CHAPTER IV

RESULTS

4.1 Study population

One hundred and twenty seven outpatients taking warfarin who came to follow up at Cardiovascular thoracic unit, The King Chulalongkorn Memorial Hospital during April 2006 to January 2007 and met the inclusion criteria were recruited into this study.

4.1.1 Demographic data

Of the 127 subjects recruited, the 107 patients who had stable warfarin maintenance dose, ranging in age from 21 to 78 years old were enrolled in this study. Table 4.1 shows demographic data of these 107 patients, i.e., gender, age, weight, height, BMI, indication for warfarin, underlying diseases, co-medications, smoking status, alcohol consumption, warfarin dose per week, INR, side effects and duration of taking warfarin.

Most of the indication for taking warfarin (94.4%) was prosthetic heart valve replacement. Other indications were Bental's operation, Pulmonary Embolism, PA thrombelectomy, commisurotomy. Moreover, most of them (64.3%) had no other diseases, no change of co-medications. Warfarin maintenance doses varied over 15-folds ranging from 4.5 to 70 mg/week.

Table 4.1 Demographic data of patients who enrolled the study

Characteristic		Frequency, (mean ± SD)	%, (range)
Total number of patients		107	
Gender	Female	61	57
	Male	46	43
Age (years)		(48.21 ± 13.06)	(21-78)
Age range	21 – 30 years	9	8.4
	31 – 40 years	24	22.4
	41 – 50 years	28	26.2
	51 – 60 years	26	24.3
	61 – 70 years	17	15.9
	71 – 80 years	3	2.8
Weight (kg)		(57.89 ± 11.19)	(30-100)
Height (cm)		(161.1 ± 7.60)	(145-180)
BMI (kg/m^2)		(22.32 ± 4.21)	(13.67-38.10)
Indications			
Mitral Valve Replacement		52	48.6
Aortic Valve Replacement		25	23.4
Double Valve Replacement		24	22.4
Others		6	5.6
Underlying diseases*			% of total diseases
No other diseases		72	64.3
CVA**		7	6.2
Pain (back, knee, leg)		6	5.3
Dyslipidemia		5	4.4
Hypertension		4	3.6
Diabetes Mellitus		3	2.7
Peptic ulcer		4	3.6
Headache		3	2.7
Gout		2	1.8
Asthma		2	1.8
Tuberculosis**		1	0.9
G-6-PD deficiency		1	0.9
CHD		1	0.9
Depression		1	0.9

* 5 patients had more than one underlying diseases

** Patients had history of that disease

Table 4.1 (Cont.) Demographic data of patients who enrolled the study

Characteristic	Frequency, (mean ± SD)	%, (range)
Co-medications		% of total drugs
Digoxin	54	29.8
Lasix	39	21.5
Potassium chloride	19	10.5
Enalapril	15	8.3
HCTZ	14	7.7
Amiloride	10	5.5
Aspirin	9	5.0
Aldactone	8	4.4
Statins*	5	2.7
Allopurinol	3	1.6
Omeprazole	3	1.6
Celecoxib	1	0.6
Indomethacin	1	0.6
Smoking status		
Never	68	63.6
Ever smoke	34	31.8
Smoking	5	4.7
Alcohol consumption		
Never	51	47.7
Ever drink	45	42.0
Drinking	11	10.3
INR	(2.1 ± 0.6)	(1.2-4.2)
INR range < 1.8	36	33.6
1.8– 3.0	65	60.8
> 3.0	6	5.6
Side effects (last month)		
No side effect	60	56.1
Bruises	19	17.8
Bleeding	25	23.3
Numb or fatigue	3	2.8
Warfarin dose (mg/day)	(3.7 ± 1.5)	(0.64 - 10)
Warfarin dose (mg/kg/wk)	(0.4614 ± 0.2043)	(0.0616 - 1.5909)
Duration of taking warfarin (years)	(7.6 ± 6.5)	(0.5 - 32)

* Simvastatin were used in 4 patients and rosuvastatin was used in 1 patient

4.2 Population allelic frequencies

Genetic polymorphisms of CYP2C9*2, CYP2C9*3 and VKORC1 C1173T and VKORC1 G-1639A were detected by Realtime PCR, LightCycler® which took approximately 1 hour/batch (about 25 samples). As shown in table 4.2, the result obtained from 127 patients taking warfarin enrolled in this study, showed that there was CYP2C9 *1 about 98.0% (95%CI 95.5%-99.2%) while no CYP2C9*2 among Thai population (0%). Allele frequency of CYP2C9*3 in Thai population was 2% (95%CI 1.0%-5.0%). Most of the genotype in Thais (96.8%) were wild type (CYP2C9*1/*1). There was 1 patient (0.8%) with CYP2C9*3/*3 and 3 patients (2.4%) with CYP2C9*1/*3.

The allelic frequencies of VKORC1 1173T and -1639A, which were haplotype group A, in Thais were 75% (95%CI 69.1% – 79.8%). There were 72 patients (56.7%) with **AA** haplotype (1173 TT and -1639 AA). In addition, the number of patients who were **AB** (1173 CT and -1639 GA) and **BB** haplotypes (1173 CC and -1639 GG) were 46 (36.2%) and 9 (7.1%), respectively. Therefore, VKORC1 1173C>T was completely correlated with VKORC1 -1639G>A, pearson's correlation = 1.0, $p<0.0001$.

Table 4.2 Prevalence of CYP2C9 and VKORC1 genotypes

		(127 patients x 2 alleles)				Observed		Predicted
	Alleles	N=254	%	[95%CI]	**Genotypes**	N = 127	%	**(HWE)**
CYP2C9	***1***	249	98.0	[95.5 – 99.2]	***1/*1***	123	96.8	122
					1/*3	3	2.4	5
	3	5	2.0	[1.0 – 5.0]	***3/*3***	1	0.8	0
						Chi-square = 1.504 , p = 0.471		
VKORC1	**C**	64	25.2	[20.2 – 30.9]	**CC**	9	7.1	8
1173C>T					**CT**	46	36.2	48
	T	190	74.8	[69.1 – 79.8]	**TT**	72	56.7	71
VKORC1	**G**	64	25.2	[20.2 – 30.9]	**GG**	9	7.1	8
-1639G>A					**GA**	46	36.2	48
	A	190	74.8	[69.1 – 79.8]	**AA**	72	56.7	71
		r = 1.0				**Chi-square = 0.108, p = 0.947**		

Allele frequencies of CYP2C9 and VKORC1 genotypes were in Hardy-Weinberg Equilibrium (HWE), p = 0.471 and 0.947, respectively. For example, calculation if CYP2C9*3 were in HWE.

The number of the *1 allele = p = (123x2) + (3*1) = 249 alleles
the number of the *3 allele = q = (1x2) + (3*1) = 5 alleles
the frequency of the *1 allele = p = 249/254 = 0.98
the frequency of the *3 allele = q = 5/254 = 0.02

The proportion of expected *1/*1, *1/*3 and *3/*3 genotypes could be predicted from Hardy-Weinberg Equilibrium; $p + q = 1$ and $(p + q)^2 = 1$ so, $p^2+2pq+q^2=1$

p^2 (the frequency of *1/*1 in the population) = (0.98) (0.98) = 0.9604
2pq (the frequency of *1/*3 in the population) = 2 x (0.98) (0.02) = 0.0392
q^2 (the frequency of *3/*3 in the population) = (0.02) (0.02) = 0.0004

Expected number of *1/*1 = 0.9604 x 127 = 122
Expected number of *1/*3 = 0.0392 x 127 = 5
Expected number of *3/*3 = 0.0004 x 127 = 0

Chi-square = 1.504, p = 0.471

Therefore, we could not reject the null hypothesis that the population is in Hardy-Weinberg equilibrium.

4.3 Effect of genetic polymorphisms on warfarin doses

One hundred and seven patients had stable dose of warfarin for at least 2 months, among these, five patients were excluded because lack of genetic data. There was statistically significant difference between the mean warfarin doses between CYP2C9 wild type group (*1/*1) and variant group (*1/*3 and *3/*3), the dosage were 0.473 ± 0.202 mg/kg/wk and 0.229 ± 0.195 mg/kg/wk, respectively (p = 0.019, 95%CI 0.040-0.448). Warfarin dose in CYP2C9 variant group was about 50% lower than in wild type group.

Table 4.3 Weekly warfarin dose for each group of CYP2C9 and VKORC1 genotypes

	Genotypes	N** (102)	Warfarin dose (mg/kg/wk)	INR
CYP2C9	*1/*1	98	0.473 ± 0.202	2.1 ± 0.6
	*1/*3	3	0.285 ± 0.196	2.4 ± 0.3
	*3/*3	1	0.062	3.5
CYP2C9¶	**Wild type group** (*1/*1)	98	0.473 ± 0.202	2.1 ± 0.6
	Variant group (*1/*3 and *3/*3)	4	0.229 ± 0.195	2.7 ± 0.6
		Sig.	**p = 0.019***	**p = 0.029***
VKORC1¶¶	**BB** (1173CC,-1639GG)	8	0.815 ± 0.345	2.0 ± 0.5
	AB (1173CT,-1639GA)	33	0.534 ± 0.150	2.0 ± 0.5
	AA (1173TT, -1639AA)	61	0.380 ± 0.139 ‡§	2.2 ± 0.6
		Sig.	**p < 0.0001***	**p = 0.199**

* Significant at p value < 0.05

** No genetic data in 5 patients

¶ Analyzed by t-test

¶¶ Analyzed by ANOVA followed by post hoc test with Dunnett T3 method

‡ p<0.0001 between AB and AA groups

§ p=0.025 between BB and AA groups

There were significant differences between warfarin maintenance doses in VKORC1 haplotypes ($p<0.0001$). Post hoc test with Dunnett T3 method, mean warfarin dose in VKORC1 AA haplotype was significant lower than those in AB haplotype, $p<0.0001$ [95%CI 0.077-0.232] and mean warfarin dose in VKORC1 AA haplotype was significant lower than those in BB haplotype, $p=0.025$ [95%CI 0.063-0.808] while there were no difference in INR level. As shown in Table 4.3, mean warfarin dose in the high dose group (BB) was about 40 mg/wk (5.7 mg/day) while in the low dose group (AA) was about 21 mg/wk (3 mg/day). Among CYP2C9*1/*1 genotype, there was trend of warfarin dosage decreasing in advanced age but there was no statistically significant difference of weekly warfarin doses among different age groups ($p=0.367$). Also among VKORC1 BB, AB and AA haplotypes, warfarin dose had trend of lowering in advanced age but the difference in weekly warfarin doses were not statistically significant among different age groups ($p=0.105$, 0.445 and 0.051, respectively). (Table 4.4)

Table 4.4 Weekly warfarin dose for different age group among CYP2C9 and VKORC1 genotypes. (N=102)

CYP2C9 / Age (yr)	*1/*1 ¶¶	(N) (98)	*1/*3 ¶	(N) (3)	*3/*3	(N) (1)
21-40	28.2 ± 12.1	(31)	-	(0)	-	(0)
41-60	26.2 ± 10.0	(49)	19.2 ± 12.4	(2)	4.5	(1)
> 60	23.9 ± 7.0	(18)	13.5	(1)	-	(0)
Sig.	**p=0.595**		**p=1.0**		**-**	

VKORC1 / Age (yr)	BB ¶¶	(N) (8)	AB ¶¶	(N) (33)	AA ¶¶	(N) (61)
21-40	52.5 ± 17.5	(3)	29.5 ± 8.3	(7)	24.3 ± 8.0	(21)
41-60	39.0 ± 3.6	(3)	32.9 ± 9.4	(18)	20.0 ± 7.1	(31)
> 60	24.2 ± 4.6	(2)	28.7 ± 6.3	(8)	18.4 ± 4.8	(9)
Sig.	**p=0.106**		**p=0.547**		**p=0.090**	

¶ Analyzed by Mann-Whitney rank sum test

¶¶ Analyzed by Kruskal-Wallis test

Table 4.5 Weekly warfarin dose for different VKORC1 combined with CYP2C9 genotypes.

VKORC1	CYP2C9	N (102)	Weekly warfarin dose		INR
			mg/wk	mg/kg/wk	
BB	*1/*1	8	40.38 ± 15.24	0.815 ± 0.345	2.0 ± 0.5
	*1/*3	0	-	-	-
	*3/*3	0	-	-	-
AB	*1/*1	32	31.27 ± 8.62	0.535 ± 0.153	1.9 ± 0.5
	*1/*3	1	28	0.509	2.3
	*3/*3	0	-	-	-
AA	*1/*1	58	21.86 ± 7.02	0.392 ± 0.129	2.1 ± 0.6
	*1/*3	2	12 ± 2.1	0.173 ± 0.041	2.5 ± 0.4
	*3/*3	1	4.5	0.062	3.5

Table 4.5 shows that approximately 60% of Thais had AA haplotype which required lower dose of warfarin. Patient with VKORC1 BB and CYP2C9*1/*1 genotypes required highest warfarin maintenance dose while patient with VKORC1 AA and CYP2C9*3/*3 genotypes required lowest warfarin maintenance dose.

Most of Thai populations are VKORC1 AA/CYP2C9*1/*1 genotypes which required about 50% lower dose from the highest group.

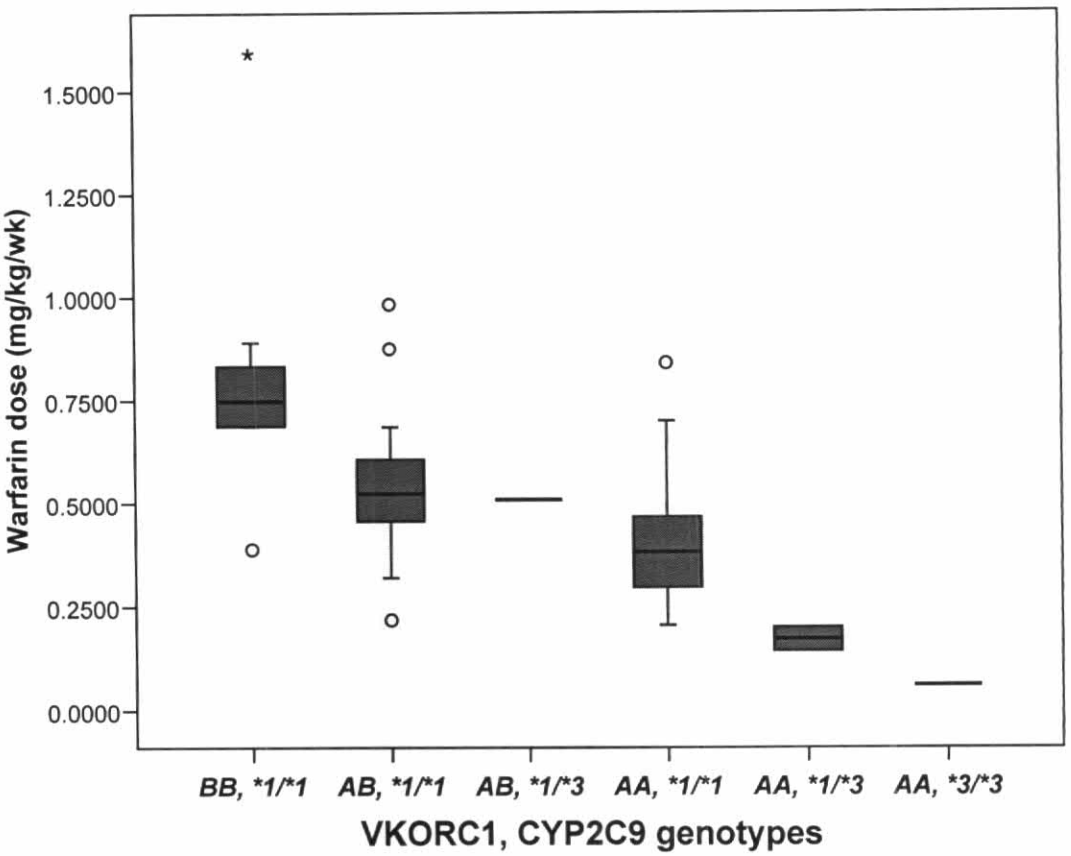

Figure 4.1 Box and whisker plot of mean weekly warfarin doses in different genotypes shows the median as a horizontal line, quartiles as a box, the maximum length of each whisker is 1.5 times of the interquartile range, outliers as a circle and extreme outliers as a star. (N=102)

Figure 4.1 shows mean weekly warfarin dose (mg/kg/wk) in different CYP2C9 and VKORC1 genotypes. Most of Thais required approximately 21 mg/wk or about 0.4 mg/kg/wk of warfarin dose. There was one patient which was an extreme outlier, she was a 24 years old woman, weight 44 kg and required 10 mg daily (70 mg/wk) of warfarin to reach the target INR of 2.0 – 3.0. Even this extreme outlier patient was excluded, the weekly warfarin doses were still significantly different among different genotypes ($p<0.0001$). Among VKORC1 AB group, warfarin dose of CYP2C9 *1/*3 was comparable to CYP2C9*1/*1.

Warfarin dose of 21 mg/wk which was the most common dosage needed was set as a reference line in figure 4.2. Weekly warfarin dose in each group was compared with the reference group and was shown as percentage of change in

warfarin dose. Dose of warfarin for VKORC1 AA/CYP2C9*1/*3 and VKORC1 AA/CYP2C9*3/*3 were reduced about 40% and 70%, respectively but for VKORC1 BB/CYP2C9*1/*1, VKORC1 AB/CYP2C9*1/*1 and VKORC1 AB/CYP2C9*1/*3 the doses of warfarin were increased about 90%, 50% and 30%, respectively.

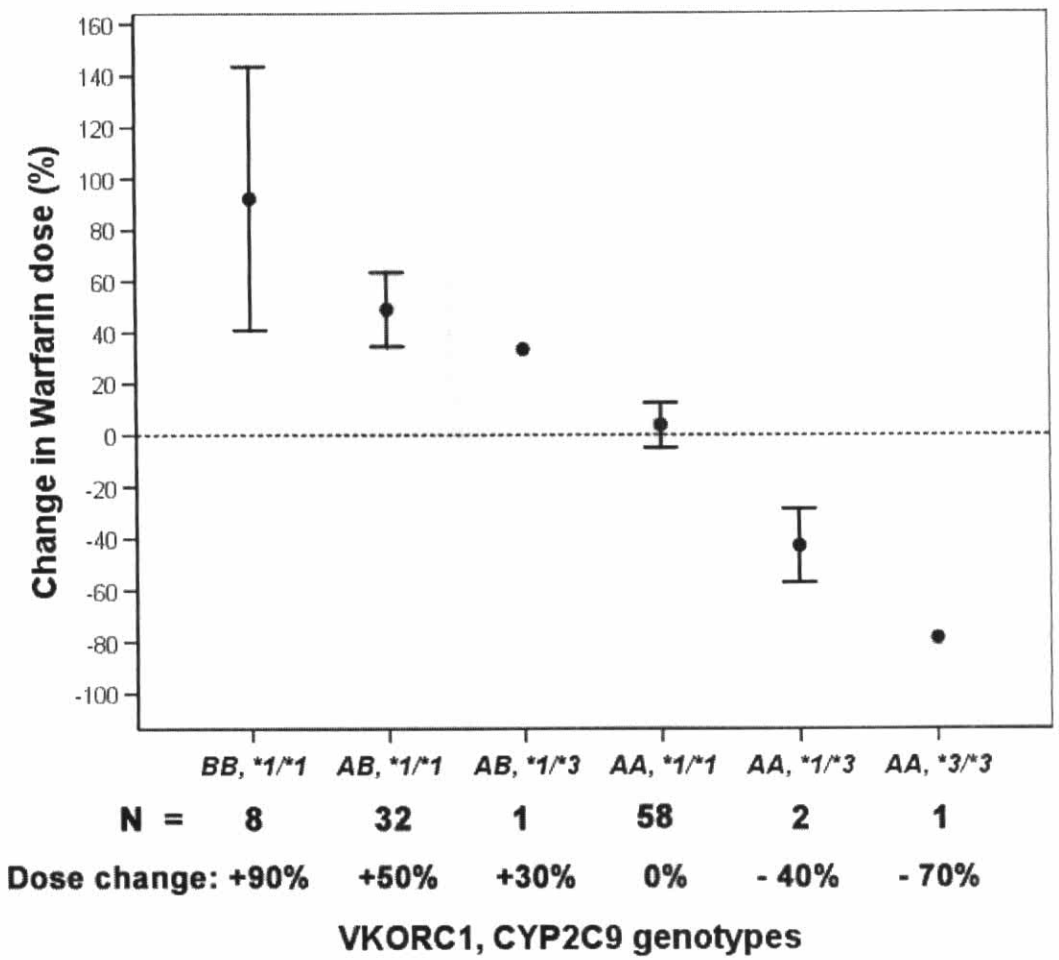

Figure 4.2 Error bar show standard error of mean weekly warfarin dose. Reference line is warfarin 21 mg/week (dot line) (N=102)

4.4 Effect of genetic factors on pharmacokinetic parameter

Warfarin was a long half life drug and taken once daily, from 102 patients with completed genetic data, 90 patients (88.2%) took the drug in the morning and 12 patients (11.8%) took the drug at bedtime. Blood was drawn in the morning before the next dose. Two patients were excluded (one took warfarin before the blood drawing time while another skip one dose before the blood drawing time). Total warfarin concentration in plasma was determined from this one point blood sample and was approximated to be the average steady state concentration (Cssav). From the 100 patients included, mean of the last warfarin daily dose was 3.80 ± 1.58 mg (range 1.5 to 10 mg).

The relationship between peak height ratio of warfarin and naproxen and warfarin concentration was found to be linear in the ranges of 150 to 3000 ng/ml (0.15 to 3.0 mg/L). Plasma concentrations of warfarin were calculated from calibration curves derived from peak height ratio of warfarin and naproxen. Mean total warfarin concentration in plasma was 975.21±480.15 ng/ml (range 310.89 to 2,889.74 ng/ml). The recovery of medium concentration (0.8 mg/L) was 66%. The intraday precision and accuracy were 3.64-6.35% and -2.91-3.55%, respectively. The interday precision and accuracy were 2.79-6.18% and -1.57-2.18%.

There were concomitant drugs which were reported as not be interfered with the determination of warfarin concentration (number of patients who took those drugs were shown in the parentheses) such as aldactone (8), allopurinol (3), alprazolam (2), amiloride (10), amlodipine (3), aspirin (9), atenolol (3), celecoxib (1), colchicine (1), digoxin (54), diltiazem (1), doxazosin (1), enalapril (15), furosemide (39), glipizide (1), hydrochlorothiazide (14), metformin (3), omeprazole (3), paracetamol (3), potassium chloride (19), rosuvastatin (1), simvastatin (4) and valsartan (2).

This study determined the clearance as the pharmacokinetic parameter. Clearance (Cl) was 0.052 ± 0.020 ml/min/kg (range 0.009 - 0.114 ml/min/kg). As shown in Table 4.6, warfarin clearance was significantly lower in variant group of CYP2C9 polymorphisms, (p=0.014) while the warfarin concentration was not significantly different (p=0.364). However, Table 4.7 shows that clearance were not significantly different in each group of VKORC1 haplotypes (p=0.459) while warfarin concentrations were significantly different (p<0.0001) because warfarin doses were statistically significant different among VKORC1 haplotypes (p<0.0001). Only CYP2C9 polymorphisms had effect on warfarin metabolism and reduced its clearance. Therefore, patients with different CYP2C9 genotypes required different warfarin doses.

Table 4.6 Comparison of warfarin concentration and clearance between CYP2C9 genotypes

N= 100**	CYP2C9*1 (96)	CYP2C9*3 (4)	Sig. ¶	95%CI
Last dose (mg/day)	3.88 ± 1.56	2.12 ± 1.25	**p = 0.030***	0.17 – 3.32
Warfarin Concentration (ng/ml)	984.16 ± 477.12	760.49 ± 158.97	**p = 0.364**	-262.99 – 710.33
Clearance (ml/min/kg)	0.053 ± 0.019	0.028 ± 0.020	**p = 0.014***	0.005-0.044

** Excluded 2 patients (one skips one dose of warfarin and another took warfarin before the blood drawing time)

Total N = 102	CYP2C9*1 (98)	CYP2C9*3 (4)	Sig. ¶	95%CI
Last dose (mg/day)	3.85 ± 1.56	2.12 ± 1.25	**p = 0.032***	0.15 – 3.29
Warfarin Concentration (ng/ml)	974.38 ± 488.50	760.49 ± 158.97	**p = 0.386**	-273.81 – 701.60
Clearance (ml/min/kg)	0.053 ± 0.020	0.028 ± 0.020	**p = 0.014***	0.005-0.045

* Significant at p value < 0.05

¶ Analyzed by t-test

Table 4.7 Comparison of warfarin concentration and clearance between VKORC1 genotypes

N = 100**	*BB* (8)	*AB* (33)	*AA* (59)	Sig. ¶¶
Last dose (mg/day)	5.81 ± 2.10	4.29 ± 1.45	3.26 ± 1.27	**p < 0.0001***
Warfarin Concentration (ng/ml)	1,466.68 ± 581.30	1,092.25 ± 462.01	843.11 ± 419.65	**p < 0.0001***
Clearance (ml/min/kg)	0.057 ± 0.013	0.054 ± 0.021	0.050 ± 0.020	**p = 0.459**

** Excluded 2 patients (one skips one dose of warfarin and another took warfarin before the blood drawing time)

Total N = 102	*BB* (8)	*AB* (33)	*AA* (61)	Sig. ¶¶
Last dose (mg/day)	5.81 ± 2.10	4.29 ± 1.45	3.24 ± 1.25	**p < 0.0001***
Warfarin Concentration (ng/ml)	1,466.68 ± 581.30	1,092.25 ± 462.01	832.03 ± 419.86	**p < 0.0001***
Clearance (ml/min/kg)	0.057 ± 0.013	0.054 ± 0.021	0.050 ± 0.020	**p = 0.520**

* Significant at p value < 0.05

¶¶ Analyzed by ANOVA followed by post hoc test with Tukey method

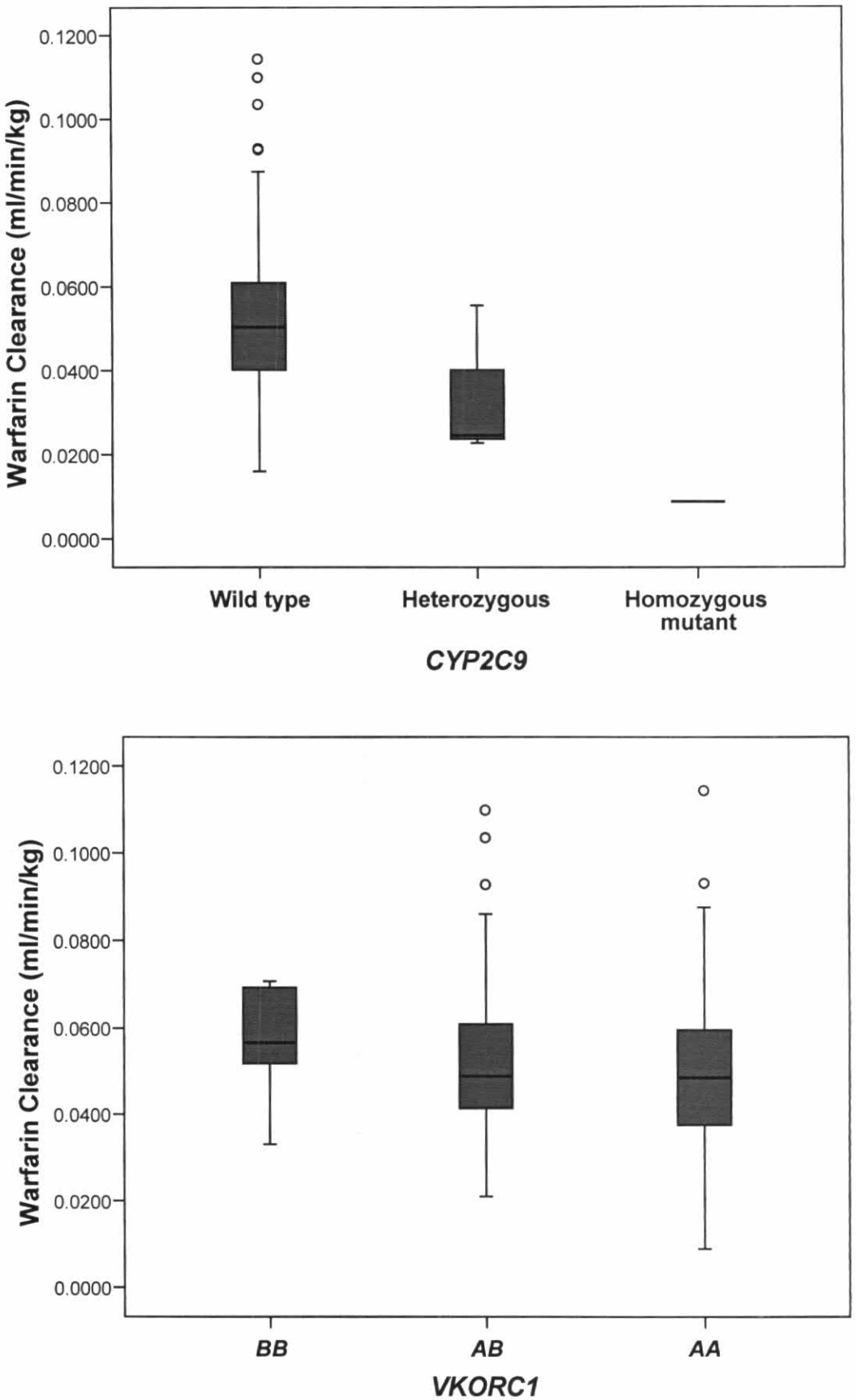

Figure 4.3 Warfarin clearance in different CYP2C9 and VKORC1 genotypes (N=100)

Time of taking warfarin was separated into 2 groups; morning group (interval 20 to 27 hours after last dose) and bedtime group (interval 6.5 to 15 hours after last dose) while all of blood samples were drawn in the morning. From 100 patients, the effect of time of taking warfarin on clearance was investigated among CYP2C9*1/*1 genotype, N=96 (all four patients with CYP2C9*3 were taking warfarin in the morning). As shown in table 4.8, warfarin concentration among patients with CYP2C9*1/*1 were not significantly different between morning and bedtime group (p=0.262). However, warfarin clearance in morning group were significantly greater than those in bedtime group, p=0.050. All patients with VKORC1 BB haplotype (8 patients) were taking warfarin in the morning. However, there was no effect of time taking warfarin on warfarin concentration and clearance among different VKORC1 haplotypes.

Table 4.8 Comparison of clearance among different group of time taking warfarin

CYP2C9		**Last dose (mg/day)**	**Warfarin Concentration (ng/ml)**	**Clearance (ml/min/kg)**
****1/*1*** **(N=96)**	**Morning (84)**	3.80 ± 1.55	962.97 ± 503.28	0.054 ± 0.020
	Bedtime (12)	4.42 ± 1.61	1,132.51 ± 334.51	0.043 ± 0.012
	Sig. ¶	**p = 0.201**	**p = 0.262**	**p = 0.050***
	[95% CI]	[-1.57 – 0.34]	[-467.68 – 128.60]	[0.000 – 0.023]
VKORC1		**Last dose (mg/day)**	**Warfarin Concentration (ng/ml)**	**Clearance (ml/min/kg)**
AB **(N= 33)**	**Morning (26)**	4.00 ± 1.34	1,039.70 ± 474.14	0.056 ± 0.023
	Bedtime (6)	5.58 ± 1.28	1,328.74 ± 339.26	0.044 ± 0.009
	Sig. ¶	**p = 0.013***	**p = 0.169**	**p = 0.188**
	[95% CI]	[-2.81 – (-0.35)]	[-707.96 – 129.88]	[-0.007 – 0.032]
AA **(N= 59)**	**Morning (53)**	3.26 ± 1.31	832.57 ± 437.72	0.051 ± 0.020
	Bedtime (6)	3.25 ± 0.88	936.28 ± 196.63	0.042 ± 0.016
	Sig. ¶	**p = 0.980**	**p = 0.571**	**p = 0.291**
	[95% CI]	[-1.09 – 1.12]	[-467.80 – 260.38]	[-0.008 – 0.026]

* Significant at p value < 0.05

¶ Analyzed by t-test

4.5 Effect of genetic factors on pharmacodynamic parameter

Clotting factor II (FII) and factor VII (FVII) activities and ratio of INR:total plasma warfarin concentration (INR:Cp) which defined as "Warfarin Sensitivity Index" were analyzed as pharmacodynamic parameters. The clotting factor could not be obtained in ten patients. There were 92 patients who had completed data of genetic, warfarin concentration and clotting factor activities. Mean of FII and FVII activities were 32.21 ± 15.14% (range 4.97 to 80.92%) and 45.28 ± 20.38% (range 11.17 to 111.38%), respectively. Sub-group analysis of time of taking warfarin in morning and bedtime group, FVII activities (short half life; approximately 6 hr.) in bedtime group were significantly higher than those in morning group, 63.79 ± 22.99% and 42.50 ± 18.57 %, respectively (p = 0.001, 95%CI -33.07-(-9.50)), while warfarin dose, plasma warfarin concentration, vitamin K-containing food within 1 week were not significantly difference (Table 4.9). However, FII activities (long half life; approximately 60 hr.) were not significantly different between taking warfarin in the morning or bedtime.

Table 4.9 Effect of time of taking warfarin on clotting factors

N = 92**	**Morning** (N=80)	**Bedtime** (N=12)	**Sig.**¶ [95%CI]
FII activity (%)	31.25 ± 15.30	38.60 ± 12.79	**p = 0.118** [-16.58 – 1.89]
FVII activity (%)	42.50 ± 18.57	63.79 ± 22.99	**p = 0.001*** [-33.07– (-9.50)]
INR	2.1 ± 0.6	1.7 ± 0.3	**p < 0.0001*** [0.22 – 0.65]
Last dose (mg/day)	3.78 ± 1.60	4.42 ± 1.61	**p = 0.204** [-1.62 – 0.35]
Weekly Dose (mg/wk)	26.02 ± 10.60	30.71 ± 11.18	**p = 0.159** [-11.25 – 1.87]
Warfarin conc. (ng/ml)	967.92 ± 512.45	1,132.51 ± 334.51	**p = 0.285** [-468.50 – 139.32]
Vit K food (meals/wk)	5.9 ± 2.9	5.3 ± 3.2	**p = 0.522** [-1.2 – 2.4]

* Significant at p value < 0.05

** Data complete included genetic data, warfarin concentration and clotting factor activities

¶ Analyzed by t-test

Patients taking warfarin in the morning (N=80) was selected to compare the effect of genetic polymorphisms on clotting factor activities. As this study reported that genetic polymorphisms were associated with warfarin dose, the effect of VKORC1 genotypes on FII and FVII activities were compared in 76 patients with CYP2C9*1/*1 (Table 4.10). Factor II activities in VKORC1 AA group was significantly lower than in VKORC1 BB & AB group, i.e., 28.50 ± 12.89 % and 36.64 ± 17.21%, respectively, p = 0.021 [95% CI 1.25 – 15.01]. While the effect of CYP2C9 genotypes on FII and FVII activities were compared in 47 patients with VKORC1 AA genotype. There were no significant different between CYP2C9*1 and CYP2C9*3, p=0.151. However, factor VII activities were not significantly different between VKORC1 BB&AB and AA group, p = 0.098. VKORC1 AA group was associated with reduction of activated clotting factor II and required lower dose of warfarin. However, there were no significant differences between FII and FVII activities among different group of CYP2C9 polymorphisms.

Table 4.10 Clotting factor activities in different genotypes

	N**	FII activity¶ (%)	FVII activity¶ (%)	INR¶
CYP2C9 *1/*1 (N=76)				
VKORC1 BB & AB	32	36.64 ± 17.21	47.51 ± 18.00	2.0 ± 0.6
VKORC1 AA	44	28.50 ± 12.89	40.42 ± 18.41	2.2 ± 0.6
Sig.		**p = 0.021***	**p = 0.098**	**p = 0.178**
[95% CI]		[1.25-15.01]	[-1.35– 15.53]	[-0.45 – 0.09]
VKORC1 AA (N=47)				
CYP2C9*1	44	28.51 ± 12.89	40.42 ± 18.41	2.2 ± 0.6
CYP2C9*3	3	17.34 ± 10.75	22.74 ± 13.73	2.8 ± 0.7
Sig.		**p = 0.151**	**p = 0.111**	**p = 0.076**
[95% CI]		[-4.21-26.55]	[-12.71-35.05]	[-1.37-0.072]

* Significant at p value < 0.05

** Sub group of patients who took warfarin in the morning

¶ Analyzed by t-test

Total plasma warfarin concentrations of patients taking warfarin in the morning were obtained from 88 patients. The effect of VKORC1 genotypes on INR:Cp were compared in 84 patients with CYP2C9*1/*1. As shown in Table 4.11, INR:Cp or Warfarin Sensitivity Index (WSI) was significantly different in each group of genetic polymorphisms. WSI in VKORC1 AA group was significantly greater than that in VKORC1 BB & AB groups, 2.99 ± 0.99 and 2.05 ± 0.98, respectively ($p<0.0001$). However, WSI was not significantly different between wild type and variant type of CYP2C9*3, $p=0.054$.

Table 4.11 Warfarin sensitivity index in different genotypes

	N**	INR:Cp ¶
CYP2C9 *1/*1 (N=84)		
VKORC1 BB & AB	34	2.05 ± 0.98
VKORC1 AA	50	2.99 ± 0.99
Sig.		**p < 0.0001***
[95% CI]		[-1.37 – (-0.51)]
VKORC1 AA (N=53)		
CYP2C9*1	50	2.99 ± 0.99
CYP2C9*3	3	4.17 ± 1.45
Sig.		**p = 0.054**
[95% CI]		[-2.39-0.02)]

* Significant at p value < 0.05

** Sub group of patients who took warfarin in the morning

¶ Analyzed by t-test

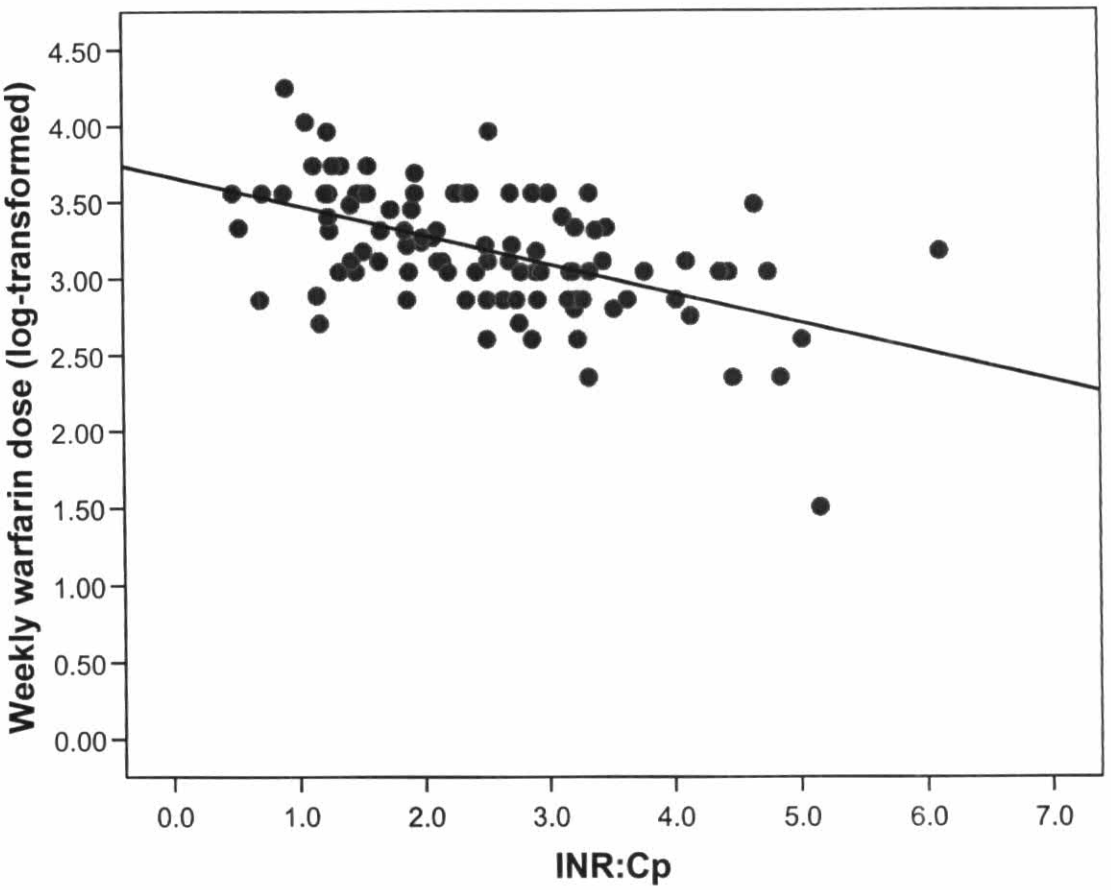

Figure 4.4 Scatter plot between log-transformed weekly warfarin dose and INR:Total plasma warfarin concentration (Cp) in μg/ml (N = 100)

The ratio of INR: total plasma warfarin concentration (INR:Cp) was negatively correlated with weekly warfarin dose in log-transformed ($r = -0.531$, $p < 0.0001$).

4.6 Estimation equations

Table 4.12 shows the entire significant model from forward stepwise linear regression while model 7 was the best fit equation.

Table 4.12 Model summary of forward stepwise linear regression

Model	Variable entered	R^2	R^2 change	Sig (F change)
1	INR:Cp	30.1%	30.1%	<0.0001
2	INR:Cp	38.8%	8.7%	<0.0001
	CYP2C9*3/*3			
3	INR:Cp	43.7%	4.9%	0.004
	CYP2C9*3/*3			
	VKORC1 AB			
4	INR:Cp	52.6%	8.9%	<0.0001
	CYP2C9*3/*3			
	VKORC1 AB			
	VKORC1 BB			
5	INR:Cp	55.3%	2.7%	0.018
	CYP2C9*3/*3			
	VKORC1 AB			
	VKORC1 BB			
	Age			
6	INR:Cp	57.3%	2.0%	0.038
	CYP2C9*3/*3			
	VKORC1 AB			
	VKORC1 BB			
	Age			
	Weight			
7	INR:Cp	59.5%	2.2%	0.027
	CYP2C9*3/*3			
	VKORC1 AB			
	VKORC1 BB			
	Age			
	Weight			
	CYP2C9*1/*3			

The coefficients and p-value of each variables which entered by forward stepwise method of model 7 were shown in table 4.13. Multicollinearity of independent factors was determined (data not shown).

Table 4.13 Coefficients of factors in the best fit equation

Factors	Sig. (p-value)	B	[95%CI]
Constant	<0.0001	3.194	[2.825 – 3.562]
INR:Cp	0.001	-0.098	[-0.152 – (-0.044)]
CYP2C9*3/*3	<0.0001	-1.385	[-1.943 – (-0.827)]
VKORC1 AB	<0.0001	0.290	[0.161 – 0.420]
VKORC1 BB	<0.0001	0.509	[0.295 – 0.723]
Age	0.038	-0.004	[-0.009 – 0.00]
Weight	0.020	0.006	[0.001 – 0.011]
CYP2C9*1/*3	0.027	-0.362	[-0.681 – (-0.043)]

Table 4.14 Estimation equations of warfarin dose

	Equations of ln warfarin dose (mg/week)	R^2	Sig.
Age	ln dose = 3.507 – (0.007 x Age)	4.8%	p =0.023
INR :Cp	ln dose = 3.685 – (0.198 x INR:Cp)	31.8%	p<0.0001
CYP2C9	ln dose = 3.207 – (0.445 x CYP2C9*1/*3) – (1.703 x CYP2C9*3/*3)	20.1%	p<0.0001
VKORC1	ln dose = 2.993 + (0.413 x VKORC1 AB) + (0.645 x VKORC1 BB)	32.4%	p<0.0001
CYP2C9 VKORC1	ln dose = 3.031 – (0.398 x CYP2C9*1/*3) – (1.527 x CYP2C9*3/*3) + (0.387 x VKORC1 AB)+ (0.607 x VKORC1 BB) (Equation 1)	48.4%	p<0.0001
Age, CYP2C9, VKORC1	ln dose = 3.331 - (0.006 x Age) - (0.360 x CYP2C9 *1/*3) – (1.499 x CYP2C9*3/*3) + (0.402*VKORC1 AB) + (0.588*VKORC1 BB) (Equation 2)	52.3%	p<0.0001
Age,weight INR:Cp, CYP2C9, VKORC1	ln dose = 3.194 – (0.004 x Age) - (0.362 x CYP2C9 *1/*3) – (1.385 x CYP2C9*3/*3) + (0.290 x VKORC1 AB) + (0.509 x VKORC1 BB) – (0.098 x INR :Cp) + (0.006 x weight) (Equation 3)	59.5%	p<0.0001

Input Age in year, weight in kg, INR:Cp in ml/mcg, 0 or 1 if present or absent of CYP2C9 *1/*3, CYP2C9 *3/*3, VKORC1 AB or VKORC1 BB group, respectively.

In the Kolmogorov-smirnov test, the distribution of weekly warfarin dose was skewed ($p = 0.038$) and had to be natural logarithmically transformed to achieved normal distribution ($p = 0.297$). As shown in table 4.14, genetic factors played the important role on the inter-individual variation of warfarin maintenance dose in Thai population. CYP2C9*3 combined with VKORC1 genotypes could explained about 50% of warfarin dose.

CYP2C9 polymorphism was associated with pharmacokinetics parameter on warfarin metabolism which is clearance while VKORC1 was associated with pharmacodynamics parameters such as Factor II, Factor VII activities and Warfarin sensitivity index. Warfarin maintenance dose was depended about one third on VKORC1. Using stepwise multiple linear regression, clinical factors including age, weight, and INR:Cp and genetic factors including CYP2C9*3 and VKORC1 could explain 59.5 % of the variance on warfarin maintenance dose.

As shown in Figure 4.5, there was a highly significant correlation between observed warfarin dose and predicted dose in log-transformed from equation 3 (pearson's correlation = 0.771, $p < 0.0001$).

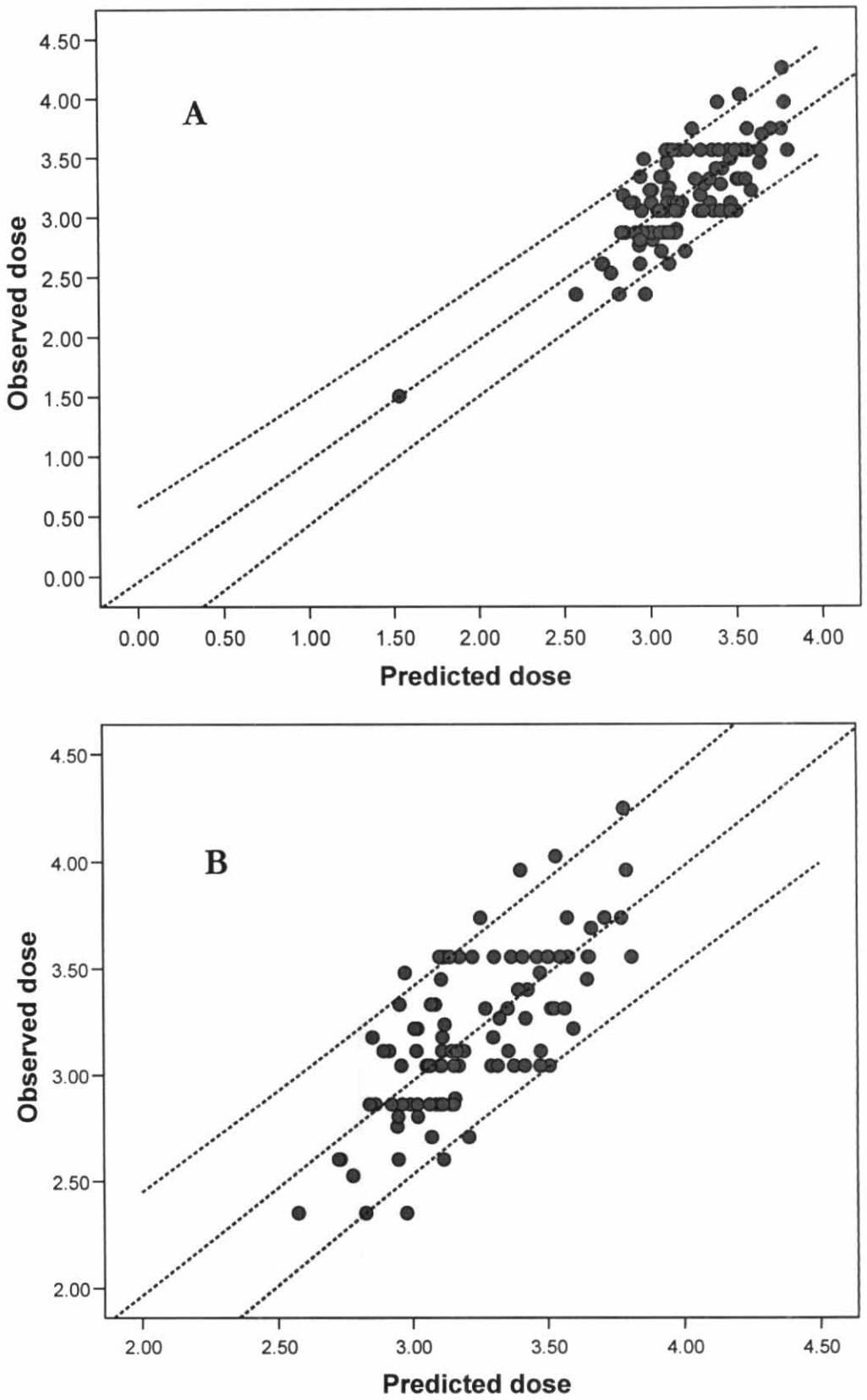

Figure 4.5 Scattered plot of warfarin dose in log-transformed and predicted dose from estimation equation. (figure 4.5A, N= 100, figure 4.5B excluded one patient who was outlier, N=99)

4.7 Association of VKORC1 on side effect of warfarin treatment

Warfarin had a narrow therapeutic index. Bleeding was a major side effect. In this study, side effects included bruises, major and minor bleeding in the past and currently. Major bleeding included cerebral hemorrhage, GI bleeding and hematuria and need to admit into the hospital. Minor bleeding included bleeding gum, epistaxis (bleeding from nose), and abnormal menstruation.

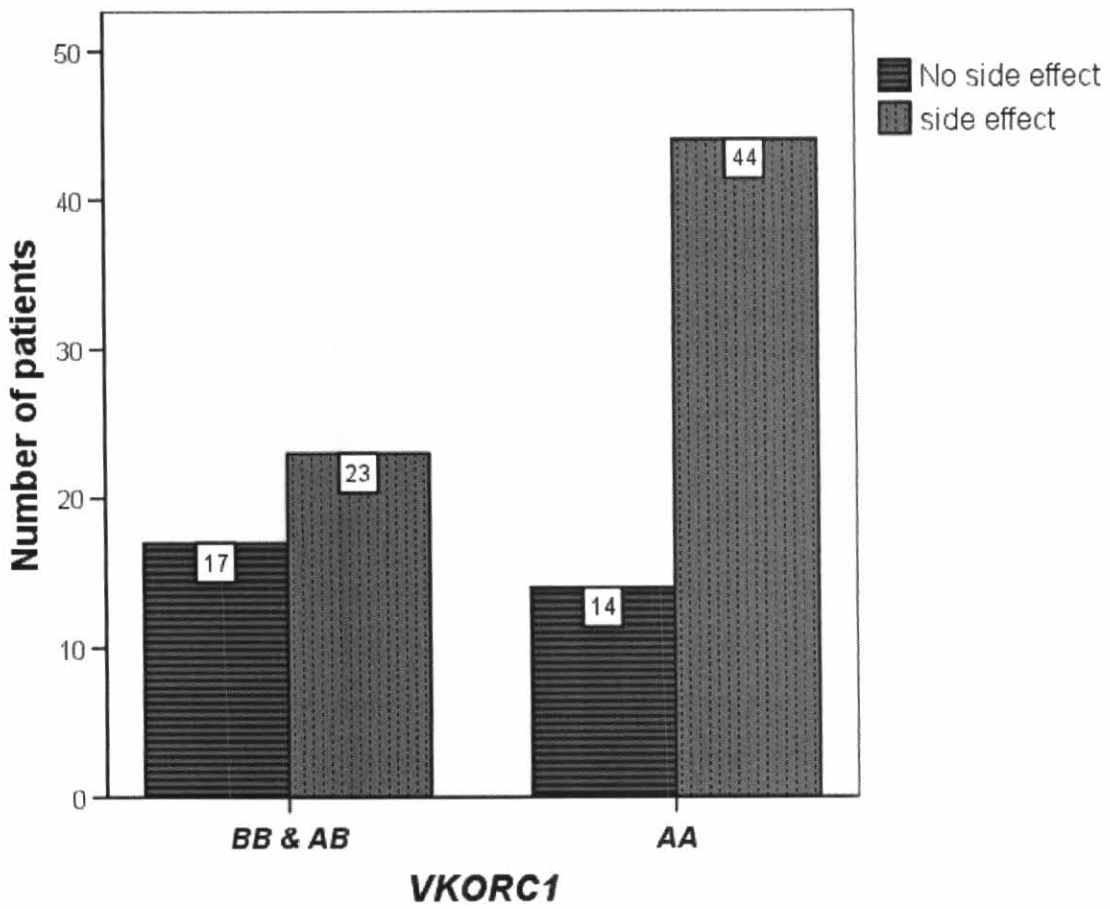

Figure 4.6 Bar chart shows comparison of side effect in different VKORC1 genotypes

Table 4.15 Comparison of side effect in different VKORC1 genotypes

	CYP2C9*1/*1 (N=98)		VKORC1 AA (N=61)	
	VKORC1 BB & AB	**VKORC1 AA**	**CYP2C9 *1**	**CYP2C9 *3**
No side effect	17 (17.3%)	14 (14.3%)	14 (23%)	1 (1.6%)
Side effects	23 (23.5%)	44 (44.9%)	44 (72.1%)	2 (3.3%)
	Chi-square = 3.691 p = 0.045*		**Chi-square = 0.130 p = 0.578**	

Among patient with CYP2C9*1/*1, VKORC1 AA haplotypes had side effects from warfarin significantly higher than patients in BB & AB groups using Chi-square test, Fisher's Exact test (Exact Sig. 1-sided, p = 0.045) (Table 4.15). Patients who had VKORC1 AA haplotype had about 2.3 folds higher risk of having side effect as compare to the BB & AB haplotypes, Odds ratio (OR)= 2.32 [95%CI = 0.97-5.54]. There were no significant differences between side effect and CYP2C9 genotypes, p = 0.578. Table 4.16 shows that major bleeding was no significantly differences in each group of VKORC1 and CYP2C9 genotypes, p = 0.317 and 0.614, respectively).

Table 4.16 Comparison of major bleeding in different VKORC1 genotypes

	CYP2C9*1/*1 (N=98)		VKORC1 AA (N=61)	
	VKORC1 BB & AB	**VKORC1 AA**	**CYP2C9 *1**	**CYP2C9 *3**
No major bleeding	36 (36.7%)	49 (50.0%)	49 (80.3%)	3 (4.9%)
Major bleeding	4 (4.1%)	9 (9.2%)	9 (14.8%)	0 (0%)
	Chi-square = 0.626 p = 0.317		**Chi-square = 0.546 p = 0.614**	

4.8 Interethnic variability of genetics polymorphisms in Asia

Allelic frequencies of CYP2C9 polymorphisms in Asian population were different from Caucasian and African-American population. There was no CYP2C9*2 in Japanese, Chinese, Korean, Malaysian and Vietnamese. In addition, CYP2C9*3 allele frequency was comparable among East Asian (Japanese, Chinese and Korean) and South East Asian (Thais, Malaysian and Vietnamese), but not for Indian (Table 4.17). One-hundred and seven Thais patients (254 alleles) were analyzed for single nucleotide polymorphisms (SNPs) of CYP2C9*2, CYP2C9*3 and VKORC1 C1173T and G-1639A.

Table 4.17 Comparison of CYP2C9*3 allele frequencies among Asians

Ethnic	Alleles	*CYP2C9*1*	*CYP2C9*3*	95% CI of variant proportion
Thais (This study)	**254**	**249 (98.0 %)**	**5 (2.0%)**	**0.7 – 4.7 %**
Chinese [44]	356	324 (95.5%)	16 (4.5%)	2.8-7.2%
Chinese* [53]	254	248 (97.6%)	6 (2.4%)	1.1-5.1%
Indian* [53]	184	161 (87.5%)	15 (8.2)%	5-13%
Japanese [54]	172	169 (98.3%)	3 (1.7%)	0.2-3.6 %
Japanese [55]	436	427 (97.9%)	9 (2.1%)	0.8-3.4 %
Japanese [56]	1,656	1,617 (97.8%)	39 (2.2%)	1.7-3.2%
Korean [57]	1,148	1,135 (98.9%)	13 (1.1%)	0.5-1.7 %
Malaysian* [53]	236	222 (94.1%)	14 (5.9%)	3.6-9.7%
Taiwan Chinese [35]	196	191 (97.4%)	5 (2.6%)	0.7-4.5 %
Vietnamese [58]	314	307 (97.8%)	7 (2.2%)	0.6-3.9 %

* population who lived in Malaysia

Table 4.18 Comparison of VKORC1 haplotype frequencies among Asians

Ethnic	N	BB	AB	AA
Thai (This study)	**127**	**9 (7.1%)**	**46 (36.2%)**	**72 (56.7%)**
Chinese [44]	178	1 (0.6%)	28 (15.7%)	149 (83.7%)
Chinese[42]	104	2 (1.9%)	19 (18.3%)	83 (79.8%)
Chinese* [22]	140	0 (0%)	35 (25%)	104 (74%)
Indian* [22]	43	28 (65%)	4 (9.3%)	3 (7%)
Japanese[45]	125	1 (0.8%)	25 (20.0%)	99 (79.2%)
Japanese [43]	93	0 (0%)	14 (15%)	79 (85%)
Japanese [56]	828	6 (0.7%)	132 (16%)	690 (83.3%)
Malaysian* [22]	84	7 (8.3%)	40 (47.6%)	35 (41.7%)

* Population who lived in Singapore

Also, allelic frequencies of VKORC1 in Asian population were different from Caucasian and African-American population. However, the VKORC1 frequencies in Thais were different from the other ethnic in Asian such as Chinese and Japanese (Table 4.18). VKORC1 haplotypes of BB, AB and AA group in Thais are 7.1%, 36.2% and 56.7%, respectively and were significantly different from the frequencies in Japanese reported by Mushiroda et al. [56] (Chi-square = 17.02, $p<0.0001$). As shown in Table 4.19, alleles frequencies of VKORC1 group A in Thai population is about 74.8% while those in Chinese and Japanese were approximately 85-90%. Mean warfarin dose in Chinese and Japanese were likely to be lower than those in Thais, Malaysian and Indian, maybe associated with different in VKORC1 group A frequency.

Table 4.19 Comparison of genetic and non-genetic factors among Asians

Ethnic	Thais (This study)	Japanese [45]	Japanese [56]	Chinese [44]	Chinese* [22]	Malaysian* [22]	Indian* [22]
N of patients	127	125	828	178	140	84	43
N of alleles	254	250	1,656	356	280	168	86
CYP2C9*3 **allele**	5 (2.0%)	7 (2.9%)	39 (2.2%)	16 (4.5%)	7%	9%	18%
VKORC1 **group A allele**	190 (74.8%)	223 (89.2%)	1,511 (91.2%)	326 (91.6%)	243 (86.8%)	110 (65.5%)	10 (11.6%)
Mean Dose (mg/day)	3.70 ± 1.50	3.28 ± 1.44	2.5 (median)	2.01 ± 0.64	3.54 ± 1.47	3.64 ± 1.36	5.96 ± 2.96
Warfarin dose (mg/kg/day)	0.066 ± 0.029	-	-	-	0.058 ± 0.025	0.059 ± 0.023	0.089 ± 0.036
Age (yo)	48.2 ± 13.1	62.5 ± 13.1	68	54.6 ± 13.2	56	56	56
INR	2.1 ± 0.6	2.1 ± 0.4	(1.5 – 2.5)	2.0 ± 0.4	(2-3)	(2-3)	(2-3)

* Population who lived in Singapore