Original Article

Warfarin dose requirement and cytochrome P450 2C9 and Vitamin K epoxide reductase complex subunit 1-1639 genetic polymorphisms in Thai patients

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ABSTRACT

Aims: The purposes of this study were to investigate the influence of genetic polymorphisms of cytochrome P450 2C9 (CYP2C9)*3 and Vitamin K epoxide reductase complex subunit 1-1639 (VKORC1-1639) G > A and patient's characteristics on warfarin dose requirement and to establish an equation for predicting the warfarin maintenance dose in Thai patients.
Settings and Design: This is an observational, retrospective study in outpatients. Ninety-one outpatients receiving warfarin at Phaholpolpayuhasena Hospital, Kanchanaburi, were recruited to this study.
Subjects and Methods: Whole blood, dose, and demographic data were collected. Blood samples were analyzed for the genetic polymorphism by restriction fragment length polymorphism technique.
Statistical Analysis Used: Differences in baseline characteristics among genotypes were evaluated by analysis of variance or Kruskal–Wallis and the Mann–Whitney U-test or Chi-square test for parametric and nonparametric variables, respectively. Association between genetic factors and warfarin dose was based on Eta test, whereas associations between warfarin dose and polymorphisms were evaluated using Pearson correlation test. Stepwise regression was used to identify factors contributing to warfarin dose requirement followed by linear regression model to develop a warfarin dosing algorithm.
Results: CYP2C9*1*1 (wild type) genotype was found in 90 patients (98.90%), and CYP2C9*1*3 was found in only 1 patient (1.10%). No CYP2C9*3*3 genotype was observed. Polymorphisms of VKORC1-1639 GG was found in 9 patients (9.89%) while GA and AA genotype were found in 30 patients (32.97%) and in 52 patients (57.14%), respectively. Patients with VKORC1-1639 AA genotype required statistically and significantly lower, average weekly warfarin dose (19.97 ± 7.61 mg) than GG genotype (37.89 ± 12.20 mg) and GA genotype (29.48 ± 11.50 mg) with the $P < 0.05$.

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Key words: Cytochrome P450 2C9 isozyme, genetic polymorphism, Vitamin K epoxide reductase complex subunit 1, warfarin

Conclusions: Using stepwise multiple linear regression, VKORC1-1639 AA, age, and weight could explain about 45.3% of the variation of warfarin maintenance dose. Multivariate analysis of the equation indicated a significant negative correlation between warfarin dose and VKORC1-1639 AA and age, but a significant positive correlation between warfarin dose and weight. This suggested that VKORC1 genotyping may be more important in warfarin dose adjustment and should be a priority for genotype measurement.

INTRODUCTION

The oral anticoagulant warfarin has been the mainstay therapeutic drug for the treatment and prevention of thromboembolism in various cerebro- and cardio-vascular diseases. Warfarin inhibits Vitamin K epoxide reductase complex subunit 1 (VKORC1), a key enzyme in the Vitamin K recycling.[1] Inhibition of this enzyme results in inhibition of the synthesis of Vitamin K-dependent clotting factors, including factor II, VII, IX, and X.[2] Warfarin is usually available as a racemic mixture of S- and R-enantiomers. The S-enantiomer exhibits 3 to 5 times more anticoagulant activity than the R-enantiomer[3] and is principally metabolized by the cytochrome P450 2C9 isozyme (CYP2C9), whereas the CYP1A2, CYP2C8, CYP2C19, and CYP3A4[4] isoforms mainly transform the R-warfarin to relatively inactive metabolite which is later excreted from the body through the kidneys.

A major problem in the clinical use of warfarin is bleeding complications. Bleedings can vary and occur at any site from the gums while brushing or from visceral organs to fatal intracranial hemorrhages. As with other drugs with narrow therapeutic window, minute variations of warfarin plasma concentration may result in serious toxicity, and hence poor outcomes. Previous studies have identified several factors associated with clinical responses of warfarin. These include age, ethnicity, food, concurrent medications, and genetics.[5]

Among more important genetic characteristics known to affect warfarin dose requirement in the population are CYP2C9 and VKORC1 gene polymorphisms.[6] As mentioned above, CYP2C9 is involved in the metabolism of warfarin, whereas VKORC1 is the molecular target of the action of warfarin. Previous research has shown differences in polymorphic frequencies of these two genes, and this may be used to predict the optimal doses in different ethnic groups.[7] It has been demonstrated that CYP2C9*2 (430 C>T) and CYP2C9*3 (1075 A>C) polymorphisms reduce the ability of the enzyme to transform warfarin into inactive metabolites by 30% and 80%, respectively. CYP2C9*2 variant is found exclusively in European and African but Asian descents while CYP2C9*3 can be frequently found in all three populations.[8] People with CYP2C9*2 and CYP2C9*3 genotypes typically require lowered warfarin doses.[7] There are also many variants of VKORC1 genes. The VKORC1*1 (wild-type) is found exclusively in native African whereas VKORC1*2 (-1639 G>A) is present in 95% of Asians. VKORC1*3 (3730 G>A) and VKORC1*4 (698 C>T) are frequently found in European and African and are rarely found in Asians.[7,9] VKORC1*2 polymorphism is associated with the lower daily dose of warfarin compared with wild type. Interestingly, VKORC1*3 and VKORC1*4 are associated with higher daily dose than normal genotype.[9,10]

Previous studies have shown that CYP2C9 and VKORC1 polymorphisms could explain almost 50% of responsiveness of patients to warfarin therapy.[9] In 2007, the U.S. Food and Drug Administration had made a change in warfarin drug label recommending that CYP2C9 and VKORC1 genotypes may be useful in determining the optimal starting dose of warfarin for individual patients.[11]

This study was aimed to establish a relationship between warfarin weekly dose requirement and CYP2C9*3 and VKORC1-1639 G>A genetic polymorphisms commonly found in Southeast Asian in Thai patients.

SUBJECTS AND METHODS

This is an observational, retrospective study in outpatients who has been receiving warfarin with stable INR at Phaholpolpayuhasena Hospital, Kanchanaburi, Thailand. The investigation was approved by the IRB for medical research involving human subjects of the Hospital, and all the participants gave informed consent.

Study participants and sample size

A total of 91 patients who had a stable warfarin dose requirement for at least three consecutive clinic visits with a target INR of 2.0–3.0 were recruited to the study. All patients provided written consent as required

by the institutional review board. Inclusion criteria were nonsmoking patients receiving warfarin for diverse thromboembolic disorders, including mitral or aortic valve replacement, rheumatic heart disease, atrial fibrillation, deep vein thrombosis, pulmonary embolism, embolic stroke, and cardiomyopathy. Exclusion criteria were made by attending physicians for patients who have had other medical conditions such as liver or renal diseases, thyroid disorders, and other malignant diseases, as well as those who were concurrently receiving medications capable of inducing or inhibiting hepatic microsomal enzymes.

Cytochrome P450 2C9 and Vitamin K epoxide reductase complex subunit 1 genotyping

On arrival at the clinic visit, a blood sample (5 ml) was taken for CYP2C9 and VKORC1 genotyping. Patient demographics of sex, age, weight, and height, as well as indications and maintenance dose for warfarin therapy, additional medical problems, and concurrent medications, were also recorded during the clinic visit. Genomic DNA sample was extracted and purified from whole, fresh blood using Promega Wizard® genomic DNA purification kit (Madison, WI) following the manufacturer's protocol. VKORC1 and CYP2C9 genotypes were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) technique. The PCR reaction was carried out in a final volume of 100 µl, containing 0.4 µM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.0), and 2.5 unit of GoTaq™ DNA Polymerase (Promega, Madison, WI). RFLP primers for the CYP2C9*3 (NG_008385) were 5′-TGCACGAGGTCCAGAGGTAC-3′, forward primer, and 5′-GGGACTTCGAAAACATGGAG-3′, reverse primer. After purification with Wizard® PCR Preps DNA purification system (Promega, Madison, WI, USA), the 141 base pair (bp) PCR fragment was restriction digested with *Kpn*I enzyme. Homozygous CYP2C9*3 alleles resulted in DNA fragment of 121 and 20 bp whereas heterozygous CYP2C9*3 yielded DNA fragment of 141, 121, and 20 bp. Forward and reverse primers for VKORC1-1639 A>G polymorphism (NG_011564.1) were 5′-GCCAGCAGGAGAGGGAAATA-3′, and 5′-AGTTTGGACTACAGGTGC CT-3′, respectively. The 290 bp fragment from VKORC1-1639 G allele created a *Msp*I restriction site and, on *Msp*I digestion resulted in 123 bp and 167 bp fragments.

Statistical analysis

For a description of age, weight, and warfarin dose, calculations of the mean and standard deviation were presented. Differences in baseline characteristics among genotypes were evaluated by analysis of variance or Kruskal–Wallis and the Mann–Whitney U-test or Chi-square test for parametric and nonparametric variables, respectively. Association between genetic factors and warfarin dose was based on Eta test, whereas associations between warfarin dose and polymorphisms were evaluated using Pearson's correlation test. Stepwise regression was used to identify factors contributing to warfarin dose requirement followed by linear regression model to develop a warfarin dosing algorithm. Multivariate linear regression adjusted for age, weight, sex was performed to investigate the influence of VKORC1 genotypes and CYP2C9 haplotypes on average daily dose of warfarin prescribed. Overlay scatter plots were also performed, comparing average daily dose of warfarin in this study with one of the other previous studies. All the analyses were performed according to the Statistical Package for Social Science (SPSS 11.5; SPSS Science, Chicago, IL). A significance level of 0.05 was used for all tests.

RESULTS

Population characteristics

The study population consisted of Thai patients with stable control on warfarin therapy. Age, weight, sex distribution, indications for anticoagulation, and VKORC1 and CYP2C9 genotype frequencies of the study participants are summarized in Table 1. A total

Table 1: Patient demographics

Patient characteristics	*n* (%)
Age (mean±SD)	60.98±14.41
Mean weekly dose (range)	24.88 (7.50-56.00)
Sex	
Female	56 (61.54)
Male	35 (38.46)
Body weight (mean±SD)	58.51±11.92
Indications for warfarin use	
Rheumatic heart disease	34 (37.36)
Heart valve replacement	11 (12.09)
Atrial fibrillation	25 (27.47)
Deep vein thrombosis	8 (8.79)
Others	13 (14.29)
CYP2C9 genotype	
*1/*1	90 (98.90)
*1/*3	1 (1.10)
VKORC1-1639 genotype	
GG	9 (9.89)
GA	30 (32.97)
AA	52 (57.14)

SD=Standard deviation, VKORC1=Vitamin K epoxide reductase complex subunit 1, CYP2C9=Cytochrome P450 2C9

of 56 females and 35 males with average age and weight of 61-year-old and 58.51 kg, respectively, were included. The mean warfarin dose required to maintain a therapeutic INR of 2–3 was 24.88 mg/week. SNP frequency for participants with CYP2C9*1*1 (wild type) and CYP2C9*1*3 were 98.9% and 1.10% (1 subject), respectively. No homologous CYP2C9*3*3 individual was found. The VKORC1-1639 frequencies of subjects with GG, GA, and AA were 9.895%, 32.97%, and 57.14%, respectively. Rheumatic heart disease and atrial fibrillation accounted for more than 50% of therapeutic indications for warfarin.

Effects of demographic variables and Vitamin K epoxide reductase complex subunit 1 and cytochrome P450 2C9 polymorphisms on warfarin, weekly dosage

The mean weekly warfarin dosage was higher in patients with CYP2C9 homozygote wild-type (CYP2C9*1*1) genotype (24.96 ± 11.27 mg) than in patients with CYP2C9*1*3 genotypes (17.50 mg). The mean weekly dose of the patients with VKORC1-1639 AA genotype (19.97 ± 7.61 mg) was significantly lower than those of patients with VKORC1-1639 GA genotype (29.48 ± 11.50 mg) and GG genotype (37.89 ± 12.02 mg) ($P = 0.000$; $P < 0.05$). There was no statistically significant difference between mean weekly doses in patients with VKORC1-1639 GG and GA genotypes ($P = 0.077$; $P > 0.05$) [Table 2]. Age, weight, and body surface area (BSA) among patients with three VKORC1-1639 G>A genotypes were not significantly different.

The influences of both VKORC1 and CYP2C9 genetic polymorphisms were also investigated. VKORC1-1639 AA and CYP2C9*1*1 genotypes were present in most subjects, and these patients required weekly warfarin at almost half the dose of VKORC1-1639 GG/CYP2C9*1*1 patients who required the highest weekly drug [Table 3]. When the weekly dose of patients with VKORC1-1639 AA and CYP2C9*1*1 was taken as a reference, one G-allele of VKORC1-1639 contributed to an approximately 50% increase in warfarin, weekly dose (i.e. ~150% for GA, ~200% for GG, Table 3) in subjects with CYP2C9 wild-type. However, a patient with VKORC1-1639 GA genotype and one CYP2C9*3 allele in the study requires a further lower weekly dose of warfarin.

VKORC1-1639 G>A was also associated with weekly warfarin doses (Eta = 0.548). As expected, patient's age was inversely and significantly associated with weekly doses of warfarin ($P = 0.000$; $P < 0.01$, $R = -0.423$). Other parameters found to be proportionally associated with warfarin doses were patient's body weight ($P = 0.005$; $P < 0.01$, $R = 0.292$) and surface area ($P = 0.005$; $P < 0.01$, $R = 0.289$).

Table 2: VKORC1 genotype of the patients in the study

	VKORC1-1639 genotype (mean±SD)			
	GG	GA	AA	*P*
Age (years)[a]	54.67±13.37	63.13±14.13	60.83±14.67	0.304
Body weight (kg)[a]	58.33±12.69	57.85±10.98	58.92±12.51	0.926
BSA (m^2)[a]	1.60±0.21	1.61±0.18	1.61±0.19	0.989
Weekly dose (mg)[b]	37.89±12.02	29.48±11.50	19.97±7.61	0.000*
Sex[c] *n* (%)				
Female	6	16	34	0.528
Male	3	14	18	

a: One-Way ANOVA, b: Kruskal-Wallis, c: Chi-Square, *$P < 0.05$
VKORC1=Vitamin K epoxide reductase complex subunit 1, SD=Standard deviation, BSA=Body surface area

Table 3: Mean weekly warfarin dose of patients with VKORC1 and CYP2C9 genetic polymorphisms

VKORC1 -1639	CYP2C9	*n* (91)	Weekly dose (mg)	Percentage of CV	Percentage relative to reference
GG	*1/*1	9	37.89±12.02	32.20	200
	*1/*3	0	-	-	-
GA	*1/*1	29	29.90±11.48	38.39	150
	*1/*3	2	17.50	-	90
AA	*1/*1	52	19.97±7.61	38.11	100 (reference)
	*1/*3	0	-	-	-

VKORC1=Vitamin K epoxide reductase complex subunit 1, CYP2C9=Cytochrome P450 2C9, CV=Coefficient of variation

Regression analysis and warfarin dosing algorithm

Univariate analysis showed that significant cofactors influencing weekly warfarin dose requirements were VKORC1 diplotype status ($P < 0.005$), age ($P = 0.000$), weight ($P = 0.007$), BSA ($P = 0.009$), and body mass index (BMI) ($P = 0.027$) [Table 4]. Age and, specifically, VKORC1-1639 AA genotype had a negative influence whereas weight, BSA, BMI, and VKORC1-1639 GA and GG genotypes had a positive effect on warfarin dose requirements.

Due to skewed distribution, natural logarithm of collected data was used to model warfarin maintenance dose algorithm in multiple stepwise regression analysis. Moreover, due to the lower frequency of CYP2C9*3 allele, the accuracy of prediction for this group could not be determined. The multiple stepwise regression model including the variables age, VKORC1 AA genotype, and weight produced the best model for estimating warfarin maintenance dose with the largest R^2 value of 45.3% [Table 5]. Using data from all the samples, the model generated

was dose (mg) = exp (3.648 − 0.457 × VKORC1 AA − 0.012 × age + 0.008 × weight) with the following keys: VKORC1 AA genotype: Input 1 for AA, 0 for AG or GG; input ages in years; and input weights in kilograms. Multivariate analysis of the algorithm revealed that the strongest influence to warfarin weekly requirement was VKORC1 AA genotype, followed by age and weight, respectively [Table 6].

DISCUSSION

The frequency of CYP2C9 and VKORC1 genotypes found in this study are consistent with other studies conducted in Thailand [Table 7] with the majority of patients having CYP2C9*1*1 and VKORC1-1639 AA genotypes. However, the VKORC1-1639 GG genotype frequency is somewhat higher in the present study. The basis for the difference is unknown but may be due to geographic variation from the results of other previous three studies in Bangkok and Chiang Mai.[12-14] However, VKORC1 genotypes among all studies is not statistically different using Chi-square method (P = 0.804; P > 0.05).

Weekly dose of warfarin in patients with VKORC1-1639 AA and CYP2C9*1*1 genotype in this study (0.345 ± 0.123 mg/kg/week) was statistically significantly lower than that of the most recent study (0.395 ± 0.124 mg/kg/week).[12] In addition, while the difference of coefficient of variation of weekly warfarin in patients with VKORC1-1639 GG and GA genotypes of the two studies was statistically significant, the data from groups of patients with VKORC1-1639 AA genotype were not [Table 8].

The equation from regression analysis in this present study is "weekly warfarin dose (mg) = exp (3.648 − 0.457 VKORC1 AA − 0.012 × age + 0.008 × weight)" with R^2 = 45.3%. We then input data of our patients and calculated the predicted weekly dose of warfarin using both our equation and formula from Sangviroon *et al*. which was "weekly warfarin dose (mg) = exp (1.846+ [0.412 × VKORC1AB] + [0.559 × VKORC1 BB] + [1.512 × CYP2C9*1*1] + [1.136 × CYP2C9*1*3] – [0.007 × age])," and compared with observed doses in the clinic. Figures 1 and 2 showed the distribution of observed doses versus predicted doses from the equation of this study and those of Sangviroon *et al*., respectively. The overlay scatter plots were then made as shown in Figures 3 and 4.

Paired t-test analyses of mean absolute different doses (P = 0.229) and mean square errors (P = 0.076) in an overlay scatter plot showed that distributions of

Table 4: Univariate analysis of cofactors influencing weekly warfarin dose

Parameters	R^2	Standardized beta	P
Sex	0.000	−0.022	0.833
Age	0.185	−0.430	0.000*
Body weight	0.079	0.281	0.007*
Height	0.018	0.135	0.201
BSA	0.075	0.274	0.009*
BMI	0.054	0.232	0.027*
Indication			
Heart valve replacement	0.001	−0.036	0.738
Rheumatic heart disease	0.038	0.196	0.063
Atrial fibrillation	0.026	−0.160	0.129
Deep vein thrombosis	0.023	0.150	0.155
Others	0.003	−0.050	0.636
VKORC1-1639 genotype			
GG	0.121	0.347	0.001*
GA	0.087	0.295	0.005*
AA	0.239	−0.489	0.000*

*P < 0.05, VKORC1=Vitamin K epoxide reductase complex subunit 1, BMI=Body mass index, BSA=Body surface area

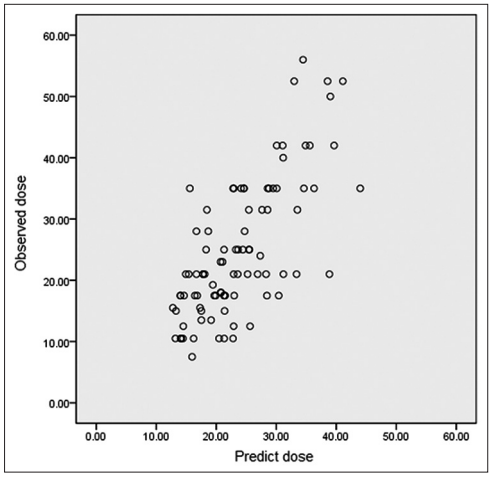

Figure 1: Scatter plots showing the administered doses and estimated doses based on the regression model of this study

Table 5: Multiple stepwise regression analysis of CYP2C9, VKORC1 genotypes, and other cofactors

Parameters	Regression model	P	R^2
VKORC1 genotype	Dose=Exp (3.648-0.445 VKORC1 AA)	0.000*	23.1
VKORC1 genotype, age	Dose=Exp (4.206-0.450 VKORC1 AA-0.014 age)	0.000*	41.6
VKORC1 genotype, age, weight	Dose=Exp (3.648-0.457 VKORC1 AA-0.012 age+0.008 weight)	0.000*	45.3

*P < 0.05, VKORC1=Vitamin K epoxide reductase complex subunit 1, CYP2C9=Cytochrome P450 2C9

all observed and predicted doses using both equations from this study and from Sangviroon *et al*. were not statistically different, $P = 0.05$ [Figures 3 and 4]. In the present study, we used three parameters which were VKORC1-1639 AA genotype, age, and weight, whereas Sangviroon *et al*. used five. This suggested that VKORC1 genotyping might be more important. In fact, it has been shown that 30% of the warfarin dose variance is explained by its target VKORC1 SNPs and a mere 12% by two nonsynonymous SNPs (*2, *3) of CYP2C9.[15] Therefore, the predicted doses of warfarin based on the equation from this study were coherent with those predicted Sangviroon *et al*. Although the predicted doses estimated either by the present study or by Sangviroon study were not different, the predicting factors used in the present study were fewer than those used in Sangviroon study. VKORC1-1639 AA, age, and weight were predicting factors employed by the present study, whereas VKORC1 AB haplotype, VKORC1 BB haplotype, CYP2C9*1/*1, CYP2C9*1/*3, and age were predicting factors employed by Sangviroon study. As a result, it should be seen and also noted that importance be given to VKORC1-1639 AA in the warfarin-treated Thai patient since the occurrence of this polymorphic genotype accounts for 50% in the Thai population. On the contrary, CYP2C9*1/*1 has been found to occur in approximately 90% for the Thai population. Consequently, warfarin dose reduction would require VKORC1-1639 AA as a major predictor and thus is needed as a priority for genotype measurement.

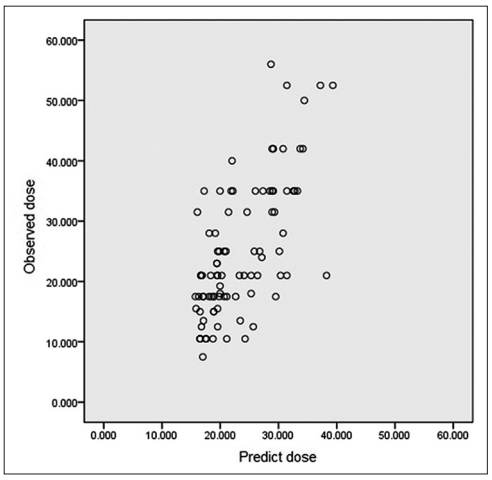

Figure 2: Scatter plot showing the administered doses and estimated doses based on the regression model of Sangviroon study

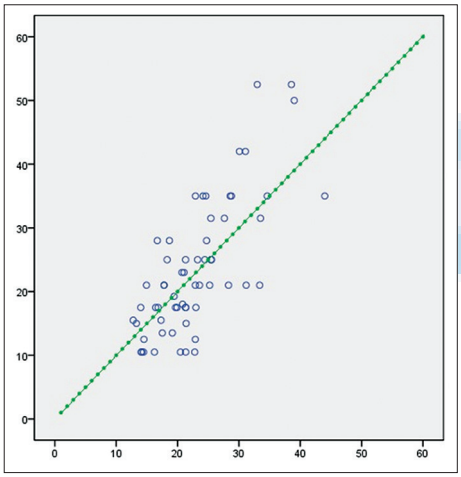

Figure 3: Overlay scatter plots showing the administered doses and estimated doses based on the regression model of this study

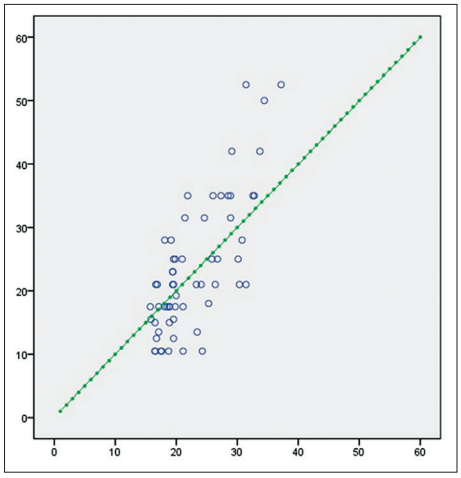

Figure 4: Overlay scatter plots showing the administered doses and estimated doses based on the regression model of Sangviroon study

Limitation of the study

This present study may fall in short of controlling confounding factors such as nutrients or food that

Table 6: Multivariate analysis of the algorithm factors

Parameter	Coefficient	SE	Standardized beta	*P*
Constant	3.648	0.265		
VKORC1-1639 AA	−0.457	0.071	−0.502	0.000*
Age	−0.012	0.003	−0.387	0.000*
Weight	0.008	0.003	0.211	0.000*

*$P < 0.05$, SE=Standard error, VKORC1=Vitamin K epoxide reductase complex subunit 1

Table 7: CYP2C9 and VKORC1 polymorphism frequency in Thai population

Genes	Klamchuen *et al.* (%)	Sangviroon *et al.* (%)	Kuanprasert (%)	This study (%)
VKORC1-1639				
GG	5.7	6.7	2.1	9.9
GA	31.1	31.5	34.3	33.0
AA	63.2	61.8	63.6	57.0
CYP2C9				
*1/*1	92.5	95.5	95.0	98.9
*1/*3	7.5	3.4	5.0	1.1
*3/*3	-	1.1	-	-

VKORC1=Vitamin K epoxide reductase complex subunit 1, CYP2C9=Cytochrome P450 2C9

Table 8: Warfarin dose comparison between this study and those from Sangviroon *et al.*

VKORC1-1639	CYP2C9	Sangviroon *et al.*			This study			*P*
		n (89)	Weekly dose (mg/kg/week)	Percentage of CV	*n* (91)	Weekly dose (mg/kg/week)	Percentage of CV	
GG	*1/*1	6	0.842±0.403	47.86	9	0.664±0.210	31.63	0.358
	*1/*3	0			0			
	*3/*3	0			0			
GA	*1/*1	27	0.552±0.151	27.35	29	0.517±0.191	36.94	0.449
	*1/*3	1	0.509		1	0.389		
	*3/*3	0			0			
AA	*1/*1	52	0.395±0.124	31.39	52	0.345±0.123	35.65	0.042**
	*1/*3	2	0.173±0.041	23.70	0			
	*3/*3	1	0.062		0			

**P* < 0.05, VKORC1=Vitamin K epoxide reductase complex subunit 1, CV=Coefficient of variation, CYP2C9=Cytochrome P450 2C9

have Vitamin K or exhibit as Vitamin K agonists, compliance and the involved adverse drug reactions. Inclusion criteria did not have a criterion for weight as well. As these confounders are usually found in observational, retrospective study as in this present study and Sangviroon study, the proof of concept (i.e., the model equations of this present study and that of Sangviroon) by prospective clinical trials are needed to clarify their use in real clinical settings.

CONCLUSION

The present study demonstrated that the model equation to predict warfarin doses was relatively comparable to that of Sangviroon. However, fewer predicting and appropriate factors were found to give comparable predicted warfarin doses. This may be economically reasonable in terms of time and cost in clinical settings.

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Conflicts of interest

There are no conflicts of interest.

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