Toxicology and Applied Pharmacology 309 (2016) 37–43

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Gene polymorphisms and the risk of warfarin-induced bleeding complications at therapeutic international normalized ratio (INR)

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ARTICLE INFO

Article history:
Received 22 June 2016
Revised 24 August 2016
Accepted 26 August 2016
Available online 28 August 2016

Keywords:
Bleeding
Cytochrome P450 subfamily 2C polypeptide 9 (CYP2C9)
Genotype
International normalized ratio (INR)
NQO1
Polymorphism
Warfarin

ABSTRACT

Background: Bleeding episodes commonly occur in patients on warfarin treatment even in those within therapeutic range of international normalized ratio (INR). The objective of this study was to investigate the effects of the 8 examined polymorphisms on the risk of bleeding complications in a sample of Iranian patients.
Methods: A total of 552 warfarin treated patients who maintained on a target INR level of 2.0–3.5 for at least three consecutive intervals were enrolled from those attended our anticoagulation clinics. Ninety-two bleeding events were observed in 87 patients. The presences of the examined polymorphisms were analyzed using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP).
Results: Patients with the T allele in NQO1*2 (CT or TT genotypes) had a higher risk of bleeding than patients with the CC genotype (adjusted OR: 2.25, 95% CI: 1.37 to 3.70, $P = 0.001$). Those who were carriers of CYP2C9 one-variant haplotypes (*1/*2 or *1/*3) were also found to be associated with the higher risk of bleeding events. Compared to reference group (*1/*1), the odds of bleeding increased for carriers of one variant allele (*1/*2 or *1/*3) (adjusted OR: 1.75, 95% CI: 1.03 to 2.97, $P = 0.039$). Variant VKORC1, Factor VII, and EPHX1 genotypes were not significantly associated with the risk of bleeding events.
Conclusion: The SNP C609T within NQO1 and haplotypes of CYP2C9 (1*2 or 1*3) are independently associated to bleeding complications of warfarin at normal INR. Further studies are required to confirm such associations in diverse racial and ethnic populations.

1. Introduction

Although newer oral anticoagulants have been approved for clinical use that do not need routine laboratory monitoring, their clinical effectiveness and safety outside clinical trial settings are lacking (Kirley et al., 2012). Warfarin, the most widely used oral anticoagulant, continues to be the mainstay of therapy for thromboembolic disorders, particularly in low socioeconomic populations, where there are access barriers to novel and more expensive medications (Desai et al., 2014; Cavallari & Duarte, 2015). However, this drug has a narrow therapeutic index and a given dosage can lead to a significant inter-individual variation; thus, it requires monitoring of prothrombin time (PT) that is expressed as the international normalized ratio (INR) (Kimmel, 2008). INR levels >3 and <2 are associated to increased risk of bleeding and thrombotic events, respectively (Wysowski et al., 2007; Oden et al., 2006). Even though an increased INR (over-anticoagulation) is a significant predictor for bleedings as a major concern for both patients and physicians (Hylek et al., 2003), bleeding episodes commonly occur within therapeutic INR (Suzuki et al., 2007; Campbell et al., 2001). There is not a doubt that fatal and major hemorrhages are of essential importance but most bleeding problems are clinically minor (Fitzmaurice et al., 2002). Even minor bleedings are important because they alert for subsequent major bleeding (Veeger et al., 2011), and may increase the number of repeat visits to clinics and sometimes emergency room (ER) that normally add an extra cost. They also can result in permanent withdrawal of warfarin therapy (Gullov et al., 1999), and depriving patients of the most effective therapy available. There are many environmental risk factors for warfarin response and warfarin-induced bleeding complications including advanced age, gender, co-morbidities, polypharmacy, inadequate social support, and reduced functional status (Kimmel, 2008; Nekkanti et al., 2012). After accounting for these patient and environmental factors, unexplained variance in warfarin response

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http://dx.doi.org/10.1016/j.taap.2016.08.026
0041-008X/

remains; there is a growing body of evidence that genetics may have a large contribution to the regulation of warfarin dose and its efficacy and toxicity. A large number of pharmacogenetic studies have used increased INR as outcome, but limited data are available regarding the predictors of warfarin-induced bleeding complications at therapeutic INR (An et al., 2014).

Two genes, the cytochrome P450 subfamily IIC polypeptide 9 (CYP2C9) and the vitamin K epoxide reductase complex subunit 1 (VKORC1), have been extensively studied regarding their effects on warfarin metabolism and bleeding complication (Aithal et al., 1999; Jorgensen et al., 2012; Yang et al., 2013). Two single nucleotide polymorphisms (SNPs) within CYP2C9, the alleles with these are referred to as *2 and *3 with the wild type being referred to as *1, are significantly linked with reduced warfarin dose requirements and increased risk of bleeding in Caucasian populations (Kimmel, 2008; Limdi et al., 2008). Several SNPs and haplotypes within the VKORC1 gene, e.g. −1639A allele, have been linked with lower warfarin dose requirements; however, a single variant (1173C > T) has been demonstrated to equally predict warfarin dose in Caucasians and African Americans (Schelleman et al., 2007). Several other genes also have been proposed to change warfarin response but are less well studied. (Suarez-Kurtz & Botton, 2013). These include genes encoding for microsomal epoxide hydroxylase (EPHX1), NADP(H):quinone oxidoreductase 1 (NQO1), and factor VII. In the present study we investigated 8 SNPs in 5 genes of interest within three pathways: pharmacokinetic, pharmacodynamic, and the coagulation pathway (Table 1). The aim of this study was to evaluate the impact of the 8 examined SNPs on the risk of bleeding complications in an Iranian sample with INR within therapeutic range during warfarin treatment.

2. Methods

2.1. Study population

A cross-sectional study was carried out for the period of six months, from September 2013 to March 2014, at Tehran Heart Center (THC). A total of 552 warfarin treated patients who maintained on a target INR level of 2.0–3.5 for at least three consecutive intervals of attending in our anticoagulation clinics, were included. Monitoring of patients with stable INR values occurred every 4 to 6 weeks and patients were enrolled if written informed consent was obtained. Exclusion criteria were previous history of a chronic condition that can influence warfarin metabolism, especially liver disease, alcohol intake, using glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), or any other medications that could have caused drug interactions. Patients were also excluded from the study if they had a known malignancy or uncontrolled hypertension. The study was approved by the local ethical committee and was conducted in agreement with the Declaration of Helsinki for research involving human subjects. All patients provided written informed consent for gathering the relevant clinical data and for DNA analyses.

2.2. Data collection and clinical information

Data on age, sex, body weight, height, valve prosthesis, warfarin therapy duration, daily warfarin dose, INR measurements, concurrent medication, co-morbidity, bleeding complications, and patient's file number were achieved by completing specific data collection forms designed for this research study. Other data collected at each visit included blood pressure, changes in warfarin dose, diet, alcohol, and medication histories. Then, we merged the gathered data with the hospital database by using patient's file number variable to obtain patient's variables such as cardiovascular risk factors and laboratory data. The occurrence of all types of bleedings were recorded and categorized as any bleeding episodes requiring medical intervention to stop or treat bleeding. All other bleeding episodes requiring no additional outpatient visits, testing, or treatment including oozing from injection site bleeding, echymoses, petechiae, or bruising, and minor gingival bleeding was categorized as minimal bleeding. We defined the duration of warfarin therapy as the number of days between warfarin initiation and the last clinic admission.

2.3. Laboratory measurements

About10 mL venous blood samples were collected; 5 mL blood was collected in tubes containing citrate anticoagulant and used for coagulation assays immediately; the rest of the sample (5 mL) placed in ethylenediamine tetraacetic acid (EDTA)-containing tubes and stored deep-frozen until later use. For determination of INR values, plasma was extracted from the samples by centrifugation at 1500 rpm for 15 min and then the PT with INR was assayed using a fully-automated coagulation analyzer – the ACL-ELITE-PRO (Instrumentation Laboratory, USA).

2.4. DNA extraction and SNP genotyping

Genomic DNA was extracted from the stored EDTA whole blood samples using 'salting out' method. DNA quantity was evaluated by calculating absorbance at $\lambda = 260$ nm, and the quality was assessed by a ratio of $\lambda = 260/280$ nm being close to 1.8. Genotyping of CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910), VKORC1 (rs9923231), VKORC1 (rs9934438) variants were carried out by restriction enzyme digestion

Table 1
Characteristics of eight single nucleotide polymorphisms genotyped in five genes of interest.

Gene/location (rs)	Type	Alternative names	Pathway	Gene product and function
VKORC1/rs9934438	Intron	1173C > T	Pharmacodynamic	VKORC1 catalyzes the rate-limiting step in vitamin K recycling that recycles the epoxide and quinone form of vitamin K to the reduced non-oxidized form.
VKORC1/rs9923231	Promoter	−1639G > A	Pharmacodynamic	
CYP2C9/rs1799853	Exon 3	430C > T Arg144Cys	Pharmacokinetic	CYP2C9 is the primary enzyme responsible for metabolizing of (*S*)-warfarin, the more potent stereo-isomers of the drug (S-warfarin vs. R-warfarin).
CYP2C9/rs1057910	Exon 7	1075A > C Ile359Leu	Pharmacokinetic	
EPHX1/rs1051740	Exon 3	28T > C Tyr113His	Pharmacodynamic	EPHX1, putative subunit of VKOR, is a critical xenobiotic-metabolizing enzyme, catalyzing both detoxification and bioactivation reactions.
EPHX1/rs2234922	Exon 4	52A > G His139Arg	Pharmacodynamic	
Factor VII/rs6046	Exon 8	10976G > A Arg353Gln	Coagulation	FVII is a zymogen that, when bound to tissue factor, initiates the coagulation.
NQO1/rs1800566	Exon 6	609C > T Pro187ser	Pharmacodynamic	NQO1 is suggested to catalyze metabolic reduction of vitamin K to KH2.

CYP2C9, cytochrome P450 subfamily IIC polypeptide 9; EPHX1, microsomal epoxide hydroxylase; KH2, vitamin K hydroquinone; NQO1, NADP(H):quinone oxidoreductase 1; VKORC1, vitamin K epoxide reductase complex subunit 1.

of PCR-amplified DNA based on a newly designed primers listed in Table 2. Genotyping of factor VII (rs6064), EPHX1 (rs1051740), EPHX1 (rs2234922), and NQO1*2 (rs1800566) variants were also performed by restriction enzyme digestion according to a previously published protocol with some modifications (Cheng et al., 2004; Cheraghi et al., 2013; Boroumand et al., 2015) (Table 2). Briefly, each PCR products of the examined polymorphisms were digested overnight using 1 U of the appropriate restriction enzyme. After incubation for 16 h at 37 °C, the PCR products and digested products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The genotypes were determined by the pattern on the digested bands.

2.5. Statistical analysis

Statistical analysis was performed by PASW Statistics for Windows, Version 18.0 (Chicago: SPSS Inc.). Variables were tested for normality by using Kolmogorov–Smirnov normality test. Warfarin dose and time on warfarin, which were skewed, presented as median and interquartile range (25th to 75th percentiles). Two groups of patients with and without bleeding were compared using the independent two-sample Student *t*-test (or Mann-Whitney *U* test if required) for the continuous variables and the chi-square test (or the Fisher exact test, as appropriate) for the categorical variables. To determine the Hardy–Weinberg equilibrium of each polymorphism studied in both groups, a chi-square analysis was used. For each polymorphism, comparisons of the allele presence (homozygous or heterozygous) or absence between the groups were also made using a chi-square test. The logistic regression model was used to determine the association between the examined genotypes and CYP2C9 haplotypes with bleeding complications of warfarin in the presence of other covariates. Before entering the model, the haplotypes were collapsed into three groups (0, 1, and 2) on the basis of the number of variant alleles (i.e., *1/*1 = 0, *1/*2 = 1, *1/*3 = 1, *2/*2 = 2, *2/*3 = 2, *3/*3 = 2), and non-normal variables such as time on warfarin were natural log-transformed. Odds ratio (OR) and 95% confidence intervals (CI) were calculated, and a p value ≤0.05 was considered statistically significant.

3. Results

Ninety-two bleeding events (with the exception of minimal hemorrhages) were observed in 87 patients, because 5 patients had two bleeding events (1 patient with both epistaxis and hematuria; 1 patient with both epistaxis and subconjunctival hemorrhage; 3 patients with both epistaxis and gingival bleeding). None of the patients had major life threatening bleeding complications. The most common site of bleeding was epistaxis 54% (50/92). In descending order of frequency, other sites of bleeding were gastrointestinal (12%), hematuria (12%), gingival (12%), subconjunctival hemorrhage (6%), and vagina (4%). Of a total of 552 patients meeting all the inclusion criteria and none of the exclusion criteria, 87 (16%) had bleeding complications at therapeutic INR. All patients were between the ages of 16 and 88 years, and had an average INR of 2.7 ± 0.4. The mean age of patients was 59 ± 13 years and 53% were female. The most frequent indication for warfarin therapy was prosthetic valve (63%) followed by atrial fibrillation (21%) and thromboembolic disease (12%), and the mean length of warfarin therapy was 1450 ± 793 (Ranging from 133 to 4432) days. The mean stable warfarin dose was 36 ± 18 mg/week. Clinical and demographic characteristics of the study patients are listed in Table 3. There was no statistically significant difference in age, sex, BMI, co-morbidities, concomitant medications, warfarin indications, dosage, and duration of therapy between patients with bleeding and those without bleeding.

All eight studied polymorphisms were in Hardy–Weinberg Equilibrium (HWE $\chi^2 < 384$ and $p > 0.05$). Frequencies of genotypes for the examined polymorphism and haplotypes of CYP2C9 in the case and control groups, separated by sex, are presented in Table 4 and Table 5. Due to the rarity of the some homozygous mutant genotypes, the clumped (mutant/heterozygous) genotypes as carriers of the minor alleles were compared to wild type genotypes. There was a statistically significant difference in the distribution of the clumped TT + CT genotypes of NQO1*2 variant, as carriers of T allele, in comparison with CC genotype among whole population and in women or men subgroups. There was also a statistically significant difference in the distribution of genotypes of CYP2C9*2 SNP among women subgroup but not among whole population or men.

Table 6 shows that after applying a binary logistic regression model with adjusting for age, sex, warfarin dose, warfarin indications, and the natural log-transformed length of warfarin therapy as well as medications used, and other polymorphisms and grouped CYP2C9 haplotypes, carriers of CYP2C9 one-variant haplotypes (*1/*2 or *1/*3) and NQO1*2 variant were found to be associated with the occurrence of bleeding events. Compared to reference group (*1/*1), the odds of bleeding increased for carriers of one variant allele (*1/*2 or *1/*3) (adjusted OR: 1.75, 95% CI: 1.03 to 2.97, $P = 0.039$). Patients with the T allele in NQO1*2 (CT or TT genotypes) had a higher risk of bleeding than patients with the CC genotype (adjusted OR: 2.25, 95% CI: 1.37 to 3.70, $P = 0.001$). Variant VKORC1, Factor VII, and EPHX1 genotypes were not significantly associated with the risk of bleeding events.

4. Discussion

The results of the present study suggest that NQO1*2 and one-variant haplotypes of CYP2C9 (*1/*2 or *1/*3) are associated with

Table 2
Primer design and RFLP fragments of the eight examined single nucleotide polymorphisms.

Gene SNP (rs)	Sequence of forward (F) and reverse (R) primers	Restriction enzyme	RFLP fragments (bp)	Reference
VKORC1 (rs9923231)	F: 5′-TGCCCACGCCATAAACTAGC-3 R: 5′-AGAGTTCCCAGA AGGGTAGGTG-3′	*Msp*I (*Hpa*II)	472, 387, 85	–
VKORC1 (rs9934438)	F: 5′-TGGGCTATCCTCTGTTC-3 R: 5′-GGTCAGTGACATGGAATC-3′	*Hin*fI	110, 60, 50	–
CYP2C9 (rs1799853)	F: 5′-AACAGAGACTTACAGAGCTC-3′ R: 5′-TCCAAGAATGTCAGTAGAGAAG-3′	*Eco*47I (*Ava*II)	333, 215, 118	–
CYP2C9 (rs1057910)	F: 5′-GTGCACGAGGTCCAGAGATGCCTTG-3′ R: 5′-TAAATCTGGAGAACACACACTGCC-3′	*Mph*1103I (*Nsi*I)	303, 275, 28	–
EPHX1 (rs1051740)	F: 5′-GATCGATAAGTTCCGTTTCACC-3′ R: 5′-ATCTTAGTCTTGAAGTGAGGAT-3′	*Eco*32I (*Eco*RV)	162, 140, 22	21
EPHX1 (rs2234922)	F: 5′-ACATCCACTTCATCCACGT-3′ R: 5′-ATGCCTCTGAGAAGCCAT-3′	*Rsa*I	210, 164, 46	21
Factor VII (rs6046)	F: 5′-ACGCAGCCTTGGCTTTCTCTC-3′ R: 5′-GGGAGACTCCCCAAATATCAC-3′	*Msp*I (*Hpa*II)	305, 220, 85	22
NQO1 (rs1800566)	F: 5′-AGTGGCATTCTGCATTTCTGTG-3′ R: 5′-GATGGACTTGCCCAAGTGATG-3′	*Hin*fI	188, 151, 85	23

CYP2C9, cytochrome P450 subfamily IIC polypeptide 9; EPHX1, microsomal epoxide hydroxylase; NQO1, NADP(H):quinone oxidoreductase 1; RFLP, restriction fragment length polymorphism; VKORC1, vitamin K epoxide reductase complex subunit 1.

Table 3
Demographic, clinical and laboratory characteristics of the study groups.

Characteristics	Patients with bleeding (n = 87)	Patients without bleeding (n = 465)	*p*-Value
Age (year)	60.5 ± 12.5	58.5 ± 12.7	0.186
Male sex	38 (43.7)	223 (48.0)	0.463
Body mass index (kg/m^2)	27.5 ± 4.2	26.9 ± 4.4	0.269
Body surface area (m^2)	1.81 ± 0.16	1.80 ± 0.19	0.498
Warfarin indication			
Atrial fibrillation	14 (16.1)	103 (22.2)	0.253
PTE, DVT, LV clot	13 (14.9)	54 (11.6)	0.546
Prosthetic valve	57 (65.5)	291 (62.6)	0.567
Other	3 (3.4)	17 (3.7)	0.871
Warfarin dose (mg/week) [median, IQR]	33 (25 to 42.5)	34 (22.5 to 43.8)	0.916
Time on warfarin (days) [median, IQR]	1423, 854 to 2033	1259, 783 to 2016	0.378
Co-morbidities			
Thyroid disease	7 (8)	48 (10.3)	0.515
Chronic kidney disease	2 (2.3)	6 (1.3)	0.619
Congestive heart failure	13 (14.9)	57 (12.3)	0.785
Diabetes	16 (18.4)	77 (16.6)	0.675
Hypertension	45 (51.7)	210 (45.2)	0.260
Current cigarette smoker	8 (9.2)	27 (5.8)	0.236
Medications			
Amiodarone	2 (2.3)	11 (2.4)	0.970
Antiplatelet agents	48 (55.2)	239 (51.4)	0.518
Antibiotics	3 (3.4)	3 (0.6)	0.530
Fasting blood sugar (mg/dl)	109.01 ± 3.48	108.82 ± 1.66	0.960
Triglyceride (mg/dl)	146.54 ± 9.18	142.13 ± 5.65	0.742
HDL-cholesterol (mg/dl)	42.35 ± 1.11	41.24 ± 0.54	0.374
LDL-cholesterol (mg/dl)	108.74 ± 4.29	108.80 ± 1.75	0.773
Total cholesterol (mg/dl)	177.65 ± 5.30	173.42 ± 2.24	0.451

Data are presented as mean ± SD or n (%) unless otherwise noted.
DVT, deep vein thrombosis; LV, left ventricular; NSAIDs, nonsteroidal anti-inflammatory drugs; PTE, pulmonary thromboembolism.

bleeding complications after the stabilization of warfarin dose in Iranian patients with therapeutic INR during warfarin treatment. Compared to reference group (*1/*1), the odds of bleeding did not show any increase for carriers of two variant alleles (*2/*2, *2/*3, or *3/*3) (adjusted OR: 0.88, 95% CI: 0.36 to 2.16, $P = 0.780$). It seems counter-intuitive but can be explained by the low number of patients with bleeding incidents with these 3 genotypes. Therefore, not enough statistical power was possible in the current study to evaluate the overall risk of these dual variants. To the best of our knowledge, the current study is the first to examine the impact of gene polymorphisms of NQO1, EPHX1, and Factor VII on bleeding complications at normal INR.

Warfarin acts by inhibiting the regeneration of a reduced form of vitamin K, Vitamin K hydroquinone (KH2), which is an essential cofactor for γ-carboxylation and activation of vitamin K-dependent clotting factors II, VII, IX, and X. During carboxylation, KH2 is oxidized to vitamin K epoxide (KO) by γ-glutamyl carboxylase (GGCX) (Tie et al., 2011). During vitamin K recycling, KO is first reduced to vitamin K (the quinone form) by vitamin K epoxide reductase (VKOR), and subsequently to KH2 by two pathways. One pathway, similar to the reduction of KO to vitamin K, also involves two free cysteine residues in the VKOR active site; therefore, is sensitive to warfarin inhibition. The second pathway uses NAD(P)H, as a cofactor, and is resistant to warfarin (Tie et al., 2011). It is postulated that NQO1 catalyzes metabolic reduction of vitamin K to vitamin K hydroquinone (Gong et al., 2008); hence, NQO1 may play a role in vitamin K recycling. However, it is noteworthy that GGCX and VKOR are the only two enzymes unequivocally identified as part of the cycle (Oldenburg et al., 2008).

NQO1, an important detoxifying cytosolic flavoenzyme, is considered an antioxidant enzyme by catalyzing a two-electron reduction of quinone compounds and detoxification of the electrophilic compounds (Zhu & Li, 2012). The human NQO1 gene, located on chromosome 16q22.16, contains six exons and five introns. Among all known SNPs in this gene, a non-synonymous SNP (rs1800566; *2) is the most widely investigated in molecular epidemiology studies. It is a C-to-T transition at nucleotide position 609 in exon 6 leading to a proline-to-serine amino acid change at codon 187 of the amino acid sequence of the protein (Traver et al., 1997) that destabilizes the enzyme due to ubiquitination and proteasomal degradation (Siegel et al., 2001). Bress et al. (2012) showed that NQO1*2 was independently associated with therapeutic warfarin dose in Hispanic–Americans but not in African–Americans. Chung et al. (2015) also reported that NQO1 gene polymorphisms influence stable warfarin doses in Korean patients. Our findings suggest that patients with the T allele in NQO1*2 (CT or TT) could be a significant independent predictor for bleeding complications at therapeutic INR. Our results are in line with previous in vitro and in vivo studies demonstrating that this variant is associated with the loss of enzyme activity and low levels of blood coagulation factors (Ross & Siegel, 2004; Shyu et al., 2010).

It is well-established that genotype variants of CYP2C9 (*1, *2 and *3) and VKORC1 (at least one of seven SNPs in linkage disequilibrium) substantially contribute to predicting warfarin dose (Klein et al., 2009). However, a recent meta-analysis have suggested that, as compared to clinical dosing, genotype-guided warfarin dosing does not result in improved outcomes regarding the occurrence of major bleeding episodes or thromboembolic events (Stergiopoulos & Brown, 2014). Although most bleeding events occur after first weeks of therapy,

Table 4
Genotype frequencies of examined polymorphisms among cases and controls in whole study group and in subgroups separated by gender.

		Patients with bleeding			Patients without bleeding			*p*-Value*		
		All (n = 87)	Male (n = 38)	Female (n = 49)	All (n = 465)	Male (n = 223)	Female (n = 242)	All	Male	Female
(VKORC1) −1639G > A	GG	24 (27.6)	9 (23.7)	15 (30.6)	168 (36.1)	84 (37.7)	84 (34.7)	0.125	0.096	0.581
	GA + AA	63 (72.4)	29 (76.3)	34 (69.4)	297 (63.9)	139 (62.3)	158 (65.3)			
(VKORC1) 1173C > T	CC	24 (27.6)	9 (23.7)	15 (30.6)	173 (37.2)	85 (38.1)	88 (36.4)	0.086	0.087	0.443
	CT + TT	63 (72.4)	29 (76.3)	34 (69.4)	292 (62.8)	138 (61.9)	154 (63.6)			
(CYP2C9) 430C > T	CC	68 (78.2)	29 (76.3)	39 (79.6)	365 (78.5)	172 (77.1)	193 (79.8)	0.968	0.935	0.990
	CT + TT	19 (21.8)	9 (23.7)	10 (20.4)	100 (21.5)	51 (22.9)	49 (20.2)			
(CYP2C9) 1075A > C	AA	60 (69.0)	30 (78.9)	30 (61.2)	352 (75.7)	169 (75.8)	183 (75.6)	0.185	0.672	0.038
	AC + CC	27 (31.0)	8 (21.1)	19 (38.8)	113 (24.3)	54 (24.2)	59 (24.4)			
(EPHX1) 28T > C	TT	52 (59.8)	24 (63.2)	28 (57.1)	264 (56.8)	130 (58.3)	134 (55.4)	0.593	0.573	0.802
	CT + CC	35 (40.2)	14 (36.8)	21 (42.9)	201 (43.2)	93 (41.7)	108 (44.6)			
(EPHX1) 52A > G	AA	48 (55.2)	18 (47.4)	30 (61.2)	298 (64.1)	136 (61.0)	162 (66.9)	0.122	0.120	0.453
	GA + GG	39 (44.8)	20 (52.6)	19 (38.8)	167 (35.9)	87 (39.0)	80 (33.1)			
(Factor VII) 10976 G > A	GG	53 (60.9)	25 (65.8)	28 (57.1)	255 (54.8)	116 (52.0)	139 (57.4)	0.326	0.110	0.879
	GA + AA	34 (39.1)	13 (34.2)	21 (42.9)	210 (45.2)	107 (48.0)	103 (42.6)			
(NQO1) 609C > T	CC	45 (51.7)	19 (50.0)	26 (53.1)	320 (68.8)	150 (67.3)	170 (70.2)	0.002	0.040	0.019
	CT + TT	42 (48.3)	19 (50.0)	23 (46.9)	145 (31.2)	73 (32.7)	72 (29.8)			

Data are presented as n (%).
* *p*-Values for cases vs. controls.

Table 5
The frequency of CYP2C9 haplotypes among cases and controls in whole study group and in subgroups separated.

	Patients with bleeding			Patients without bleeding			*p*-Value*		
	All (n = 87)	Male (n = 38)	Female (n = 49)	All (n = 465)	Male (n = 223)	Female (n = 242)	All	Male	Female
CYP2C9							0.088	0.890	0.075
*1/*1	42 (48.3)	21 (55.3)	21 (42.9)	276 (59.4)	129 (57.8)	147 (60.7)			
*1/*2	13 (14.9)	6 (15.8)	7 (14.3)	55 (11.8)	28 (12.6)	27 (11.2)			
*1/*3	25 (28.7)	8 (21.1)	17 (34.7)	78 (16.8)	38 (17.0)	40 (16.5)			
*2/*2	5 (5.7)	3 (7.9)	2 (4.1)	27 (5.8)	16 (7.2)	11 (4.6)			
*2/*3	1 (1.1)	0 (0.0)	1 (2.0)	16 (3.4)	5 (2.2)	11 (4.6)			
*3/*3	1 (1.1)	0 (0.0)	1 (2.0)	13 (2.8)	7 (3.1)	6 (2.5)			

Data are presented as n (%).
* *p*-Values for cases vs. controls.

previous pharmacogenetic studies and guidelines have focused on dose-titration phase, when an individual's proper warfarin dose is not established and is titrated empirically according to INR results (Klein et al., 2009). Hence, less is known about the contribution of these warfarin dose-related variants to bleeding risk after stabilization (An et al., 2014; Aithal et al., 1999; Limdi et al., 2008; Margaglione et al., 2000; Higashi et al., 2002; Kawai et al., 2014).

It has been reported that knowledge of genotype was associated with a marked reduction in hospitalization for bleeding or thrombosis in a cohort of outpatients initiating warfarin (Epstein et al., 2010). In our study, carriers of one-variant haplotypes (*1/*2 or *1/*3) in CYP2C9 gene showed a significant increased risk for bleeding complications of warfarin at therapeutic INR. In accordance with our findings, Sanderson et al. (2005), in the first systematic review and meta-analysis of this topic, reported that subjects who carry at least one copy of a variant allele have an increased risk ratio of bleeding compared with non-carriers of that allele; (*2), (*3), and (*2 or *3) showed RR (95%CI) of 1.91 (1.16 to 3.17), 1.77 (1.07 to 2.91), and 2.26 (1.36–3.75), respectively. In a more recent meta-analysis, Yang et al. (2013) also found both *2 and *3 carriers to be related to significantly higher risk for the total hemorrhages. In contrast, in another meta-analysis by Jorgensen et al. (2012) no significant effect of *2 or *3 on the risk of warfarin bleeding events was showed whereas they reported an increased bleeding risk for homozygous *3 patients (Jorgensen et al., 2012). Notably, most bleeding complications in the previous studies occurred before stabilization and were claimed to be related to supra-therapeutic INRs (Higashi et al., 2002; Molden et al., 2010). In a recent study on Korean patients with INR blood level at therapeutic range, no association was found between bleeding complications of warfarin and two dose-related gene variants (*3 and VKORC1 rs9934438) (An et al., 2014). The investigators proposed that patients with mutant genotype of CYP2C9 or VKORC1 had dose adjustment according to INR measurement, and concluded that following the stabilization of warfarin anticoagulation, these genotypes may not expected to be associated with bleeding complications (An et al., 2014). Unlike CYP2C9 (*1, *2 and *3), studies on association between VKORC1 genotypes and hemorrhagic complications are homogenous and comparable; in consistent with our result, no association of VKORC1 genotypes and hemorrhagic complications has ever been shown (An et al., 2014; Yang et al., 2013; Limdi et al., 2008; Kawai et al., 2014; Wadelius et al., 2009).

We found that advanced age was independently associated with increased risk of bleeding complications of warfarin therapy even at normal-range INR. This is in agreement with the literature (Choudhry et al., 2006; Fang et al., 2006). In the ISCOAT study (Palareti et al., 1996), 36% of minor hemorrhages and 36% of major bleeds occurred while the INR was between ≥2 and <3 and all the cases of fatal intracranial bleeding, in which an INR value at the time of the event was available, occurred at an INR of <4.5. Palareti et al. (1996) reported that the annual risk of major bleeding was 2.9% for patients >70 years, whereas no major bleeds occurred in patients <50 years of age.

The strength of this study is that we included a cohort of Iranian patients on warfarin with a target INR level of 2.0 to 3.5 who maintained this INR range for at least 3 consecutive intervals. This gave us the unique opportunity to assess effects of warfarin-related genes on the risk of bleeding complications at normal INR. However, this study has several limitations. First, this study is limited by its cross-sectional nature, but it remains one of the few studies to include multiple demographic, clinical, and genetic factors in an adjusted regression model. Second, due to small sample size, our study was not sufficiently powered to detect small to modest effect sizes. Finally, our results from a single-center may not necessarily be generalized to other ethnic groups.

Table 6
Logistic regression analysis showing odds ratios for bleeding complications.

Variables		Adjusted[a] OR	95% CI for OR	*p*-Value
Age		1.03	1.01 to 1.05	0.015
Female sex		1.23	0.75 to 2.04	0.415
(VKORC1) 1173C > T	CC	1.00 (Ref.)	–	–
	CT	2.71	0.45 to 16.38	0.278
	TT	3.36	0.32 to 35.13	0.312
(VKORC1) −1639G > A	GG	1.00 (Ref.)	–	–
	GA	0.67	0.11 to 4.14	0.666
	AA	0.34	0.03 to 3.57	0.369
(Factor VII) 10976G > A	GG	1.00 (Ref.)	–	–
	GA	0.82	0.48 to 1.37	0.441
	AA	0.63	0.20 to 2.01	0.438
(EPHX1) 52A > G	AA	1.00 (Ref.)	–	–
	AG	1.49	0.90 to 2.47	0.126
	GG	1.74	0.42 to 7.25	0.446
(EPHX1) 28T > C	TT	1.00 (Ref.)	–	–
	TC	0.83	0.46 to 1.49	0.527
	CC	0.97	0.45 to 2.09	0.944
NQO1 T allele carriers		2.25	1.37 to 3.70	0.001
CYP2C9 (*1/*1)		1.00 (Ref.)	–	–
CYP2C9 (*1/*2 or *1/*3)		1.75	1.03 to 2.97	0.039

OR, odds ratio; CI, confidential interval; VKORC1, vitamin K epoxide reductase complex subunit 1; EPHX1, microsomal epoxide hydrolase 1; NQO1, NADP(H):quinone oxidoreductase 1; CYP2C9, cytochrome P450 2C9; Ref., the reference category.
[a] Logistic regression model adjusted for warfarin dose and duration, warfarin indications, medications used, and the other variables shown in the table.

5. Conclusion

Our findings suggest that the SNP C609T within NQO1 and haplotypes of CYP2C9 (1*2 or 1*3) are independently associated to bleeding complications of warfarin at normal INR. The knowledge of these genotypes could change clinical care particularly after the warfarin dose-titration phase.

Conflict of interests

The authors declare no conflict of interests with respect to the research, authorship, and/or publication of this article.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This work was part of the MSc thesis of the first author at Tehran Heart Center (THC) with the support of Tehran University of Medical Sciences (TUMS) and Kerman University of Medical Sciences.

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