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Rapid and Short Communication

Pharmacodynamic resistance to warfarin associated with a Val66Met substitution in vitamin K epoxide reductase complex subunit 1

Dominic J. Harrington[1], Sarah Underwood[2], Colin Morse[2], Martin J. Shearer[1], Edward G. D. Tuddenham[3], Andrew D. Mumford[2]

[1]The Centre for Haemostasis and Thrombosis, St. Thomas' Hospital, London, United Kingdom
[2]Department of Haematology, Bristol Royal Infirmary, Bristol, United Kingdom
[3]Haemostasis and Thrombosis, MRC Clinical Sciences Centre, Imperial College, London, United Kingdom

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Summary

The gene encoding vitamin K epoxide reductase complex subunit 1 (VKORC1), a component of the enzyme that is the therapeutic target site for warfarin, has recently been identified. In order to investigate the relationship between VKORC1 and warfarin dose response, we studied the VKORC1 gene (*VKORC1*) in patients with warfarin resistance. From a study group of 820 patients, we identified 4 individuals who required more than 25 mg of warfarin daily for therapeutic anticoagulation. Three of these had serum warfarin concentrations within the therapeutic range of 0.7–2.3 mg/l and showed wild-type *VKORC1* sequence. The fourth warfarin resistant individual had consistently high (≥5.7 mg/l) serum warfarin concentrations, yet had no clinically discernible cause for warfarin resistance. *VKORC1* showed a heterozygous 196G→A transition that predicted a Val66Met substitution in the VKORC1 polypeptide. This transition was also identified in 2 asymptomatic family members who had never received warfarin. These individuals had normal vitamin-K dependent coagulation factor activities and undetectable serum PIVKA-II and vitamin K_1 2,3 epoxide suggesting that their basal vitamin K epoxide reductase activity was not adversely affected by the VKORC1 Val66Met substitution. The association between a nucleotide transition in *VKORC1* and pharmacodynamic warfarin resistance supports the hypothesis that VKORC1 is the site of action of warfarin and indicates that *VKORC1* sequence is an important determinant of the warfarin dose response.

Keywords

Warfarin resistance, vitamin K, vitamin K epoxide reductase, pharmacogenetics, coagulation factor

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Introduction

The coumarin derivative warfarin is an anticoagulant that is prescribed widely for the treatment and prevention of thrombosis (1). However, the therapeutic response to warfarin is influenced by a variety of pharmacokinetic and pharmacodynamic factors so that there is wide variation between individuals in dose requirement (2). Since warfarin also has a narrow therapeutic index and may be associated with adverse events such as bleeding (3), it is desirable to develop strategies to predict the warfarin dose response in patients before initiation of anticoagulation (4).

Warfarin exerts its anticoagulant effect by inhibiting the vitamin K epoxide reductase enzyme complex (VKORC) that recycles vitamin K 2,3-epoxide to vitamin K hydroquinone (5). This cofactor is required by γ-glutamyl carboxylase for the post-translational modification of coagulation factors II, VII, IX, X and other polypeptides (6). A component of the VKORC termed VKORC1, has now been identified as a therapeutic target site of warfarin (7, 8). Point mutations were identified within the gene encoding VKORC1 (*VKORC1)* in individuals who required large doses of warfarin to maintain therapeutic anticoagulation (7). More recently, polymorphic loci within *VKORC1* have also been shown to influence warfarin dose requirements in non-warfarin resistant individuals (9). However, the relationship between the primary structure of VKORC1 and the mechanism of action of warfarin is poorly understood. Similarly, the effect of *VKORC1* point mutations on VKORC activity in individuals, who are not exposed to warfarin, is unreported. To evaluate the relationship between *VKORC1* genotype, warfarin dose requirement and vit-

Correspondence to:
Dr. Andrew Mumford
Department of Haematology
Bristol Royal Infirmary
Bristol BS2 8HW, United Kingdom
Tel.: + 44 117 928 2655, Fax: + 44 117 928 4036
E-mail: a.mumford@bristol.ac.uk

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amin K metabolism, we studied *VKORC1* in warfarin resistant patients from our anticoagulation clinic.

Materials and methods

Study subjects

Patients who required daily maintenance doses of warfarin >25 mg were identified from the anticoagulation clinic records at the Bristol Royal Infirmary (UK). Each subject was assessed clinically and after informed consent, venous blood was obtained for coagulation factor assays, measurement of serum concentrations of warfarin, phylloquinone (vitamin K_1; K_1), vitamin K_1 2,3-epoxide (K_1O), undercarboxylated prothrombin (PIVKA II) and for extraction of genomic DNA. Subjects were investigated after a minimum period of 6 weeks within the desired therapeutic range of INR without change in the maintenance warfarin dose. Venesection was performed 18 hours after ingestion of warfarin.

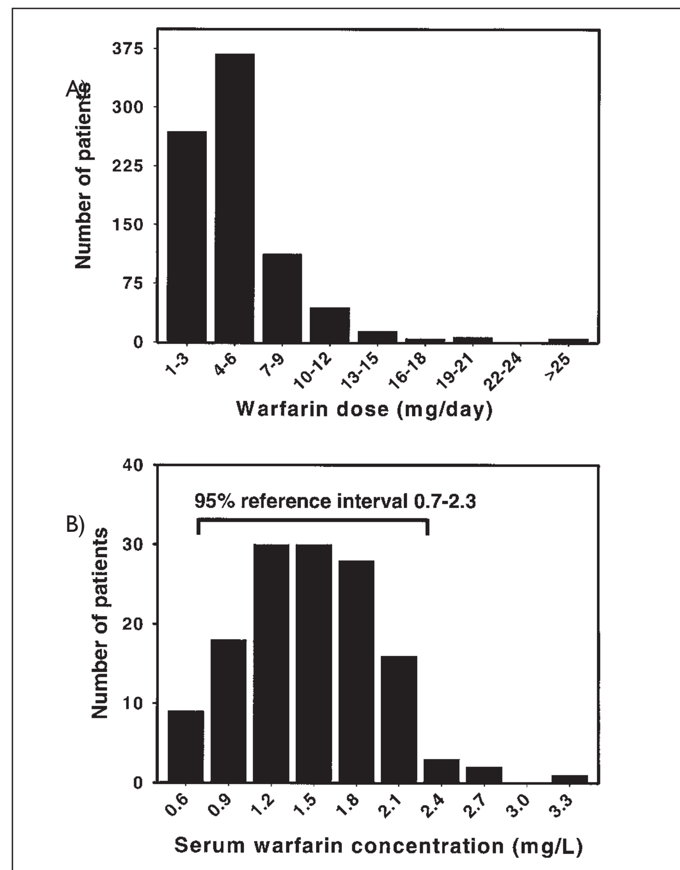

Figure 1: Warfarin dose requirements and serum warfarin concentrations for the study and reference populations. A. Warfarin dose requirements for the study population of 820 patients stably anticoagulated within a target INR range of 2.0 to 3.0. Four subjects required daily warfarin doses >25 mg. B. Serum warfarin concentrations in a reference population of 137 patients attending the anticoagulation clinic at Guy's hospital (UK), who were stably anticoagulated within target INR range of 2.0–4.0. The median (± SD) warfarin concentration was 1.4 (± 0.63) mg/l. The therapeutic range for serum warfarin concentration (0.7–2.3 mg/l) was defined as the middle 95% reference interval in this population.

Coagulation factor, warfarin, vitamin K and PIVKA II assays

INR values were calculated from prothrombin times obtained using Innovin thromboplastin (Dade-Behring, Marberg, Germany). Factor II, VII, IX and X activities were determined by one-stage coagulation assays on a CA-1500 coagulometer (Sysmex Corporation, Kobe, Japan). Protein C activity was measured using a chromogenic substrate (Berichrom®, Dade-Behring) and free Protein S antigen by ELISA (READS, Corgenix, Westminster, CO, USA).

Assays for serum warfarin, K_1 and K_1O were performed at The Centre for Haemostasis and Thrombosis, St. Thomas' Hospital, London by HPLC as previously described (10, 11). A reference population of 137 patients from the Guy's Hospital (UK) anticoagulation clinic who were well-controlled within an INR range of 2.0 to 4.0 defined the therapeutic reference range for serum warfarin concentration. PIVKA II was determined by ELISA using a conformation-specific monoclonal antibody that selectively binds under-carboxylated prothrombin (12).

VKORC1 mutation detection

The VKORC1 coding sequence was amplified by PCR from genomic DNA and was sequenced using oligonucleotide primers and experimental conditions described previously (7).

Results

Study subjects and coagulation assays

The anticoagulation clinic at the Bristol Royal Infirmary (UK) comprised 820 subjects that required a median daily warfarin dose of 4 mg (range 1–32 mg) to maintain stable anticoagulation within a target INR range of 2.0–3.0 (Fig. 1A). Four subjects required daily maintenance doses >25mg warfarin and were selected for further investigation. Poor compliance was reasonably excluded and no concurrent medication, co-morbid disorders or dietary factors could be identified to explain warfarin resistance. The reference anticoagulation clinic population showed a median (±SD) serum warfarin concentration of 1.4 (± 0.63) mg/l (Fig. 1B). The therapeutic range for serum warfarin concentration was defined as the 95% reference interval of this group (0.7–2.3 mg/l).

Three of the subjects with warfarin resistance from the study population had serum warfarin concentrations within the therapeutic range. The serum K_1 concentrations in these subjects were also within our laboratory normal range (0.15–1.55 µg/l) and the serum K_1O and PIVKA II were elevated to levels consistent with therapeutic anticoagulation (13; data not shown).

The fourth warfarin resistant subject (I-1, kindred 4) was a 64-year-old Afro-Caribbean female with a history of recurrent pulmonary emboli. Initiation of anticoagulation using conventional warfarin loading regimes was ineffective and daily maintenance doses of 27–35 mg were eventually required to achieve therapeutic anticoagulation. Once established within the therapeutic range, anticoagulation control was stable and there was no history of bleeding during treatment. There was no family history of abnormal bleeding or thrombosis, and no family members had been prescribed warfarin. Subject I-1 showed serum warfarin concentrations of 7.3, 5.7 and 5.8 mg/l measured

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Table 1: **Laboratory characteristics of the individuals from the study kindred 4.**

Plasma/Serum Measurement	Subject			Reference range
	I-1*	II-1**	III-1**	
PT(s)	26	9.5	11	9.5–12.0
INR	2.2	0.9	1.1	-
Factor II:C	14	139	96	50–200
Factor VII:C	20	143	93	50–200
Factor IX:C	31	146	88	50–200
Factor X:C	19	143	106	50–200
Protein C activity	45	115	80	70–148
Free Protein S antigen	32	65	66	55–109
Warfarin (mg/L)	6.3	-	-	0.7–2.3
K_1 (µg/L)	0.63	0.27	0.62	0.15–1.55
K_1O (µg/L)	2.52	<0.05	<0.05	<0.05
PIVKA II (AU/ml)***	>10	<0.2	<0.2	<0.2

* Results obtained during prolonged anticoagulation treatment with 32mg warfarin daily. All assay results are from a single time point with the exception of warfarin concentration that represents the mean of 3 measurements from plasma specimens obtained at 6 weeks intervals with INR values of 2.2, 2.1 and 1.7.
** Subjects not treated with warfarin
*** AU/ml = Arbitrary Units/ml with 1AU equivalent to approximately 1µg purified PIVKA-II

at 6-week intervals corresponding to INR measurements of 2.2, 2.1 and 1.7 respectively. Coagulation factor assay results and serum K_1, K_1O and PIVKA II concentrations for the proband I-1 and the available family members (daughter II-1 and grandson III-1) are shown in table 1.

VKORC1 sequence analysis

The *VKORC1* sequence in the 3 subjects with serum warfarin concentrations within the therapeutic range corresponded to the published wild-type sequence (NCBI accession number: AY587020). However, subject I-1 from kindred 4 was heterozygous for 196G→A transition within exon 2 of *VKORC1* that predicted a Val66Met substitution in the VKORC1 polypeptide. This transition was also present in both subjects II-1 and III-1.

Discussion

The wide variation in warfarin dose required by our subjects is similar to that identified in other studies (2, 4), and highlights the heterogeneity amongst patients in therapeutic response to warfarin. We have arbitrarily defined warfarin resistance as a daily requirement of >25 mg. This approximated to the highest half-percentile of the distribution of warfarin dose and was predicted to identify individuals who were candidates for rare *VKORC1* alleles.

Warfarin resistance that is unexplained by excessive vitamin K ingestion, poor drug absorption or drug interaction has been recognised previously as a heritable trait (14–19). The origin of warfarin resistance in many of these reports is incompletely understood. However, in one kindred, affected individuals showed increased warfarin clearance (pharmacokinetic warfarin resistance; 19). Pharmacokinetic resistance to warfarin may also account for the high doses required by the 3 subjects from our study population who showed serum warfarin concentrations within the therapeutic reference range.

In contrast, the resistant subject I-1 from kindred 4 showed serum warfarin concentrations that were consistently greater than the therapeutic reference range. It was unlikely that these measurements lay within the normal distribution of therapeutic serum warfarin concentrations since the mean concentration in subject I-1 was 6.3 mg/l. This was >7.5 standard deviations higher than the median level of the therapeutic reference range (Fig. 1B) and was identified as a statistical outlier by Grubbs test (P = <0.01). Elevated serum warfarin concentrations of this magnitude are usually associated with anticoagulation to supra-therapeutic INR values (13, 20) yet subject I-1 was stably anticoagulated throughout the study period in the INR range 1.7–2.2.

The association of high dose requirement and increased serum levels of warfarin indicated pharmacodynamic warfarin resistance in subject I-1. Since the serum K_1 concentration was normal, it was unlikely that this had arisen through high dietary vitamin K intake as has been previously identified as a cause of warfarin resistance (21). Instead, these findings are consistent with a defect in the site of action of warfarin. A similar mechanism has been proposed in previous reports of warfarin resistance associated with the same pattern of biochemical abnormality (14–17). These reports include a severe phenotype kindred in which affected individuals required daily warfarin doses of up to 145mg and showed corresponding plasma warfarin concentrations of 50 mg/l (15). Other kindreds showed a moderate phenotype with warfarin doses and serum warfarin concentrations similar to subject I-1 in the current study (17).

Our demonstration of a *VKORC1* 196G→A transition in subject I-1 complements the previous report that identified other *VKORC1* mutations in patients with warfarin resistance (7). This transition is unlikely to be polymorphic within *VKORC1* since it

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was not identified in population studies of *VKORC1* sequence (7, 9). The 196G→A transition is predicted to cause a Val66Met substitution in the VKORC1 polypeptide. This is a non-conservative substitution that lies in a conserved region of VKORC1 and is within a putative cytoplasmic loop of the polypeptide that contains three of the four previously identified amino acid substitutions associated with warfarin resistance (Val29Leu, Val45Ala and Arg58Gly; 7, 22). The Val66Met substitution has also now been identified independently in an unrelated kindred with warfarin resistance (J. Oldenburg, personal communication). Together, these observations suggest that the Val66Met substitution is responsible for the warfarin resistance phenotype in our subject. The association between an amino-acid substitution in VKORC1 and pharmacodynamic, but not pharmacokinetic, warfarin resistance supports the hypothesis that VKORC1 is the molecular site of action of warfarin (7, 8). However, since the other members of kindred 4 with the 196G→A transition have not been challenged with warfarin, we are unable to confirm hereditary transmission of warfarin resistance through this allele.

We found no evidence of a defect in the function in VKORC in subjects II-1 and III-1 from kindred 4 despite heterozygosity for *VKORC1* 196G→A. This was demonstrated by normal serum K_1 and K_1O concentrations, normal activities of the vitamin K dependent clotting factors and absent serum PIVKA-II. The PIVKA-II assay allows detection of undercarboxylated prothrombin to a lower threshold of approximately 200 ng/ml (0.2% of total prothrombin). Our inability to detect serum PIVKA-II in subjects II-1 and III-1 therefore allows us to exclude even subclinical vitamin K deficiency that may be insufficient to impair coagulation factor activity (12). The apparent preservation of function of the mutant VKORC1 is at first sight surprising since point mutations in *VKORC1* have also been identified in individuals with familial vitamin K dependent clotting factor deficiency type II (FMVKCDII) in which VKORC activity is impaired (7). However, FMVKCDII is associated with homozygosity for deleterious point mutations in *VKORC1* causing amino-acid substitutions in a different putative VKORC1 domain (22). Significant impairment of VKORC activity may be prevented by expression of the normal *VKORC1* allele in heterozygous 196G→A members of our kindred. Alternatively, the VKORC1 domain that interacts with warfarin may not be obligatory for the reductase activity of VKORC. Finally, the postulated defect in vitamin K recycling may only manifest when the dietary vitamin K intake is very low as has been demonstrated in a previous individual with familial pharmacodynamic warfarin resistance (16).

This report suggests that point mutations in *VKORC1* may be an important, though rare, cause of warfarin resistance in anticoagulation clinic populations. Further investigation is required to fully characterise the structure–function relationship for VKORC1 and to determine the relationship between *VKORC1* genotype and other pharmacogenetic determinants of the warfarin dose response.

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