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Plasma vitamin K concentrations depend on *CYP4F2* polymorphism and influence on anticoagulation in Japanese patients with warfarin therapy

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ABSTRACT

Introduction: Warfarin is characterized by a large inter-individual variability in dosage requirement. This study aimed to analyze the contribution of the *CYP4F2* genetic polymorphism and plasma vitamin K concentration on the warfarin pharmacodynamics in patients and to clarify the plasma vitamin K concentration affecting warfarin sensitivity index in rats.

Materials and Methods: Genetic analyses of selected genes were performed and plasma concentrations of warfarin, vitamin K1 (VK1) and menaquinone-4 (MK-4) were measured in 217 Japanese patients. We also assessed the association of plasma VK1 and MK-4 concentrations with the warfarin sensitivity index (INR/Cp) in rats.

Results: Patients with the *CYP4F2* (rs2108622) TT genotype had significantly higher plasma VK1 and MK-4 concentrations than those with CC and CT genotypes. The multiple linear regression model including *VKORC1*, *CYP4F2*, and *CYP2C9* genetic variants, age, and weight could explain 42% of the variability in warfarin dosage. The contribution of *CYP4F2* polymorphism was estimated to be 2.2%. In contrast, plasma VK1 and MK-4 concentrations were not significantly associated with warfarin dosage in patients. Nevertheless, we were able to demonstrate that the warfarin sensitivity index was attenuated and negatively correlated with plasma VK1 concentration by the oral administration of VK1 in rats, as it resulted in a higher VK1 concentration than that in patients.

Conclusions: The plasma VK1 and MK-4 concentrations are significantly influenced by *CYP4F2* genetic polymorphism but not associated with warfarin therapy at the observed concentration in Japanese patients. The *CYP4F2* polymorphism is poorly associated with inter-individual variability of warfarin dosage requirement.

Introduction

Warfarin is a widely prescribed anticoagulant for the prevention of thromboembolism and stroke. Because there are large intra- and inter-individual variabilities in dosage requirements, the treatment using warfarin is complicated and requires a time-consuming procedure to determine the adequate dosage for each patient, which consists of monitoring the anticoagulant status using the international normalized ratio of prothrombin time (PT-INR). Warfarin dosing variability is attributable to several genetic and clinical factors, including race, age, body weight, height, and polymorphisms of the gene encoding the enzyme associated with the warfarin pharmacokinetics and pharmacodynamics [1–8].

Variations in the gene of the warfarin target protein, vitamin K epoxide reductase complex subunit 1 (VKORC1), cause clotting factor deficiencies and warfarin resistance [9,10]. Several studies have investigated the association of the −1639G>A (rs9923231) polymorphism in the promoter region of *VKORC1* with warfarin dosing variability [11–16]. Polymorphisms in *CYP2C9*, the primary enzyme metabolizing (*S*)-warfarin, result in a large variability in the warfarin pharmacokinetics [17–19]. *CYP2C9**2 (rs1799853) and *CYP2C9**3 (rs1057910) independently predict a low warfarin dose requirement. Furthermore, *VKORC1* and *CYP2C9* genetic polymorphisms and clinical factors cumulatively predict approximately 50% of the variability in warfarin doses for patients [3,4,7,16].

The genetic variant (V433M, rs2108622) of *CYP4F2* was recently shown to affect warfarin dose requirements [20]. Patients with the *CYP4F2* TT genotype require approximately 1 mg/day more warfarin than those with the CC genotype, corresponding to a 4%–12% increase in the warfarin dose per T allele. A genome wide association study in

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Japanese patients also demonstrated the association of the *CYP4F2* polymorphism with the therapeutic warfarin dose [21]. Some studies have demonstrated that the *CYP4F2* rs2108622 polymorphism accounts for approximately 2%–7% of the inter-individual warfarin dose variability [5,22]. However, some studies have also demonstrated that the effect of the *CYP4F2* rs2108622 polymorphism on warfarin response is non-significant [23–25]. In addition, a recent meta-analysis on the impact of the *CYP4F2* polymorphism on coumarin dose requirements suggests a lower contribution of this polymorphism than that of the *VKORC1* and *CYP2C9* polymorphisms [26].

The role of CYP4F2 has recently been identified: this enzyme is responsible for the metabolism of vitamin K1 (phylloquinone, VK1) [27] and menaquinone-4 (MK-4), a form of vitamin K2 [28]. Furthermore, the *CYP4F2* rs2108622 polymorphism reduces CYP4F2 protein concentration and oxidation activity of VK1 and MK-4 in carriers of the minor allele. Vitamin K is a fat-soluble vitamin and co-substrate for γ-glutamyl carboxylase in the vitamin K cycle. VK1 is synthesized and stored in various green vegetables, whereas vitamin K2 is produced by bacteria and intestinal microflora and is enriched in fermented foods [29]. Among the vitamin K2 homologues, MK-4 is majorly found in food and is obtained from dietary VK1 from various animal tissues [30,31]. Variability in vitamin K intake influences the instability of oral anticoagulant therapies [32–34]; however, the degree to which vitamin K influences warfarin anticoagulant therapy has not been well established. Therefore, it is necessary to evaluate the vitamin K status in patients simultaneously with *CYP4F2* polymorphism.

The aim of this study was to examine the contribution of plasma vitamin K concentration and *CYP4F2* genetic polymorphism as well as *VKORC1* and *CYP2C9* polymorphisms on the warfarin pharmacokinetics and pharmacodynamics in Japanese patients and to clarify the degree to which plasma vitamin K concentration affects warfarin pharmacodynamics in rats.

Methods

Patients

We attempted to replicate the associations of polymorphisms with warfarin therapy in subjects from the previously recruited population [7]. A total of 217 adult patients were included in this study from the Shizuoka General Hospital. Predefined inclusion criteria was followed: patients on anticoagulation therapy with warfarin for at least 3 months; having a stable anticoagulation status, which was determined from the results (within the therapeutic range) of a minimum of 3 consecutive INR measurements; and having a stable daily dosage during 1 month before blood sampling. We excluded patients who were co-administered amiodarone and/or anticancer agents. This study was approved by the Ethics Committee of the University of Shizuoka and Shizuoka General Hospital. Written informed consent was obtained from the patients after the purpose and protocol of the study were explained. Whole venous blood (5 mL) was collected into a tube containing EDTA-2Na after at least 12 hours of warfarin administration. The sample was used to isolate genomic DNA, and plasma was harvested by centrifugation (1,500 ×*g* for 10 min) to determine the warfarin and vitamin K concentrations.

Genetic Analysis of the Polymorphisms

Leukocyte genomic DNA was extracted directly from the blood specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Subjects were genotyped for *CYP4F2* (rs2108622), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), and *VKORC1* −1639G>A (rs9923231) polymorphisms by the polymerase chain reaction-restriction fragment length polymorphism method, as previously described [7,12,35,36].

Measurement of Plasma Warfarin and Vitamin K Concentrations

The plasma concentrations of warfarin and vitamin K (VK1 and MK-4) were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described previously [36], with minor modifications. A simple chromatographic assay was used for the simultaneously quantifying the warfarin in human plasma. In brief, 10-μL aliquot of plasma was spiked with 90 μL of chilled internal standard solution (500 ng/mL in acetonitrile, warfarin-d5; Toronto Research Chemicals, Ontario, Canada). The mixture was vortex-mixed for 1 min to precipitate proteins and centrifuged at 3,000 ×*g* for 10 min. A 55-μL aliquot of supernatant was placed into another tube and diluted with 45 μL of 5 mM ammonium acetate (pH 4.0), and a 10-μL aliquot was injected into the HPLC column. Separation was achieved using a Kinetex C18 column (100 × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA) at 40 °C, using an isocratic solvent system of 45:55 (v/v) acetonitrile: 5 mM ammonium acetate (pH 4.0), with a flow rate of 0.2 mL/min. The concentration of warfarin in the plasma samples was quantified using the peak area of standard samples (100–1,500 ng/mL) for calibration. The lower limit for quantifying warfarin in plasma was 100 ng/mL. At this concentration, the coefficient of variation (CV) of the assay was 8.5%. The extraction recovery rate of warfarin was 88.1%, and the CV was 7.5%.

Animals

Male Sprague–Dawley rats (6 weeks of age; Japan SLC, Hamamatsu, Japan) weighting between 180 and 230 g were purchased and housed under standard laboratory conditions (23 ± 1 °C in a 12-h light–dark cycle). Rats were fed standard non-purified diets (Oriental Yeast, Tokyo, Japan) and allowed free access to food and tap water. Animal care and experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka.

Animal Experimental Protocol

After a 1-week acclimation, the rats were divided into five vitamin K groups as follows: (1) VK1, 0.25 mg/kg/day (low dose); (2) VK1, 1 mg/kg/day (high dose); (3) MK-4, 0.25 mg/kg/day (low dose); (4) MK-4, 1 mg/kg/day (high dose); (5) vehicle, (1% ethanol solution) as control group. VK1 and MK-4 (Wako, Osaka, Japan) were suspended in 1% ethanol solution and were orally administered twice a day for 7 days. Warfarin sodium (Wako, Osaka, Japan) was suspended in 1% ethanol solution and was orally administered once a day at a dose of 0.2 mg/kg for 7 days. After 7 days, it might be considered to reach a steady state, blood samples were collected from the abdominal portion of vena cava under diethyl ether anesthesia 12 h after the final administration of vitamin K. For the PT assay, the venous blood was collected into non-heparinized syringe and assay was performed immediately using the INRatio2 meter (Alere, San Diego, USA). To determine the warfarin and vitamin K concentrations, the venous blood was collected into a heparin sodium-containing tube. Then, plasma was harvested by centrifugation (1,500 ×*g* for 10 min).

Statistics Analysis

The warfarin sensitivity index was determined by calculating the PT-INR response per plasma warfarin concentration. Genotyping data for deviation from the Hardy–Weinberg equilibrium were tested using the chi-square test. The differences between any pairs of the medians were determined using the Mann–Whitney U test. Data among *CYP4F2* and *VKORC1* genotype groups were compared by the Mann–Whitney test with Bonferroni correction. The relationship between the plasma vitamin K concentration and warfarin sensitivity index was analyzed by the linear regression model and Spearman's