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Effect of *VKORC1* −1639 G>A polymorphism, body weight, age, and serum albumin alterations on warfarin response in Japanese patients

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ABSTRACT

Introduction: To establish individualized warfarin therapy, we investigated the contribution of genetic variations of vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*) −1639 G>A and Cytochrome P450 2C9 gene (*CYP2C9*) and clinical factors on warfarin sensitivity in Japanese patients.
Materials and Methods: Genetic analyses of *VKORC1* −1639 G>A and *CYP2C9* *2, *3, and *4 were performed in 259 Japanese patients and 341 healthy subjects. We selected 259 patients who have been prescribed warfarin with a 1.5–3.0 range of prothrombin time normalized as an international normalized ratio for at least 3 months and investigated factors that contribute to individual variability in warfarin dose. Furthermore, multivariate analysis was performed to investigate a warfarin dosing algorithm.
Results and Conclusions: There were great inter-individual differences in warfarin maintenance dose in 259 patients, ranging from a minimum dose of 0.75 mg/day to a maximal dose of 8.00 mg/day. *VKORC1* −1639 G>A polymorphism, body weight, age, and serum albumin were found to affect the inter-individual variability. The dosing algorithm of warfarin maintenance dose was investigated by multivariate linear regression. The regression equation was able to account for 33.2% ($R^2_{Adj} = 0.332$) of the overall variability in warfarin dose.

Introduction

Warfarin is one of the most widely prescribed anticoagulants to prevent and treat venous and arterial thromboembolism. Warfarin has a very narrow therapeutic index—increased anticoagulant effect puts the patients at a risk of bleeding, while decreased anticoagulant effect puts them at a risk of thromboembolic disorders such as heart attack and stroke. In addition, warfarin exhibits large inter-individual and inter-ethnic differences in the dose required for its anticoagulation effect. Therefore, it needs long time to determine the adequate dosage for each patient within the optimal prothrombin time normalized as an international normalized ratio (PT-INR) range.

Vitamin K epoxide reductase (VKORC1) recycles vitamin K 2,3-epoxide to vitamin K hydroquinone, a cofactor that is essential for γ-glutamyl carboxylation of clotting factors II, VII, IX, and X [1–3]. Warfarin, a coumarin derivative, exerts its anticoagulant effect by inhibiting VKORC1 [3,4]. Genetic variants in the gene encoding VKORC1 result in altered sensitivity to warfarin [5]. Since the rare mutations in *VKORC1* gene were identified as causes for clotting factor deficiencies and warfarin resistance [6,7], many clinical studies have investigated the role of *VKORC1* genetic polymorphisms on warfarin dose requirements [5]. Rieder *et al.* [8] categorized eight haplotypes into H1-H8 as combinations of 10 genetic variants of *VKORC1*. In their study, patients with H1 or H2 haplotype required low warfarin dose and patients with H7-H9 haplotypes required high warfarin dose. The *VKORC1* −1639 A allele was categorized into H1 and H2, and the G allele was categorized into H7-H9. This single nucleotide polymorphism (SNP) is located at the promoter region of *VKORC1* gene [9]. A *VKORC1* −1639 G>A SNP qualitatively changed the expression of the VKORC1 protein [8]. VKORC1 promoter with *VKORC1* −1639 G allele showed 44%

Abbreviations: VKORC1, Vitamin K epoxide reductase complex subunit 1; CYP2C9, Cytochrome P450 2C9; PT-INR, prothrombin time normalized as an international normalized ratio; SNP, single nucleotide polymorphism; V_{max}, maximum velocity; K_m, Michaelis constant; FDA, Food and Drug Administration; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; ANOVA, analysis of variance; ANCOVA, analysis of covariance; MD, maintenance dose; GGCX, γ-glutamylcarboxylase.

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increase of promoter activity compared with the A allele [9]. Therefore, a *VKORC1* −1639 G>A SNP causes a reduction in promoter activity and consequently reduces expression of both mRNA and VKORC1. Therefore, this SNP may be one of the important factors for determining individual differences in warfarin dose.

Cytochrome P450 (CYP) has been known to play an important role in biological oxygenation reactions. CYP2C9, one of the major isoforms of CYP family, metabolizes *S*-warfarin to 7-hydroxywarfarin and 6-hydroxywarfarin [10]. The human *CYP2C9* gene is approximately 55 kb long and located on chromosome 10q24.2 [11,12]. *CYP2C9* *1/*1 genotype is widely present and is defined as the wild type genotype [13]. Among 24 genetic variants in the human *CYP2C9* gene that have been reported previously [14], *CYP2C9* *2 (Arg144Cys) and *CYP2C9* *3 (Ile359Leu) alleles have a high frequency of appearance in Caucasians and are likely to influence the variation of warfarin dose [15]. In *S*-warfarin 7-hydroxylation, CYP2C9 enzyme activity with the *CYP2C9* *2 variant showed 50% reduction in V_{max} (maximum velocity) and a higher K_m (Michaelis constant) compared with the wild type [13,16–18]. With the *CYP2C9* *3 variant, CYP2C9 showed a markedly higher K_m and a reduction of approximately 90% of the intrinsic clearance (V_{max}/ K_m) compared with the wild type [13,16–18]. These reports suggest that *CYP2C9* *2 and *3 variants play a role in influencing the therapeutic dose of warfarin in patients [16,19].

Genotyping both *VKORC1* and *CYP2C9* may therefore improve the warfarin dosage adjustment process in a patient. In August 2007, the Food and Drug Administration (FDA) approved labeling changes for warfarin following reported effects of *VKORC1* and *CYP2C9* on dose requirements [20]. FDA recommended that patients who possess variants in these genes be considered for a lower initial dose to avoid the risk of bleeding.

There are few reports describing the effects of these SNPs in Japanese patients treated with warfarin. Therefore, in this study, we investigated the factors that influence warfarin maintenance dose and determined the warfarin dosing algorithm for warfarin responsiveness in Japanese patients.

Patients, Materials, and Methods

Patients and healthy subjects

This study was approved by the Ethics Committee of the University of Shizuoka, Shizuoka, Japan and the Shizuoka General Hospital, Shizuoka, Japan. A total of 259 Japanese patients receiving anticoagulation therapy with warfarin was recruited from the Shizuoka General Hospital, and a total of 341 unrelated healthy Japanese volunteers was participated in this study. Written informed consent was obtained from each patient and healthy subjects after a detailed briefing of the study purposes and protocols. The patient's inclusion criteria were as follows: (1) warfarin dose has remained constant for at least 3 months and PT-INR has been within the range of 1.5–3.0 during the same period (n = 253), (2) the anticoagulation treatment was well controlled before changing hospitals, even though they do not meet the above criteria (n = 6). We excluded patients who were co-administered amiodarone and/or anticancer agents.

Genotyping

Whole venous blood (5 mL) was obtained from 259 patients and 341 unrelated healthy subjects using EDTA-2Na Venoject II tubes (Terumo, Tokyo, Japan). Leukocyte genomic DNA was extracted directly from the blood specimen using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping the −1639 G>A polymorphism of *VKORC1* according to the method reported by Sconce *et al.* [21]. Briefly, genomic DNA (100 ng) was amplified in PCR buffer containing 200 μM dNTP mixture (Applied Biosystems, Foster City, CA), 0.3 μM each of forward primer (5′-GCC AGC AGG AGA GGG AAA TA-3′) and reverse primer (5′-AGT TTG GAC TAC AGG TGC CT-3′), 0.5 units HotStar *Taq* plus DNA polymerase (Qiagen), and 1.5 mM $MgCl_2$. Amplification was performed by i-Cycler thermal cycler (Bio-Rad, Hercules, CA). The PCR conditions consisted of an initial denaturation/enzyme activation step at 95 °C for 5 min, amplification for 30 cycles at 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 1 min and final extension step at 72 °C for 10 min. The amplified PCR products were digested with 5.0 units *Msp* I (New England BioLabs, Beverly, MA) at 37 °C for 2 h. The digested products were analyzed on 3% agarose gel (agarose S, Nippon Gene, Tokyo, Japan).

A PCR-RFLP method was used for genotyping *CYP2C9* *2 and *3 based on the method reported by Moridani *et al.* [22]. A multiplex PCR was performed. Briefly, genomic DNA (100 ng) was amplified in PCR buffer containing 200 μM dNTP mixture, 0.3 μM each of two sets of primer (*2-forward primer: 5′-GGA GGA TGG AAA ACA GAG ACT TA-3′, *2-reverse primer: 5′-TGA GCT AAC AAC CAG GAC TCA T-3′, *3-forward primer: 5′-GCT GTG GTG CAC GAC GTC CAG AGA TGC-3′ and *3-reverse primer: 5′-ACA CAC ACT GCC AGA CAC TAG G-3′), 0.4 units of HotStar *Taq* plus DNA polymerase and 1.5 mM $MgCl_2$. The PCR conditions consisted of an initial denaturation/enzyme activation step at 95 °C for 5 min, 30 cycles of amplification at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s, and a final extension step at 72 °C for 10 min. *CYP2C9* *2 genotype was determined as follows, the PCR products (5 μL) were digested with 5.0 units of *Ava* II (New England BioLabs) at 37 °C for 2 h. The digested products were analyzed on a 4% agarose gel. *CYP2C9* *3 genotype was determined as follows: the PCR product (5 μL) was digested with 2.5 units of *Nsi* I (New England BioLabs) at 37 °C for 2 h. The digested products were analyzed on a 4% agarose gel. The *4 allele (1076T>C) located next to the *3 (1075A>C) allele cannot be distinguished from *3 allele by RFLP method, all samples identified to have at least one *3 allele were directly sequenced to confirm the genotype. Sequencing was performed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and Big Dye® Terminator Cycle Sequencing Kit (v.1.1) according to the manufacturer's instructions.

Statistical analyses

Fisher's exact test was used for comparison of the allelic frequencies of *VKORC1* and *CYP2C9* variants between Japanese patients and healthy subjects. Genotyping data for deviation from the Hardy-Weinberg equilibrium were tested using the chi-square test. Warfarin dose among five genotype groups was compared by Mann-Whitney test with Bonferroni correction. Correlations between PT-INR and age and between warfarin dose and age were compared by the linear regression analysis.

Spearman's rank correlation tests followed by multiple regression analysis were performed to assess the contribution of patients' height, body weight, age, serum albumin, and PT-INR on the overall variability of maintenance dose of warfarin. Student's *t*-test followed by multiple regression analysis was performed to assess the contribution of gender and *CYP2C9* variants on warfarin dose, respectively. One-way analysis of variance (ANOVA) followed by multiple regression analysis was performed to assess the contribution of *VKORC1* variant and clinical indications for anticoagulant therapy on warfarin dose, respectively. The covariates with p-value <0.15 determined by these univariate analyses were followed by two multivariate analyses performed by analysis of covariance (ANCOVA) and multivariate linear regression analysis. ANCOVA was performed to identify factors contributing to the overall variability of maintenance dose of warfarin. Multivariate linear regression