http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 13 (18): 906-910, 2010
ISSN 1028-8880

VKORC1 Gene Analysis of Some Iranian Sensitive Patients to Warfarin

[1]Z. Kianmehr, [1]P. Ghadam, [2]S. Sadrai, [3]B. Kazemi and [4]R.A. Sharifian
[1]Department of Biology, Faculty of Sciences, Alzahra University, Tehran, Iran
[2]Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[3]Cellular and Molecular Biology Research Center,
Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[4]Department of Hematology, Tehran University of Medical Sciences, Imam Hospital, Tehran, Iran

Abstract: VKORC1 is a component of the enzyme that is the therapeutic target site of warfarin. In order to investigate the relationship between *VKORC1* gene and warfarin dose response, we studied this gene in 22 clinically sensitive patients and 36 clinically normal patients as control group that in previous studies their blood warfarin levels were determined by HPLC and genotyped for *CYP2C9*. In the majority of these patients, the results had shown that the clinical phenotype was according to the *CYP2C9* genotype, but there were sensitive patients with normal *CYP2C9* genotype. So in this study for investigation of another reason of sensitivity to warfarin in these patients, *VKORC1* was chosen. In order to determine the impact of *VKORC1* 1173C>T and 3730G>A polymorphisms in warfarin dose variability, a protocol of RFLP based PCR with *HinfI* and *SsiI* was used. Polymorphisms of *VKORC1* were effective factors in sensitivity to warfarin in the majority of them. However, in some of sensitive patients, there was inconformity of clinical phenotypes with *CYP2C9* and *VKORC1* genotypes. In conclusion, our findings suggest that *CYP2C9* and *VKORC1* are not the only effective factors in sensitivity to warfarin and more studies are necessary for investigation of sensitivity reasons in these patients.

Key words: Warfarin, *VKORC1*, Warfarin sensitivity, anticoagulant drug, pharmacogenetic, RFLP based PCR

INTRODUCTION

Warfarin is currently the most widely used oral anticoagulant drug for the prevention and treatment of patients with arterial and venous thromboembolic disorders (Hirsh *et al*., 2001). However, the use of this drug is accompanied by considerable problems because of the high interindividual variability, as well as intraindividual variability in dose requirement (Takahashi *et al*., 2003). To achieve and maintain an optimal warfarin dose, the prothrombin time and the International Normalized Ratio (INR) are monitored and doses are adjusted to maintain the patient's INR within a narrow therapeutic range. The target INR depends on the indication of warfarin treatment. For most indications, the ideal INR value lies between 2.0 and 3.0 (Horton and Bushwick, 1999). The large variability in warfarin response is multifactorial.

Polymorphisms in the gene encoding the cytochrome P-450 2C9 enzyme (CYP2C9) are known to contribute to variability in sensitivity to warfarin (Kim *et al*., 2009). CYP2C9 is the enzyme primarily responsible for the metabolic clearance of the S-enantiomer of warfarin. A number of polymorphisms in CYP2C9 have been identified, but the most important are CYP2C9*2 (R144C) and CYP2C9*3 (I359L). However, allelic variants of CYP2C9 cannot fully explain the large interindividual and interethnic differences in warfarin dose requirements, suggesting that additional factor(s) may contribute to this variability (Takahashi *et al*., 2004).

The presence of polymorphisms in the Vitamin K epoxide reductase subunit 1 gene (VKORC1) has been recently identified as another cause of variability in the response to warfarin in many populations (Kim *et al*., 2009). VKORC1 recycles vitamin K epoxide to the reduced form of vitamin K, an essential cofactor in the formation of the active clotting factors II, VII, IX and X through γ-glutamyl carboxylation .Warfarin inhibits VKORC1 activity by reducing the regeneration of vitamin K and thus exerting its anticoagulation effect (Cain *et al*., 1997). Several studies have been shown association between VKORC1 polymorphisms and reduction of warfarin dose requirement and warfarin sensitivity (D'Andrea *et al*., 2005; Geisen *et al*., 2005; Yuan *et al*., 2005; Lee *et al*., 2006). To date, numerous Single Nucleotide Polymorphisms (SNPs) have been identified in the

Corresponding Author: P. Ghadam, Department of Biology, Faculty of Sciences, Alzahra University, Tehran, Iran
Tel: +982188044051/2718

VKORC1 gene. Among these SNPs, two common polymorphisms, a C>T transition at position 1173 (rs9934438) in intron 1 and 3730G>A transition in the 3'-untranslated region [UTR] (rs7294) in *VKORC1* gene, affect the interindividual variability of warfarin dose (D'Andrea *et al*., 2005). In some of populations the SNP 1173C>T has been reported to be associated with the low dose warfarin phenotype (Limdi *et al*., 2008) and patients carrying at least one 1173T allele have an increased risk of bleeding compared to those with the CC genotype (Reitsma *et al*., 2005). In addition, it has been shown that the SNP 3730G>A was associated with higher warfarin dose requirement (D'Andrea *et al*., 2005).

Genetic polymorphisms involved in both pharmacokinetic (e.g., CYP2C9) and pharmacodynamic (e.g., VKORC1) factors. Therefore, appear to interplay in the overall interindividual variability of warfarin doses; moreover, the contribution of each factor may differ among different ethnic populations (Yin and Miyata, 2007).So for the first time in this study, the effect of VKORC1 1173C>T and 3730G>A polymorphism in variability of warfarin requirement in some of Iranian warfarin sensitive patients was investigated.

MATERIALS AND METHODS

This project has been conducted at Alzahra University since 2008.

In this study, 58 treated patients with warfarin were recruited from Imam Khomeini Hospital (Tehran-Iran) for participation in this trial. The study group clinically sensitive patients (22 patients) were selected by participating centers according to the following criteria:

- Adult-patients who need less than 10.5 mg week^{-1} of warfarin to keep the INR in therapeutic range (INR = two - three)
- Patients who were on usual dose of warfarin (5-15 mg day^{-1}) but they had bleeding or INR>4 either in seven days after cessation of warfarin or three days after receiving 3 mg day^{-1} vitamin K1 intravenously

The first criterion was chosen from previous reports (Linder, 2001) and the second was chosen by our group while encountered such patients in clinic. The control group (36 adult patients) was randomly selected from patients who took warfarin for various causes and showed normal response to warfarin. In order to determine the impact of 1173C>T and 3730G>A SNPs in VKORC1, a protocol of RFLP (Restriction Fragment Length Polymorphism) based PCR was used. DNA was extracted from whole blood samples using the DNG-Plus™ kit (Fermentas).

Primers 1173F (5'TGACATGGAATCCTGACGTG3') and 1173R (5'GAGCTGACCAAGGGGGAT3') for amplification of 361 base pairs (bp) from intron one of VKORC1 gene (D'Andrea et al., 2005), also primers 3730F (5'AGCCTGATGTGGCTCAGTTT3') and 3730R (5'GTGTGGCACATTTGGTCCATT3') for amplification of 226 bp from the 3'UTR of VKORC1 gene were selected (Li *et al*., 2006). DNA samples were amplified by PCR in a final volume of 15 μL, with 7.5 μL PCR Master Mix (Ampliqon), 0.5 mM each primer, 0.3 μg genomic DNA. Amplification conditions consisted of an initial denaturation of 10 min at 94°C, followed by 30 PCR cycles in 30 sec at 94°C, 30 sec at 58°C and, 90 sec at 72°C with a final extension of 5 min at 72°C. PCR products of these fragments (2 μg from each) were digested with 10 units of Hinf I and SsiI (AciI), respectively in a final volume of 30 μL in the appropriate digestion buffer at 37°C for 16 h. The digested products were visualized on 8% poly acryl amide gels stained with ethidium bromide.

All the Statistical analyses were performed using SPSS for Windows, version 11.5. Multiple comparisons between different CYP2C9 and VKORC1 haplotypes were made using univariate analysis (ANOVA), the unequal variance t-test and Post-Hot tests. Results are presented as Mean±SD unless stated otherwise. A p-value of less than 0.05 was taken as statistically significant.

RESULTS

PCR product of 1173C>T region contained 361 bp. There was one recognition site in position eight for restriction enzyme HinfI in wild type genotype (homozygote CC) so that two fragments (353 and 8 bp) are formed upon HinfI digestion. The 1173T allele created another HinfI recognition site in position 59 and three fragments (310, 43 and 8 bp) in mutant homozygous (TT) and four fragments (353, 310, 43 and 8 bp) in heterozygote genotype (CT) were resulted by HinfI digestion. PCR product of 3730G>A region contain 226 bp. There was one recognition site in position 195 for restriction enzyme SsiI in wild type genotype (homozygote GG) so that two fragments (195 and 31 bp) are formed upon SsiI digestion. In the present of mutation (3730A allele), SsiI has no recognition site and thus one fragment (226 bp) in mutant homozygote (AA) and three fragments (226, 195 and 31 bp) in heterozygote genotype (GA) are formed upon SsiI digestion. There was not wild type genotype (GG) in our samples.

The genotype information was achieved from the band cleavage patterns (Fig. 1, 2). Genotype distributions of CYP2C9 allele variants (*1*1,*1*2, *2*2), 1173C>T and

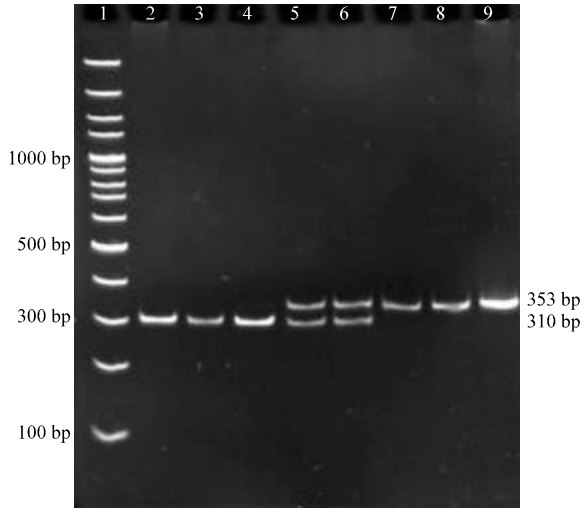

Fig. 1: Acryl amide gel electrophoresis of PCR-RFLP in 1173C>T region with restriction enzyme *HinfI*. Lane 1: DNA ladder molecular size marker (100 bp), Lanes 2, 3, 4: 1173 TT, Lanes 5, 6: 1173 CT, Lanes 7, 8, 9: 1173 CC

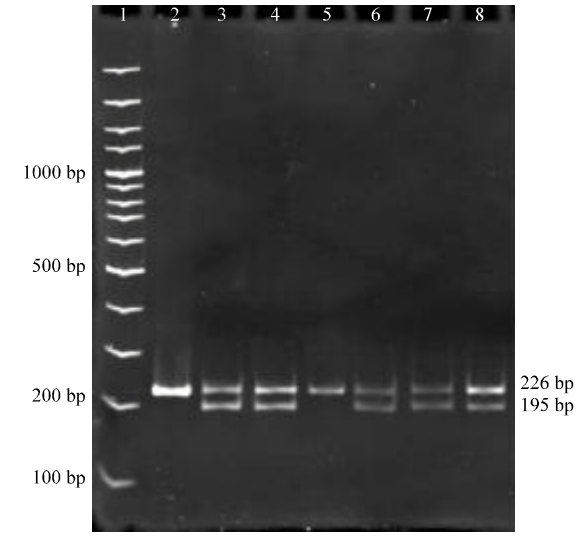

Fig. 2: Acryl amide gel electrophoresis of PCR-RFLP in 3730G>A region with restriction enzyme *SsiI*. Lane1: DNA ladder molecular size marker (100 bp), Lanes 2, 5: 3730 AA , Lanes 3, 4, 6, 7 and 8: 3730 GA

3730G>A SNPs in VKORC1, mean weekly warfarin dose requirements and blood warfarin concentrations in all patients are summarized in Table 1. As shown, mean weekly warfarin doses in carriers of CYP2C9*1*2 and *2*2 are significantly lower (p = 0.006) like patients with 1173CT and TT, in contrast to carriers of 3730AA that need higher doses. Mean concentration of warfarin in patients blood with CYP2C9*1*1 is lower than others with *1*2 variant. Mean concentration of warfarin in the blood of TT carriers is lower than CT and CC carriers (Table 1). The frequencies of the allele variants CYP2C9 and 1173C>T and 3730G>A SNPs in VKORC1 gene between sensitive and normal patients were shown in Table 2. There were significant differences ($p<0.001$) in mean warfarin doses between sensitive and normal patients. According to these results, 68.1 and 81.8% of sensitive patients to warfarin have CYP2C9 variant and VKORC1 1173T allele respectively. The frequency of CYP2C9*2*2 was as expected low (6.9%) whereas the frequency of homozygote for VKORC1 1173TT genotype was 17.2% (Table 2).

Table 1: Relationship between *CYP2C9* and *VKORC1* genotype distributions and pharmacokinetics of warfarin

Genotype	INR	Warfarin dose (mg week^{-1})	Warfarin Conc. (μg mL^{-1})
CYP2C9			
*1*1(n = 32)	2.4±1	28.9±13.9	1.5±0.9
*1*2(n = 22)	3.5±1.8	18.2±10.1	1.6±0.9
*2*2 (n = 4)	3.9±2.1	16.3±5.3	0.5±0.08
p-value	0.009	0.006	0.176
VKORC1 1173 C>T			
CC (n = 21)	2.7±1.5	26.42±15.82	1.7±1
CT (n = 27)	3±1.5	24.8±10.2	1.5±0.7
TT (n = 10)	3.2±1.5	15.03±6.2	1.1±1.1
p-value	0.634	0.052	0.311
VKORC1 3730G>A			
GG (n = 0)	-	-	-
GA (n = 51)	2.9±1.4	23.2±13.5	1.4±0.8
AA (n = 7)	3.1±2.3	28.2±10.7	2.1±1.4
p-value	0.785	0.349	0.229
Total	2.9±1.5	23.8±13.2	1.5±0.9

n: No. of patients, INR: International Normalized Ratio, Conc: Concentration. A p-value of less than 0.05 was taken as statistically significant. *In literatures, CYP2C9 genotypes are shown by this symbol

Table 2: Relationship between *CYP2C9* and *VKORC1* genotype distributions and warfarin sensitivity in patients

Genotype groups	Sensitive■	Normal■■	Total in each genotype group
CYP2C9			
*1*1	7 (31.8%)	25 (69.4%)	32 (55.2%)
*1*2	12 (54.5%)	10 (27.8%)	22 (37.9%)
*2*2	3 (13.6%)	1 (2.8%)	4 (6.9%)
VKORC1 1173C>T			
CC	4 (18.2%)	16 (44.4%)	20 (34.5%)
CT	11 (50%)	17 (47.2%)	28 (48.3%)
TT	7 (31.8%)	3 (8.3%)	10 (17.2%)
VKORC1 3730G>A			
GG	-	-	-
GA	22 (100%)	29 (80.6%)	51 (87.9%)
AA	-	7 (19.4%)	7 (12.1%)

■No. of patients (percent of total sensitive patients), ■■No. of patients (percent of total normal patients)

DISCUSSION

The use of the same fixed dose of warfarin for all patients is unachievable because the responsiveness of

Table 3: Relationship between Clinical phenotype and *CYP2C9/VKORC1* genotypes

	Clinical phenotype		
CYP2C9 genotype	CYP2C9 (n)	VKORC1 1173C>T (n)	VKORC1 3730G>A(n)
Sensitive patients with mutant CYP2C9 (n = 15)	*1*2 (12) *2*2 (3)	CC(1), CT(7), TT (3)	GA (12) TT(4), GA (3)
Normal patients with mutant CYP2C9 (n = 11)	*1*2 (10) *2*2 (1)	CC (4), CT (6) CC (1)	GA (8), AA(2) GA (1)
Sensitive patients with wild type CYP2C9 (n = 7)	*1*1 (7)	CC (3), CT (4)	GA(7)
Normal patients with wild type CYP2C9 (n = 25)	*1*1 (25)	CC(11), CT(11), TT(3)	GA (20), AA(5)

n: No. of patients

different patients to warfarin is highly variable. Therefore, some patients require less warfarin dose than others warfarin sensitive patients and some of them need more warfarin dose warfarin resistant patients. In many populations CYP2C9 andVKORC1 polymorphisms are important to sensitivity to warfarin (Yuan *et al*., 2005; Kirchheiner and Brockmoller, 2005; Scott *et al*., 2008; Wen *et al*., 2008).

In our previous studies (Ghadam *et al*., 2009), all patients that used in this study were genotyped for CYP2C9*2 and *3 and in another study the concentration of warfarin in the patient's blood was determined by HPLC (Sadrai *et al*., 2008). In these population, there were sensitive patients but with wild type CYP2C9 genotype (*1*1). This study was designed to investigate the effect of VKORC1 gene polymorphisms and their relationship to warfarin dose and sensitivity to warfarin in these Iranian warfarin sensitive patients with CYP2C9 *1*1. As it was shown in Table 3, on the basis of CYP2C9 genotype and clinical phenotype, a total of 58 warfarin treated patients were divided in four different groups that genotyped for 1173C>T and 3730G>A SNPs in VKORC1 gene. Consist to the previously published observations (Aithal *et al*., 1999; Henne *et al*., 1998; Kamali *et al*., 2004) our results showed that, majority of sensitive patients have mutation in CYP2C9. Also like studies Takahashi *et al*. (2006) and Osman *et al*. (2006) studies our findings confirmed that VKORC1 polymorphism in 1173C>T is effective factor in sensitivity to warfarin in the majority of sensitive patients. But, in contrast to the previous findings which showed that the SNP 3730G>A was associated with higher warfarin dose requirement (D'Andrea *et al*., 2005) in this study, the patients with SNP 3730G>A need normal or even lower level of warfarin.

Therefore, the VKORC1 1173T allele accounts for low dosage requirements of most patients with CYP2C9 *1*1. However, in some sensitive patients with wild type CYP2C9, there was not mutation in 1173 position of VKORC1. This means that these polymorphisms in CYP2C9 and VKORC1 are not the only effective factors in sensitivity to warfarin, so more investigations should be done in the Iranian patients who have normal CYP2C9 and VKORC1 genotypes but with sensitivity to warfarin until recognized the cause(s) of their sensitivity.

ACKNOWLEDGMENTS

The Vice Chancellor for Research of Alzahra University supported this study. The authors wish to thank Imam Khomeini hospital, Cellular and Molecular Biology Research Center of Shahid Beheshti University, M.C and the Biopharmacutics and Pharmacokinetics Laboratory of Tehran University of Medical Sciences.

REFERENCES

Aithal, G.P., C.P. Day, P.J. Kesteven and A.K. Daly, 1999. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. Lancet, 353: 717-719.

Cain, D., S.M. Hutson and R. Wallin, 1997. Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum membrane. J. Biol. Chem., 272: 29068-29075.

D'Andrea, G., R.L. D'Ambrosio, P. Di Perna, M. Chetta and R. Santacroce *et al*., 2005. A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. Blood, 105: 645-649.

Geisen, C., M. Watzka, K. Sittinger, M. Steffens and L. Daugela *et al*., 2005. VKORC1 haplotypes and their impact on the inter-individual and inter-ethnical variability of oral anticoagulation. Thromb. Haemost., 94: 773-779.

Ghadam, P., R. Sharifian, Z. Jomeh Farsangi, Z. Kianmehr and M. Lak, 2009. CYP2C9 gene analysis of some Iranian hypersensitive patients to warfarin. Pak. J. Biol. Sci., 12: 1160-1163.

Henne, K.R., A. Gaedigk, G. Gupta, J. Steven Leeder and A.E. Rettie, 1998. Chiral phase analysis of warfarin enantiomers in patient plasma in relation to CYP2C9 genotype. J. Chromatogr. B Biomed. Sci. Applied, 710: 143-148.

Hirsh, J., J.E. Dalen, D.R. Anderson, L. Poller, H. Bussey, J. Ansell and D. Deykin, 2001. Oral anticoagulants: Mechanism of action, clinical effectiveness and optimal therapeutic range. J. Am. Chest Phys., 119: 8S-21S.

Horton, J.D. and B.M. Bushwick, 1999. Warfarin therapy: Evolving strategies in anticoagulation. Am. Fam Physician., 3: 635-646.

Kamali, F., T.I. Khan, B.P. King, R. Frearson and P. Kesteven *et al*., 2004. Contribution of age, body size and CYP2C9 genotype to anticoagulant response to warfarin. Clinical. Pharmacol. Therapeutics., 75: 204-212.

Kim, H.S., S.S. Lee, M. Oh, Y.J. Jang and E.Y. Kim *et al*., 2009. Effect of CYP2C9 and VKORC1 genotypes on early-phase and steady-state warfarin dosing in Korean patients with mechanical heart valve replacement. Pharmacogenetics Genomics., 19: 103-112.

Kirchheiner, J. and J. Brockmoller, 2005. Clinical consequences of cytochrome P450 2C9 polymorphisms. Clin. Pharmacol. Ther., 77: 1-16.

Lee, S.C., S.S. Ng, J. Oldenburg, P.Y. Chong and S. Rost *et al*., 2006. Interethnic variability of warfarin maintenance requirement is explained by VKORC1 genotype in an Asian population. Clin. Pharmacol. Ther., 79: 197-205.

Li, T., L.A. Lange, X. Li, L. Susswein and B. Bryant *et al*., 2006. Polymorphisms in the VKORC1 gene are strongly associated with warfarin dosage requirements in patients receiving anticoagulation. J. Med. Genet., 43: 740-744.

Limdi, N.A., D.K. Arnett, J.A. Goldstein, T.M. Beasley and M.J. Rieder *et al*., 2008. Influence of CYP2C9 and VKORC1 polymorphisms on warfarin dose, anticoagulation attainment and maintenance among European American and African Americans. Pharmacogenomics, 9: 511-526.

Linder, M.W., 2001. Genetic mechanisms for hypersensitivity and resistance to the anticoagulant Warfarin. Clin. Chim. Acta, 308: 9-15.

Osman, A., C. Enstrom, K. Arbring, P. Soderkvist and T.L. Lindahl, 2006. Main haplotypes and mutational analysis of vitamin K epoxide reductase (VKORC1) in a Swedish population: A retrospective analysis of case records. J. Thromb. Haemost., 4: 1723-1729.

Reitsma, P.H., J.F. van der Heijden, A.P. Groot, F.R. Rosendaal and H.R. Buller, 2005. A C1173T dimorphism in the VKORC1 gene determines coumarin sensitivity and bleeding risk. PLoS Med., 2: e312-e312.

Sadrai, S., P. Ghadam, R. Sharifian, E. Nematipour, Z. Kianmehr and Sh. Shahriari, 2008. Pharmacokinetic analysis of warfarin in iranian warfarin sensitive patients. Int. J. Pharmacol., 4: 149-152.

Scott, S.A., L. Edelmann, R. Kornreich and R.J. Desnick, 2008. Warfarin Pharmacogenetics: CYP2C9 and VKORC1 genotypes predict different sensitivity and resistance frequencies in the Ashkenazi and Sephardi Jewish populations. Am. J. Human Genet., 82: 495-500.

Takahashi, H., G.R. Wilkinson, R. Padrini and H. Echizen, 2004. CYP2C9 and oral anticoagulation therapy with acenocoumarol and warfarin: Similarities yet differences. Clin. Pharmacol. Ther., 75: 376-380.

Takahashi, H., G.R. Wilkinson, E.A. Nutescu, T. Morita and M.D. Ritchie *et al*., 2006. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. Pharmacogenet Genomics, 16: 101-110.

Takahashi, H., G.R. Wilkinson, Y. Caraco, M. Muszkat and R.B. Kim *et al*., 2003. Population differences in S-warfarin metabolism between CYP2C9 genotype-matched Caucasian and Japanese patients. J. Clin. Pharmacol. Ther., 73: 253-263.

Wen, M.S., M.T.M. Lee, J.J. Chen, H.P. Chuang and L.S. Lu *et al*., 2008. Prospective study of warfarin dosage requirements based on CYP2C9 and VKORC1 genotypes. Clin. Pharmacol. Therapeutics, 81: 83-89.

Yin, T. and T. Miyata, 2007. Warfarin dose and the pharmacogenomics of CYP2C9 and VKORC1: Rationale and perspectives. Thromb. Res., 120: 1-10.

Yuan, H.Y., J.J. Chen, M.T. Lee, J.C. Wung, Y.F. Chen and M.J. Charng, 2005. A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity. Hum. Mol. Genet., 14: 1745-1751.