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Single Nucleotide Polymorphism in Cytochrome P450 2E1 among Korean Patients on Warfarin Therapy

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Abstract – This study was designed to assess the distribution of cytochrome P450 2E1 (CYP2E1) polymorphism among Korean patients on warfarin therapy. CYP2E1 polymorphism was analyzed at 5' flanking region of CYP2E1 gene using restriction fragment length polymorphism method. Patient characteristics including the measured internal normalized ratio (INR) were also evaluated. Based on the warfarin dose and the bleeding cases, the patients were grouped as the regular dose control, the regular dose bleeding, the low dose control, and the low dose bleeding. Total 96 patients were evaluated for both *Pst* I and *Rsa* I loci of the CYP2E1 gene and the results showed that both loci were tightly linked. Thirty-three patients (34.4%) were heterozygotes and 4 patients (4.2%) were homozygote. There was no significant difference in patient characteristics in the dose and bleeding case groups. CYP2E1 polymorphism showed a little difference among the groups but was not statistically significant, however, lower INR value was observed in homozygote genotype groups. It was also revealed that genotype allele frequencies of CYP2E1 in Korean was close to other Asian groups but was significantly different from other Caucasian and African-American populations.

Key words □ CYP2E1, polymorphism, warfarin

INTRODUCTION

There are increased interests and reports in usage of genetic information that is available as the result of the Human genome project. This involves identifying genetic mutations that may be important factors in outbreaks of diseases and in drug metabolism. Recently, development of genotyping methods including polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) and single strand conformational polymorphism (SSCP) expedite the research in this field (Itoh *et al.*, 2000).

The cytochrome P450 enzymes (CYP) play a critical role in the metabolism of many therapeutic drugs. Individual susceptibility and variability in drug response is thought to be due to the differences in CYP enzyme activity. About 30 isozymes of human CYP enzymes were found and many of them including CYP2C9, CYP2C19, and CYP2E1 are known to be involved in major drug metabolism (Umeno *et al.*, 1988, Hayashi *et al.*, 1991, Terelius *et al.*, 1991, Iwahashi *et al.*, 1994, Coon *et al.*, 2003; Ingelman-Sundberg, 2004). Moreover, these CYP enzymes, CYP2C9, CYP2C19 and CYP2E1, known to contain non-functional alleles and these alleles vary substantially in population of different racial origin (Hamdy *et al.*, 2002, Tanaka, 1999). We have reported the genetic variations in CYP2C9 (Lee *et al.*, 2003) and CYP2C19 (data not shown) among Korean patients under warfarin therapy. In the series of these studies, we report here the allele frequency in the regulatory 5'-flanking region of CYP2E1 that was analyzed using PCR-RFLP in Korean patients on warfarin therapy.

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PATIENTS AND METHODS

Subjects and clinical data collection

Subjects were recruited from patients visiting the anticoagulation clinic to monitor INR regularly and to adjust warfarin dose at Samsung Medical Center (SMC), Seoul, Korea. As a case-control study, the case group was the patients with experi-

ence of bleeding complications and the control group was the patients with no bleeding events during follow-up visits. The low dose group was on warfarin less than 10 mg/week for longer than 3 months without apparent causes for low-dose requirement such as drug interactions or hepatic insufficiency. The patients did not have any major organ failures and bleeding tendency. The low dose was defined based on the previous study (Furuya *et al.*, 1995; Aithal *et al.*, 1999; Scordo *et al.*, 2002). It was estimated by approximately 2 standard deviations below the mean weekly dose of warfarin in all patients under warfarin therapy in SMC anticoagulation service. Patients on warfarin dose greater than 10 mg/week were included in the regular dose group. Review of bleeding complications included experiences of minor or major bleeding events that were reviewed. Database of the SMC anticoagulation service was reviewed for demographic characteristics, the weekly warfarin dose, the measured INR, indications, bleeding events and concomitant medications. Patient characteristics, mean warfarin dose per week and anticoagulant effect of INR were compared among the groups (Table I).

DNA isolation

Chromosomal DNA was purified from 2 ml of blood samples using Genome whole blood kit (Bio101, Carlsbad, CA, USA) according to manufacture's protocol. The kit consisted of cell lysis/denaturation solution, RNase Mix, salt-out mixture, cell suspension solution and blood washing solution. A crude extract of genomic DNA was obtained from 2 ml whole blood for polymerase chain reaction (PCR) analysis. Whole blood packed cells were mixed with blood washing solution and centrifuged at 1,500×*g* for 15 min. The supernatant was aspired and the cell pellet was washed twice. The final cell pellet was resuspended in cell suspension solution. RNase Mix, denaturing solution, and protease solution were added. After incubation at 55°C for 2h, salt-out mixture was added and centrifuged at 12,000×*g* for 10 min. To the supernatant, 2 ml TE buffer and 8 ml of 100% ethanol were added and mixed. The ethanol phase was eliminated and DNA was dried in the air and dissolved in sterile TE (10 mM Tris and 1 mM EDTA, pH 8.0).

Amplification of DNA by polymerase chain reaction and genotyphing

The SNP loci were amplified using PCR. The PCR reaction was carried out in a 50 μl solution consisting of 1× *Taq* buffer (10 mM Tris-Cl, 50 mM KCl, and 15 mM $MgCl_2$, pH 8.3), 0.2 mM of 4 dNTPs, 80 nmol of each primer, 125 ng of chromosomal DNA as a template and 1.25 U of *Taq* polymerase (Takara-Korea, Seoul, Korea). For *Pst* I locus amplification, forward primer 5'-CCAGTCGAGTCTACATTGTCAGTT-3' and reverse primer 5'GAGTTATGCCATTCTATACTTGTA-3' were used. For *Rsa* I locus amplification, forward primer 5'-AGAG AAAAACTGGGTTAGAATGCA-3' and reverse primer 5'-TT CATTCTGTCTTCTAACTGGCAA-3' were used. Thirty-five cycles of PCR (94°C for 40 sec, 55°C for 50 sec, and 72°C for 40 sec) were followed by a 10 min incubation at 72°C. To minimize error in the reverse transcription and PCR procedures, all reactions were carried out in duplicate with each primer set. The amplified cDNA fragments were analyzed by agarose gel electrophoresis and were visualized by ethidium bromide staining.

Genotyping

For genotyping of CYP2E1 at *Pst* I locus and *Rsa* I locus PCR products described above were digested with 2 U of *Pst* I (Promega, USA) or *Rsa* I (Promega, USA), respectively. Reaction mixtures were incubated at 37°C for 1 hr and the resulting DNA fragments were analyzed by electrophoresis on 3% low melting point agarose gel and DNA products were visualized by ethidium bromide staining. The SNP pattern was assessed by the number and size of DNA bands.

RESULTS AND DISCUSSSION

The PCR product generated for *Pst* I sites in the regulatory

Table I. Characteristics of patients analyzed for CYP2E1 polymorphisms.

	Regular dose (n=79)		Low dose (n=17)	
	Control	Bleeding	Control	Bleeding
Patients (n=96) n (%)	47 (49.0)	32 (33.3)	10 (10.4)	7 (7.3)
Age (year, mean±SD)	62.1±13.2	66.3±11.8	75.6±9.3	71.1±11.7
Weight (Kg, mean±SD)	61.8±12.9	63.1±9.4	53.1±10.2	55.6±10.2
Dose (mg/week, mean±SD)	30.3±11.9	29.4±10.1	8.2±2.8	9.5±3.4
Dose (mcg/kg/week, mean±SD)	502.6±212.2	468±150.6	161.4±62.9	177±66.0
INR (mean±SD)	2.1±0.4	2.4±1.0	2.3±0.4	2.1±0.8

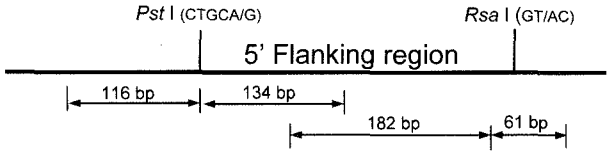

Fig. 1. A. Schematic diagram of CYP2E1 polymorphic sites. Both *Pst* I and *Rsa* I polymorphic sites were located in 5' flanking region of the gene. A wild type CYP2E1 gene contains *Rsa* I site but not *Pst* I site.

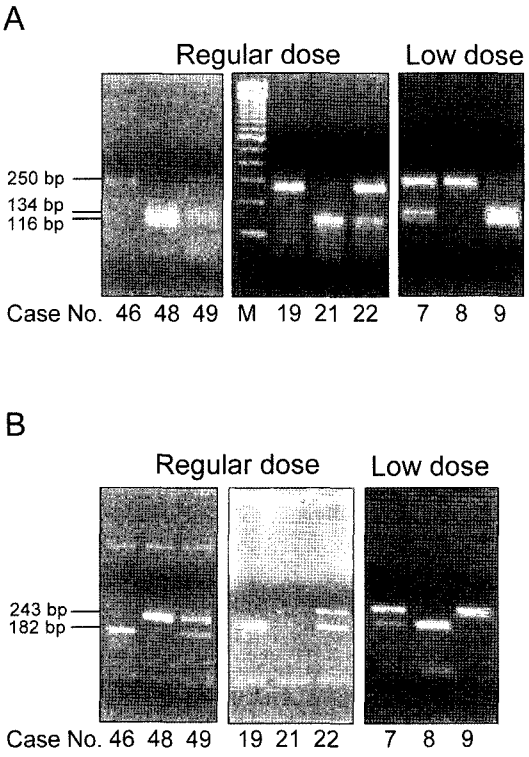

Fig. 2. Electrophoretic analysis of restriction fragment length polymorphism of CYP2E1 genetic variations at *Pst* I (A) and *Rsa* I (B) loci. Cases 46, 48 and 49 and 52 were regular dose control group; cases 19, 21 and 22 were regular dose bleeding groups; case 7 was low dose control group; and case 8 and 9 were low dose bleeding group. M; molecular weight marker.

5' flanking region of CYP2E1 gene analysis was 250 bp. Wild type in this region does not contain *Pst* I restriction site, however ***G***TGCAG → ***C***TGCAG mutation generates *Pst* I site, thus restriction digestion with *Pst* I would generate 134 bp and 116 bp DNA fragment. Therefore, a homozygote or heterozygote mutation would generate 2 or 3 DNA bands, respectively (Fig. 1 and Fig. 2A). In the case of *Rsa* I region, wild type will show 2 DNA bands since it contains *Rsa* I restriction site. A C→ G mutation destroys *Rsa* I restriction site, thus, a homozygote mutation would generate a single DNA band and a heterozygote mutation would generate 3 DNA bands (Fig. 1 and Fig. 2B). It was reported that the 5'-flanking region of CYP2E1 gene is important in its binding of trans-acting factor and in its transcriptional regulation (Hayashi *et al.*, 1991).

The polymorphism distributions for CYP2E1 at *Pst* I and *Rsa* I sites were completely linked each other. Among 96 patients analyzed in this study, 59 (61.5%) cases were wild types (Table II). Thirty-three (34.4%) and 4 (4.2%) cases showed heterozygote and homozygote genotypes, respectively. It was noticeable that INR in homozyogtes were lower than other genotype groups (1.7 vs 2.4 and 2.0).

CYP2E1 genetic variations were further assessed for wild type, heterozygote and homozygote group by the dose group and the bleeding complications (Table III). Regular dose control group and regular dose bleeding groups showed 68.1% and 62.5% of wild types; 29.8% and 34.4% of heterozygote genotype; and 2.1% and 3.1% of homozygote genotypes. Low dose groups (10 cases in control and 7 cases in bleeding groups) were too small to obtain statistically significant distribution. Patient characteristics in various genotypes were similar in the age and weight. The lower measured INR were observed in homozygote genotype groups (Table III).

This is the first report regarding the CYP2E1 genotype distribution in Korean patients on warfarin therapy. The significance of the relationship between lower INR and homozygote

Table II. CYP2E1 genotyping and characteristics of patients.

	Wild type c1/c1	Heterozygote c1/c2	Homozygote c2/c2
Patients (n =96), n (%)	59 (61.5)	33 (34.4)	4 (4.2)
Age (year, mean±SD)	64.5±13.4	66.5±12.4	73.8±5.0
Weight (Kg, mean±SD)	60.8±11.9	61.3±11.8	58.2±9.7
Dose (mg/week, mean±SD)	27.3±12.7	25.9±13.3	12.5±7.8
Dose (mcg/kg/week, mean±SD)	452.8±214.5	421.2±206.8	213.1±118.7
INR (mean±SD)	2.4±0.7	2.0±0.6	1.7±0.4

Table III. Distribution of patients base on CYP2E1 polymorphism and dosage groups.

	Regular dose						Low dose					
	Control (n=47)			Bleeding (n=32)			Control (n=10)			Bleeding (n=7)		
	Wild type	Heterozygote	Homozygote	Wild type	Heterozygote	Homozygote	Wild type	Heterozygote	Homozygote	Wild type	Heterozygote	Homozygote
Patients (n=96), n (%)	32 (68.1)	14 (29.8)	1 (2.1)	20 (62.5)	11 (34.4)	1 (3.1)	6 (60.0)	3 (30.0)	1 (10.0)	1 (14.3)	5 (71.4)	1 (14.3)
Age (year, mean±SD)	61.9±13.0	62.0±13.9	70.8	65.4±13.2	66.7±8.2	78.6	78.1±4.0	73.4±14.8	67.1	46.5	74.5±6.9	78.6
Weight (Kg, mean±SD)	60.8±13.0	64.2±12.8	62.6	63.7±9.2	63.2±9.1	49.1	51.9±10.0	49.2±2.3	72.0	57.0	56.6±11.7	49.1
Dose (mg/week, mean±SD)	30.0±12.6	31.5±10.7	26.0	29.5±9.6	31.3±9.2	8.0	9.2±2.8	6.4±2.2	8.0	9.3	9.9±4.0	8.0
Dose (mcg/kg/week, mean±SD)	505.5±232.9	502.3±163.4	415.3	463.1±127.3	505.8±163.1	162.9	185.1±64.7	130.7±43.5	111.1	163.7	182.5±77.4	162.9
INR (mean±SD)	2.2±0.4	2.0±0.4	1.9	2.7±1.0	2.0±0.7	1.3	2.5±0.4	2.1±0.5	2.4	3.5	2.0±0.6	1.3

Table IV. CYP2E1 allele frequency in different ethnic groups.

Ethnic group	Genotype		Allele frequency	Reference
	c1c1/c1c2/c2c2	(%)		
Korean	67/38/4	(61.5/34.9/3.7)	0.211	This study
Japanese	53/29/4	(61.6/33.7/4.7)	0.215	Watanabe *et al.*, 1995
	103/68/7	(57.9/38.2/3.9)	0.230	Ueno *et al.*, 1996
Chinese	141/88/3	(58.5/36.5/5.0)	0.232	Chao *et al.*, 1995
African-American	45/20/0	(69.2/30.8/0)	0.150	McCarver *et al.*, 1998
Caucacian	54/4/0	(93.1/7/0)	0.034	McCarver *et al.*, 1998
Caucacian (Spanish)	482/33/3	(92.9/6.4/0.6)	0.038	Vidal *et al.*, 2004
Swedish	358/27/1	(92.7/7.0/0.3)	0.038	Persson *et al.*, 1993
Caucacian	65/5/0	(92.9/6.1/0)	0.036	Kim *et al.*, 1995
Caucacian (Australian)	236/1/0	(99.6/0.4/0)	0.002	Griese *et al.*, 2001
Egyptian	231/4/0	(98.3/1.7/0)	0.009	Hamdy *et al.*, 2002
Korean	317/143/21	(65.9/29.7/4.4)	0.192	Lee *et al.*, 1997

genotype distribution need to be further elucidated. Korean population shared very similar allele frequencies with Japanese and Chinese populations in CYP2E1 (Table IV). In addition, the genotype distribution and allele frequency for Asian population were significantly different from Caucasian, African-American, or Egyptian populations. The frequency of *Pst* I/*Rsa* I single nucleotide polymorphism in CYP2E1 was higher in Asian than other ethnic groups.

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