Effect of VKORC1 and CYP2C9 polymorphisms on warfarin dose requirement in Bangladeshi population

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Abstract: Warfarin, an oral anticoagulant is one of the most widely prescribed drugs in modern medicine. Large inter-individuals variability due to age, gender, diet, concurrent drug interactions and variations in *CYP2C9* and *VKORC1* genes make the management of warfarin therapy challenging and yet no study has been conducted on the Bangladeshi population. The aim of the study was to identify the role of VKORC1 and CYP2C9 polymorphisms in Bangladeshi population in dose requirement of warfarin. We studied 87 heart valve replacement patients who were prescribed warfarin for minimum of 6 months with a target International normalized ratio of 2.0-3.5. Genotyping of VKORC1rs9923231 (-1639 G>A), CYP2C9*2 and CYP2C9*3 was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. The frequencies of GG, AG and AA genotypes of VKORC1rs9923231 in the studied population were 87.4%, 8%, and 4.6% respectively whereas the frequencies of the CYP2C9*1/3 and CYP2C9*3/3 were 4.6% and 3.4% respectively. The CYP2C9*2 was not found in the studied population. The results of this study indicate that comparatively higher daily maintenance doses of warfarin were required to achieve the target INR for patients carrying both GG genotype of VKORC1rs9923231 and wild type variant of CYP2C9*3 whereas minimum dose were required for patient having AA genotype of VKORC1rs9923231 and *3/*3 variant of CYP2C9.

Keywords: VKORC1, CYP2C9, polymorphism, warfarin dose adjustment, Bangladeshi population.

INTRODUCTION

Warfarin is the most commonly prescribed oral anticoagulant for the treatment and prevention of thrombotic diseases such as myocardial infarction, ischemic stroke, atrial fibrillation, deep vein thrombosis (Daly, 2014; Natarajan *et al*., 2013) and in patients who have undergone valve replacement surgery (Kulik *et al*, 2006). Warfarin has a narrow therapeutic index and its over- or under-dose related hemorrhagic or thrombotic complications could be devastating. The clinical optimization of warfarin dose is very difficult due to large inter-individual variability (Flockhart *et al*., 2008; Saminathan *et al*., 2010).

Warfarin dose requirement can be influenced by various factors including age (Beyth *et al*., 2002), weight, height (Wells *et al*., 1994), ethnicity (Kamali *et al*., 2004), vitamin-K enriched diet (Absher *et al*., 2002), drug interactions (Gan *et al*., 2003) and individual genetic profile (Scordo *et al*., 2002). The effectiveness and safety of warfarin is dependent on maintaining the prothrombin time, expressed as the international normalized ratio (INR), within the therapeutic range. A number of studies have been reported which shows a sharp increment in bleeding risk when the INR is above the upper limit whereas the risk of thromboembolic events increases when the INR falls below it (McBride *et al*., 1996). The target INR recommended for high-risk aortic valve replacement (AVR), Mitral valve replacement (MVR) is 2.5-3.5 and that for low risk AVR is 2.0-3.0 (ACC, 2006). The current clinical practice utilizes the INR for the optimization of the dose of warfarin in individual patients. The factors that can affect the dose of warfarin and the identification of biomarkers for dose prediction are the prime concern to the warfarin pharmacogenetic researchers (Liu *et al*., 2007).

The more active warfarin form, S-warfarin is metabolized to its inactive metabolite- 7-hydroxywarfarin that is mainly catalyzed by CYP2C9 (Breckenridge *et al*., 1974). VKORC1 (vitamin K epoxide reductase complex 1) catalyzes the conversion of vitamin K epoxide to reduced vitamin K and the anti-coagulant effect of warfarin is shown due to the non-competitive inhibition of the action of VKORC1 by it (Li *et al*., 2004; Rost *et al*., 2004). The pharmacodynamics of warfarin and its dosage requirements are affected by the genetic variants in the VKORC1 gene and it may account for 20−30% of variation in dose requirement (Sandanaraj *et al*., 2009).

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Many studies were conducted in different population to determine the effect of CYP2C9 polymorphic variants on the pharmacodynamics and pharmacokinetics of warfarin (Lee *et al*., 2002; Scordo *et al*., 2001; Yoon *et al*., 2001; Zhao *et al*., 2004). In particular, CYP2C9*2 and *3 polymorphisms have been associated with decreased activity and patients positive for one or both of these require lower doses of warfarin. Approximately 7-10% of warfarin dose variation was found for CYP2C9 polymorphisms (Lee *et al*., 2002, Taube *et al*., 2000 Aithal *et al*., 1999). Bangladesh is a country with more than 160 million people and even though no statistics is available for people suffering from cardiovascular diseases, the number of the patients has been increasing day by day. A portion of these patients undergoes valve replacement surgery and treatment with warfarin. The time period of therapy is from minimum 3 months to lifelong warfarin intake. Till now, no study has been conducted to observe the genetic polymorphism of CYP2C9 and VKORC1 genes in Bangladeshi population and its possible relation with dose adjustment of warfarin. In this study 87 heart valve replacement patients taking warfarin were investigated in relation to genetic polymorphisms of both the CYP2C9 and VKORC1 genes.

METHODS

Subject and Study design

This study was conducted on 87 Bangladeshi patients treated with warfarin at the cardiac unit of the National Heart Foundation and Research Institute (NHFRI), Bangladesh. The study protocol was approved by the NHFRI and written consent was obtained from each patient. During patients recruitment the following inclusion criteria were maintained: the investigated patients had undergone heart valve replacement surgery and were taking warfarin at a fixed dose (maintenance dose) for two months, patients agreed to give blood samples required for DNA extraction. Patients having a previous history of any invasive malignancy and those who refused to give consent and share their data were excluded from this study. No patients had any reported hepatic and renal impairments at any time during the study. A single non-fasting blood sample was obtained from each patient after 12 hours of his or her last warfarin dose. Trained nurses in the presence of expert physicians collected the demographic characteristics, and lifestyle factors through interviews. The study was conducted in accordance with the declaration of Helsinki and its subsequent revisions (WMADH, 2008).

Identification of VKORC1 and CYP2C9 genome variation

Venous blood (3 ml) was collected from each patients and genomic DNA was extracted by a chemical method previously established in our laboratory (Islam *et al*., 2013). Genotyping of the VKORC1rs9923231, CYP2C9*2 (C470T), CYP2C9*3 (A1075C) was carried out by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) [27-29]. Briefly, 25µl PCR reaction volume was used, containing 1µl of genomic DNA (50–70 ng/µl), 5µl of 5X GoTaq reaction buffer, 4µl of $MgCl_2$ (25 mM), 2µl of dNTPs (2.5mM), 1µl of each primer (10µM), 0.1µl of GoTaq DNA polymerase (5U/µl) (Promega Corporation, USA), and 10.9µl of nuclease free water. After PCR amplification, 20µl PCR products were digested (overnight at 37 °C) with approximately 2 units of MspI, AvaII and StyI for VKORC1, CYP2C9*2 and CYP2C9*3 respectively and finally the products were visualized on 2% agarose gel after ethidium bromide staining (Sconce *et al*., 2005; Ozawa *et al*., 1999; Aynacioglu *et al*., 1999). PCR primers, PCR conditions, restriction enzymes used and length of the expected fragments on digestion to different VKORC1 rs9923231, CYP2C9*2 and CYP2C9*3 alleles are presented in table 1. Samples found heterozygous and mutant homozygous were analyzed twice and subject to direct sequencing by using our previous reported method (Islam *et al*., 2013) for confirmation.

STATISTICAL ANALYSIS

Unpaired t-test was used to compare the effect of allelic frequency of each variant on steady dose requirement. Results are presented as mean ± standard deviation (SD). A p value of <0.05 was considered statistically significant.

RESULTS

We analyzed the genotype and allele frequency of VKORC1rs9923231, CYP2C9*2 and CYP2C9*3 polymorphisms for the assessment of warfarin metabolism in 87 heart valve replacement Bangladeshi patients. The demographic data of the patients were summarized in table 2. The frequency of VKORC1 rs9923231 (G>A) allele was 8.62% and that of its wild type allele was 91.38% among the studied patients. The frequency of CYP2C9*3 allele was 5.75% whereas CYP2C9*2 allele was absent in the studied population. After confirming the genotypes, we compared daily warfarin maintenance dose among the patients, taking the patients having both the wild type of VKORC1 and CYP2C9 as a controls. These patients comprise 87.4% of the total study population (group 1) and 7 patients carried VKORC1 AG and CYP2C9 *1/*3 genotypes (group 2) and 4 patients had VKORC1AA and CYP2C9*3/*3 genotypes (group 3). We found the mean warfarin daily maintenance doses for patients of group 1, group 2 and group 3 were 5.07±0.57, 3.5±1.13 and 2.2±0.63 mg respectively to maintain the target INR 2.0-3.5 (table 3). The dose requirement for group 2 and group 3 patients were 30.97% and 56.61% less respectively than that for group 1 patients ($P<0.005$).

Table 1: Primer design, restriction enzyme used and DNA fragments found for VKORC1 and CYP2C9 gene

Allele	Primers	PCR (35 cycles)	RE	DNA fragements	Reference
VKORC1 rs9923231	FP-5'-GCCAGCAGGAGAGGGAAATA-3' RP-5'-AGGCACCTGTAGTCCAAACT-3'	94°C 1 min 59°C 1 min 72°C 1 min	MspI	NH-168,122 HE -290,168,122 MH -290	[27]
CYP2C9*2	FP-5'-TGCATGTGCCTGTTTCAGCA-3' RP-5'-ACCCTTGGTTTTTCTCAACTC-3'	94°C 30 sec 58°C 30 sec 72°C 2 min	AvaII	NH 259, 57 HE 316, 259, 57 MH 316	[28]
CYP2C9*3	FP-5'-AGGAAGAGATTGAACGTGTGA-3' RP-5'GGCAGGCTGGTGGGGAGAAGGCCAA-3'	94°C 1 min 62°C 1 min 72°C 1 min	StyI	NH 130 HE130, 104, 26 MH 104, 26	[29]

Table 2: Demographic data of the patients

Gender	Age (years)	Weight (kg)	BMI (kg/m^2)
Male (66)	38.33±12.88	51.88±10.25	21.27±9.25
Female (21)	38.32±13.08	51.42±10.36	21.59±8.65

All data shown are presented as mean ±Standard Deviation (SD)

Table 3: Relationship between mean daily warfarin maintenance dose and genotype in Bangladeshi patients

Variable	Number of patients (%)	Mean warfarin maintenance dose (mg/day)
	87	
VKORC1 GG +CYP2C9 *1/*1 (Group 1)	76(87.4)	5.07±0.57
VKORC1 AG +CYP2C9 *1/*3 (Group 2)	7(8.0)	3.5±1.13**
VKORC1 AA +CYP2C9 *3/*3 (Group 3)	4(4.6)	2.2±0.63**

All data shown are presented as mean± SEM. *P<0.05, **P<0.005 analyzed by unpaired t-test

DISCUSSION

Warfarin is still the main oral anticoagulant drug although its narrow therapeutic window and higher inter-individual variability complicate the dose adjustment. The individualized dosing of warfarin is required for each patient to maintain the optimal INR depending on its response. This study was designed to investigate the frequency of VKORC1, CYP2C9 gene polymorphisms and comparing with warfarin maintenance dose in heart valve replacement patients of Bangladesh origin.

Patients carrying GG and AA genotype for VKORC1 rs9923231 polymorphism required the highest and lowest doses of warfarin whereas the highest and lowest dose were required for *1/*1 and *3/*3 genotypes of CYP2C9*3 polymorphism that are consistent with other previous studies (Schelleman *et al*., 2007). The usual dose of warfarin for the patients ranges from 1.25 mg to 7.5 mg. This indicates that warfarin dosage requirements greatly differ among individuals and the corresponding pharmacodynamics also varies greatly. The starting dose for every patient was adjusted according to the INR value that required at minimum 4 days and at maximum 10 days.

The frequency of rs9923231 allele is 8.62% in Bangladeshi population. This minor allelic frequency is widely varied among different ethnic groups. The Whites have a major proportion of G allele (Sconce *et al*., 2005, Aquilante *et al*., 2006) and similar trend is found to be present among Caucasians (Schelleman *et al*., 2007). The African-Americans have the highest proportions of G allele (Schelleman *et al*., 2007) whereas higher A allele was found among Asians. Chinese and Japanese have the high frequency of AA genotype than other Asians (Miao *et al*., 2007; Yuan *et al*., 2005; Kimura *et al*., 2007). The Asian population usually needs the low dose of daily warfarin compare to Whites and Blacks due to the presence of high A allelic frequency (Aquilante *et al*., 2006; Limdi *et al*., 2010). However, in the Asian subcontinent, India and Bangladesh present a unique distribution of allelic frequency of VKORC1, which resembles more with the Caucasians and this is due to the population substructures of this region (Reich *et al*., 2009). The population substructure in Indian subcontinent is found to have ancestral origin among Middle Eastern, Central Asians and Europeans (Reich *et al*., 2009). Moreover, the south part of the continent is divergent in genotype from both the north part of the continent and eastern Asia. Therefore, the genotype frequency of VKORC1 in the population of Indian subcontinent is different from other East Asian countries and here G allelic frequency is found to be more prevalent in Indian population (Rathore *et al*., 2010). We report the same trend for Bangladeshi population and thus dose of

warfarin in these Indian and Bangladeshi patients will also differ with other Asians.

Compared with the corresponding wild-types, CYP2C9*3 significantly reduces drug clearance whereas the effects of CYP2C9*2 on drug metabolism are comparatively less severe (Rettie and Jones, 2005; Krämer and Testa, 2008). The studies in different ethnic populations revealed the absence of CYP2C9*2 allele (0%) in population of Chinese, Japanese and Koreans (Yoon et al., 2001; Wang et al., 1995; Sullivan-Klose et al., 1996; Nasu et al., 1997; Haug et al., 1997). North Indian population has an allele frequency of 4.9% whereas that is 10% in Americans and 19% in British population (Aithal et al., 1999; Sullivan-Klose et al., 1996; Haug et al., 1997; London et al., 1996; Stubbins et al., 2002). The frequency for CYP2C9*2 in Bangladeshi patients was in accordance to those found in the major ethnic groups of Chinese and Japanese patients. CYP2C9*3 has been detected in all ethnic groups with varying allelic frequency (Xie et al., 2002). Similarly, Africans have an overall allele frequency of 1.3%, whereas Caucasians exhibit a significant prevalence of heterogeneity in CYP2C9*3 allelic distribution with frequency of about 10% in Americans and British (Sullivan-Klose et al., 1996; Stubbins et al., 1996). The frequency of CYP2C9*3 in our study population is 5.75%. Such variations in genotype within Bangladeshi patient, reflects allelic frequency similar to the Caucasians.

CONCLUSION

Warfarin daily maintenance dose was affected by rs9923231 and CYP2C9*3 variants whereas variant CYP2C9*2 was absent in the study population of Bangladesh where genotyping data is not yet considered during the prescription of warfarin. These results indicate that, VKORC1 and CYP2C9 genotype should be considered in designing dosage regimen and delivering warfarin as an anticoagulant.

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