statistics using common SNPs that exhibited more than 0.05 in their allelic frequencies. Haplotype analysis was performed using IIPGA tools (https://innateimmunity.net/IIPGA2/Bioinformatics). Twenty variants identified by direct DNA sequencing in 50 healthy Koreans were applied to select tagging SNPs. The program Tagger was used to select tagging SNPs, which combines the simplicity of pairwise methods with the potential efficiency of multimarker approaches (http://www.broad.mit.edu/mpg/tagger/) [22]. Warfarin dose requirements were determined with stable INR values for three consecutive measurements within 3 month intervals and expressed as the mean mg day^{-1} ± standard deviation (SD) [18]. The values between two different genotypes were analyzed using a Wilcoxon rank-sum test. Odds ratios (OR) and 95% CI were estimated using a logistic regression model adjusting for weight, age, aspirin, congestive heart failure/cardiomyopathy, INR-increasing drugs (amiodarone, fluconazole, doxifluridine) and INR-decreasing dietary supplements. All statistical analysis was performed using the SAS program (version 9.1.3; SAS Institute, Cary, NC, USA). A $P < 0.05$ was considered statistically significant.

Results

Thirty-nine variants of *CYP2C9* were identified in 50 Korean individuals and their allele frequencies are presented in Table 1. Of these, 13 SNPs were identified in the 5′-UTR, two in coding regions, 21 in introns, and two in the 3′-UTR. Seven of the 39 SNPs were newly identified in the present study as rare variants (four in the 5′-UTR and three in introns). Two SNPs found in the coding regions included *CYP2C9*3* at 6% frequency, and a silent base pair change, g.1425 A>T, in exon 9 at 5% frequency. No significant deviations from Hardy-Weinberg equilibrium were detected for the identified variants. A program at http://www.cbrc.jp/research/db/TFSEARCH.html was used to examine the creation or elimination of transcriptional factor binding elements introduced by the mutation. No significant novel mutations for altered transcriptional binding sites were found in our analysis, except a variant -1565C>T which disrupted a possible binding motif sequence of signal transducers and activator of transcription (STAT) as well as an Elk-1 with 89 and 86% probability, respectively. To predict a possible acceptor or donor site for splicing events, a sequence analysis program at http://www.fruitfly.org/seq_tools/splice.html was used. One variant, *IVS4-115A>G* was predicted as a possible splicing donor site with 89% probability and this variant was predicted to be a haplotype with -1565C>T, -1188T>C, IVS3+197G>A, IVS3-334C>T, IVS3-65G>C and IVS5-73A>G.

Thirty-six subjects who required a low warfarin dose without having *CYP2C9*3* and *VKORC1 1173C>T* mutations

Table 1

Single nucleotide polymorphisms (SNPs) found in the *CYP2C9* gene in a Korean population*

Site	Nucleotide changeand position†	Amino acid change	Nucleotide change and flanking sequence	Subject number (*n*) w/w	w/m	m/m	Minor allele frequency (95% CI)
5-UTR	-3089G>A		TGATTCCAACC(**G/A**)TATTACATTTTG	24	21	5	0.31 (0.182, 0.438)
5-UTR	-2787G>T‡		GTGTGGGTGCA(**G/T**)GGAAAGAGGC	49	1	0	0.01 (0, 0.038)
5-UTR	-2665~2664delTG		TCAGTGAC(**DEL/TG**)TGGAGGGCTTAA	24	21	5	0.31 (0.182, 0.438)
5-UTR	-1911T>C		GGACCAAGTTA(**T/C**)TGCTTTTCTTTGC	45	4	1	0.06 (0, 0.126)
5-UTR	-1885C>G		CCTGTATAAAGG(**C/G**)TTCTCCAAGGCC	45	4	1	0.06 (0, 0.129)
5-UTR	-1875G>C‡		GGCTTCTCCAAG(**G/C**)CCTTTGACTTAC	49	1	0	0.01 (0, 0.038)
5-UTR	-1746C>T‡		TAGACTGAATTA(**C/T**)GAAATCCTGAAT	49	1	0	0.01 (0, 0.038)
5-UTR	-1565C>T		GCTTCCTCATTC(**C/T**)GGAAATGGGTC	46	4	0	0.04 (0, 0.094)
5-UTR	-1537G>A		TTTATTGTAAGCA(**G/A**)AGGTAATTGAGA	45	4	1	0.06 (0, 0.126)
5-UTR	-1520C>T		ATTGAGAGATT(**C/T**)AAAAGGGACATG	49	1	0	0.01 (0, 0.038)
5-UTR	-1188T>C		ACCTCCCATCTT(**T/C**)TATTGCATCCAC	17	23	10	0.43 (0.292, 0.567)
5-UTR	-981G>A		TGCAGTGATGGA(**G/A**)AAGGGAGATCC	45	4	1	0.06 (0, 0.126)
5-UTR	-641A>T‡		CTGCCTTCAGGA(**A/T**)TTTTTTTTAGGG	49	1	0	0.01 (0, 0.038)
Intron 1	IVS1+83T>C		CCTAGAGGTACA(**T/C**)GTTACAAGAGG	48	2	0	0.02 (0, 0.059)
Intron 1	IVS1-22T>C		CTTCGTTTGCTG(**T/C**)TATCTCTGTCTA	49	1	0	0.01 (0, 0.038)
Intron 2	IVS2+73T>C		GACTTACAGAGC(**T/C**)CCTCGGGCAGA	48	2	0	0.02 (0, 0.059)
Intron 3	IVS3+197G>A		GCATGATTGTGC(**G/A**)TACAGTGTGGG	41	8	1	0.10 (0.017, 0.183)
Intron 3	IVS3+239C>T		ATCCCATGTTCTC(**C/T**)TGAACTTTGCT	24	21	5	0.31 (0.182, 0.438)
Intron 3	IVS3+265T>C		TTTTGCTTTCAAA(**T/C**)AAGAAATGATG	45	4	1	0.06 (0, 0.126)
Intron 3	IVS3-334C>T		TCTCAGTGCCTTG(**C/T**)TGTCTACTGACT	41	8	1	0.10 (0.017, 0.183)
Intron 3	IVS3-122T>A‡		ATGCATGCCGAAC(**T/C**)CTTTTTTGCTGT	49	1	0	0.01 (0, 0.038)
Intron 3	IVS3-65G>C		ACTACTATTATCT(**G/C**)TTAACAAATACA	46	4	0	0.04 (0, 0.094)
Intron 4	IVS4-115A>G		TTCCCCATCAAG(**A/G**)TATACAATATAT	46	4	0	0.04 (0, 0.094)
Intron 4	IVS4-50T>C‡		GGTATATGGTATG(**T/C**)ATGCTTTTATTAA	49	1	0	0.01 (0, 0.038)
Intron 5	IVS5-73A>G		ATAACTATGTGA(**A/G**)TAATTTTGAATTC	41	8	1	0.10 (0.017, 0.183)
Intron 6	IVS6+95A>G		TAGAGAAGCTTC(**A/G**)TTATTTAAACTTT	48	2	0	0.02 (0, 0.059)
Intron 6	IVS6+152A>G		ATGGTGATTACA(**A/G**)TGGGATATCTTGG	49	1	0	0.01 (0, 0.038)
Intron 6	IVS6+214G>A‡		TTGGGAGGCTGA(**G/A**)GTGGGTGGATCA	49	1	0	0.01 (0, 0.038)
Intron 6	IVS6-137T>A		ATCCTCTCTTTAAG(**T/A**)TTGCATATACTT	49	1	0	0.01 (0, 0.038)
Exon 7	1075A>C	I359L	GGTCCAGAGATAC(**A/C**)TTGACCTTCTC	45	4	1	0.06 (0, 0.126)
Intron 7	IVS7+38C>T		CAACTCCATGTTTT(**C/T**)GAAGTCCCCA	45	5	0	0.05 (0, 0.11)
Intron 8	IVS8+53A>T		AGATAACTTTTTTG(**A/T**)TCCATTGGAAC	45	4	1	0.06 (0, 0.126)
Intron 8	IVS8+147C>T		GTGTACACCCTG(**C/T**)TCATGATACATCC	24	21	5	0.31 (0.182, 0.438)
Intron 8	IVS8-112G>A		TTCATCTCTTCTAC(**G/A**)ATACACTGAAC	45	4	1	0.06 (0, 0.126)
Intron 8	IVS8-109A>T		ATCTCTTCTACGAT(**A/T**)CACTGAACAGT	17	24	9	0.42 (0.283, 0.557)
Intron 8	IVS8-98T>A		ATACACTGAACAG(**T/A**)TATTGCATATTC	49	1	0	0.01 (0, 0.038)
Exon 9	1425A>T	N474N	CAGTTGTCAATGG(**A/T**)TTTGCCTCTGTG	46	3	1	0.05 (0, 0.11)
3'UTR	7G>C		GTCTGAAGAAGA(**G/C**)CAGATGGCCTG	49	1	0	0.01 (0, 0.038)
3'UTR	396T>A		CTTCTTTTATGCA(**T/A**)AATGTAGGTCAG	45	4	1	0.06 (0, 0.126)

Parentheses with bold letters indicate the nucleotide change. *The reference sequence used was GenBank accession no. NC_000010.9. †Position is indicated in respect to the start codon ATG of the *CYP2C9* gene; the A in ATG is +1 and the next base toward to 5' is -1. ‡Variant alleles newly identified in the present study.

[18] were selected for full DNA sequencing of the *CYP2C9* gene. The influences of *CYP2C9* variants on low warfarin dose requirement were investigated. Of the 24 variants found in the 36 patients, five variants were not found in the normal 50 subjects (Table 2). Among five variants, two were *CYP2C9*13* and **14* variants each identified in one individual as a hetrerozygous mutation. The other three variants included two from the promoter region and one from the intron region. These three alleles exhibited no significant consequences in our sequence analysis using molecular software. Twenty SNPs were found only in 50 normal subjects and five SNPs were only observed in the 36 patients. All of these 25 SNPs except *CYP2C9*13* and **14* displayed no significant consequences in our software analysis. The degree of linkage disequilibrium (LD) (Figure 1) was analyzed by the Haploview program based on Gabriel's block definition [23] and seven Tagging SNPs (Figure 1B) were determined using SNPs found in 50 normal subjects. Variants with a frequency greater than 5% were included in the Haploview analysis to get a clear boundary of the LD block. The *CYP2C9* locus could be divided into two LD blocks between -2665__2664delTG and -1911T>C. Therefore recombination may have occurred between these two boundaries. However, the actual recombination would be a rare occurrence as it was too minor to divide the LD block. There were different frequencies of the haplotype between the group of 36 patients and the 50 healthy normal subjects. The most sig-

Table 2

Genetic polymorphisms of the *CYP2C9* gene in 36 patients who required for low warfarin dose*

Site	Nucleotide change and position†	Amino acid change	Nucleotide changeand flanking sequence	Subject number (n) w/w	w/m	m/m	Allelic frequency (95%CI)
5-UTR	-3089G>A		TGATTCCAACC(**G/A**)TATTACATTTTG	23	9	4	0.24 (0.097, 0.375)
5-UTR	-2665~-2664delTG		TCAGTGAC(**DEL/TG**)TGGAGGGCTTAA	23	9	4	0.24 (0.097, 0.375)
5-UTR	**-1924A>G‡**		GTTTCATGAGTC(**A/G**)GGGACCAAGTT	35	0	1	0.03 (0, 0.082)
5-UTR	-1565C>T§		GCTTCCTCATTC(**C/T**)GGAAATGGGTC	26	9	1	0.15 (0.035, 0.27)
5-UTR	**-1322T>C‡**		AAGTATTGACAT(**T/C**)AATGATCTAGTA	35	1	0	0.01 (0, 0.052)
5-UTR	-1188T>C§		ACCTCCCATCTT(**T/C**)TATTGCATCCAC	14	16	6	0.39 (0.23, 0.548)
Intron	IVS1+83T>C		CCTAGAGGTACA(**T/C**)GTTACAAGAGG	34	2	0	0.03 (0, 0.082)
Intron	**IVS1+125T>C‡**		CTTTGAAAGGCT(**T/C**)TTGTTGCCTTTT	35	1	0	0.01 (0, 0.052)
exon2	**269T>C‡**	L90P	CCCTGATTGATC(**T/C**)TGGAGAGGAGT	35	1	0	0.01 (0, 0.052)
Intron	IVS1-22T>C		CTTCGTTTGCTG(**T/C**)TATCTCTGTCTA	35	1	0	0.01 (0, 0.052)
Intron	IVS2+73T>C		GACTTACAGAGC(**T/C**)CCTCGGGCAGA	34	2	0	0.03 (0, 0.082)
exon3	**374G>A‡**	R125H	AGGAGATCCGGC(**G/A**)TTTCTCCCTCAT	35	1	0	0.01 (0, 0.052)
Intron	IVS3+197G>A§		GCATGATTGTGC(**G/A**)TACAGTGTGGG	26	9	1	0.15 (0.026, 0.252)
Intron	IVS3+239C>T		ATCCCATGTTCTC(**C/T**)TGAACTTTGCT	25	8	4	0.24 (0.1, 0.38)
Intron	IVS3-334C>T§		TCTCAGTGCCTTG(**C/T**)TGTCTACTGACT	26	9	1	0.15 (0.035, 0.27)
Intron	IVS3-65G>C§		AACTACTATTATCT(**G/C**)TTAACAAATAC	26	9	1	0.15 (0.035, 0.27)
Intron	IVS4-115A>G§		TTTCCCCATCAAG(**A/G**)TATACAATATA	26	9	1	0.15 (0.035, 0.27)
Intron	IVS4-50T>C		TGGTATATGGTATG(**T/C**)ATGCTTTTATTA	35	1	0	0.01 (0, 0.052)
Intron	IVS5-73A>G§		ATAACTATGTGA(**A/G**)TAATTTTGAATTC	26	9	1	0.15 (0.035, 0.27)
Intron	IVS6+95A>G		TAGAGAAGCTTC(**A/G**)TTATTTAAACTTT	34	2	0	0.03 (0, 0.082)
Intron	IVS6+152A>G		ATGGTGATTACA(**A/G**)TGGGATATCTTGG	34	2	0	0.03 (0, 0.082)
Intron	IVS7+38C>T		CAACTCCATGTTTT(**C/T**)GAAGTCCCCA	34	2	0	0.03 (0, 0.082)
Intron	IVS8+147C>T		GTGTACACCCTG(**C/T**)TCATGATACATCC	24	8	4	0.24 (0.1, 0.38)
Intron	IVS8-109A>T		ATCTCTTCTACGAT(**A/T**)CACTGAACAGT	14	16	6	0.39 (0.23, 0.548)

Parentheses with bold letters indicate the nucleotide change in the flanking sequence. *The reference sequence used was GenBank accession no. NC_000010.9. †Position is indicated in respect to the start codon ATG of the *CYP2C9* gene; the A in ATG is +1 and the next base toward to 5′ is −1. ‡Unidentified *CYP2C9* variants in the 50 normal subjects are indicated in bold letters. §*CYP2C9* variants exhibited significantly different allele frequencies compared with the 50 normal healthy subjects.

nificant difference between the two groups was from three variants, *-1565C>T*, *IVS3-65G>C* and *IVS4-115A>G*, which were found in perfect LD, exhibiting 4% frequency in the normal group and 15% in the 36 patients (data not shown due to less than 5% frequency). These three variants were predicted to construct a haplotype together with -1188T>C, IVS3+197G>A, IVS3-334C>T, and IVS5-73A>G as reported previously in the Japanese population as *CYP2C9*1e* (hereafter referred to as *CYP2C9*1e*) [20]. To study further the association between the *CYP2C9*1e* and warfarin dose, a genotyping method for the detection of *IVS4-115A>G* was developed using pyrosequencing (data not shown) as a marker SNP of *CYP2C9*1e*. The relationship between the haplotype and warfarin sensitivity was investigated (Figure 2). Regardless of the *CYP2C9*3*, **13*, and **14* genotypes, the effect of heterozygous mutation of *IVS4-115A>G* on warfarin dose sensitivity appeared to be insignificant. However, *IVS4-115A>G* tended to affect the warfarin dose requirement in patients homozygous for this mutation ($P = 0.18$) (Figure 2A). The odds ratio for *CYP2C9*1e* between the low dose *vs.* the normal dose groups was insignificant (OR 1.75, 95% CI 0.79, 3.87, $P = 0.17$). However, when the factors affecting warfarin dose were adjusted, the individuals carrying the *CYP2C9*1e* had a 2.5-fold increased odds of low dose requirement (OR 2.54, 95% CI 1.05, 6.16, $P = 0.04$). Patients homozygous for the *IVS4-115A>G* mutation showed warfarin dose requirement levels similar to the individuals carrying *CYP2C9*3*, **13* and **14* genotypes (Figure 2B). Since one of the SNPs in *CYP2C9*1e*, -1565C>T, was predicted to alter possibly a transcriptional binding site, gel shift assay was performed (Figure 3). A representative of three different experiments was presented. All experiments consistently indicated a specific binding pattern to wild-type probe only and shifted bands disappeared after the addition of only wild-type cold competitor. Mutated cold competitor (1565T) addition did not change any shifted bands. The probe containing a single mutation at 1565C>T did not exhibit any binding shift as shown in the wild-type. A computer search revealed that this region contained a consensus binding motif (TTC-CNNNAA) for STAT proteins [24]. Addition of a cold competitor known as the core sequence of STAT binding sequence (5′-TGGGGCCAGATTCCGAGAAGACAGCAT-3′) [24] did not affect the band shift or density, suggesting that the binding is likely to originate from the other nuclear proteins rather than the STAT proteins (data not shown).

Discussion

The fixed mean daily dose of warfarin in Asians has been reported as low compared with Caucasians [25, 26]. This is

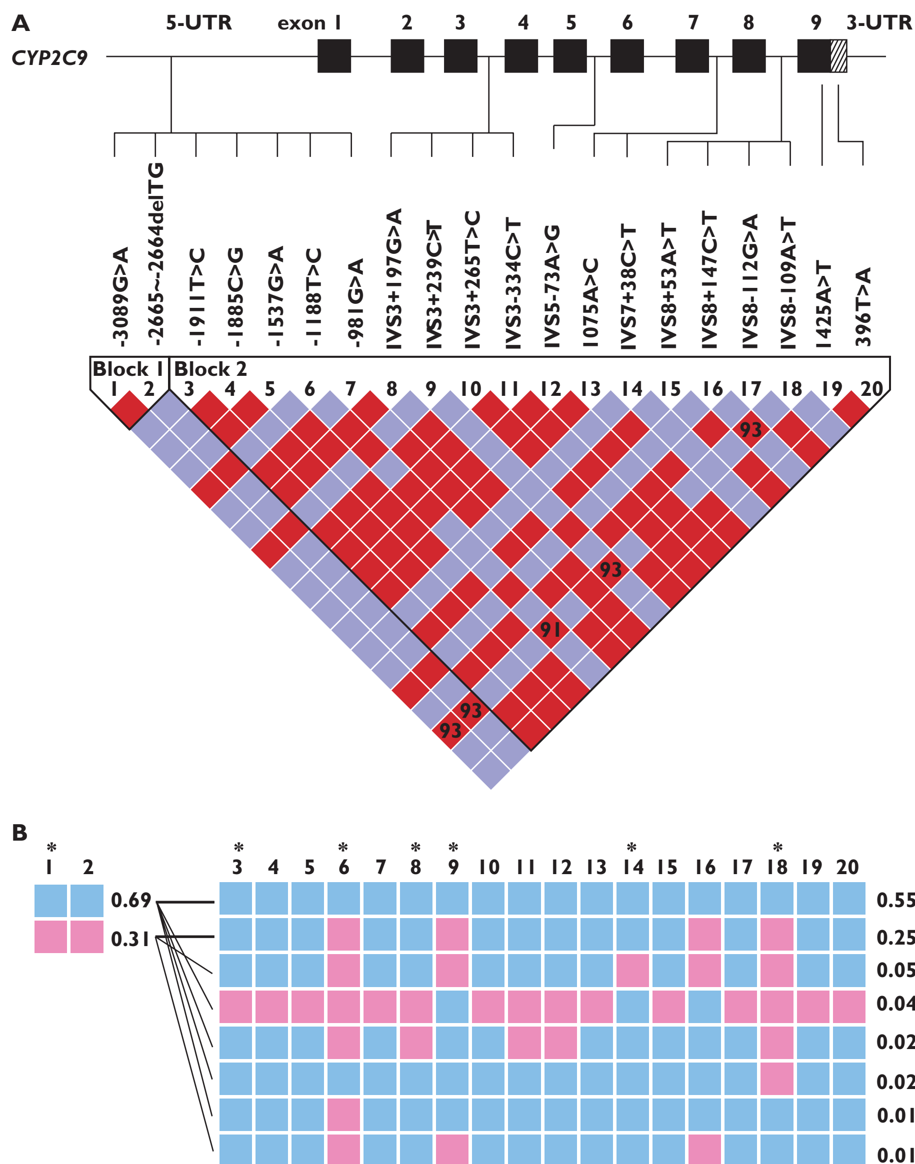

Figure 1

Linkage disequilibrium (LD) map of *CYP2C9* single nucleotide polymorphisms obtained from normal healthy subjects ($n = 50$). *CYP2C9* variants at >5% were included in the LD analysis using the statistics |D′| and r^2 values [23]. A) Sequential arrangement of *CYP2C9* SNPs along with their locations in the *CYP2C9* gene. The red colour depicts a significant linkage between the pair of SNPs. Numbers inside the square are the D′ value multiplied by 100. B) *CYP2C9* SNPs and their occurrence in common haplotype structures in two blocks. Frequency of each haplotype is shown at the edge. The blue square indicates the most common allele and the pink colour represents the variant allele. The selected tag SNPs are indicated by the star symbol. The thick black lines between haplotypes indicate the most common crossings from block 1 to block 2 and thinner lines mark the less common crossings.

consistent with Asians having lower INR values for anticoagulation than Caucasians [27, 28]. This phenotype is not fully explained with the current genotypes of *CYP2C9* and *VKORC1* even after adjustment for low body weight and other factors present in Asian people, suggesting that unidentified genetic factors or other environmental factors are involved in Asians. Although *CYP2C9*2* and **3* are important genetic factors for predicting warfarin dose in Caucasians, these alleles are not critical factors for Asians due to their having very low frequencies. Furthermore, there are still large inter-individual variations in warfarin doses in people with *CYP2C9*1/*1* genotype.

Full DNA sequencing of the *CYP2C9* gene in 50 individuals from a Korean population exhibited similar variant frequencies compared with other Asian reports [17, 20]. A set of seven tag SNPs of *CYP2C9* were determined for the first time in Koreans. It is suggested that these tag SNPs would be required to track all important haplotypes in the *CYP2C9* gene in Koreans. Although no novel mutations were detected in our sequencing analysis, two variants

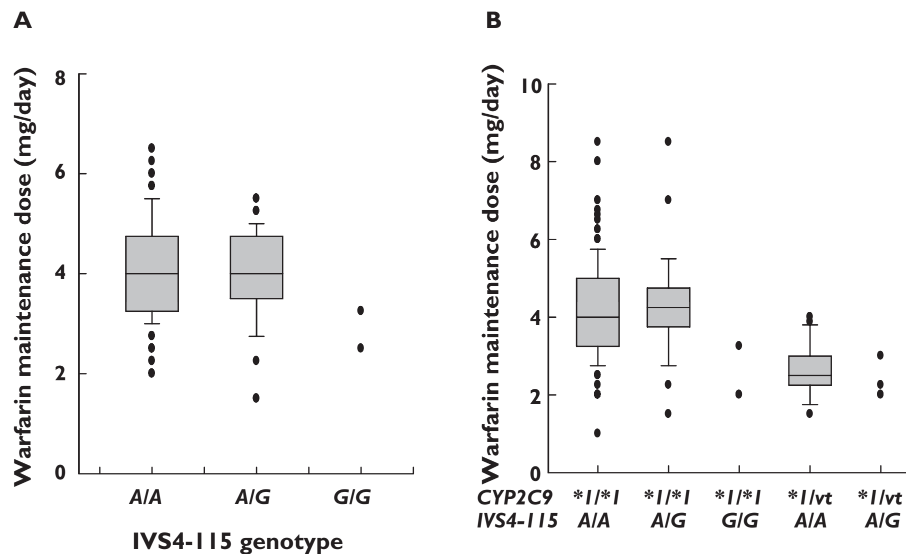

Figure 2

Influence of the *CYP2C9*1e* haplotype on warfarin maintenance dose. A) All patients with the *IVS4-115A>G* genotype only, which was used for the detection of *CYP2C9*1e*. B) Patients stratified by *CYP2C9* variants and *IVS4-115A>G*. All *CYP2C9* variants (vt) were heterozygous for mutations of *3, *13, and *14. Boxes extend from the 25th to the 75th percentiles and vertical lines extending from the 10th to 90th percentiles.

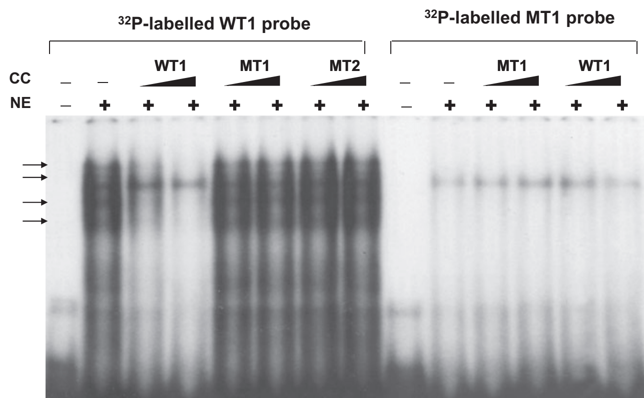

Figure 3

Electrophoretic mobility shift assays (EMSA) of the oligonucleotide containing a -1565C>T change with human liver nuclear extract (NE). EMSAs demonstrate the binding of human liver nuclear proteins (indicated by arrows) to wild-type oligonucleotide probe containing -1565C only. EMSAs were performed as described in Methods. Oligonucleotides for wild-type (WT) and MT-1 (-1565T) were labelled with [^{32}P]-dATP. Oligonucleotides were incubated with nuclear extract at 4°C for 30 min, followed by 5% polyacrylamide gel electrophoresis. Nonradiolabeled cold competitors (CC) were added at 20 and 50-fold excess to determine the specificity of binding complex.

were quite intriguing: one in the 5′-UTR (*-1565C>T*) disrupted a palindromic sequence motif in the promoter region and the other in intron 4 (*IVS4-115A>G*) had a possible implication in splicing events (predicted at 89% by a splicing prediction program (http://www.fruitfly.org/seq_tools/splice.html). These two variants were found to construct a haplotype with five other intron variants having 4% frequency in 50 randomly selected subjects, and 15% frequency in patients who required low warfarin maintenance doses. These variants were previously reported as a haplotype, known as *CYP2C9*1e*, which includes -1565C>T, -1188T>C, IVS3+197G>A, IVS3-334C>T, IVS3-65G>C, IVS4-115A>G, and IVS5-73A>G [20]. Interestingly, a SNP in the *CYP2C9*1e* haplotype, IV3-65G>C, has been reported to be associated with warfarin low dose requirement in Taiwanese Chinese [29] and Han Chinese [30]. Frequency of IVS3-65G>C in the normal population is somewhat inconsistent, since Wang *et al.* reported a 4.5% frequency in healthy normal Chinese subjects (n = 995) [30] and Blaisdell *et al.* reported a 4.3% frequency in 23 Asian samples obtained from Human Genetic Cell Repositories sponsored by the National Institute of health housed at the Coriell Institute [17]. However healthy Taiwanese subjects (n = 69) showed 10.1% frequency [29] and normal Japanese subjects (n = 263) exhibited 11.8% frequency [20]. Further study would be necessary to determine the allele frequency in Asian populations. Although the frequency of this allele has been shown to be inconsistent in the literature, association of this haplotype with wafarin low dose requirement appears to be consistent as shown in two independent studies [29, 30] and in the present study. Since this haplotype has not been identified in Caucasians [31], it appears to exist preferentially in Asian populations. As reported in the previous study [29], this haplotype was not found with the *CYP2C9*3* variant, suggesting that this haplotype segregated differently from the *CYP2C9*3* variant. A sequence analysis program for splicing prediction indicated a possible splicing donor site for variant *IVS4-115A>G* with 89% probability. However,

since *CYP2C9* is a large gene spanning more than 50 kb, predicting any obvious changes in splice sites is currently impossible. In our gel shift assay the oligonucleotide probe containing a -1565C>T mutation disrupted its specific binding to nuclear proteins, suggesting that there might be a regulation mechanism related to this mutation (Figure 3). Shintani *et al.* reported that the promoter construct having a -1565C>T mutation exhibited approximately 15% decreased luciferase activity compared with the wild-type with a slight decreased intrinsic clearance of phenytoin [32]. These data would be supportive of the interpretation of warfarin sensitivity, but our assay of transcriptional activity did not show significant changes between the wild-type and variant promoters in luciferase assays (data not shown) in HepG2 cells. It could be possible that HepG-2 cells may not contain enough transcriptional machinery associated with the bound protein in the present gel-shift assay.

Among the *CYP2C9* polymorphisms, *CYP2C9*2* and **3* appear to cause functional consequences in most clinical drug responses as well as side effects. *CYP2C9*3* (I359L) exhibited a significant decrease in activity for most *CYP2C9* substrates *in vivo*, resulting in approximately 50% of the wild-type activity in heterozygous individuals, with a statistically insignificant impact in some cases, and an approximately 5- to 10-fold reduction in homozygous individuals with statistically significant potency. In the present study, one of our goals was to find *CYP2C9* variants or haplotypes responsible for the low warfarin dose requirement. Therefore, individuals carrying *CYP2C9*3* were excluded in the resequencing of *CYP2C9*, since *CYP2C9*3* is a commonly known allele for low dose requirement of warfarin in many population studies. Although *CYP2C9*3* is a common cause for the low dose requirement in Asia, the possibility of new variants in *VKORC1* in addition to *CYP2C9*1e* in the low dose group cannot be ruled out. In order to compare the distribution of *CYP2C9*1e* haplotype in the low and high dose groups, one of the variants comprising *CYP2C9*1e* haplotype, IVS4-115A>G, was genotyped. The low dose group (<3 mg day^{-1}, $n = 36$) exhibited 10 alleles of IVS4-115G which comprised of three alleles with *CYP2C9*1/*3* and seven alleles with *CYP2C9*1/*1*, resulting in 14% frequency of the IVS4-115A>G. All these 10 alleles were of the *VKORC1 1173* T/T genotype. The high dose group (>5.5 mg day^{-1}, $n = 34$) showed five individuals having a heterozygous mutation of IVS4-115A>G, resulting in 7% frequency of the IVS4-115A>G. Interestingly, all these five individuals were found to have the *VKORC1 1173* C/T genotype which has been shown to require a higher warfarin dose than the *VKORC1 1173* T/T genotype, suggesting that the high doses of warfarin in these patients may be due to the influence of the *VKORC1* genotype and not the *CYP2C9* genotype.

Our observations revealed that two individuals having homozygous mutation of *CYP2C9*1e* showed a low warfarin dose requirement compared with the wild type, similar to the extent of the *CYP2C9*3* heterozygous mutation. This phenotype/genotype relationship is in accordance with previous reports [29, 30]. Although we suggested possible changes of splicing and promoter function caused by the haplotype *CYP2C9*1e*, the present study does not provide a clear cellular mechanism on how *CYP2C9*1e* causes warfarin sensitivity. However, in addition to our results, the association of *CYP2C9*1e* with warfarin dose was repeated in two other independent studies in geographically different regions. Further evaluation with different substrates and more subjects with this genotype are necessary to derive a decisive answer on the consequence of *CYP2C9*1e* in Asians. The clinical relevance of these variants with respect to warfarin dose could be valuable markers in Asian populations.

Competing interests

There are no competing interests to declare.

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