

ORIGINAL ARTICLE

Pharmacodynamic resistance to warfarin is associated with nucleotide substitutions in *VKORC1*

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Summary. *Background:* Vitamin K epoxide reductase subunit 1 (*VKORC1*) is the molecular target of coumarin anticoagulants and mutations in *VKORC1* have been identified previously in individuals who required high warfarin doses. *Objective:* Detailed characterization of the relationship between variation in *VKORC1* and the warfarin resistance phenotype. *Patients and methods:* Serum warfarin concentration and coagulation parameters were determined in 289 subjects who required warfarin doses > 20 mg day⁻¹. The *VKORC1* sequence was studied in selected study subjects. *Results:* Twenty-eight out of 289 (10%) subjects had serum warfarin > 2.3 mg L⁻¹ during stable therapeutic anticoagulation indicating pharmacodynamic warfarin resistance. Detailed analysis of 15 subjects from this group showed that eight out of 15 (53%) had nucleotide substitutions in *VKORC1* predictive of p.V66M, p.L128R, p.V54L or p.D36Y. *VKORC1* was normal in the remaining seven out of 15 (47%) subjects and in nine out of nine (100%) subjects with high warfarin dose requirement not caused by pharmacodynamic resistance. At referral, subjects with *VKORC1* mutations received a median warfarin dose of 32 mg day⁻¹ (range 22–55) and had a median serum warfarin concentration of 4.6 mg L⁻¹ (range 2.6–9.0). *VKORC1* substitutions were associated with a requirement for high warfarin doses but not with adverse clinical events. Family members with *VKORC1* nucleotide substitutions and not receiving warfarin had undetectable PIVKA-II and K₁ epoxide (K₁O). *Conclusions:* Nucleotide variations in *VKORC1* are a common cause of pharmacodynamic warfarin resistance but are not associated with adverse outcome during anticoagulation. Mutations

associated with warfarin resistance do not cause a discernible defect in *VKORC1* reductase function.

Keywords: Anticoagulation, Vitamin K₁, *VKORC1*, warfarin resistance.

Introduction

Warfarin is a clinically effective oral anticoagulant that is used widely for the treatment and prevention of thrombosis [1,2]. However, warfarin also has a narrow therapeutic index and bleeding caused by excessive anticoagulation remains a significant source of morbidity and death [3]. These difficulties are compounded by the wide inter-patient variation in the dose of warfarin required to achieve therapeutic anticoagulation [4].

The safety of oral anticoagulant therapy may be improved by prediction of warfarin dose requirement before starting anticoagulation and by definitive investigation of subjects who display an abnormal warfarin dose response once therapy has started [1]. This may be valuable for individuals who require high warfarin doses as warfarin resistance is associated with prolonged loading and therefore presents greater risk of anticoagulation failure. Identifying the cause of warfarin resistance once treatment has started may allow contributory factors to be corrected or may enable the design of safer individualized dosing regimes [1].

The warfarin dose response is affected by complex factors that include patient demographic and acquired determinants. However, variation within genes whose products mediate warfarin metabolism or the synthesis of the vitamin K dependent clotting factors (VKDFs) also influences the warfarin dose response [5]. Informative genetic markers include the common cytochrome P-450 2C9 isoenzyme (*CYP2C9*) *2 and *3 alleles which are associated with lower warfarin dose requirement than *CYP2C9* *1 [6,7]. A more significant effect arises from variation within the gene encoding vitamin K epoxide reductase subunit 1 (*VKORC1*) [6–8]. *VKORC1* mediates reduction of phyloquinone 2,3 epoxide (vitamin

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K₁O; K₁O) to phyloquinone (vitamin K₁; K₁) which is the essential co-substrate for γ -glutamyl carboxylation of the VKDCFs [9]. VKORC1 activity is a major determinant of the rate of carboxylation of the VKDCFs and the VKORC1 protein is the molecular target of coumarin and 1,3-indandione anticoagulants [10,11]. Within the VKORC1 gene (*VKORC1*), a common population haplotype *VKORC1* *2 is associated with decreased hepatic expression of VKORC1. Accordingly, individuals who are homozygous for *VKORC1* *2 require lower warfarin doses compared with individuals with the haplotypes *VKORC1* *1, *3 or *4 [6–8].

Genotype analysis of polymorphic alleles within *CYP2C9* and *VKORC1* is an effective way of predicting warfarin dose requirement in anticoagulation clinic populations [5]. However, the size of the effect of these common variations is small and does not account fully for the wide inter-individual variation in warfarin dose response [4,5]. Polymorphic variation in *CYP2C9* and *VKORC1* does not explain the highly abnormal warfarin dose response in rare individuals with warfarin sensitivity or resistance.

It has been recognized previously that some individuals who require very high doses of warfarin have rare nucleotide variations in *VKORC1* [10]. However, warfarin resistance associated with *VKORC1* mutations has only been reported in isolated case reports [8,12–14] or in short series without a full description of the clinical phenotype [10,15]. Warfarin resistance has diverse etiology [16] and the prevalence of genetic variation in *VKORC1* in affected individuals is unknown. In order to characterise the relationship between variation in *VKORC1* and the warfarin resistance phenotype, we conducted a systematic analysis of 289 subjects with high warfarin dose requirement. We now report that nucleotide substitutions within *VKORC1* are frequent in individuals with pharmacodynamic warfarin resistance and provide a detailed description of the associated clinical phenotype.

Methods

Selection of study subjects

Two control cohorts of subjects receiving warfarin were identified from anticoagulation clinics at our Centres. We first selected 820 consecutive patients attending the Bristol Royal Infirmary anticoagulation clinic with INR 2–4 and no changes in warfarin dose for at least 4 weeks before recruitment. This cohort was used to identify the distribution of warfarin dose requirement and to define a diagnostic threshold for *warfarin resistance*. Using the same recruitment criteria, 137 subjects were then selected from the anticoagulation clinic at Guy's Hospital for determination of serum warfarin concentration and establishment of a laboratory reference range for this parameter.

The study cohort of 289 subjects comprised all patients who were referred to the Centre for Haemostasis and Thrombosis at St Thomas' Hospital in 2004–2007 for investigation of abnormal warfarin dose response and who fulfilled our

diagnostic criteria for warfarin resistance. Our centre is the unique provider of this laboratory service in the UK and during the study period, referrals were received from 100 anticoagulation clinics. For all subjects with pharmacodynamic warfarin resistance and for first degree relatives of subjects with nucleotide substitutions in *VKORC1*, referring clinicians were requested to seek informed consent for *VKORC1* sequence analysis. Sequencing was performed on all available subjects from these groups who gave written consent.

Laboratory evaluation of warfarin resistance

Study subjects were evaluated by determining INR (Innovin thromboplastin; Dade-Behring, Marburg, Germany) and serum warfarin concentration during a period of stable warfarin prescription [13]. For detailed analysis of study subjects with *VKORC1* nucleotide substitutions, serum K₁, K₁O and under-carboxylated prothrombin (PIVKA-II) concentrations were determined as described previously [17,18]. One-stage activity assays for the VKDCFs were performed on a CA-1500 coagulometer (Sysmex, Kobe, Japan). The dose response to warfarin during initiation of anticoagulation in selected subjects was obtained from anticoagulation clinic records from the referring institution. The warfarin pharmacokinetic study was performed by administration of 0.5 mg kg⁻¹ sodium warfarin orally 36 h after the previous routine warfarin dose. Serum warfarin, K₁ and K₁O concentrations were then determined from blood samples obtained at baseline and at multiple time-points up to 96 h.

VKORC1 genetic analysis

Genomic DNA (gDNA) was extracted from whole blood using the Nucleon DNA isolation kit (Tepnel Life Sciences, Manchester, UK). For mutation detection, amplicons containing the *VKORC1* coding sequence and splice sites were generated by PCR from gDNA and sequenced with a model 3700 DNA analyser using an ABI PRISM Big Dye V2 reaction kit (Applied Biosystems, Foster City, CA, USA). Nucleotide variations were identified by comparison with the *VKORC1* cDNA reference sequence NM_024006.

VKORC1 haplotypes were determined by PCR amplification of selected fragments of *VKORC1* followed by restriction endonuclease digestion to detect the presence or absence of tag-SNPs. The *VKORC1**2 haplotype was defined by the SNP *VKORC1* g.1173C>T (c.173 + 1000; rs9934438) and was identified by digestion of a *VKORC1* intron 1 amplicon with *StyI*. The *VKORC1**3 haplotype was defined by *VKORC1* g.3730G>A (c.492 + 134; rs7294) and was identified by *BfaI* digestion of a *VKORC1* 3'-untranslated region amplicon. Finally, the *VKORC1**4 haplotype was defined by *VKORC1* g.698C>T (c.173 + 525; rs17708472) and was identified by *MwoI* digestion of a *VKORC1* intron 1 amplicon. Subjects without these markers were considered to have the ancestral *VKORC1**1 haplotype defined by the NCBI sequence AY587020 [19]. Nucleotide numbering accords with guidelines

from the human genome variation society (<http://www.hgvs.org>) in which the A nucleotide of the ATG initiation codon is designated +1. PCR primer sequences and experimental conditions are described previously [19] and are available on request.

Results

Diagnostic criteria for warfarin resistance

In order to establish criteria for the diagnosis and classification of warfarin resistance, we first studied the relationship between warfarin dose, INR and serum warfarin concentration in control anticoagulation clinic populations at our Centres. In a survey of 820 stably anticoagulated patients within an INR range of 2–4, the median daily warfarin dose was 4 mg (range 1–32 mg) and 1% of individuals required a warfarin dose of $> 20 \text{ mg day}^{-1}$. We therefore defined *warfarin resistance* as a prolonged warfarin requirement of $> 20 \text{ mg day}^{-1}$ to achieve therapeutic anticoagulation or failure to achieve therapeutic anticoagulation with this dose. In the second control cohort of 137 stably anticoagulated subjects, who were also within an INR range of 2–4, the median (\pm SD) serum warfarin concentration was $1.4 (\pm 0.63) \text{ mg L}^{-1}$ and the middle 95% reference range was 0.7–2.3 mg L^{-1} . The upper limit of this range was chosen to define provisional diagnostic criteria to distinguish subjects with high warfarin dose requirement arising from *pharmacodynamic resistance* (Table 1).

Referral practice for investigation of warfarin resistance

Within the study cohort of 289 subjects referred to our Centre with warfarin resistance, 261 out of 289 (90%) showed serum warfarin concentrations $\leq 2.3 \text{ mg L}^{-1}$ on at least one serum sample during a period of stable warfarin prescription. This group included 13 subjects in whom poor compliance was suspected by referring clinicians and who had multiple time-point determinations of serum warfarin concentration (median number of determinations 2.2, range 2–5). According to our diagnostic criteria, these subjects were classified provisionally as *poor compliance, poor absorption or pharmacokinetic resistance*.

The remaining 28 subjects in our study cohort showed serum warfarin concentrations $> 2.3 \text{ mg L}^{-1}$ on the initial referral serum specimen. For two subjects within this group, this was confirmed on a sample from a second time-point during stable anticoagulation. These subjects were classified as *pharmacodynamic resistance* (Table 1).

Genetic analysis of VKORC1

We have previously shown that a predicted p.V66M substitution in VKORC1 was associated with pharmacodynamic, but not pharmacokinetic warfarin resistance [13]. We therefore studied the VKORC1 coding sequence in our study subjects with pharmacodynamic resistance. Amongst the 15 subjects in this group who gave informed consent for genetic analysis, seven out of 15 (47%) subjects showed wild-type VKORC1 sequence but the remaining eight out of 15 (53%) subjects showed non-synonymous nucleotide substitutions in the VKORC1 coding sequence. These comprised heterozygous substitutions predictive of p.V66M (four subjects), p.L128R (two subjects), p.V54L (one subject) and a homozygous substitution predictive of p.D36Y (one subject). We also studied VKORC1 in nine randomly selected subjects with warfarin resistance but serum warfarin concentration $\leq 2.3 \text{ mg L}^{-1}$ and who were therefore classified as poor compliance, poor absorption or pharmacokinetic resistance. Within this sample, nine out of nine (100%) subjects showed a normal VKORC1 coding sequence.

Demographic details, indication for anticoagulation and VKORC1 genotype for the detailed-study cohort of 15 subjects with pharmacodynamic warfarin resistance are shown in Table 2. At the time of referral, these subjects were prescribed a median warfarin dose of 32 mg day^{-1} (range 22–55) and had a median serum warfarin concentration of 4.6 mg L^{-1} (range 2.6–9.0). At referral, five out of 15 (33%) of subjects in this group had sub-therapeutic INR measurements. The relationship between serum warfarin concentration and INR in these 15 study subjects at referral for investigation and in control subjects is shown in Fig. 1. This demonstrates that there was wide variation in serum warfarin concentration amongst control subjects with comparable INR values. However, the study subjects with VKORC1 nucleotide substitutions predictive of p.V66M, p.L128R and p.V54L showed a proportionately higher serum warfarin concentration relative to INR [mean ratio of serum warfarin concentration to INR (\pm SD ratios) 3.54 ± 1.48] compared with control subjects without warfarin resistance [mean ratio (\pm SD ratio) 0.51 ± 0.48] and to individuals with pharmacodynamic warfarin resistance without VKORC1 substitutions [mean ratio (\pm SD ratio) 1.23 ± 1.34]. Subject 8 (homozygous p.D36Y) had a laboratory phenotype that was intermediate between controls and study subjects with other VKORC1 substitutions. Although there was a clear trend of difference in serum warfarin concentration to INR ratio between these groups, in this small

Table 1 Classification of the likely mechanism of warfarin resistance using serum warfarin concentration determined during a period of stable warfarin prescription in which the subject shows an international normalized ratio (INR) < 4.0

Diagnostic classification	Serum warfarin concentration (mg L^{-1})	Number of patients (%) $n = 289$
Poor compliance, reduced absorption or pharmacokinetic resistance (<i>Increased metabolic clearance of warfarin</i>)	≤ 2.3 at any sample time point	261 (90)
Pharmacodynamic resistance (<i>Reduced anticoagulant effect of warfarin</i>)	> 2.3 at every sample time point tested	28 (10)

Table 2 Demographic details, indication for anticoagulation and laboratory features of 15 subjects with pharmacodynamic warfarin resistance

Subject	Age (years): gender	Indication for anticoagulation	Warfarin dose at referral (mg day ⁻¹)	Serum warfarin concentration at referral (mg L ⁻¹)	<i>VKORC1</i> genotype [†]	Predicted amino-acid substitution [‡]	<i>VKORC1</i> haplotype	Long-term anticoagulation control
1	34 F	Recurrent VTE*	28	2.98	WT	–	–	–
2	68 F	Recurrent VTE	35	7.29	c.196G>A	p.V66M	*1/*3	Therapeutic INR with warfarin 27–35 mg day ⁻¹
3	20 F	Recurrent VTE	38	3.62	WT	–	–	–
4	80 M	Atrial fibrillation	35	7.11	c.383T>G	p.L128R	*3/*3	Sub-therapeutic INR with warfarin 40 mg day ⁻¹ and phenindione 250 mg day ⁻¹ . Therapeutic INR with acenocoumarol 15–20 mg day ⁻¹
5	78 M	Recurrent VTE	36	2.90	c.196G>A	p.V66M	*3/*4	–
6	71 F	Atrial fibrillation	22	4.63	c.196G>A	p.V66M	*3/*3	Therapeutic INR with warfarin 24–35 mg day ⁻¹
7	49 F	Recurrent VTE	24	4.76	WT	–	–	–
8	54 M	Cardiac thrombus	32	4.17	c.106G>T	p.D36Y	*1/*1	Therapeutic INR with warfarin 25–35 mg day ⁻¹
9	81 M	Atrial fibrillation	32	7.02	c.160G>C	p.V54L	*3/*1	Therapeutic INR with warfarin 35–38 mg day ⁻¹
10	38 F	Recurrent VTE	30	3.18	WT	–	–	–
11	51 M	APS**	30	7.39	c.196G>A	p.V66M	*1/*1	Therapeutic INR with warfarin 30–42 mg day ⁻¹
12	53 F	VTE	55	5.57	WT	–	–	–
13	19 F	VTE	24	2.56	WT	–	–	–
14	56 M	Recurrent VTE	22	2.55	WT	–	–	–
15	75M	VTE	40	9.00	c.383T>G	p.L128R	*2/*3	Sub-therapeutic INR with warfarin 40 mg day ⁻¹ . Coumarins abandoned.

Daily warfarin dose and serum warfarin concentration on the day of referral to our laboratory and the subsequent daily coumarin dose to achieve therapeutic anticoagulation are indicated. *VTE, venous thromboembolic disease. **APS, antiphospholipid syndrome. [†]Nucleotide numbering according to reference sequence NM_024006 with the A of the ATG initiation codon designated as +1. [‡]Amino acid numbering according to reference sequence NP_076869 with the ATG initiation codon designated as +1.

sample population these differences did not reach statistical significance.

Detailed phenotypic characterization of p.L128R, p.V54L and p.D36Y variants

We have previously reported the phenotype of a subject with a predicted p.V66M substitution [13]. However, the phenotype of subjects with p.L128R, p.D36Y and p.V54L has been described incompletely. We therefore performed detailed analyses and, where possible, family studies for the subjects with these substitutions.

Before initiation of warfarin, study subjects four and 15 (p.L128R), nine (p.V54L) and eight (p.D36Y) had no history of abnormal bleeding or had previously received coumarin anticoagulants. During warfarin loading using standard regimes [2], most subjects from our control population achieved an INR >2.0 within 5 days of daily treatment and after a cumulative warfarin dose of 10–40 mg (Fig. 2). However, for subjects with *VKORC1* substitutions, standard warfarin loading achieved a median INR increment of only 0.25 (range 0.2–0.4) after exposure to a median cumulative warfarin dose of 65 mg (range 60–80) during 7 days of standard INR-adjusted warfarin loading (Fig. 2).

Subsequent anticoagulation in subjects four, eight, nine and 15 required rapid warfarin dose escalation yet all subjects experienced frequent episodes of sub-therapeutic anticoagulation. Stable long-term anticoagulation with warfarin was achieved in subjects nine (p.V54L) and eight (p.D36Y) at doses indicated in Table 2. Subject four (p.L128R) failed to achieve therapeutic anticoagulation with warfarin 40 mg day⁻¹ or phenindione 250 mg day⁻¹ but was successfully anticoagulated with acenocoumarol 15–20 mg day⁻¹. Coumarin anticoagulants were abandoned in subject 15 (p.L128R) after failure to achieve therapeutic anticoagulation with warfarin 40 mg day⁻¹ (Table 2). No study subjects experienced thrombosis, bleeding or other adverse events during coumarin therapy.

VKDCF activities and serum K₁, K₁O and PIVKA-II concentrations were determined in subjects four, nine and 15 (incomplete data) during warfarin treatment and in subject eight at seven days after temporary warfarin withdrawal (Table 3). No subjects showed elevated serum K₁ concentration that may cause pharmacodynamic warfarin resistance. Subjects four, nine and 15 showed reduced VKDCF activities and increased serum K₁O and PIVKA-II concentrations consistent with current warfarin therapy. The low serum PIVKA-II concentration in subject eight is likely to represent

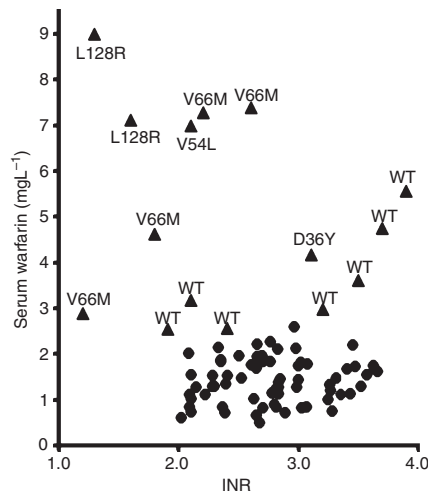


Fig. 1. Relationship between international normalized ratio (INR) and serum warfarin concentration. INR and serum warfarin concentration were determined in 66 subjects from a reference anticoagulation clinic population (●) and the 15 subjects with pharmacodynamic warfarin resistance who underwent *VKORC1* sequence analysis (▲). For all control subjects, the assays were performed during a period of stable warfarin prescription with INR < 4 (WT, wild-type *VKORC1* sequence). For the pharmacodynamic warfarin-resistant study subjects, data were determined at the time of referral to our laboratory and not all study subjects had achieved therapeutic anticoagulation despite daily warfarin doses > 20 mg.

residual undercarboxylated prothrombin generated during previous warfarin treatment but still present in plasma at the time of sampling (Table 3). A pharmacokinetic study in subject four showed a peak serum warfarin concentration of 6.8 mg L^{-1} and serum warfarin half-life of 58 h. These parameters are comparable to those from control individuals (data not shown) indicating that warfarin resistance in subject four was not as a result of reduced absorption or increased metabolism of warfarin.

For subjects four (p. L128R) and nine (p.V54L), family studies were also performed to identify individuals with *VKORC1* nucleotide substitutions who did not require long-term anticoagulation. Two family members with p.L128R (4ii-sister and 4iii-daughter) and one family member with pV54L (9 ii-son) were identified. No family members had a history of abnormal bleeding and none had been previously exposed to warfarin. Coagulation assay results and serum K_1O concentrations in these family members were normal and none had detectable serum K_1O or PIVKA-II (Table 3).

Discussion

We report a study of 289 consecutive subjects who were referred for investigation of high warfarin dose requirement in which we first defined diagnostic criteria for warfarin resistance using reference anticoagulation clinic populations. We then used these criteria to investigate systematically our study cohort. According to our diagnostic criteria, subjects with a high warfarin dose requirement were classified as warfarin resistant only if they required warfarin doses of $> 20 \text{ mg day}^{-1}$

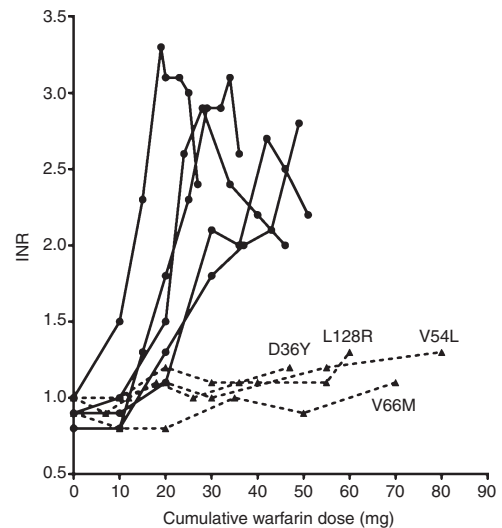


Fig. 2. Relationship between international normalized ratio (INR) and cumulative warfarin dose during initiation of anticoagulation. During warfarin loading at the start of anticoagulation therapy, study subjects received daily warfarin treatment at a dose determined by daily INR measurements in accordance with standard treatment algorithms in their referring institution. The relationship between INR and cumulative warfarin dose during the first 7 days of treatment are shown for five consecutive subjects from our anticoagulation clinic who did not have warfarin resistance (●) and from subjects with pharmacodynamic warfarin resistance associated with the predicted p.V66M (subject 2), p.L128R (subject four), p.D36Y (subject eight) and p.V54L (subject nine) *VKORC1* substitutions (▲).

to achieve therapeutic anticoagulation or if they failed to achieve therapeutic anticoagulation with this dose. If the serum warfarin concentration was $> 2.3 \text{ mg L}^{-1}$ during a period of stable warfarin dose prescription with INR < 4.0 then subjects were classified as *pharmacodynamic resistant*. This classification reflects current understanding of the metabolism of warfarin [20] and assists in the clinical management of warfarin-resistant subjects [1]. Investigation of warfarin resistance by determining serum warfarin concentration at a single time point is a validated diagnostic approach. In most circumstances, this identifies the cause of warfarin resistance without the need for detailed warfarin pharmacokinetic studies [21].

According to our diagnostic criteria, 90% of our study subjects had warfarin resistance because of poor compliance, poor absorption or increased warfarin metabolism. We have not attempted to distinguish these diagnoses systematically in the current study. However, a subset of 20 subjects within this group has been studied further with multiple time-point determinations of serum warfarin concentration to detect poor compliance or with pharmacokinetic studies to identify poor absorption or increased metabolism of warfarin (data not shown). These limited studies suggest that warfarin resistance in most subjects with serum warfarin $\leq 2.3 \text{ mg L}^{-1}$ arises from poor compliance with warfarin and that poor absorption and increased metabolism are uncommon. Other authors have also identified poor compliance as a common cause of high warfarin dose requirement in anticoagulation clinic populations [22,23].

Table 3 Coagulation factor activity and vitamin K metabolite concentrations in study subjects and family members with *VKORC1* nucleotide substitutions

Assay	Study subject								Reference range
	L128R				D36Y	V54L			
	4*	4.ii	4.iii	15*	8 [†]	9*	9.ii		
PT (s)	14.2	10.2	10.0	12.8	11.4	11	11	9.5–12.0	
INR	1.4	1.1	1.0	1.2	1.0	1.1	1.1	–	
Factor II:C (iu dL ⁻¹)	52	101	105	-	118	85	109	50–200	
Factor VII:C (iu dL ⁻¹)	52	161	158	-	124	68	99	50–200	
Factor IX:C (iu dL ⁻¹)	71	122	119	-	119	148	101	50–200	
Factor X:C (iu dL ⁻¹)	30	103	134	-	132	75	116	50–200	
K ₁ (µg L ⁻¹)	0.18	0.78	0.91	0.78	0.33	0.48	0.33	0.15–1.55	
K ₁ O (µg L ⁻¹)	0.21	<0.12	<0.12	1.75	<0.12	0.61	<0.05	<0.05	
PIVKA-II (AU mL ⁻¹)	5.83	<0.20	<0.20	26.46	0.31	8.00	<0.2	<0.2	

*Analysis performed during warfarin treatment. [†]Analysis performed 7 days after cessation of warfarin.

Our study has focused on warfarin-resistant subjects with serum warfarin concentration consistently >2.3 mg L⁻¹ when within, or below, the therapeutic INR range. The likely mechanism of warfarin resistance in these subjects is reduced anticoagulant effect of warfarin (pharmacodynamic resistance). We have demonstrated that within our referral practice, only 28 out of 289 (10%) of subjects satisfied our criteria for pharmacodynamic warfarin resistance. Although some subjects with less marked pharmacodynamic resistance may have been misclassified using our criteria, these data demonstrate that pharmacodynamic resistance is an uncommon cause of high warfarin dose requirement. This is consistent with previous literature in which pharmacodynamic resistance with high serum warfarin concentration is described infrequently [16]. Amongst previous reports, pharmacodynamic warfarin resistance has been associated with high dietary intake of vitamin K [24,25]. No subjects from our study showed elevated serum K₁ concentration, suggesting that high dietary K intake is also an uncommon explanation of warfarin resistance.

In contrast, eight out of 15 (53%) of our evaluable study subjects with pharmacodynamic warfarin resistance showed nucleotide substitutions in *VKORC1* yet nine out of nine (100%) subjects with warfarin resistance not attributable to pharmacodynamic causes had normal *VKORC1*. This demonstrates that only pharmacodynamic warfarin resistance is commonly associated with substitutions in *VKORC1*. Subjects with *VKORC1* substitutions showed high serum warfarin concentration to INR ratios compared with controls and this may be a diagnostically useful parameter identifying subjects with likely *VKORC1* substitutions. We did not identify the cause of the high warfarin dose requirement in the seven out of 15 (47%) subjects with pharmacodynamic resistance but without *VKORC1* substitutions. However, our diagnostic threshold for pharmacodynamic resistance corresponded to the 95% centile of serum warfarin concentration in a control cohort receiving warfarin. Therefore, some study subjects with moderately elevated serum warfarin concentration but without *VKORC1* substitutions are likely to represent outliers from this

control population. A classification of pharmacodynamic warfarin resistance in these subjects may therefore be inappropriate.

Our data also indicate that subjects with warfarin resistance associated with *VKORC1* substitutions may be safely and stably anticoagulated with warfarin at daily doses of 30–60 mg with appropriate INR monitoring. Also, that exposure to large doses of coumarins is not associated with any discernible adverse events. This clinical phenotype is similar to previous case descriptions of pharmacodynamic warfarin resistance unrelated to excessive vitamin K intake. These previous reports include descriptions of individuals [26,27] and families [28,29] with warfarin resistance in which affected subjects required daily warfarin doses similar to those in our study cohort for therapeutic anticoagulation. In common with our findings, anticoagulation in these subjects was seldom associated with adverse events and anticoagulation control was satisfactory once therapeutic INR values were achieved. *VKORC1* is therefore a candidate locus for warfarin resistance in these historical cases.

The *VKORC1* nucleotide substitutions observed in our study were not identified as polymorphisms and all were predicted to cause significant substitutions of conserved amino acids within the *VKORC1* protein. Within our study cohort, the p.V66M and p.L128R substitutions occurred in subjects from more than one family. *VKORC1* haplotype analysis confirmed that the p.V66M substitution occurred on different background haplotypes suggesting that the causative mutation is recurrent and unlikely to have arisen from a common founder event. We cannot exclude a common founder event for p.L128R as subjects 4 and 15 shared a *VKORC1* *3 allele. Heterozygous p.L128R, p.V66M and p.V54L substitutions have also been identified by other authors in warfarin-resistant subjects [10,15]. Similarly, the homozygous p.D36Y substitution has been associated with warfarin resistance in subjects of Jewish or Ethiopian origin [12,14]. Variant *VKORC1* proteins with p.L128R and other *VKORC1* substitutions not present in our study cohort also showed resistance to inhibition by warfarin in expression experiments [10]. These observations support a

causal association between the *VKORC1* nucleotide substitutions observed in our study and warfarin resistance.

The positions of the predicted nucleotide substitutions identified in our study are also informative about the structure and function of the *VKORC1* protein. *VKORC1* is likely to be expressed on the endoplasmic reticulum with three transmembrane domains (TM 1–3), a cytoplasmic loop (CL; residues 31–100) between TM1 and TM2 and a small luminal loop (residues 121–127) between TM2 and TM3 [30]. Bioinformatic [31] and site-directed mutagenesis [32] approaches have identified cysteine residues 132 and 135 in TM3 as the likely redox center in *VKORC1* responsible for catalyzing reduction of K_1O and a TYA motif at residues 138–140 also in TM3, as the coumarin binding site [33]. The p.L128R substitution shown by us to confer severe warfarin resistance is in close proximity to the TYA motif and may cause steric hindrance to warfarin binding. Our anecdotal observation that subject four (p.L128R) showed less marked resistance to acenocoumarol than warfarin may indicate that impairment of binding of this structurally different coumarin is less marked.

It is also of interest that the p.D36Y, p.V54L and p.V66M substitutions identified in our study lie within the *VKORC1* CL that is distant from the putative redox and warfarin binding motifs in TM3. The *VKORC1* CL is the site of other mutations in warfarin-resistant individuals (Table 4) and contains conserved cysteine residues at 44 and 51 which suggests a functional significance for this domain [31]. Although binding of the 4-hydroxycoumarin ring portion of coumarins to the TYA motif in TM3 is essential for *VKORC1* inhibition, the varying components at the 3-position also contribute to the binding and potency of coumarin anticoagulants [34,35]. It is likely therefore that the *VKORC1* protein possesses an accessory binding site for the 3-substituent of 4-hydroxycoumarins. We suggest that the *VKORC1* CL is one candidate site for this and that amino-acid substitutions in this domain reduce

binding of this structural part of the coumarins and cause warfarin resistance.

Our data also gives insight into residues that mediate the K_1O reductase activity of *VKORC1*. We have demonstrated that family members with *VKORC1* substitutions show normal VKDCF activities and have undetectable serum K_1O or PIVKA-II. Our PIVKA-II assay allows detection of under-carboxylated prothrombin to a lower threshold of 200 ng mL^{-1} (0.2% of total prothrombin). These findings therefore indicate that without warfarin, these variant *VKORC1* proteins have normal K_1O reductase activity. This is unexpected as a homozygous p.R98W substitution in the *VKORC1* CL has also been identified in subjects with familial VKDCF deficiency (Table 4) [10]. This substitution reduces *VKORC1* reductase activity and causes increased serum K_1O and PIVKA-II concentration [10]. One explanation is that substitutions at different sites within *VKORC1* are required for the familial VKDCF deficiency and warfarin resistance phenotypes. Alternatively, expression of a normal allele in our subjects with heterozygous *VKORC1* substitutions may be sufficient to preserve reductase activity. This is supported by observations that the vitamin K requirement of heterozygous warfarin-resistant rats which also have nucleotide substitutions in *VKORC1*, was increased by only 2- to 3-fold compared with 20-fold in homozygous-resistant rats [36]. It follows that a postulated defect in *VKORC1* reductase activity may only become manifest when dietary vitamin K intake is low as has been reported in a previous individual with heritable pharmacodynamic warfarin resistance [37].

This report highlights the importance of nucleotide substitutions in *VKORC1* in the pathogenesis of warfarin resistance. Further structural characterization of the effects of these informative *VKORC1* substitutions on vitamin K_1 metabolism and the interaction with coumarin anticoagulants are now required.

Table 4 Previously reported *VKORC1* mutations associated with human disease phenotypes

cDNA nucleotide substitution*	Predicted amino acid substitution [†]	<i>VKORC1</i> protein domain [‡]	Phenotype	Reference
c.76G > C	p.A26P	TM1	Warfarin resistance	15
c.85 G > T	p.V29L	TM1	Warfarin resistance	10
c.106G > T	p.D36Y	CL	Warfarin resistance	This study, 12, 14, 15
c.121G > T	p.A41S	CL	High warfarin dose requirement	8
c.134T > C	p.V45A	CL	Warfarin resistance	10
c.160G > C	p.V54L	CL	Warfarin resistance	This study, 15
c.172G > A	p.R58G	CL	Warfarin resistance	10
c.196G > A	p.V66M	CL	Warfarin resistance	This study, 13, 15
c.292C > T	p.R98W	CL	Vitamin K dependent coagulation factor deficiency	10
c.383 T > G	p.L128R	TM3	Warfarin resistance	This study, 10
c.452G > A	p.R151Q	CT	Warfarin resistance	10

*Nucleotide numbering according to reference sequence NM_024006 with the A of the ATG initiation codon designated as +1. [†]Amino acid numbering according to reference sequence NP_076869 with the ATG initiation codon designated as +1. [‡]Predicted *VKORC1* protein domains according to the model proposed by Tie *et al.* [30]. TM1, transmembrane domain 1; CL, cytoplasmic loop; TM3, transmembrane domain 3; CT, cytoplasmic tail.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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