J Hum Genet (2006) 51:249–253
DOI 10.1007/s10038-005-0354-5

ORIGINAL ARTICLE

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Association of *VKORC1* and *CYP2C9* polymorphisms with warfarin dose requirements in Japanese patients

Received: 11 October 2005 / Accepted: 28 November 2005 / Published online: 24 January 2006

Abstract Warfarin is the most commonly used oral anticoagulant for treatment of thromboembolism, but adjustment of the dose appropriate to each patient is not so easy because of the large inter-individual variation in dose requirement. We analyzed single nucleotide polymorphism (SNP) genotypes of the *VKORC1* and *CYP2C9* genes using DNA from 828 Japanese patients treated with warfarin, and investigated association between SNP genotype and warfarin-maintenance dose. Five SNPs in *VKORC1*, 5′ flanking−1413A > G, intron 1−136T > C, intron 2+124C > G, intron 2+837T > C and exon 3 343G > A, were in absolute linkage disequilibrium, and showed a significant association with daily warfarin dose of these patients. The median warfarin dose of patients with homozygosity for the minor allele was 4.0 mg/day, which is significantly higher than those heterozygous for the minor allele (3.5 mg/day) or those homozygous for the major allele (2.5 mg/day; $P = 5.1\times10^{-11}$ in the case of intron 1−136T > C SNP). We then genotyped the *CYP2C9* gene for the Japanese common genetic variant, *CYP2C9*3* and, based on the genotype of these two genes, classified patients into three categories, which we call "warfarin-responsive index." The median warfarin daily dose varied significantly in this classification according to the warfarin-responsive index (2.0 mg/day for index 0 group, 2.5 mg/day for index 1 group, and 3.5 mg/day for index 2 group; $P = 4.4\times10^{-13}$). Thus, analysis of the combination of *VKORC1* and *CYP2C9* genotypes should identify warfarin-sensitive patients who require a lower dose of drug, allowing personalized warfarin treatment.

Keywords Pharmacogenomics · Warfarin · *VKORC1* · Vitamin K epoxide reductase · *CYP2C9* · Cytochrome P450 2C9 · SNP

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Introduction

The application of pharmacogenomic/pharmacogenetic information to clinical treatment is expected to help in the prediction of efficacy and/or toxicity of drugs, leading to appropriate therapeutic regimens for individual patients, thus contributing to improvement of our medical care. In an attempt to identify genetic parameters that predict efficacy or risk of adverse reactions for various drugs, many investigators have conducted and are conducting association studies using genetic polymorphisms detected in genes encoding drug-metabolizing enzymes, transporters, receptors, and proteins involved in the drug-signaling pathway. However, only a few useful examples have so far been applied to routine clinical practice for prediction of efficacy/toxicity prior to drug administration (Lesko and Woodcock 2004).

Warfarin is the most commonly used oral anticoagulant in the world. Although this agent is indispensable

for treatment of thromboembolism, it is not so easy to adjust the appropriate dose to each patient due to the large inter-individual variation in the requirement for this drug. An insufficient dose will result in failure to prevent thrombosis, while overdose increases the risk of unexpected bleeding. The maintenance dose of warfarin is usually determined by monitoring prothrombin time using an international normalization ratio (INR). An INR of 1.5–2.5 is recommended for Asian populations (Matsuyama et al. 2002).

Although clinically available warfarin is a racemic mixture, (*S*)-warfarin is five times more potent as an anticoagulant than (*R*)-warfarin (Breckenridge et al. 1974). (*S*)-Warfarin is primarily metabolized to the 7-hydroxylated form in humans, principally by cytochrome P450, subfamily IIC, polypeptide 9 (*CYP2C9*) (Rettie et al. 1992). To date, more than 50 variants in the *CYP2C9* gene have been described (Allele Nomenclature Committee home page: http://www.imm.ki.se/CYPalleles), of which two single-nucleotide polymorphisms (SNPs), *CYP2C9*2* (R144C) and *CYP2C9*3* (I359L), have been well verified in relation to the wild-type allele *CYP2C9*1*. No Asians so far studied have the *CYP2C9*2* allele (Kimura et al. 1998; Takahashi et al. 2003; Kirchheiner and Brockmoller 2005). Previous studies have demonstrated that the warfarin 7-hydroxylase activity of the *CYP2C9*3* variant allele is approximately 10-fold lower than that of *CYP2C9*1* in in vitro analysis (Lee et al. 2002) and that carriers of the variant allele required a lower maintenance dose of warfarin (Takahashi et al. 2003; Kirchheiner and Brockmoller 2005). Warfarin exerts its anticoagulant effects by interfering with regeneration of vitamin K by reduction of its 2,3-epoxide in the vitamin K cycle, leading to inhibition of gamma-carboxylation of vitamin K-dependent clotting factor II (prothrombin), VII, IX and X. This vitamin K epoxide reductase is encoded by vitamin K epoxide reductase complex subunit 1 (*VKORC1*) (Rost et al. 2004; Li et al. 2004). Rare mutations that lead to amino acid changes in the VKORC1 protein have been found in familial cases in vitamin K-dependent clotting factor defective patients as well as in those with warfarin resistance, although these mutations have not been identified in the general population (Rost et al. 2004; Harrington et al. 2005). Recently, several groups have demonstrated that non-coding SNPs in *VKORC1* influenced warfarin sensitivity (D'Andrea et al. 2005; Wadelius et al. 2005; Yuan et al. 2005; Rieder et al. 2005; Sconce et al. 2005). These data strongly suggest that differences in genetic variations in both *CYP2C9* and *VKORC1* could explain the diversity in warfarin sensitivity and dose requirement. Interestingly, recent reports indicate an additive effect on warfarin dose requirement in combinations of variant forms of *VKORC1* and *CYP2C9* in Caucasian patients (D'Andrea et al. 2005; Sconce et al. 2005).

Furthermore, it is well known that there are significant differences in warfarin dose requirement in different ethnic groups; for example, Chinese patients were re-

Table 1 Patient characteristics ($n = 828$)

Age (years), median (range)	68 (19–92)
Gender (%)	
Male	67
Female	33
Daily warfarin dose (mg), median (range)	2.5 (0.5–10.5)
CYP2C9 genotype, *n* (%)	
*1/*1	790 (95%)
*1/*3	37 (4%)
*3/*3	1 (<1%)

Table 2 Daily maintenance dose of warfarin in patients with different *VKORC1* genotypes

Genotype	No. of patients	Dose (mg/day)		*P* value
		Median	Range	
5′ Flanking−1413A > G				
GG	6	4.0	2.0–8.0	9.1×10^{-11}
GA	133	3.5	1.0–10.5	
AA	689	2.5	0.5–10.0	
Intron 1−136T > C				
CC	6	4.0	2.0–8.0	5.1×10^{-11}
CT	132	3.5	1.0–10.5	
TT	690	2.5	0.5–10.0	
Intron 2 + 124C > G				
GG	6	4.0	2.0–8.0	5.1×10^{-11}
GC	132	3.5	1.0–10.5	
CC	690	2.5	0.5–10.0	
Intron 2 + 837T > C				
CC	6	4.0	2.0–8.0	5.1×10^{-11}
CT	132	3.5	1.0–10.5	
TT	690	2.5	0.5–10.0	
Exon 3 343G > A				
AA	6	4.0	2.0–8.0	9.1×10^{-11}
AG	133	3.5	1.0–10.5	
GG	689	2.5	0.5–10.0	

ported to require a warfarin dose nearly 40% lower than that required by Caucasian patients (Zhao et al. 2004). In addition, the therapeutic maintenance dose of warfarin for Japanese was also 31% lower than that for Caucasians (Takahashi et al. 2003). These phenotypic differences in dose requirement cannot be explained only by SNPs in *CYP2C9* (Takahashi et al. 2003), suggesting that interethnic variability in *VKORC1* might play an important role. We describe here the results of association studies using the SNPs in *VKORC1* to identify genetic variations that might confer sensitivity to warfarin in Japanese patients. Based on the genotypes for *VKORC1* SNPs significantly associated with warfarin sensitivity, in combination with genotypic information of *CYP2C9* polymorphisms, we developed a prediction system that was able to determine the dose appropriate to each patient.

Materials and methods

Subjects

A total of 828 patients with warfarin treatment was recruited at Tokushukai Hospital, Japan. Clinical conditions for anticoagulation therapy with warfarin varied, but it was used for prevention or treatment of thromboembolic diseases. In each patient, the orally administered dose of warfarin (1–4 times a day) was clinically adjusted based on anticoagulation response, as measured by INR. All subjects were Japanese and gave written informed consent to participate in the study in accordance with the process approved by the Ethical Committees at The Institute of Medical Science at the University of Tokyo, Tokyo, Japan.

SNP genotyping

SNPs were genotyped by direct sequencing of PCR products from warfarin-treated patients using a capillary sequencer (ABI3730, Applied Biosystems, Foster City, CA), as previously described (Iida et al. 2001), and/or the TaqMan assay (Morris et al. 1996). The probe sets for the TaqMan assay were obtained from Applied Biosystems. DNA extraction and PCR primer design were performed as previously described (Ohnishi et al. 2001).

Statistical analysis

Differences in daily maintenance dose of warfarin in the different genotype groups were evaluated by Mann–Whitney's *U* and Kruskal–Wallis tests using SPSS software (version 12.0, SPSS, Chicago, IL). A 5% two-tailed significance level was used in all tests.

Results

Patient characteristics

Table 1 summarizes clinical information for the 828 warfarin-treated patients in the present study. The median maintenance daily dose of warfarin was 2.5 mg/day. For the *CYP2C9* gene, only *CYP2C9*3*, a common Japanese genetic variant, was evaluated. As a result, 790 subjects (95%) were found to be homozygous for *CYP2C9*1*, 37 patients (4%) were heterozygous for *CYP2C9*3* and 1 was homozygous for *CYP2C9*3*. The observed allele frequencies of *CYP2C9*1* and *CYP2C9*3* were comparable to those previously reported in healthy Japanese subjects (Kimura et al. 1998).

Association of *VKORC1* and *CYP2C9* SNPs with warfarin dose

We genotyped the five known SNPs, 5′ flanking −1413A > G, intron 1 −136T > C, intron 2 + 124C > G, intron 2 + 837T > C and exon 3 343G > A in patients receiving warfarin treatment. The genotypes of these five SNPs in the *VKORC1* gene for 828 patients were completely concordant except for one subject. As shown in Table 2, the median warfarin daily dose was 4.0 mg for patients homozygous for the minor allele, 3.5 mg for those heterozygous for the minor allele and 2.5 mg for

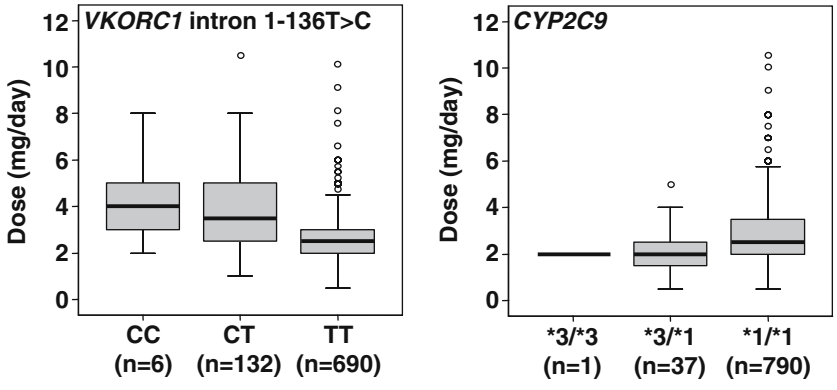

Fig. 1 *Box* and *whisker plot* showing daily warfarin dose for patients with different genotypes for *VKORC1* [single nucleotide polymorphism (SNP): intron 1 −136T > C] and *CYP2C9* (allele: **1*, **3*). The *horizontal line* indicates the median dose, the *box* covers the 25–75 percentiles and the maximum length of each whisker is 1.5 times of the interquartile range. Points outside them show up as outliers

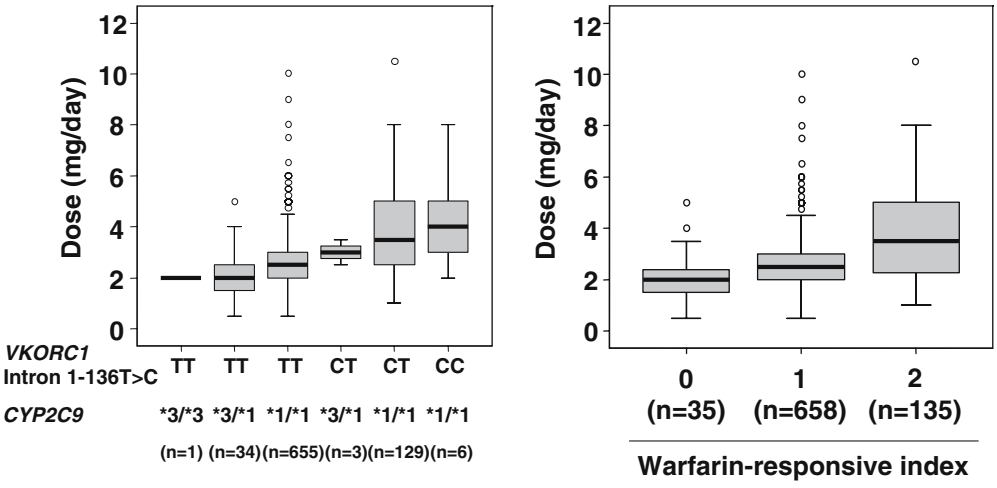

Fig. 2 *Box* and *whisker plot* showing daily warfarin dose for patients classified by our definition of warfarin-responsive index on the basis of a combination of two SNP genotypes of *VKORC1* (SNP: intron 1−136T > C) and *CYP2C9* (allele: **1*, **3*)

those homozygous for the major allele. The differences in warfarin dose between the three genotypic groups were significant [$P = 9.1\times10^{-11}$ for 5′ flanking−1413A > G and exon 3 343G > A, and $P = 5.1\times10^{-11}$ for the three other SNPs (Kruskal–Wallis test)]. The five significant SNPs were used to infer *VKORC1* haplotypes from all patients, yielding two common haplotypes, H1 and H2, with frequencies of 0.91 and 0.09, respectively. The frequencies for the H1/H1, H1/H2 and H2/H2 genotypes were 0.83, 0.16 and 0.0072, respectively, which was consistent with the single SNP results due to absolute linkage disequilibrium (LD) in the five SNPs. In addition, the median warfarin dose in the patient group of *CYP2C9*3* carriers (2.0 mg/day) was significantly lower than that required for *CYP2C9*1* homozygotes (2.5 mg/day; $P = 3.9\times10^{-4}$, Mann–Whitney's *U* test; Fig. 1).

To further examine whether genetic information from two genes can increase the power of prediction of warfarin sensitivity, we combined genotypes of *VKORC1* and *CYP2C9*, and analyzed their effect on warfarin dose. It is notable that patients with a TT genotype for intron 1−136T > C of *VKORC1* required a lower daily dose of warfarin than those carrying the CT or CC genotypes, both in extensive and poor metabolizers of *CYP2C9* (Fig. 2). We then classified patients into three groups according to the genotypes of these two genes and constituted a genetic index termed "warfarin-responsive index." In the case of *VKORC1*, we assigned a score of 0 to individuals with the TT genotype, which appeared to contribute to warfarin sensitivity, and 1 to individuals with CC or CT genotypes. For *CYP2C9*, we assigned a score of 1 to individuals homozygous for *CYP2C9*1* (*CYP2C9*1/*1*) and 0 to individuals with *CYP2C9*1/*3* and *CYP2C9*3/*3*. As shown in Fig. 2, the median warfarin daily dose varied significantly in the three groups as classified by the warfarin-responsive index (2.0 mg/day for the group with score 0, 2.5 mg/day for those with score 1, and 3.5 mg/day for the group with score 2; $P = 4.4\times10^{-13}$, Kruskal–Wallis test).

Discussion

Our study revealed that five known SNPs of *VKORC1* were in absolute LD in the Japanese population and that they were significantly associated with daily warfarin dose (Table 2). In the case of intron 1−136T > C, patients with allele T, in either heterozygous or homozygous form, were adapted to a lower daily dose of warfarin than patients with the CC genotype (Fig. 1). Recently, several groups have demonstrated that polymorphisms in *VKORC1* are associated with warfarin sensitivity (D'Andrea et al. 2005; Wadelius et al. 2005; Yuan et al. 2005; Rieder et al. 2005; Sconce et al. 2005). In a Swedish study, weekly warfarin doses varied significantly, and were associated with four *VKORC1* SNPs (5′ flanking−1413A > G, intron 1−136T > C, intron 2 + 837T > C and exon 3 343G > A), similar to our results (Wadelius et al. 2005). Yuan et al. (2005) demonstrated that Chinese patients homozygous for the major allele of the −1413A > G polymorphism required a lower warfarin dose than those with other genotypes. The authors also suggested that the promoter polymorphism abolished the E-box consensus sequences, leading to a 44% increase in promoter activity for the minor allele compared to the major allele. Thus, one of these SNPs should be a good indicator for defining warfarin-insensitive patients.

Ethnicity appears to be an important factor in determination of warfarin-maintenance dose requirement. It has been reported that Chinese, Malay and Japanese patients required a 30–40% lower warfarin-maintenance dose than Caucasian patients (Takahashi et al. 2003; Zhao et al. 2004). These interethnic differences may be attributed to genetic differences. In the present study, the frequency of allele G in 5′ flanking−1413A > G of *VKORC1* in the Japanese population (0.09) was much lower than that in the Swedish population (0.61; Wadelius et al. 2005). Thus, the frequency of the AA genotype associated with warfarin sensitivity in Japanese patients (0.83) was much higher than that in

Caucasians (0.14) but comparable to Chinese (0.82; Yuan at al. 2005), suggesting that *VKORC1* genotype frequencies could account for the interethnic differences in warfarin dose requirements.

The benefit of *CYP2C9* genotyping prior to drug administration is still under discussion because of the relatively small effect on warfarin dose requirement and interethnic differences (Wadelius et al. 2005; Yuan et al. 2005). However, in the present study, we have shown that prediction of more effective and safer warfarin dose can be achieved using information on the combination of *VKORC1* and *CYP2C9* genotypes, as translated into the warfarin-responsive index. For example, the warfarin daily dose requirement for patients with *CYP2C9*3*/**1* and TT genotype of *VKORC1* intron 1−136T > C (index score 0) is estimated to be approximately 2-fold lower than that for patients with *CYP2C9*1*/**1* and *VKORC1* CC genotype (index score 2). Although prospective clinical studies are essential to assess the new classification index, our prediction system may be useful in identifying warfarin-sensitive patients who require a lower dose of drug, and represents a step towards the goal of personalized medicine.

Acknowledgments We thank S. Kawauchi and A. Ohno for their excellent technical assistance; and all members of the SNP Research Center, The Institute of Physical and Chemical Research, for their contribution to the completion of our study.

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