Prevalence of CYP2C9 and VKORC1 Mutation in Patients with Valvular Heart Disease in Northern Thailand

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Background: *Warfarin has been widely used for the prevention and treatment of thromboembolism. Warfarin therapy depends on interaction between physiological, environmental, and genetic factors. Vitamin K epoxide reductase (VKORC1) and cytochrome P450 2C9 (CYP2C9) enzyme conjointly determine the warfarin maintenance dose. The prevalence of CYP2C9 and VKORC1 variants varies among ethnic groups. The purpose of the present study was to investigate the prevalence of CYP2C and VKORC1 in the Northern Thai population.*
Material and Method: *Patients with valvular heart disease who regularly took a steady maintenance warfarin dose for at least one month were recruited into the present study. Patients who had taken amiodarone or an anti-inflammatory drug were excluded. Clinical data were obtained from medical records. Five milliliters of whole blood was drawn from each patient for gene analysis and prothrombin time with international normalized ratio (INR) measurement.*
Results: *From 242 patients, CYP2C9 *1/*1 was found in 230 patients (95%) and CYP2C9 *1/*3 was found in 12 patients (5%). Neither mutant CYP2C9*2 allele nor individuals homozygous for CYP2C9*3 were observed. Regarding VKORC1, haplotype AB was found in 83 patients (34.3%) and haplotype AA was found in 154 patients (63.6%). Haplotype BB (wild type) was found in five patients (2.1%).*
Conclusion: *The prevalence of CYP2C9 *1/*1 is high while the prevalence of CYP2C9*2 and CYP2C9*3 is very low. VKORC1 haplotype AA is the most common among the Northern Thai population. Further study regarding pharmacogenetic and non-genetic factors to develop warfarin-dosing algorithm is warranted.*

Keywords: *Pharmacogenomics, Polymorphisms, Warfarin, Anticoagulant*

J Med Assoc Thai 2009; 92 (12): 1597-601
Full text. e-Journal: *http://www.mat.or.th/journal*

Warfarin has been widely used for the prevention and treatment of thromboembolism. Warfarin treatment is dependent upon interaction between physiological, environmental, and genetic factors[1]. Several studies have suggested clinical application of genotypic information on warfarin dosing adjustment[2, 3]. The US Food and Drug Administration has recently revised product labeling of warfarin on the potential effect of genetic polymorphism on drug dosing[4]. Recently, a pharmacogenetic guidance warfarin dosing study was shown to have better accuracy and efficiency of warfarin dose initiation[5].

Vitamin K epoxide reductase (VKORC1) is the key enzyme of the vitamin K cycle and the molecular target of warfarin. Warfarin interferes with clotting factor synthesis by inhibition of the C1 subunit of the VKORC1 enzyme complex, which results in reduction

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of vitamin K1 epoxide[6]. The degree of suppression depends upon the patient's haplotype. There are three haplotypes (AA, AB, and BB) of VKORC1, all of which are associated with warfarin dose requirements. Patients with haplotype B require a higher maintenance dose of warfarin than those with haplotype A[7]. Warfarin is a mixture of S-warfarin and R-warfarin. S-warfarin is 5 times more potent than R-warfarin. Metabolism of S-warfarin occurs via the cytochrome P450 2C9 (CYP2C9) enzyme, whereas R-warfarin is metabolized by cytochrome P450 1A2 (CYP1A2) and cytochrome P450 3A4 (CYP3A4). Three genetic variants (*1, *2 and *3 allele) have been identified in CYP2C9 and are associated with different enzyme activity on warfarin metabolism. The *2 allele and *3 allele variants can cause *in vitro* reduction in enzymatic activity by 30 and 80% respectively and had led to increased anticoagulant efficacy of warfarin, thereby decreasing the required warfarin dosage for therapeutic range maintenance[8]. The importance of CYP2C9 and VKORC1 to patient-specific dose requirement has been established in several studies[5,7,8].

The prevalence of CYP2C9 and VKORC1 variants correlates with membership of ethnic groups. The purpose of the present study was to investigate the prevalence of CYP2C and VKORC1 in Thai patients with valvular heart disease requiring warfarin treatment.

Material and Method

The study population was patients with valvular heart disease from the cardiology outpatient department of Maharaj Nakorn Chiang Mai hospital who regularly took a steady maintenance warfarin dose for at least one month. Patients who currently took oral amiodarone or anti-inflammatory agents (*i.e.* NSAIDs or steroid) at the time of the present study were excluded. After obtaining written informed consent, 247 patients were recruited into the present study. Clinical data were obtained from medical records. Five milliliters of whole blood was drawn from each patient for gene analysis and prothrombin time with international normalized ratio (INR) measurement. INR was measured by CoaguChek system (Roche Diagnostic, USA).

DNA samples

DNA samples were extracted using the High Pure PCR Template Preparation Kit protocol (Roche Applied Science, Mannheim, Germany).

*Preparation of the PCR Mix for CYP2C9*2 and CYP2C9*3*

Polymerase Chain Reaction (PCR) contained 5 ml of DNA (~100 ng), 4 ml of reagent mix containing primers and probes of CYP2C9*2 and CYP2C9*3, 3 ml of Roche master containing FastStart Taq DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs, Final volume was adjusted with sterile distilled water to 20 ml. Control DNA samples (wild type, heterozygous and mutant) for human CYP2C9*2 and CYP2C9*3 and negative control sample were run with the patient samples.

Preparation of the PCR Mix for VKORC1 C1173T and VKORC1 G-1639A

PCR contained 5 ml of DNA (~100 ng), 4 ml of reagent mix containing primers and probes of VKORC1 C1173T and VKORC1 G-1639A and 3 ml of Roche master containing FastStart Taq DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs and final volume was adjusted with sterile distilled water up to 20 ml. Control DNA samples (wild type, heterozygous and mutant) for human VKORC1 C1173T and VKORC1 G-1639A and negative control samples were run with the patient samples. PCR reactions were performed in a Real-time PCR thermal cycler as described above for CYP2C9 genotyping reactions.

Tm analysis

374 bp and a 180 bp fragments of the CYP2C9 gene, and 176 bp and a 289 bp fragments of the human VKORC1 gene were amplified with specific primers. Human CYP2C9*2 and VKORC1 C1173T were detected with a SimpleProbe® (detected in channel 530) and human CYP2C9*3 and VKORC1 G-1639A were analyzed with LighCycler® Red 640 labeled hybridization probes (detected in channel 640). Hybridization of the probes to the target DNA resulted in fluorescence resonance energy transfer (FRET) between two fluorophores. During melting of the final product, the sequence alteration was detected as a change in the melting temperature (Tm) of the sensor probe. In the presence of the wild type DNA, hybridization probes formed an exact match with the target, resulting in a higher stability of the complex and the highest melting peak temperature. Whereas, in the presence of the mutant DNA, hybridization probes formed a mismatch with the target, resulting in a lower stability of the complex and the lowest melting peak temperature. Therefore, the heterozygous samples showed two distinct melting peaks of wild type and mutant DNA. The melting peak

temperature of CYP2C9*2, CYP2C9*3, VKORC1 1173 and VKORC1-1639 genotypes was analyzed using Tm Calling mode of the LightCycler® Software 4.05.

Statistical analysis

Continuous variables with a Gaussian distribution were expressed as the mean ± SD and compared by means of student t-test. Categorical variables were expressed as percentages of the study population and compared by the χ^2 test. All calculations were performed using SPSS software version 14.0 statistical package (Chicago, IL). Statistical significance was considered for $p < 0.05$.

Results

From 247 valvular heart patients, five patients were excluded from the present study due to absent fluorescence peak after gene amplification. The mean age was 51 ± 10 years old and 160 (66%) were female. Ninety-one (37%) patients had a mechanical valve. Among 242 patients, CYP2C9*1/*1 was found in 230 patients (95%) and CYP2C9*1/*3 was found in 12 patients (5%). Neither mutant CYP2C9*2 allele nor individuals homozygous for CYP2C9*3 were found in this study population (Table 1). Regarding VKORC1, haplotype AB of VKORC1 was found in 83 patients (34.3%) and haplotype AA was found in 154 patients (63.6%). Haplotype BB (wild type) was found in five patients (2.1%). The distribution of VKORC1 genotype was similar among CYP2C9 genotype *1/*1 and *1*3.

Discussion

From this first pharmacogenetic study of warfarin in Northern Thailand, the authors found a similar prevalence of genetic variations of CYP2C9 in this population compared to a Korean population[9]. Neither Mutant CYP2C9*2 allele nor homozygous CYP2C9*3 were found in the present study, which is consistent with a rare prevalence of these genotype in the other studies of East Asian populations[10,11]. The prevalence of mutant CYP2C9*2 allele and homogenous CYP2C9*3 were reported to be higher in Caucasians[2]. The ethnic difference of CYP2C9 variant frequency has been demonstrated in various studies[9,12] where the variant of CYP2C9 in Asians was significantly lower than in Westerners. Furthermore, the gene-dose effect of defective CYP2C alleles on the *in vivo* CYP2C9 activity was different among Asian and Westerners. Takahashi and colleague demonstrated that the unbound clearance of warfarin was significantly lower in Japanese patients with homozygous and heterozygous of CYP2C9*3 than patients with homozygous CYP2C9*1[13] while there was no difference among Caucasian patients with CYP2C9*1 homozygous and patients with heterozygous CYP2C9*2 or CYP2C9*3 genotypes. The difference of warfarin clearance in genotype-matched has shown the significantly higher warfarin clearance of Japanese than Caucasian patients with homozygous CYP2C9*1[14].

The prevalence of VKORC1 haplotype AA and AB in the presented population was 97.9%, which was higher than the prevalence reported in the Westerners population[7,15]. Similarly, Reider et al demonstrated that Asian-Americans had a significantly higher proportion of group A haplotype than in European-Americans[7].

Although the pharmacogenomic effects of CYP2C9 and VKORC1 on warfarin dosage have been addressed in several studies[3,7,16,17], the non-genetic factors also play significant roles on warfarin dose. These factors include age, gender, body size, diet, and the presence of co-morbidities and medications, which can account for variation of warfarin dose,

Table 1. The allelic frequency distribution of variant CYP 2C9 and VKORC1 genotype

VKORC1 haplotype	CYP2C (genotype)					
	Rapid metabolizer	Intermediate metabolizer		Poor metabolizer		
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
High (BB)	2%	-	0.5%	-	-	-
Medium (AB)	33%	-	1.0%	-	-	-
Low (AA)	60%	-	3.5%	-	-	-

Data presented in number (%) of the patients

ranging from 4.7 to 14.6%[17]. Dosing algorithm using both pharmacogenetic and non-genetic factors could account for 54.2-62% of the warfarin dose variability[2,19,20]. Furthermore, time to steady state of warfarin varies by genotype; 3-5 days for CYP2C9*1/*1 to 12-15 days for CYP2C9*1/*3. This variation knowledge may guide the frequency of dose adjustment.

Until now, the selection of warfarin dosage has relied upon an iterative change in daily dosage until the desired therapeutic effect is achieved. During this process of iteration towards an optimal INR the patients are still at risk from thromboembolism and bleeding, some of which could be fatal. Warfarin dosing guided by pharmacogenetics and non-pharmacogenetic factors could be beneficial for a more proper starting dose and this could lead to a faster achievement of the best maintenance dose to minimize the patient risk.

The present study has some limitations. It recruited only patients with valvular heart disease, mostly from Northern Thailand. Therefore, the results may not be applied to other population. The present study regarding the genetic, clinical, and environmental factors that may affect warfarin dose in larger populations to develop the dose algorithm for warfarin are warranted.

Acknowledgement

The authors wish to thank Prof. Apichard Sukonthasarn for helping in manuscript preparation. We also wish to thank Mrs. Pornapa Thitasarn for research assisting.

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ความชุกของ CYP2C9 และ VKORC1 mutation ในผู้ป่วยโรคลิ้นหัวใจในภาคเหนือของประเทศไทย

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***ภูมิหลัง**: ยาวาร์ฟาริน เป็นยาที่ใช้อย่างแพร่หลายในการป้องกันและรักษาภาวะลิ่มเลือดอุดตัน โดยการใช้ยาวาร์ฟารินต้องคำนึงถึงปัจจัยด้านพยาธิสภาพสิ่งแวดล้อม และ พันธุกรรม เอนไซม์ Vitamin K epoxide reductase (VKORC1) และ cytochrome P4502C9 (CYP2C9) มีความสำคัญต่อขนาดยาที่ใช้ความชุกของจีน CYP2C9 และ VKORC1 มีความแตกต่างกันไปในแต่ละเชื้อชาติ การศึกษานี้เพื่อหาความชุกของจีนทั้ง 2 ชนิด ในประชากรภาคเหนือของประเทศไทยรวมถึงผลของจีนต่อขนาดของยาวาร์ฟาริน*

***วัสดุและวิธีการ**: ผู้ป่วยโรคลิ้นหัวใจที่ได้รับยาวาร์ฟารินในขนาดคงที่อย่างน้อย 1 เดือน จะได้รับเข้าร่วมการศึกษา ผู้ที่ได้รับ amiodarone และ ยาในกลุ่ม anti-inflammatory drug จะถูกคัดออก ข้อมูลทางคลินิกได้จากทะเบียนประวัติ ผู้ป่วยจะได้รับการตรวจเลือดสำหรับวิเคราะห์จีน และ prothrombin time ร่วมกับ INR*

***ผลการศึกษา**: จากผู้ป่วย 242 รายพบว่ามี CYP2C9 *1/*1 230 ราย (ร้อยละ 95) และพบ CYP2C9 *1/*3 12 ราย (ร้อยละ 5) ไม่พบ mutant CYP2C9*2 หรือ hemozygous CYP2C9*3 ในแง่ของ VKORC1 พบ haplotype AB ในผู้ป่วย 83 ราย (ร้อยละ 34.3) haplotype AA 154 ราย (ร้อยละ 63.6) และ haplotype BB 5 ราย (ร้อยละ 2.1)*

***สรุป**: ความชุกของจีน CYP2C9 *1/*1 สูง แต่ CYP2C9 *2 และ CYP2C9 *3 มีความชุกต่ำความชุกของ VKCRC1 นั้นพบ haplotype AA มากที่สุด การศึกษาการใช้ปัจจัยพันธุกรรมและปัจจัยอื่น ๆ เพื่อสร้างวิธีการปรับขนาดของยาวาร์ฟารินควรจะจัดเป็นการศึกษาขั้นต่อไป*