RESEARCH

Cardiovascular Therapeutics

Novel VKORC1 Mutations Associated with Warfarin Sensitivity

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Warfarin is widely used anticoagulant drug for the prophylaxis and treatment of venous and arterial thromboembolic disorders and exerts its anticoagulant effect by inhibiting the vitamin K epoxide reductase. To determine the impact of genetic variants of the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) on the anticoagulant response to warfarin, polymorphisms in exon 1, exon 3, and 3′-untranslated region (3′ UTR) were assessed. Results: Sixty patients (34 males and 26 females) with stable INR (2–3) were selected from cardiology and anticoagulant clinic. Three VKORC1 frameshift mutations were detected. The first frameshift mutation was nucleotide deletion (91delCC) in exon 3 (1 patient). The second variation was nucleotide addition (51addCT) in exon 3 (2 patients). All the 3 patients reported bleeding during warfarin use, while no other bleeding was reported during the study period. Warfarin maintenance dose was significantly different between 3 patients with mutations and patients without mutations. The use of a fixed-dose warfarin for all patients and in range INR may not be sufficient for warfarin monitoring. Many factors including unknown ones may also play an important role in highly variable response among patients. Our data for the first time, suggested a new possible call for screening to reduce the risk of bleeding and guide for dosing.

Introduction

Warfarin is the most commonly prescribed oral anticoagulant, but there is interindividual and interethnical variability in the dose required attaining a therapeutic response. Warfarin exerts its anticoagulant effect through inhibition of the vitamin K epoxide reductase encoded by the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) [1]. Pharmacogenetic analysis of VKORC1, has confirmed its influence on warfarin maintenance dose. The effect of several genetic polymorphisms in VKORC1 on warfarin dose has been identified. Two common polymorphisms, 1173C>T in intron 1 and 3730G>A in the 3′-untranslated region, affect interindividual variability of warfarin dose [2].

Race appears to be an important determinator of oral anticoagulants maintenance dose requirement. Asian patients may require lower doses than caucasian patients to obtain the same degree amount of anticoagulation [3,4]. Dietary habits or polymorphisms of the cytochrome P450 2C9 (CYP2C9) are not enough to explain the differences in maintenance dose requirement of warfarin between race groups [5,6].

An understanding of genetic and ethnic factors that contribute to the variabilility of warfarin dose may be helpful to control and prevent thromboembolic diseases during therapy and to predict the serious bleeding complications. In this study, for the first time, we investigated variants in the exon 1, exon 3, and 3′-untranslated region (3′ UTR) of VKORC1 and correlation between

variants with warfarin maintenance doses in Iranian patients receiving warfarin.

Materials and Methods

Patients

Stable patients were recruited from cardiology and anticoagulant clinics of Masih Daneshvari, a tertiary care teaching hospital. A stable patient was defined as one whose warfarin dose requirement had remained constant for at least a minimum period of 1 month, and with an international normalization ratio (INR) within the range of 2.0 to 3.0. Patients whose target INR was not 2–3 were also excluded. Dosage requirement was not known at the time of patient eligibility evaluation. All patients who were stable on any dose, based on the target INR range, could enter the study. Patients with abnormal liver, thyroid, and renal function, and those receiving concurrent therapy known to interact with warfarin were excluded.

Ethical permission for the study was obtained from the ethical review board of National Research Institute of Tuberculosis and Lung Disease (NRITLD). All patients signed written consent prior to their participation in the study.

On arrival at the clinic, a blood sample (5 mL) was taken for INR measurement, and VKORC1 genotyping. Demographics of sex, age, weight, and height, as well as indication for warfarin therapy, warfarin maintenance dose, and concurrent medications were recorded during the clinic visit.

DNA Extraction and Analysis

DNA was extracted from whole blood using sodium per chlorate method [7]. Amplifications of exon 1, exon 3, and 3′ UTR of VKORC1 gene were achieved using sense and antisense oligonucleotide designed (Table 1). PCR was carried out on 50 μL volume in a gradient model thermal cycler (Corbett research, Australia). PCR reaction contained 1X PCR buffer, 0.1-μg genomic DNA, 20 pmol of each of forward and reverse primers, 125-μM deoxyribonucleoside triphosphates (dNTPs), 1.5-mM MgCl2, and 1.5 U Taq polymerase (CinnaGen, Iran). PCR condition was follow: denaturing at 94°C for 30 min, annealing (Table 1) for 30 min and extension at 72°C for 30 min. These stages repeated for 30 cycles. Reaction was incubated for 5 min at 94°C and 72°C before and after cycling, respectively [8]. PCR products were subjected for electrophoresis on 1.5% agarose gel [9].

Mutation Detection

Each PCR product was screened for mutation based on hetero duplex formation using confirmation sensitive gel electrophoresis (CSGE) method [10]. Briefly, PCR product of each sample was mixed with sequence confirmed PCR product and incubated at 72°C for 15 min. and electrophoresed on CSGE gel, gel was stained by ethidium bromide. Hetero duplex and homoduplex bands were formed and mutated nucleotide, heteroduplex bands, confirmed by sequencing using dideoxy chain termination method.

Sample Size Calculation

A sample size of 60 was calculated to produce a 90% confidence interval equal to the sample proportion plus or minus 0.08000 when the estimated proportion is 0.19000 [11,12,13,14].

Statistical Analysis

All statistical analysis was performed using SPSS software (version 17.0). Two-sided significance tests were used throughout and a P value of less than 0.05 was considered significant. Mann-Whitney Test was used for univariate analysis. The relationship between variables and the maintenance dose of warfarin was assessed by Kendall's tau˙b correlation coefficient. The association between VKORC1 mutation and warfarin maintenance dose were evaluated using unpaired student t-test.

Table 1 Primers used for amplification and sequencing of the VKORC1 gene, fragment size, and polymerase chain reaction conditions

Primer	Position	Sequence	Amplicon size	Annealing temperature
$VKEX_1$ F	5263–5565	5′CTCCGTGGCTGGTTTTCT 3′	303 bp	58°C
$VKEX_1$ R		5′CCGATCCCAGACTCCAGAAT3′		
$VKEX_3$ F	8659–8984	5′AGTGCCTGAAGCCCACAC 3′	326 bp	58°C
$VKEX_3$ R		5′ACCCAGATATGCCCCCTTAG 3′		
VK_3UTR F	8845–9312	5′AGCCTGATGTGGCTCAGT TT 3′	467 bp	57°C
VK_3 UTR R		5′ATAACCACCCTTAAACGCAG 3′		

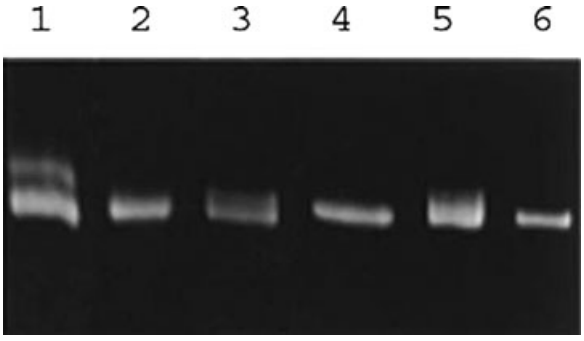

Figure 1 Mutations in exon 3 (326 bp) detected by CSGE. Lane 1, 3, and 5 represent the mutations in the patients 1, 3, and 5. Lane 6 represents control.

Results

Sixty patients (34 males and 26 females) met the inclusion criteria and related data were analyzed. The mean ±SD age and weight of all patients was 56.35 ± 17.08 years and 72.92 ± 13.76 kg, respectively. The mean ± SD of warfarin maintenance dose was 4.31 ± 1.83 mg/day (Range: 1.25–8 mg/day). There was no correlation between sex, weight, height, and body mass index (BMI) with warfarin maintenance dose.

Three patients showed novel mutations in exon 3 of VKORC1 detected by CSGE (Figure 1). The first mutation, was nucleotide deletion (91delCC) in exon 3. This mutation produces a frameshift that results in premature translation termination at genomic position 8852 (patients 1).

The second mutation, noted in two affected patients, was nucleotide addition (51addCT) in exon 3, resulting in a frameshift and premature termination at genomic position 8693 (patients 3 & 5) (Table 2). These patients had no family relationship and the mean of their warfarin dose was significantly lower than other patients (2.50 ± 0.00 mg/day compared to 4.40 ± 1.83 mg/day, *P*-value < 0.001). The 3 patients had minor bleeding during the study period, while no rise in their INRs was detected at the time of bleeding. Demographical and clinical data of patients with new mutations are shown in Table 3.

Table 3 Characteristics of patients with new mutations in VKORC1 gene

Variables	Patient 1	Patient 3	Patient 5
Demographics			
Age, (year)	52	26	54
Weight, (kg)	90	65	75
Sex	female	female	female
Indications			
AF	−	−	−
DVT	+	−	+
PE	−	+	−
Maintenance warfarin dose, (mg/day)	2.5	2.5	2.5

Discussion

Warfarin, is accompanied with a high risk of bleeding, due to it's interindividual and intraindividual variability in response within patients, especially during the initiation period. Recently, a sensitivity analysis has shown that the use of a pharmacogenetic algorithm predicts the initial dose of warfarin better than a clinical algorithm or a fixed-dose approach [15].

Several previous studies have established that Asian population require significantly lower maintenance doses of warfarin to effect the same degree of anticoagulation obtained in patients of European countries [3,4,16]. VKORC1 genotype is emerging as an important genetic factor influencing dose and response of coumarin anticoagulant drugs at least in studies involving patients of European descent [2,17–20]. Veenstra DL et al. demonstrated that VKORC1 genotype is the single most important genetic factor influencing warfarin dose in Hong Kong Chinese [21]. D'Andrea G and coworkers described that polymorphisms in the VKORC1 gene may associate with an interindividual variability in the dose-anticoagulant effect of warfarin. They reported polymorphisms in the intron 1 and 3'UTR, and mutations in the exon 1 and 3 [2]. In this study, we investigated variants in the exonic and 3'-untranslated regions of VKORC1 to determine the basis between interindividual and interethnical differences in warfarin dose requirements in Iranian patients. We identified 2 VKORC1 frameshift mutations, 91delCC (in 1 patient) and 51addCT (in 2 patients) among 60 patients (3.3%). These 3 patients were probably more susceptible to warfarin since their warfarin doses were significantly lower (17.5 mg/week) than other patients. Klein et al. reported the value of genetic information on warfarin dose estimation, and confirmed that the most advantage was observed in patients

Table 2 VKORC1 frameshift mutations

Mutation	Exon	Genomic position	Nucleotide change	Amino acid change
51addCT	3	8693	Add nt 51–52	Glycine(35)leucine
91deleteCC	3	8852	Delete nt 91–92	Tryptophan(156)threonine

receiving 21 mg per week or lower [15]. Although, our identified mutations were not included in their genetic data. These patients had minor bleeding during the study period, while no rise in their INRs was detected at the time of bleeding. Rosand J, et al. reported severe bleeding in patients with INRs of 3.0 or less [22]. Thus, even life threatening bleeding may develop irrespective of the degree of INR elevation [23].

In a case control study, Reitsma PH et al. demonstrated that genetic polymorphisms in VKORC1 can affect the occurrence of adverse bleeding effects [24]. Our findings are compatible with a possible association between sensitivity to warfarin with resultant bleeding effect with VKORC1 new mutations in our patients. It should be confessed that only 3 patients could not strongly confirm (if any) clinical relevance. This should be considered a secondary finding which was not our main objective and could be studied in a designed study with a definite duration of follow up of the patients to evaluate the rate of bleedings.

Conclusion

The low number of patients with mutations that we found here makes the conclusion difficult. However, this might trigger a new study with a large perspective using more sensitive methods to address the polymorphisms and mutations that may confirm our result and thus will help physicians to determine more accurate dosages and reduce the chances of bleeding for the warfarin treatment in patients with specific genotypes.

If such studies confirmed our findings, then a new era would begin in warafarin monitoring advancement. Provided that an easy genotyping in clinical settings could be available.

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Conflict of Interest

The authors declare no conflict of interest.

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