Original Article

Frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 genetic variants in the Northern Indian population

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Summary **Dose requirements for oral anticoagulants in thromboembolic events are influenced by polymorphisms in *VKORC1* and *CYP2C9* genes. The Indian population comprises multiple ethnic groups but no data is available on allele frequencies of these genes for North Indians. The present study aimed at establishing the allele and genotype frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 alleles in the North Indian population. One hundred and two healthy subjects from the Northern Indian region were genotyped for *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 by polymerase chain reaction and restriction fragment length polymorphism. Allele frequencies were compared with that of the HapMap populations. The allele frequencies for *VKORC1* -1639 A, *CYP2C9**2 and *CYP2C9**3 were found to be 14.22%, 4.90% and 3.92% respectively. This report also describes the inter-ethnic differences in the Northern Indian frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 alleles with that of other populations and HapMap project data. *VKORC1* -1639 G>A allele is present at moderately high frequency in the Northern Indian population. The frequencies of *CYP2C9**2 and *CYP2C9**3 alleles are also found to be different from other populations.**

Keywords: *VKORC1*, *CYP2C9*, polymorphism, genotype, allele frequency, North Indians

1. Introduction

Oral anticoagulants, warfarin/acitrom and related coumarins (acenocoumarol and phenprocoumon), are widely used in thromboembolic prophylaxis. These drugs have a narrow therapeutic index, and their use is associated with increased risk of clot formation when treatment is subtherapeutic or bleeding when supratherapeutic. In recent years, common genetic variations in vitamin K epoxide reductase (VKOR) and certain cytochrome P450 genes have been discovered that significantly influence oral anticoagulant maintenance dose requirements.

VKOR converts vitamin K epoxide to vitamin K and vitamin K hydroquinone in the vitamin K cycle. Acitrom (or warfarin) inhibits VKOR (*1*), specifically the VKORC1 subunit (*2,3*), thereby diminishing available vitamin K and vitamin K hydroquinone in the tissues. Single nucleotide polymorphisms (SNPs) in the *VKORC1* gene have been linked to reduced efficacy of vitamin K recycling as a result of lower VKOR activity (*3*). Therefore, common polymorphisms in *VKORC1* have been shown to greatly influence dose requirements for oral anticoagulants. The *VKORC1* -1639 G>A variant has been genotyped in a number of different populations. This polymorphism has pronounced differences in its frequency by ethnic group as it is actually the majority allele (around 90%) in Japanese populations and appears to explain the lower warfarin dose requirement for individuals of Japanese descent (*4*). This variant is also quite common in Caucasians, with an allele frequency typically around 40% in predominantly Caucasian populations (*5*). However, there is limited information for Indian populations as only a single study on a small number of Indians living

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in Taiwan has been reported so far (*6*).

CYP2C9 is primarily responsible for oxidative metabolism of narrow therapeutic index compounds like oral anticoagulants (*7*). Multiple single base pair substitution polymorphisms have been identified in the gene coding for CYP2C9 protein. Variability in the metabolism and the therapeutic effect of CYP2C9 substrates can be attributed to the genetic polymorphisms of *CYP2C9* gene. About 34 variant alleles have been reported (*8*). The two most important variants shown to have clinical implications for warfarin dosing and prevention of adverse events are *CYP2C9**2 (Chromosome 10, Exon 3, 430 C>T, rs1799853, Arg144Cys) and *CYP2C9**3 (Chromosome 10, Exon 7, 1075 A>C, rs1057910, Ile359Leu). Individuals with the *2 and *3 variants, who are more likely to need lower doses of warfarin, take a longer time to reach the target International Normalized Ratio (INR) on starting warfarin therapy and have an increased risk of bleeding complications (*9*,*10*). In the Indian context, there are two reports of *CYP2C9**2 and *3 allelic frequency distribution only on subjects from South India (*11*,*12*).

Studies across different populations have shown wide differences in the distribution of the above three alleles in different ethnic groups. The Indian population comprises multiple ethnic groups and no data is available for North Indians. Hence, the present study was aimed at establishing the allele and genotype frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 in the North Indian population. This report also compares North Indian *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 frequencies with those of other populations in the HapMap project data.

2. Materials and Methods

2.1. *Subjects and DNA Extraction*

Unrelated healthy volunteers of North Indian origin (n = 102), of either sex (males = 51, females = 51) between the age of 18-60 years with a mean age (S.D.) 28.5 (± 7.8) years were included in the study. North Indian ethnicity was based on place of residence in the last three generations, food habits and mother tongue (Hindi or related languages). Blood samples were collected in ethylenediaminetetraacetic acid and genomic DNA was extracted from peripheral blood leukocyte pellets using the standard salting-out method (*13*). The quality and quantity of DNA was checked by gel electrophoresis and spectrophotometry using the NanoDrop Analyzer (ND-1000) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ratio of absorbance at 260 and 280 nm for DNA was between 1.7 and 1.9. The isolated DNA was stored at −70°C.

2.2. *Genotyping of VKORC1 (-1639 G>A, rs9923231) allele*

Genotyping of *VKORC1* (-1639 G>A) was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) as described by Sconce *et al.* (*14*). Sequences for the forward and reverse primers were 5'-GCCAGCAGGAGAGGGA AATA-3' and 5'-AGTTTGGACTACAGGTGCCT-3', respectively. The PCR conditions consisted of 25 cycles of 1 min at each of the following: 94°C, 59°C, and 72° C. The 5'-untranslated region (UTR) polymerase chain reaction products (10 µL) were digested with 2 units of restriction enzyme Msp1 (Fermentas, MA, USA) in a final volume of 30 µL in the appropriate digestion buffer at 37°C for at least 16 h. The digested products were visualized on 10% polyacrylamide gels stained with ethidium bromide.

2.3. *Genotyping of CYP2C9 430 C>T (CYP2C9*2, rs1799853) and CYP2C9 1075 A>C (CYP2C9*3, rs1057910) allele*

*CYP2C9**2 which is responsible for amino acid change Arg144Cys was detected by PCR-RFLP as described (*15*) using primers (5'-CACTGGCTGA AAGAGCTAACAGAG-3') and (5'-GTGATATGG AGTAGGGTCACCCAC-3') to amplify a 372-bp amplicon in a 50 µL PCR mix comprising 10 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, 50 mM KCl, 200 mM dNTPs, 0.2 mM of each of the primers, 2.5 units of Taq DNA polymerase (Bangalore Genei, Bangalore, India), and 1 µL of genomic DNA. PCR was performed with an initial denaturation for 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 10 sec at 60°C, 1 min at 72°C, and a terminal extension for 7 min at 72°C. Twenty µL of PCR product were digested overnight with restriction endonuclease Sau96I (New England Biolabs, Ipswich, MA, USA), and analysis was done by 3% agarose gel electrophoresis. Wild type alleles (Arg) were cut into fragments of 179, 119, and 74 bp, whereas mutant alleles (Cys) showed fragments of 253 and 119 bp because of loss of one restriction site.

*CYP2C9**3 which codes for the amino acid change Ile359Leu was detected by a PCR-RFLP assay (*13*) using primers (forward) 5'-AGGA AGAGATTGAACGTGTGA-3' and (reverse) 5'-GGCAGGCTGGTGGGG AGAAGGCCAA-3'. PCR conditions were the same as described above. A 130-bp amplicon was digested with StyI (New England Biolabs); wild type alleles (Ile) remained uncut, but mutant alleles (Leu) were cleaved into two fragments of 104 and 26 bp.

For quality control, 10% of the samples were genotyped by other laboratory personal and no discrepancy was observed.

2.4. *Statistical analysis*

Genotype frequencies in the study population were checked for Hardy-Weinberg equilibrium. 95% confidence intervals (CI) were calculated using Confidence Interval Analysis software version 1.0. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. *Allele and genotype frequencies of VKORC1 alleles in the North Indian population*

The *VKORC1* -1639 G>A minor allele frequency in the study population ($n = 102$) was found to be 14.22% (95% CI: 10.08-19.67) (Table 1). Out of 102 subjects, 2 subjects were homozygous for *VKORC1* (-1639 AA) (1.96%, 95% CI: 0.54-6.87) and 25 subjects were heterozygous having both the alleles (-1639 GA) (24.51%, 95% CI: 17.19-33.68) (Table 1). The genotype frequencies of *VKORC1* -1639 G>A in the study population were found to be in Hardy-Weinberg equilibrium.

3.2. *Frequencies of CYP2C9*2 and *3 in the North Indian population*

None of the patients were homozygous for *2C9**2 or *2C9**3. The *CYP2C9**2 mutant allele frequency in our study population ($n = 102$) was found to be 4.9% (95% CI: 2.68-8.79) (Table 2). Out of 102 subjects, 2 subjects were homozygous (TT) for *CYP2C9**2 (1.96%, 95% CI: 0.54-6.87) and 6 subjects were heterozygous (CT) (5.88%, 95% CI: 2.72-12.24). The *CYP2C9**3 allele frequency in the study population ($n = 102$) was found to be 3.92% (95% CI: 2.00-7.55) (Table 3). Out of 102 subjects, 8 subjects were heterozygous (AC) (7.8%, 95% CI: 4.03-14.72) but no homozygous (CC) was detected in 102 subjects (Table 3).

Table 1. *VKORC1* -1639 G>A genotype and allele frequencies in the North Indian population

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
GG	35	40	73.53	64.23-81.12
GA	14	11	24.51	17.19-33.68
AA	2	0	1.96	0.54-6.87
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
G	175		85.78	80.33-89.92
A	29		14.22	10.08-19.67

* Total number of subjects genotyped for *VKORC1* -1639 G>A allele was 102 (males = 51 and females = 51); ** Total number of alleles = 204.

Table 2. *CYP2C9* 430 C>T (*CYP2C92) frequencies in the North Indian population**

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
CC	47	47	92.16	85.28-95.97
CT	4	2	5.88	2.72-12.24
TT	2	0	1.96	0.54-6.87
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
C	194		95.10	91.2-97.32
T	10		4.90	2.68-8.79

* Total number of subjects genotyped for *CYP2C9**2 allele was 102 (males = 51 and females = 51); ** Total number of alleles = 204.

Table 3. *CYP2C9* 1075 A>C (*CYP2C93) genotype and allele frequencies in the North Indian population**

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
AA	46	48	92.16	85.28-95.97
AC	5	3	7.84	4.03-14.72
CC	0	0	0	0.00-3.63
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
A	196		96.08	92.45-98.00
C	8		3.92	2.00-7.55

* Total number of subjects genotyped for *CYP2C9**3 allele was 102 (males = 51 and females = 51), ** Total number of alleles = 204.

4. Discussion

The present study performed genotyping on 204 chromosomes (102 individuals) for polymorphisms in *VKORC1* and *CYP2C9* genes. This group is considered to represent individuals from all the states of Northern India. All the blood samples were randomly collected from healthy relatives of patients coming to our institute from a large capture area of North India. The number of subjects was enough to establish frequencies of minor alleles as this number was higher than that available in the HapMap data.

The *VKORC1* -1639 G>A minor allele frequency in the study population was 14.22% which was significantly different from East Asians as well as Caucasian populations (*16*). The frequency of *VKORC1* -1639 A was significantly higher in Chinese (CHB, 94.6%), Japanese (JPT, 90.1%) and European (CEU, 39.8%) populations (*16*). Our frequency in North Indians was similar to Indians in Taiwan (mostly South Indians) (*6*), but moderately lower than the Gujarati Indian population of USA (GIH, 19.3%), the migrants from a state in the Western part of India (*16*).

The frequencies of *CYP2C9* (*CYP2C9**2 and *CYP2C9**3) in the study population were also compared to previously reported studies on Indians and five major population data groups from the HapMap project (*16*). The *CYP2C9**2 frequency was higher in European population (CEU, 10.4%) but 0% in Chinese, Japanese and African populations (*16*). The *CYP2C9**2 allele frequency in North Indians is comparable to that of South Indians but there are no reports about frequency in Gujarati Indians. Regarding *CYP2C9**3, the frequencies in North Indians were lower than Indians in South and West India. The frequency was slightly higher in European and Chinese populations (CEU, 5.8% and CHB, 5.4%) than the North Indian population of our study (16). The *CYP2C9**3 frequency was lower in the Japanese population (JPT, 2.3%) and there are no reports about the African population (*16*) (Table 4).

A clinically important observation that the oral anticoagulant daily dose requirement is highest in the black population, with the white population generally requiring an intermediate dose and the Asian population the lowest dose, led to investigation of the role of genetics in oral anticoagulant dose requirements. There are many reports associating oral anticoagulant dose requirement with patient genotypes. The G allele frequency of *VKORC1* A>G has been found in concordance with the clinical observation that Chinese patients require smaller doses of warfarin than their Caucasian counterparts to achieve the same degree of anticoagulation (*5*). Lee *et al*. (*6*) reported that the mean weight-normalized warfarin dose was lower for Chinese and Malays than for Indians.

The *CYP2C9**2 genotype is absent and *3 has a greatly decreased frequency in East Asians, whereas African Americans carry a lower frequency of *2 than whites (*17*). In individuals with African heritage the frequency of *CYP2C9**2 and *3 is much lower in blacks than in whites. About 20% of the Caucasian population is heterozygous (*1*2) and 2% is homozygous (*2*2) for *CYP2C9**2 genotype. A smaller proportion of the population is homozygous (*3*3) or heterozygous (*1*3 or *2*3) for the *3 genotype. For *CYP2C9**2, enzyme activity decreases by 30%, and for *3 it decreases by 80% (*18*). The lowest enzyme activity is seen in individuals carrying two *3 alleles. The decreased enzyme activity is associated with lower drug dose requirements. Patients with the *CYP2C9**1*2 genotype required, on average, a 20% lower warfarin dose to maintain a target INR between 2 and 4 compared to the anticoagulated patient population studied (*19*). Japanese patients have been shown to require a lower warfarin dose, and those who are of wild-type *CYP2C9* genotype can clear warfarin more efficiently than their white counterparts (*20*). Based on these facts, it appears that warfarin maintenance dose in Indians should be higher than other Asian populations but lower than Caucasians. However, there is also significant variation in frequency of *2C9**3 in different Indian populations and that may result in variation of dosage requirements for anticoagulation therapy also.

In conclusion, we report frequencies of three important genetic variants, namely *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 in the North Indian population which are different from other populations. These factors may be taken into consideration for genome-based dosing regimens for oral anticoagulant therapies in the future.

Table 4. Minor allele frequencies of *VKORC1* -1639 G>A, *CYP2C92 and *3 in the North Indian and HapMap selected populations**

SNP ID	Major/Minor allele	Number of subjects					
		NI	CEU	CHB	GIH	JPT	AFR
rs9923231 (*VKORC1*)	G/A	14.2	39.8	94.6	19.3	90.1	2.2
rs1799853 (*CYP2C9**2)	C/T	4.9	10.4	0	n.r.	0	0
rs1057910 (*CYP2C9**3)	A/C	3.9	5.8	5.4	13.1	2.3	n.r.

Data of the minor allele frequencies of various populations were obtained from the HapMap project (*16*). Abbreviations: NI, North Indian (the present data); CEU, CEPH (Utah residents with ancestry from Northern and Western Europe); GIH, Gujarati Indians in USA; JPT, Japanese in Tokyo, Japan; CHB, Han Chinese in Beijing, China; AFR, African; n.r., not reported.

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