Original Article

DOI: 10.5582/ddt.2017.01031

Genotyping of *CYP2C9* and *VKORC1* polymorphisms predicts south Indian patients with deep vein thrombosis as fast metabolizers of warfarin/acenocoumarin

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Summary **Deep vein thrombosis (DVT) is a life-threatening disease. Warfarin and acenocoumarol are anticoagulants used to treat DVT and vary among individuals in terms of treatment response/toxicity. Single nucleotide polymorphisms (SNPs) in *CYP2C9* and *VKORC1* play a role in the pharmacokinetics and dynamics of warfarin and acenocoumarol and they determine the efficacy of treatment by controlling drug clearance in treated individuals. The aim of the current study was to genotype the critical SNPs of *CYP2C9* and *VKORC1* genes in a south Indian population in order to understand the metabolizer phenotype of patients with DVT. *CYP2C9* (rs1799853, rs1057910, rs1057909, rs28371686) and *VKORC1* (rs9923231) SNPs were genotyped in 124 cases of DVT. Genomic regions of these SNPs from genomic DNA were amplified with PCR and directly sequenced using Sanger sequencing except for the SNP rs1799853, which was detected using Sau96I restriction endonuclease-based digestion of variant alleles. Among south Indian patients with DVT, 6.5% (8/124) had the rs1799853 SNP of *CYP2C9* and 11% (14/124) had the rs1057910 SNP while 16% (20/124) had the rs9923231 SNP of *VKORC1* which were associated with the response to warfarin treatment. None of the patients tested positive for poor drug metabolizing genotypes of the *CYP2C9* gene and only 1.6% of the south Indian population was sensitive to warfarin treatment. Genotyping results suggest that a relatively greater amount of the therapeutic drug is required to achieve/maintain the international normalized ratio (INR) in south Indian patients with DVT.**

Keywords: Warfarin, deep vein thrombosis, anti-coagulants, SNP, pharmacogenetics, CYP2C9, VKORC1

1. Introduction

Deep vein thrombosis (DVT) often occurs in large veins such as the femoral or popliteal vein and is one of the leading causes of mortality and morbidity (*1*). Though both genes and environment are widely considered as two major risk factors that cause DVT, numerous genetic analyses of various populations have revealed a close association among genetic factors responsible for the risk of DVT/thromboembolism (*2*). Coumarins are widely used in therapeutics as anti-coagulants with a narrow range because of variability among individuals in terms of pharmacokinetics and pharmacodynamics due to genetic and environmental factors (*3*). A patient's response to coumarin is influenced by various factors such as age, sex, vitamin K intake, and medication taken (*1,3*). In addition, defective alleles of certain genes that render individuals to be poor metabolizers of

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therapeutic drugs result in internal bleeding and stroke during clinical practice (*4*).

Genetic variations in *CYP2C9* (cytochrome P450 2C9, drug-metabolizing enzyme) and *VKORC1* (vitamin K-epoxide reductase 1, drug target) have been found to account for 30-50% of the variability in the drug dose (*5*). Warfarin is a widely used first-generation anti-coagulant and its derivative acenocoumarol is a second-generation anti-coagulant, and both share the same metabolizing gene pathways and result in similar pattern adverse reactions in patients (*6-8*). Genome-wide association studies have verified that these genes are associated with the metabolism of the drugs (*9*). Recently several studies have identified polymorphisms in these two genes (*10,11*).

CYP2C9 ranks amongst the most important drug-metabolizing enzymes in humans. Since several of the drugs (notably warfarin) are metabolized by CYP2C9, the activity of this enzyme is an important factor for therapeutic response, clearance, and toxicity in an individual. Therefore, genetic polymorphisms that affect the enzyme efficiency need to be studied in order to prescribe the right dose of drugs and avoid drug overdosing based on individual genetic variations. Various single nucleotide polymorphisms (SNPs) such as *CYP2C9*2* (rs1799853), **3* (rs1057910), **4* (rs1057909), and **5* (rs28371686) have been identified in individuals with impaired CYP2C9-mediated metabolism and lower warfarin dose requirements (*10*). Compared to individuals with the *CYP2C9*1/*1* wild-type allele, individuals with the *CYP2C9*1/*2*, *CYP2C9*1/*3*, *CYP2C9*2/*2*, *CYP2C9*2/*3*, and *CYP2C9*3/*3* variants require lower warfarin doses (*12*). *CYP2C9*2* and **3* were well-documented variant alleles that have been found to play major roles in drug clearance (*13*). *In vitro* studies have also revealed that individuals with *CYP2C9*2* have 12% less catalytic activity and those with **3* have 5% less catalytic activity than individuals with the wild-type allele (*14,15*). *CYP2C9*2* and **3* have also been associated with an increased risk of excessive anticoagulation and bleeding events among patients treated with warfarin (*16*).

Similar to the effect of SNPs on *CYP2C9* gene efficiency, SNPs in the non-coding region of *VKORC1* gene are associated with sensitivity to coumarin derivatives (*17*). The presence of non-coding variants in *VKORC1* results in differential expression of the VKOR protein that determines the drug dosage in patients. In Asians, *VKORC1* polymorphisms have been associated with warfarin response, accounting for 11% to 32% of the variability in dose response when compared to the wild-type allele (*18*). The *VKORC1* SNP, –1639 G>A (rs9923231) has been found to be an important tag for low-dose haplotypes (variant allele) and high-dose haplotypes (wild allele) (*19*).

Together, the non-coding SNPs in *VKORC1* and *CYP2C9*2* and **3* variants are linked to a reduced dose requirement for warfarin, and *CYP2C9*3* has a similar effect on acenocoumarol (*20,21*). The combined effect of the *CYP2C9* and *VKORC1* alleles on the required dose of warfarin and acenocoumarol has been intensively studied in several populations and the therapeutic dose has been determined depending on the patient's allelic variant (*22,23*). However, the allelic status of *CYP2C9* and *VKORC1* and their distribution in south Indian patients with DVT was not definitively ascertained. Doing so would help to determine the optimal drug dose based on the genotype. The current study analyzed the frequency of allelic variants of the *CYP2C9* and *VKORC1* genes in the south Indian population with DVT; among them many were found to be fast metabolizers. In addition, a meta-analysis of several individual/consortium studies was performed to ascertain the distribution of the *CYP2C9* and *VKORC1* genotype frequency in the current study population in comparison to other populations.

2. Materials and Methods

2.1. *Collection of patient blood samples and extraction of genomic DNA*

Potential subjects were outpatients/inpatients ≥ 18 years of age with a stable anticoagulation status who were being monitored. No exclusion criteria were used. This study was approved by the Institutional Ethics Committee, Rajiv Gandhi Government General Hospital and Madras Medical College, Chennai (approval No. 04092010), and this study was conducted within the ethical framework of the Dr. ALM PG Institute of Basic Medical Sciences, Chennai. Informed consent was obtained from all participants in this study. Patients (*n* = 124) on long-term maintenance therapy (July 2011 – March 2013) with acenocoumarol were recruited from Rajiv Gandhi Government General Hospital, Chennai, India, and data on age, gender, and weight were collected for each patient. Blood samples were collected with EDTA and blood was drawn irrespective of drug dosage and period of administration since this study sought to determine the allelic distribution of drug-metabolizing genes. Genomic DNA was extracted using standard phenol:chloroform extraction. The quality and quantity of the genomic DNA were respectively checked using 0.7% agarose gel electrophoresis and NanoDrop (Thermo Inc., USA).

2.2. *Determination of allelic variants using Sanger sequencing*

A polymerase chain reaction (PCR) amplicon of 473 bp covering rs1799853, which is referred as *CYP2C9*2*, was amplified using a forward primer: 5'-CATGGCTGCCCAGTGTCAGC-3' and a reverse primer: 5'-TCCCATGTTCTCCTGAACTTTGCT-3'

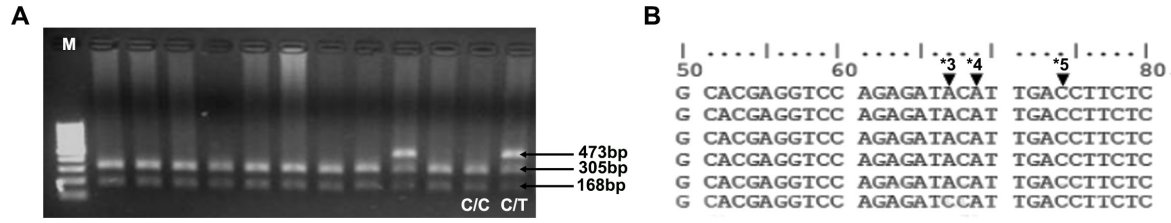

Figure 1. Genotyping gel electrophoresis for *CYP2C9*2* (rs1799853) by restriction digestion (A). The PCR fragment (473 bp) was digested with the restriction endonuclease Sau96I when the patient carried the wild-type allele (CC) and yielded two fragments 305 bp and 168 bp in size; M, DNA 100 bp ladder. **Multiple sequence alignment of *CYP2C9* indicating the nucleotide position of 3 SNPs in *CYP2C9* (*CYP2C9*3* (rs1057910), **4* (rs1057909), and **5* (rs28371686)) (B).

and digested with Sau96I restriction endonuclease, which digested the fragment when the PCR amplicon carried the wild-type allele (C allele). Two fragments 305 bp and 168 bp in size were yielded. Other *CYP2C9* variants **3* (rs1057910), **4* (rs1057909) and *5 (rs28371686) were amplified using a forward primer: 5'-GTGTGATTGGCAGAAACCGGAGC-3' and a reverse primer: 5'-TCTCACCCGGTGATGGTAGAGG -3' with an amplicon size of 256 bp. The SNP rs9923231 (–1639 G>A) located in the promoter region of *VKORC1* was amplified with PCR using a forward primer: 5'-GTTCCAGGGATTCATGCAGGGACA-3' and a reverse primer: 5'-TTGCCCTGACACCTAGTGG CTG-3' with a fragment length of 579 bp. All of the PCR reactions were carried out in 20 µL with the following temperature cycles: 94ºC 2 min for initial denaturation, followed by 40 cycles of 94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 30 sec, and 7 min of final extension at 72ºC. Amplified PCR fragments were purified and sequenced using the internal reverse primer 5'-GATACTATGAATTTGGGGACTTCG-3' for *CYP2C9* and a PCR reverse primer for *VKORC1*.

2.3. *Statistical analysis and meta-analyses*

A confidence interval for proportion was calculated using the online software Stats Calculator at *www.allto.co.uk/tools/statistic-calculators*. The URL for the data presented herein: Online Mendelian Inheritance in Man (OMIM), *http://ncbi.nlm.nih.gov/Omim/* (for *CYP2C9* and *VKORC1*-dependent blood clotting deficiency disorders). Studies with complete genotype data were selected for meta-analysis. MeSH terms like CYP2C, VKORC1, SNPs, Warfarin, Acenocoumarol, Anti-coagulant, and Pharmacogenetics were used to screen publications in the NCBI – PubMed public database. The most relevant studies were selected. Studies featuring only one of the *CYP2C9* variants (**2* or **3*) were excluded, and studies that analyzed both variants were selected for meta-analysis to avoid allelic frequency bias. Studies on the *VKORC1* non-coding variant that featured both the coding and non-coding forms or the non-coding form alone were considered acceptable since sensitivity to the drug is highly dependent on the level of the *VKORC1* gene product, which is based on the transcription efficacy determined by the promoter polymorphism. Meta-analysis was performed using comprehensive meta-analysis software (Biostat, USA). A binary random-effects model with no control group was used and the impact of heterogeneity among studies was estimated using I^2 testing. Heterogeneity was regarded as statistically significant with $p < 0.05$ or $I^2 > 50\%$.

Table 1. Genotype frequency of *CYP2C9* variants

Items	*CYP2C9* variants			
	rs1799853	rs1057910	rs1057909	rs28371686
Wild type	93.5% (116)	89% (110)	100% (124)	100% (124)
Heterozygous	6.5% (8)	11% (14)	-	-
Mutant	-	-	-	-

3. Results

3.1. *Identification of frequencies of variant alleles of CYP2C9 in south Indian patients with DVT*

Variant alleles of *CYP2C9* (*CYP2C9*2*, **3*, **4*, and **5*) were screened for in south Indian patients with DVT (n = 124) and only the *CYP2C9*2* and **3* variants were found in south Indian patients with DVT (Figure 1). The homozygous wild type allele (CC) of the *CYP2C9*2* variant (rs1799853) was present in 93.5% patients (116/124) and the CT heterozygous allele was present in 6.5% patients (8/124). Similarly, the homozygous wild-type allele AA for rs1057910 was present in 89% (110/124) patients and the heterozygous allele (AC) was present in 11% (14/124) patients with DVT. *CYP2C9*2* and **3* homozygous mutant alleles were not found among subjects (Table 1).

The frequencies of *CYP2C9* genotypes were analyzed among all of the 124 south Indian patients with DVT, and warfarin and acenocoumarol metabolizer status was categorized by combining the various allelic frequencies identified in the SNPs of *CYP2C9* summarized in Table 2. Data revealed that 82% (102/124) of south Indian patients with DVT were normal metabolizers, 18% (22/124) were intermediate metabolizers, and none were poor metabolizers.

Table 2. Predicted metabolizer frequency (*n* = 124) in south Indian patients with DVT

Metabolizer category	Genotypes (*n*)			Frequency (%)			95% CI
	Male	Female		Male	Female		
NM (*1/*1)	53	49	102	51	49	82	75.24 - 88.76 (± 6.76)
IM (*1/*2, *1/*3, *1/*5)	12	10	22	55	45	18	11.24 - 24.76 (± 6.76)
PM (*2/*2, *2/*3, *3/*3)	-	-	-	-	-	-	-

NM, normal metabolizers; IM, intermediate metabolizers; PM, poor metabolizers.

Table 3. Genotype and allele frequency of *VKORC1*

Genotypes	Number of subjects*		Frequency % (*n*)	95% CI
	Male	Female		
GG	54	50	84 (104)	77.55 - 90.45 (± 6.45)
GA	9	9	14.5 (18)	8.3 - 20.7 (± 6.2)
AA	2	-	1.5 (2)	-0.64 - 3.64 (± 2.14)
Allele	Number of alleles		Frequency %	95% CI
G	226		91	87.44 - 94.56 (± 3.56)
A	22		9	5.44 - 12.56 (± 3.56)

* In total, 124 subjects (males 65 and females 59) were genotyped for the *VKORC1* –1639 G>A allele.

Table 4. Prevalence of genotype frequency with respect to warfarin sensitivity

Genotype combinations		Prevalence *n* (%)	Warfarin sensitivity
VKORC1 –1639 G>A	*CYP2C9*		
A/A	*1/*3, *2/*2, *2/* 3, *3/*3	1 (0.8)	Very high
G/A	*3/*3	-	
A/A	*1/*1	1 (0.8)	High
G/A	*2/*3	-	
G/G	*3/*3	-	
A/A	*1/*1	-	Moderate
G/A	*1/*2, *1/*3, *2/*2	5 (4)	
G/G	*2/*3	-	
G/G	*1/*2, *1/*3, *2/*2	16 (13)	Mild
G/A	*1/*1	13 (10.4)	Normal
G/G	*1/*1	88 (77)	Less than normal
Total		124 (100)	

3.2. *Allele and genotype frequencies of VKORC1 alleles in south Indian patients with DVT*

The *VKORC1* –1639 G>A promoter polymorphism (rs9923231) was found in 16% (20/124) of the south Indian patients with DVT. Among 124 patients, 84% (104/124) were homozygous for the wild-type allele (GG), 14.5% (18/124) were heterozygous for the mutant allele (GA), and only 1.5% (2/124) were homozygous for the mutant allele (AA) (Table 3).

3.3. *Analyses of a combination of CYP2C9 and VKORC1 genotypes associated with warfarin sensitivity in South Indian patients with DVT*

The genotype combinations of *CYP2C9* with *VKORC1* variants were analyzed to classify warfarin/acenocoumarol sensitivity in the study population. Only 1.6% (2/124) of the patients with DVT were highly sensitive to warfarin treatment, 4% (5/124) were carriers with moderate sensitivity, and 13% (16/124) were carriers with mild sensitivity. Interestingly, about 81.4% (101/124) of the study population carried allelic combinations that would classify them as normal metabolizers and suggest they were unlikely to be sensitive to warfarin treatment (Table 4).

3.4. *Meta-analysis of CYP2C9 and VKORC1 genotypes in various populations*

A meta-analysis of the minor allele frequency (MAF) of *CYP2C9* (rs1799853 and rs1057910) SNPs and the

VKORC1 SNP (rs9923231) was performed in different populations from several individual/consortium studies (Table 5). The MAF of respective SNPs and confidence interval have been graphically presented as a forest plot (Figure 2). A binary random-effects model with no control group was used and a significant heterogeneity for *CYP2C9*2* (I^2 = 99.3%, $p < 0.001$), *CYP2C9*3* (I^2 = 91.3%, $p < 0.001$) and *VKORC1* rs9923231 (I^2 = 99.3%, $p < 0.001$) was observed. The meta-analysis revealed that MAFs were less frequent in the south Indian population than in other populations, and a similar frequency was reported for the north Indian population. Interestingly, the southwest Chinese population had a lower frequency of the variant allele *CYP2C9*2* compared to the study population but the same was not true for *CYP2C9*3* and *VKORC1*. Similarly, Swedes and a mixed European population studied by the International Warfarin Pharmacogenetics Consortium had a lower frequency of the variant allele in *CYP2C9* gene (**3*) than the study population. However, most studies reported a higher frequency of the polymorphic allele (the *VKORC1* non-coding variant) compared to populations on the Indian sub-continent (north and south Indians).

Table 5. Minor allele frequencies of *VKORC1* –1639 G>A and *CYP2C9*2* observed in the south Indian population (current study) and in other populations

SNP	Study	Population	MAF
VKORC1			
rs9923231 (G/A)	Current study	South Indian	0.088
	1000Genomes	European	0.387
		African	0.054
		American	0.410
		South Asian	0.145
	Hapmap	European	0.398
		African	0.022
		Han Chinese	0.951
		Japanese	0.901
		GIUS	0.193
	Rathore SS, *et al*.	North Indian	0.142
	Takeuchi F, *et al*.	Swedish	0.402
	Bodin L, *et al*.	French	0.420
	IWPC	Mixed	0.514
	Gu Q, *et al*.	SWC	0.917
	Oner Ozgon G, *et al*.	Turkish	0.500
	Anton A, *et al*.	Spanish	0.408
	Borobia AM, *et al*.	Spanish	0.372
	Scott, *et al*.	AJ	0.533
		SJ	0.500
CYP2C9			
rs1799853 (C/T)	Current study	South Indian	0.016
	1000 Genomes	European	0.124
		African	0.008
		American	0.099
		South Asian	0.034
		Caucasians	0.129
		Hispanic	0.065
	Hapmap	European	0.103
		African	-
		Han Chinese	-
		Japanese	-
	Rathore SS, *et al*.	North Indian	0.049
	Takeuchi F, *et al*.	Swedish	0.109
	IWPC	Mixed	0.080
	Gu Q, *et al*.	SWC	0.000
	Oner Ozgon G, *et al*.	Turkish	0.130
	Anton A, *et al*.	Spanish	0.165
	Borobia AM, *et al*.	Spanish	0.163
	Scott, *et al*.	AJ	0.127
		SJ	0.194
rs1057910 (A/C)	Current study	South Indian	0.028
	1000 Genomes	European	0.072
		African	0.002
		American	0.037
		South Asian	0.109
		Caucasians	-
		Hispanic	-
	Hapmap	European	0.058
		African	0.000
		Han Chinese	0.044
		Japanese	0.033
	Rathore SS, *et al*.	North Indian	0.039
	Takeuchi F, *et al*.	Swedish	0.109
	IWPC	Mixed	0.040
	Gu Q, *et al*.	SWC	0.098
	Oner Ozgon G, *et al*.	Turkish	0.100
	Anton A, *et al*.	Spanish	0.070
	Borobia AM, *et al*.	Spanish	0.081
	Scott, *et al*.	AJ	0.159
		SJ	0.138

1000 Genomes and Hapmap results were obtained from NCBI dbSNP. IWPC, International Warfarin Pharmacogenetics Consortium; GIUS, Gujarati Indians in the US; SWC, southwest Chinese; AJ, Ashkenazi Jews; SJ, Sephardi Jews.

4. Discussion

DVT is a potentially life-threatening condition due to numerous risk factors such as age, sex, vitamin K intake, and medications. The annual global incidence of DVT, and especially that in veins of the leg, is estimated to be 1.6 per 1,000 with a 10-year recurrence rate of 30% (*24,25*). Although certain risk factors have been identified, DVT is mainly caused by acquired factors including age, hospitalization, pregnancy, hormone therapy, cancer and surgery, and genetic risk factors including mutations and SNPs present in the genes actively involved in drug transport and metabolism. A number of various family and twin studies have revealed that genetic factors account for more than 60% of the risk for developing DVT (*26,27*). Regardless of ideal traditional treatment strategies with anticoagulants, a post-thrombotic syndrome often develops in one in four patients within a year while DVT recurs in one-third of patients within five years (*28,29*).

Although warfarin and acenocoumarol are widely used anticoagulants, they have a narrow range of therapeutic use because of variability among individuals. The combined action of *CYP2C9* and *VKORC1* are essential for the clearance of the anticoagulant drug given to patients with DVT. Several individual studies and GWAS have established the importance of genetic variants in the efficiency with which anticoagulants such as s-warfarin and acenocoumarol are cleared from the circulation (*9*). The dosage requirements of the anticoagulants are determined based on different combinations of alleles present in *CYP2C9* and *VKORC1*

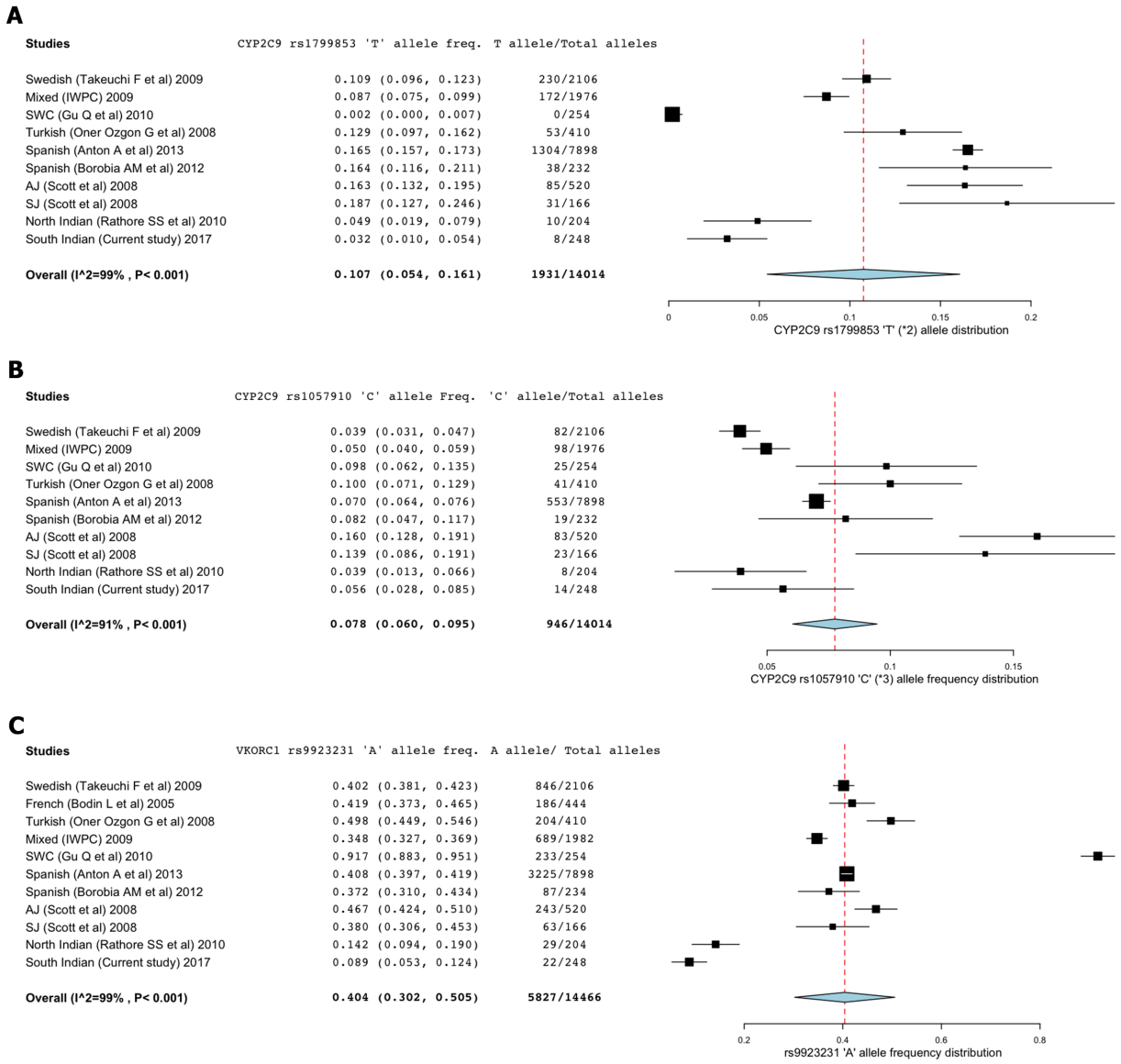

Figure 2. Forest plot of *CYP2C*2* (A), *CYP2C*3* (B), and *VKORC1* promoter SNP (C) among the south Indian population with DVT and other populations. A forest plot generated with comprehensive meta-analysis software with allelic proportion models was analyzed using a random-effects model. IWPC, International Warfarin Pharmacogenetics Consortium, SWC; Southwest Chinese; AJ, Ashkenazi Jews; SJ, Sephardi Jews.

that influence the rate of drug clearance.

The current study determined the allele frequency of *CYP2C9* polymorphisms (rs1799853, rs1057910, rs1057909, and rs28371686) and the *VKORC1* promoter polymorphism (rs9923231) in 124 south Indian patients with DVT. Results indicated that 93.5% of south Indian patients carry homozygous wild alleles in *CYP2C9*, suggesting that they are normal metabolizers. A relatively lower frequency of the mutant alleles of this gene (4%) has previously been reported in the north Indian population (*30*). The *CYP2C9*2* variant is reported to be more frequent among Caucasian populations, with ~1% of the population being homozygous carriers while 22% are heterozygous; corresponding figures for the *CYP2C9*3* allele are 0.4% and 15%, respectively (*31*). In contrast, only 6.5% of the population in the current study was heterozygous for the *CYP2C9*2* variant and 11% was heterozygous for the **3* variant, and none of the population was homozygous for variant alleles. The *CYP2C9*4* and **5* variants were not noted, suggesting that these variants are very rare in the south Indian population. The current results indicate that most south Indian patients with DVT have a *CYP2C9* wild-type allele. Similarly, 84% of the south Indian patients with DVT had the normal allele (GG) for *VKORC1* rs9923231 and 16% had a mutant allele (GA, AA). Likewise, Rathore *et al.* reported that 14% of north Indian patients with DVT had a mutant allele (GA and AA) for VKORC1 (*30*). Results of a meta-analysis also indicated that Indian patients with DVT have a lower frequency of minor alleles than other populations.

In conclusion, combined genotype analyses of both *CYP2C9* and *VKORC1* genetic variants suggested that most south Indian patients with DVT examined in this study were normal metabolizers. None of the patients in the current study exhibited a poor drug-metabolizing genotype with regard to *CYP2C9*. Only 1.6% of the south Indian population is sensitive to warfarin treatment. Combined genotype analysis of *CYP2C9* and *VKORC1* polymorphisms from the south Indian population with DVT suggested that an increase in the anti-coagulant drug dose may be necessary for Indian patients with DVT to achieve/maintain the international normalized ratio (INR). Overall, the results of the current study suggest that the drug therapy should be personalized for South Indian patients since this group displays a distinct genotype.

Acknowledgements

The authors wish to thank all of the physicians and nurses for their considerable help in collecting clinical samples. The authors also wish to thank all of the patients who participated in this study. AK and VP thank UGC for providing research fellowships.

The study was supported by a DBT-India research grant (BT/PR10023/AGR/36/27/2007) and a research grant from the Indian Council of Medical Research (ICMR) (No.82/15/2012/PHGEN(TF)/BMS).

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(Received June 20, 2017; Revised July 31, 2017; Accepted August 10, 2017)