Research Paper

Genetic variation and haplotype structure of the gene *Vitamin K epoxide reductase complex, subunit 1* in the Tamilian population

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ABSTRACT

Objective: To study the genetic variation and haplotype structure of *Vitamin K epoxide reductase* complex, subunit 1 *(VKORC1)* gene in the Tamilian population. **Materials And Methods:** The study was performed on 210 unrelated, healthy volunteers of the Tamilian population, of either sex between the age group of 18-60 years. Five ml of venous blood sample was collected using sodium ethylene diamine tetra acetic acid (EDTA) as anticoagulant. DNA was extracted using phenol-chloroform extraction method. Eight single nucleotide polymorphisms (SNPs) *VKORC1* rs9923231 (G), rs7196161 (T), rs2884737 (T), rs17708472 (C), rs9934438 (C), rs8050894 (G), rs23596121 (C), and rs7294 (A) were studied using real-time quantitative Polymerase Chain Reaction (qPCR) method and they were included for constructing five-major haplotype blocks of *VKORC1* gene. **Results:** The major alleles of *VKORC1* rs9923231 (G), rs7196161 (T), rs2884737 (T), rs17708472 (C), rs9934438 (C), rs8050894 (G), and rs23596121 (C), were found to be at frequencies of 90.0%, 89.2%, 90.9%, 94.1%, 90.7%, 89.5% and 91.2%, respectively. The variant allele of *VKORC1* rs7294 (A) was more frequent (83.6%) in the Tamilian population. The frequencies of haplotypes HAP1 (GTTCCGCA), HAP2 (ACGCTCTG), HAP3 (GTTTCGCG), HAP4 (GTTCCGCG) and HAP5 (GCTCCCCG) were found to be 80.0%, 7.4%, 4.7%, 1.5% and 1.1%, respectively. **Conclusion:** In the present study the allele- frequency distributions, genotype and haplotype frequencies of the VKORC1 gene was considered. The findings of this study provide the genetic information required for learning the association of VKORC1 genetic variation and oral anticoagulant dose variability among patients receiving oral anticoagulants in the Tamilian population.

Key words: Genotype, haplotype, *Vitamin K epoxide reductase complex subunit 1*

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INTRODUCTION

Oral anticoagulants (OAs) such as warfarin, acenocoumarol and phenprocoumon are widely prescribed for patients to prevent thromboembolic events in various conditions.[1] These drugs have a narrow therapeutic index and need careful monitoring for initiation of therapy and maintenance. The actions of OAs are significantly dependent on the enzyme (vitamin k epoxide reductase) that is responsible for converting vitamin K epoxide to vitamin K hydroquinone which is the reduced form of vitamin K. This step is crucial in the mechanism of blood coagulation.

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Vitamin K epoxide reductase complex, subunit 1 (VKORC1) is a significant molecule for cardiovascular diseases, since it is the target of oral anticoagulant drugs. The enzyme vitamin k epoxide reductase (VKOR), encoded by *VKORC1*, is specifically inhibited by coumarin oral anticoagulants and leads to diminished availability of reduced vitamin K, thereby inhibiting the clot formation.[2]

In recent years, studies had described genetic variation in the gene *VKORC1* encoding the VKOR enzyme leading to altered function of the enzyme VKOR.[3] Hence common genetic variation in the gene leads to inter-individual variability in susceptibility to developing adverse drug reactions such as bleeding manifestations and other adverse reactions to OA.[3] The most widely studied single nucleotide polymorphism (SNP) of *VKORC1* is -1639 G>A in the promoter region and has been described in a number of populations.[4-6] This polymorphism has been identified as the major allele in the Asian population.[7] The presence of this allele results in reduced dose requirement for oral anticoagulants. Other than *VKORC1* 5' flanking region polymorphisms, there are SNPs in the non-coding regions that produce significant variation in the oral anticoagulant dose requirement.[8-10]

Further, Reider *et al*.,[11] demonstrated a significant contribution of *VKORC1* haplotypes to inter-individual variability in warfarin dose. The ten most common SNPs [at positions 381 (C>T), 861 (C>A), 2653 (G>C), 3673 (G>A), 5808 (T>G), 6009 (C>T), 6484 (C>T), 6853 (G>C), 7566 (C>T), and 9041 (G>A) of the *VKORC1* reference sequence (GenBank accession number AY587020)] were used to construct the haplotypes. Among the haplotype groups, a low dose [Group A (H1-2.9 mg/day, H2- 3.0 mg/day)] and high dose [Group B (H7-6.30 mg/day, H8-4.8 mg/day, H9- 5.5 mg/day)] haplotypes were identified. These haplotype frequencies also vary among the different ethnic populations. Haplotype A is more frequent in Asians (89%); whereas Haplotype B is more frequent in Caucasians (58%).[12]

The Tamilian population is a south Indian sub-population that constitutes about 5.8% of the total population of India and resides in two states (Tamil Nadu, Pondicherry).[13] This population shares a common ancestry (Dravidians), but the present day population differs from one another in terms of language, culture and dietary habits with limited admixture. David Reich *et al*.,[14] found that the ancestral south Indians were distinct from the ancestral north Indians and east Indians.

In our previous studies we have reported that the variants in the genes encoding drug-metabolizing enzymes and drug transporters were significantly different in the Tamilian population as compared to other ethnic populations.[15-17] In the present study we aimed to establish the allele, genotype and haplotype frequencies of the *VKORC1* gene in the Tamilian population which may provide us with the basic information needed for further pharmacogenetic studies of VKORC1 among the Tamilian population.

MATERIALS AND METHODS

Study subjects

The study was conducted on 210 unrelated healthy volunteers from Tamil Nadu and Pondicherry. Both genders, aged between 18 and 60 years, were included in the study. Their nativity as Tamilian was assessed based on their family history of three generations from Tamil Nadu and Pondicherry and Tamil as their mother tongue. The study protocol was approved by the Institute Ethics Committee and the study was conducted according to the declaration of Helsinki. The study was explained to all the study participants and written informed consent was obtained.

Genotyping of Vitamin K epoxide reductase complex, subunit 1

Five milliliters of venous blood was collected using sodium ethylene diamine tetra acetic acid (EDTA) as anticoagulant. DNA was extracted by using standard phenol: chloroform method. Genotyping of *VKORC1* was carried out by real-time thermo cycler (7300 Applied Biosystems; Life Technologies Corporation, Carlsbad, CA, USA) using TaqMan SNP genotyping assays [Table 1]. The Real-Time Polymerase Chain Reaction (RT- PCR) was carried out in duplicate in a 20-μL final volume that contained 10 μL of TaqMan

Table 1: Real-Time PCR conditions and TaqMan genotyping assay ID

VKORC1 SNP	TaqMan assay ID	Location	PCR conditions		
			Initiation	Denaturation	Annealing/Extension
rs9923231	C__30403261_20	Chr.16: 31107689	95°C (10 min) (To activate the AmpliTaq Gold DNA Polymerase)	92°C (15 sec)	60°C (1 min)
rs7196161	C__30996661_30	Chr.16: 31110981			
rs2884737	C__16147492_20	Chr.16: 31105554			
rs17708472	C__32928084_10	Chr.16: 31105353			
rs9934438	C__30204875_10	Chr.16: 31104878			45 Cycles
rs8050894	C__2847860_10	Chr.16: 31104509			
rs2359612	C__26291751_10	Chr.16: 31103796			
rs7294	C__7473918_10	Chr.16: 31102321			

universal PCR master mix (2x), 0.5 µL of 20x working stock of SNP genotyping assay and 4.5 µL of genomic DNA diluted in DNAase free water and 5 µL of MilliQ water (Millipore Corporate Headquarters, Billerica, MA, USA). The thermocycler conditions are given in Table 1. The allelic discrimination analysis was performed using 7300 SDS software Version 1.4.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat 3 software (GraphPad Software Inc., San Diego, CA, USA). Hardy–Weinberg equilibrium was tested by Chi-square test to compare the observed genotype frequencies with the expected genotype frequencies calculated from the observed allele frequencies. Chi-square test was used for comparisons of different ethnic groups. $P < 0.05$ was considered statistically significant. Pair-wise linkage disequilibrium (LD) pattern and haplotype frequencies were estimated using HAPLOVIEW 4.1.[18] Haplotypes were estimated by accelerated expectation-maximization (EM) algorithm in HAPLOVIEW. The confidence interval range for LD was set between 0.7 and 0.98. D' values from 0.7-1.0 indicate strong LD between pair of SNPs. Whereas D' value <0.7 indicates moderate LD and D' value of <0.2 indicates no LD.

RESULTS

A total of 204 [95 men, 109 women; age (±SD) 35.8 ± 7.9 years] samples of healthy volunteers were included for the analysis; six samples were lost during processing of DNA. The frequency distributions of VKORC1 genotypes [Table 2] were found to be in Hardy-Weinberg equilibrium [4 × 3 contingency table; rs9923231- $\chi^2 = 0.53$, $P = 0.46$, rs7196161- $\chi^2 = 0.07$, $P = 0.79$, rs2884737- $\chi^2 = 0.07$, $P = 0.78$, rs17708472- $\chi^2 = 0.14$, $P = 0.70$, rs9934438- $\chi^2 = 0.04$, $P = 0.85$, rs8050894- $\chi^2 = 0.04$, $P = 0.0.84$, rs2359612- $\chi^2 = 0.13$, $P = 0.72$, rs7294, $\chi^2 = 0.06$, $P = 0.79$)].

The allele frequency of *VKORC1* in Tamilians was compared with other ethnic populations [Table 3]. The most studied SNPs (rs9923231, rs9934438, rs2359612 and rs7294) were compared with other ethnic populations. The results reveal that the frequency distribution of Han Chinese, Caucasians and African Americans were significantly different from the Tamilian population ($P < 0.001$). The frequency distributions of rs9923231 and rs9934438 among North Indians were not significantly different from the Tamilian population which is in south India ($P > 0.05$). The haplotype structure

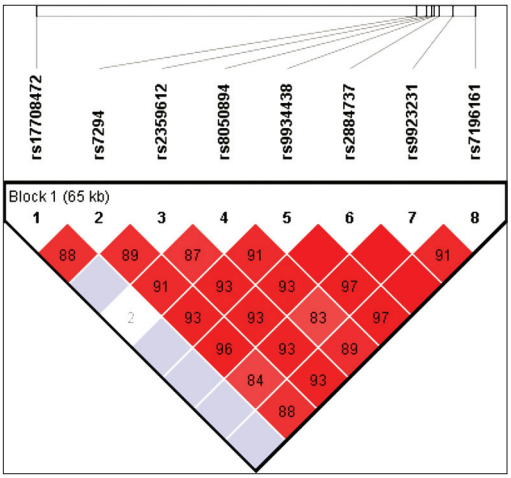

Figure 1: Linkage disequilibrium pattern of *VKORC1* genetic variants in the Tamilian population. The single nucleotide polymorphisms (SNPs) in Chromosome 16 were positioned according to the order and orientation. Each of the variant is given with its specific chromosomal position and the rsID. LD pattern of the rs9923231, rs7196161, rs2884737, rs1770847, rs9934438, rs8050894, rs23596121and rs7294 in the Tamilian population. Red and pink colors represent a very strong LD pattern (D'>0.8) and white color represents moderate to low LD (D' <0.8 to > 0.5)

Table 2: Genotype and allele frequencies of *VKORC1* in the Tamilian population

VKORC1	SNP Positions	%Genotype frequency (95% CI)			%Allele frequency (95% CI)	
rs9923231	-1639 G>A	GG	GA	AA	G	A
		81.4 (75.4-86.1)	17.2 (12.6-22.9)	1.5 (0.5-4.2)	90.0 (86.6-92.5)	10.0 (7.5-13.4)
rs7196161	296 C>T	CC	CT	TT	C	T
		1.0 (0.3-3.5)	19.6 (14.7-25.6)	79.4 (73.3-84.3)	10.8 (8.1-14.1)	89.2 (85.8-91.8)
rs2884737	497 T>G	TT	TG	GG	T	G
		82.8 (77.1-87.4)	16.2 (11.8-21.8)	1.0 (0.3-3.5)	90.9 (87.7-91.8)	9.1 (6.6-12.2)
rs17708472	698 C>T	CC	CT	TT	C	T
		88.7 (83.6-92.4)	10.8 (7.2-15.8)	0.5 (0.1-2.7)	94.1 (91.4-96.0)	5.9 (3.9-8.6)
rs9934438	1173 C>T	CC	CT	TT	C	T
		82.4 (76.5-86.9)	16.7 (12.1-22.4)	1.0 (0.3-3.5)	90.7 (87.4-93.1)	9.3 (6.8-12.5)
rs8050894	1542G>C	GG	GC	CC	G	C
		79.9 (73.8-84.8)	19.1 (14.3-25.1)	1.0 (0.3-3.5)	89.5 (86.1-92.0)	10.5 (7.9-13.9)
rs2359612	2255C>T	CC	CT	TT	C	T
		83.3 (77.6-87.8)	15.7 (11.3-21.3)	1.0 (0.3-3.5)	91.2 (88.0-93.6)	8.8 (6.4-11.9)
rs7294	3730G>A	GG	GA	AA	G	A
		2.5 (1.0-5.6)	27.9 (22.2-34.5)	69.6 (62.9-75.5)	16.4 (13.1-20.3)	83.6 (79.6-86.8)

Table 3: Distribution of widely studied *VKORC1* allele frequencies among different ethnic groups

Population	% Allele Frequency (95% CI)			
	rs9923231	rs9934438	rs2359612	rs7294
Tamilian+	10.0 (7.5-13.4)	9.3 (6.8-12.5)	8.8 (6.4-11.9)	83.6 (79.6-86.8)
Han Chinese[19,20]	92.4 (90.0-94.2)	91.5 (90.2-92.6)	87.9 (86.4-89.3)	5.8 (3.2-10.4)
Hispanic[21]	33 (24.5-42.7)	NA	NA	NA
Japanese[22,23]	91.8 (89.4-93.6)	91.5 (87.8-95.5)	92.5 (86.5-92.8)	90.1 (87.7-95.4)
Egyptian[24]	46.2 (41.3-51.1)	NA	NA	NA
Caucasian*	30.1 (26.0-34.5)	19.9 (16.5-23.8)	19.9 (16.5-23.8)	36.3 (31.9-40.8)
European American[25]	NA	37.5 (33.0-42.2)	NA	NA
African American*	43.8 (33.4-54.6)	10.2^{ns} (7.7-13.4)	19.4 (14.9-24.7)	43.2 (33.3-53.6)
Iranian[26]	55.6 (49.3-61.5)	NA	NA	NA
North Indian[27]	14.2^{ns} (10.1-19.6)	NA	NA	NA
Indonesians[28]	77 (71.6-81.6)	NA	NA	NA
Argentine[29]	49.5 (42.6-56.3)	NA	NA	NA

+present study, *Frequency data obtained from NCBI dbSNP (http://www.ncbi.nlm.nih.gov/). NA- Data not available in the population. ^{ns}Not significantly different, Tamilian vs. other ethnic population $P<0.0001$

Table 4: *VKORC1* Haplotype frequency in the Tamilian population

Haplotype No	rs9923231	rs7196161	rs2884737	rs17708472	rs9934438	rs8050894	rs2359612	rs7294	% Frequency	95% Confidence Interval
HAP1	G	T	T	C	C	G	C	A	80.0	73.8-84.8
HAP2	A	C	G	C	T	C	T	G	7.4	4.5-11.8
HAP3	G	T	T	T	C	G	C	G	4.7	2.7-8.8
HAP4	G	T	T	C	C	G	C	G	1.5	0.7-4.9
HAP5	G	C	T	C	C	C	C	G	1.1	0.5-4.2
HAP6	A	T	T	C	C	G	C	A	0.7	0.2-3.5
HAP7	A	C	G	C	T	C	C	G	0.7	0.2-3.5
HAP8	G	T	T	T	C	G	C	A	0.5	0.1-3.1
HAP9	G	T	T	C	C	C	C	A	0.5	0.1-3.1
HAP10	G	C	T	C	C	G	C	A	0.5	0.1-3.1
HAP11	A	C	G	C	T	G	T	G	0.5	0.1-3.1
HAP12	G	T	T	C	C	G	T	A	0.5	0.1-3.1
HAP13	G	T	T	T	C	C	C	G	0.5	0.1-3.1
HAP14	A	C	G	C	T	C	T	A	0.3	0-2.7
HAP15	G	T	T	C	T	G	C	A	0.2	0-2.3
HAP16	A	C	T	C	C	G	C	A	0.2	0-2.3
HAP17	A	C	G	T	T	C	T	G	0.2	0-2.3

and pair-wise LD pattern of the eight *VKORC1* SNPs were identified [Figure 1] and their frequencies in the Tamilian population were established [Table 4] by accelerated expectation-maximization (EM) algorithm. A strong LD pattern (D'>0.8) was observed between all the SNPs except between rs7294 and other SNPs, where only a moderate LD (D' <0.8) was observed. But the LD between rs17708472 and rs7294 was found to be strong (D'>0.8).

DISCUSSION

The present study investigated the baseline allele and genotype frequencies, LD pattern, Hardy- Weinberg equilibrium and haplotype structures of OA dose-altering *VKORC1* genetic variants rs9923231, rs7196161, rs2884737, rs9934438, rs8050894, rs2359612 and rs7294 on Chromosome 16 in the Tamilian population. Other than the *VKORC1* genetic variation, the two common genetic variants (*CYP2C9*2* and *CYP2C9*3*) in the cytochrome p450 2C9 (CYP2C9) gene were known to significantly reduce the oral anticoagulant dose.[30]

The frequencies of these polymorphisms are significantly different in other ethnic populations. In our previous study we have established the frequencies of *CYP2C9* variants in the Tamilian population.[15] The previous studies have reported that Asians, African Americans, Caucasians and Japanese with variant alleles for *CYP2C9*2, CYP2C9*3* and *VKORC1* rs9923231 G>A are sensitive to OA and they require low maintenance dose.[3,6] Those carrying the homozygous wild type allele required a higher or intermediate OA dose. They take a long time to achieve the stable dose with international normalized ratio (INR) values within the therapeutic range (2.0 to 3.0/3.5).[31]

It has been reported that patients with varying degrees of warfarin resistance carry mutations at least in one copy of the *VKORC1* gene.[32] More recent findings reveal that *VKORC1* genetic variation has a greater impact than *CYP2C9* genetic variation on warfarin dose variance.[33] Haplotype analyses have shown that most of the non-coding SNPs are in strong linkage disequilibrium. Based on haplotypes, individuals may be divided into two groups, haplotypes A (H1 and H2) and B (H7, H8 and H9), which are associated with lower and higher warfarin dose requirements, respectively. The haplotype frequencies were found to have a significant influence on the OA dose requirement in Asian populations.[11]

A previous study has established the haplotype frequency (296 (C>T), 776 (C>A), 3673 (G>A), 5723 (T>G), 1173 (C>T) and 698 (G>A) in Malaysian Indians and found that the TCGTCA (H7) is more frequent in Indians as compared to the Chinese and Malays. The H7 haplotype group has been associated with higher dose requirement,[34] and our study on the Tamilian population is in line with this observation. Hence, OA dose requirement in Tamilian subjects may be higher in comparison with the Chinese and Japanese. According to another study conducted in Malaysia, the mean dose of warfarin was 3.7 mg, and the mean daily dose of warfarin was significantly higher in Indians than the Chinese and Malay patients, 4.9 mg/day versus 3.5 and 3.3 mg/day, respectively.[34] Consequently, it is clear that other than inter-individual difference, studying the inter-ethnic variation is also important in the required OA dose variation throughout the world.

Other than the *CYP2C9* and *VKORC1* genes, studies have reported that variation in the *CYP4F2*, *GGCX* and *EPHX1* genes significantly influences the OA dose requirement.[23,35,36] But the association of these genetic variations is negligible when compared to the *CYP2C9* and *VKORC1*. Hence, it is important to establish the baseline data on genetic variations of the functionally important *VKORC1* gene, to initiate genetic studies for a particular population/cohort before conducting pharmacogenetic studies on OAs.

In association with the previous study reports the United States food and drug administration (US FDA) updated the OAs' label with including the new table with the range of expected therapeutic warfarin doses based on *CYP2C9* and *VKORC1* genotypes. Based on the pharmacogenetic information many studies have proposed OA dose-predicting algorithms for calculating the maintenance dose and initial dose of OAs using multivariate statistical techniques.[37-41] But the proposed algorithms appear to be specific for each population group, very likely due to differences in allele, genotype and haplotype frequencies.

In India very few studies have reported the association of genetic variation and OA dose requirement.[37,41] In the present study we have established the haplotype frequency in the Tamilian population, information that, to the best of our knowledge has not been reported before.

In conclusion, we report allele and genotype frequencies of eight important SNPs in the *VKORC1* gene, their linkage disequilibrium pattern and haplotype structure in the Tamilian population. The reported data may provide the baseline information for studying the association of haplotypes with warfarin and acenocoumarol dose requirement in Tamilian patients.

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