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The Study on Association of Calcium Channel SNPs with Adverse Drug Reaction of Calcium Channel Blocker in Korean

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Abstract – Rapid advances in pharmacogenomic research have provided important information to improve drug selection, to maximize drug efficacy, and to minimize drug adverse reaction. The SNPs that are the most abundant type of genetic variants have been proven as valid biomarkers to give information on the prediction of pharmacokinetic/pharmacodynamic properties of drugs based on genotype. In order to elucidate a correlation between SNPs of calcium channel encoding gene and adverse reactions of calcium channel blockers, we investigated SNPs in CACNA1C gene known as a binding site of calcium channel blocker. 96 patients with hypertension who had taken or are taking an antihypertensive drug, 1,4-dihydropyridine (DHP) were included for analysis. These patients were composed of 47 patients with adverse drug reactions (ADR) such as edema from calcium channel blockers and 49 patients without ADR as a control group. The exons encoding the drug binding sites were amplified by PCR using specific primers, and SNPs were analyzed by direct sequencing. We found that there was no SNP in the exons encoding DHP binding site, but four novel SNPs in the exon-intron junction region. However, four novel SNPs were not associated with the ADR of calcium channel blockers. In conclusion, this study showed that ADR from calcium channel blockers may not be caused by SNPs of the binding sites of calcium channel blockers in CACNA1C gene.

Key words □ Calcium channel blocker, Adverse Drug Reaction, SNP, CACNA1C gene, Korean

INTRODUCTION

Rapid advances in pharmacogenomic/pharmacogenetic research have identified many opportunities for the drug development and personalized treatments for subsets of patients defined as a specific genotype (Lesko and Woodcock, 2004). The constant findings of genetic variants rise interests for the development of personalized treatments and partially, it has been applied in a clinical practice (Marsh and McLeod, 2006). Actually, genotyping tests have been carried out in many clinical trials during drug development and the pharmacogenetic information relevant to the drug has been provided to physicians through Physicians Desk Reference in the Unite States. Recently U.S. Food and Drug Administration have recommended industries to submit pharmacogenetic data for New Drug Application (Little, 2005).

According to age, sex, disease status and so on, there are inter-individual different responses to medicine (Mango *et al*., 2005). And the genetic variations of metabolic enzyme, transporter, receptor and drug target are also one of the reasons to show inter-individual different response. Therefore these genetic variations can provide an important clue that explains the difference in drug efficacy and adverse drug reaction (ADR), and be an important biomarker to predict the different response to the drug (Solus *et al*., 2004).

Hypertension has been listed as one of the major disease in Korean. Calcium channel blockers (CCBs) were the most commonly prescribed for hypertensive patients. However, it is reported that CCBs have produced adverse events such as edema in 15~39% of the patients and that CCBs have been withdrawn in 10% of Korean patients due to ADR (Kim *et al*., 2000).

There are three types of CCBs; phenyl alkylamines, benz (othi)azepines and 1,4-DHPs. Among them, 1,4-DHPs have been the most widely used (De Leeuw and Birkenhager, 2002;

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Muntwyler and Follath, 2001). 1,4-DHPs bind the specific sites of cardiac and smooth muscle and prevent Ca influx into the cells through L-type Ca channel (Barone *et al.*, 1997). Amlodipine and cilnidipine have been known as the third-generation of 1,4-DHP and long lasting antihypertensive effect (Furukawa *et al.*, 1997; Mason *et al.*, 2003; Uneyama *et al.*, 1997).

In order to select the candidate gene to identify the association of adverse reaction from CCBs and genetic polymorphisms, we analyzed the type and frequency of adverse reaction caused by CCBs. Because common ADRs such as edema and flush induced by CCBs could be resulted from the excessive vessel dilation, we investigated the L-type calcium channel containing the binding site of 1,4-DHP. According to the previous study, at least five classes of voltage-gated calcium channels have been identified: L, N, P/Q, R and T type (Ertel *et al.*, 2000). It has been reported that 1,4-DHP binds the L-type calcium channel (Birnbaumer *et al.*, 1994; Hofmann *et al.*, 1994). L-Type channel is the predominant type in heart and vascular tissue and plays a central role in cardiac and smooth muscle excitation-contraction coupling (Birnbaumer *et al.*, 1994). L-Type calcium channel is a complex of the main, pore-forming subunit alpha-1 and four additional subunits: alpha-2, delta, beta and gamma (Uneyama *et al.*, 1997). Among the subtype of alpha-1, 1,4-DHP binds to the alpha 1C subunits (CACNA1C) with high affinity (Birnbaumer *et al.*, 1994; Hofmann *et al.*, 1994). The alpha 1C subunits consist of four homologous repeats (I~IV), each of which is composed of six putative transmembrane segments (S1-S6) (Liu *et al.*, 2000). According to the previous reports, the major binding site of 1,4-DHP is the S5 and S6 transmembrane segment of repeat III, the S6 transmembrane segment of repeat IV, the S5 -S6 linkers of repeat III and IV at the CACNA1C (Hockerman *et al.*, 1997; Regulla *et al.*, 1991; Singer *et al.*, 1991; Striessnig *et al.*, 1998). And these regions are encoded by exon 24, 25, 26, 27, 34, 35 and 36 (Hering *et al.*, 1998).

The primary purpose of this study is to identify the correlation between ADRs produced by CCBs and SNPs of relevant gene. Here we determined specific exons encoding 1,4-DHP binding site in the CACNA1C gene. In addition, we analyzed the exon 8 that is known to have various SNPs.

MATERIALS AND METHODS

Study population

In order to identify the effect of the polymorphism of CACNA1C gene on ADRs of CCBs, a case-control study was conducted from June 2004 to November 2004 in Asan Medical Center (Seoul, Korea). The case was defined as hypertensive patient with adverse reactions against CCBs. Among CCBs, Amlodipine were used for most study subjects (60% and 63%, respectively in the case and control group) and Cilnidipine were taken in 25% and 29% of the case or control subjects. And small number of patients (7 and 4, each of group) were taking other CCBs, such as nifedipine, lercanidipine hydrochloride, felodipine, and diltiazem hydrochloride. Although the study subjects were not taking the same CCB, the proportional distribition of using drug in each group were similar. Thus, it doesn't look like having significant impact on the occurrence of ADR between groups. The recruited cases were from hypertension cohort established from Asan Medical Center in 1999 and 2002. During the research period, 48 cases and 52 controls were enrolled, at the beginning, but one of the case group and three of the control group were not available for genetic analysis. Demographic information, adverse reaction, and blood pressure control were checked using questionnaire and medical chart. Written informed consent approved by the ethics committee of Asan Medical Center was obtained from each subject for participation in this study. Blood was drawn into an EDTA-treated tube and kept in refrigeration and DNA was isolated within 72 hours.

Genomic DNA Isolation

The buffy coat was obtained from whole blood using Histopaque-1077 (Sigma). Then we extracted genomic DNA using a DNA purification Kit (Promega). The isolated DNA was diluted in DNA rehydration solution (Promega) and stored at -20°C before using.

PCR or DNA Amplification

The primers for Polymerase Chain Reaction were presented in Table I. These primers to amplify specific exons of CACNA1C gene were designed from primer 3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Each 20 μl PCR reaction contained 20 ng of genomic DNA, 0.25 μM of forward/reverse primer, 10 mM of each dNTP mixture (Applied Biosystem), 2 μl of reaction PCR buffer with 1.5 mM $MgCl_2$ and 1 unit of AmpliTaq® Gold (Applied Biosystem). PCR was performed at 94 for 2 minutes, followed by 35 cycles at 94°C for 10 seconds, 55~60 for 30 seconds, 72°C for 90 seconds, and final extension at 72°C for 5 minutes. PCR products were confirmed by electrophoresis in 2% agarose gel (Bioneer) stained with ethidium bromide (Nalgene).

DNA Sequencing

Table I. The Primer for PCR amplification

Exon		Primer Sequence	size (b.p)	Exon		Primer Sequence	size (b.p.)
8	F	cct ctg ttc aac cac aga tt	359	27	F	cca agg tca cac agc cag ta	467
	R	ata cag cca gg aat agc aga			R	cag tat ctg cac ggc ctc tc	
24	F	ccc ctc tca gcc caa tta ct	388	34	F	ggt tgc atg gga aga ctg tt	309
	R	ccc tac cct ggt ctg aag gt			R	att cta cca cca ggc cac ag	
25	F	agt gct ggg cta tgg aga tg	334	35	F	gta act ccc tct ggg gaa gg	379
	R	gag ctt cag tgg gca gaa ac			R	tct gat agg gtt gcc ctg ac	
26	F	gaa gtt caa gcc agg cag tc	385	36	F	cag gtt ctc agg ctg gta gg	375
	R	ggg gag gac cca ggt aaa ta			R	att gat cca ccc agt gtt gg	

After individual PCR products displayed a clear single band, PCR products were purified and then sequenced. The proper amount of sample according to the size of PCR fragment was taken for sequencing PCR. The total reaction volume for sequencing was 10 μl in a mixture containing 1 μl of PCR products (10ng/1 μl), 2 μl of Bigdye® (Terminator 3.0 cycle sequencing kit, Applied Biosytem), 1.5 μM of forward or reverse primer. Cycle conditions for sequencing PCR were 1 minute at 96°C for denaturation, 5 seconds at 57°C for annealing, 1 minute at 60°C for elongation for 25 cycles. The products of sequencing PCR were purified using ethanol (Sigma). Then, 10 μl of Hi-Di formamide® (Applied Biosystem) were added and denatured at 95°C for 2 minutes. Sequencing was performed using ABI Avant 3100 DNA Sequencer programmed by BigDye Terminator 3.0 cycle sequencing kit standard (Applied Biosytem).

Detection of SNPs by direct sequencing

The samples were sequenced with BigDye terminator v3.0 cycle sequencing Kit on the ABI Avant 3100 DNA sequencer. Sequences were analyzed by Lasergene software (DNAStar, Madison, WI).

Statistical Analysis

Statistical analysis was performed by HapAnalyzer. This software consists of the following minimum analysis components; Hardy-Weinberg equilibrium (HWE) Test, SNP (or haplotype)-phenotype association, and linkage disequilibrium (LD) test. This software is available from National Geonome Research Institute (http://hap.ngri.re.kr/).

RESULTS

The baseline clinical characteristics for the patients

The baseline clinical characteristics in 100 patients are shown in Table II. BMI and duration of administration in case group are significantly different from control. However, means of age, systolic BP, diastolic BP, kinds of CCBs, smoking and medication didn't show significant differences between case and control. Although three patients of the control group and one of the case group were not available for genetic analysis, they didn't make any difference in the comparison of clinical profiles between groups.

Adverse drug reaction by Ca channel blocker

Table III showed the frequency of adverse reaction followed by each CCB. Multiple answers of ADRs were allowed. As shown in the table III, the most frequent ADRs were edema of lower limb in Amlodipine and flush in Cilnidipine. However, Kim *et al*. (2000) reported that headache was the most common ADR, followed by flushing and edema. These results may be due to the difference in the age of study subjects, since they enrolled relatively younger patients than those in our study and patients has been reported one of factors to affect the incidence

Table II. Baseline characteristics of 100 patients

	Case (n=48)		Control (n=52)		p-value
Age (years)	61.9 ± 9.3		64.0 ± 9.6		
BMI (kg/m^2)	25.0 ± 2.6		26.3 ± 2.5		<.01.
Systolic BP (mmHg)	137.8 ± 17.9		136.3 ± 18.1		
Diastolic BP (mmHg)	81.5 ± 12.7		83.0 ± 9.3		
CCB drugs					
Amlodipine	29	60%	33	63%	
Cilnidipine	12	25%	15	29%	
Others	7	15%	4	8%	
Duration of administration (months)	64.5 ± 10.6		67.4 ± 8.9		<.05
Smoking					
Never	33	69%	40	77%	
Ever	13	27%	6	12%	
Current	2	4%	6	12%	
Medication					
antihypertensive drug	47	98%	51	98%	
non	1	2%	1	2%	

Table III. Adverse reactions against CCBs in hypertensive patients

	Amlodipine (n=29)		Cilnidipine (n=12)		Others (n=7)		Total (n=48)	
	n	(%)	n	(%)	n	(%)	n	(%)
edema (lower limb)	14	(48%)	4	(33%)	2	(29%)	20	(42%)
flush	8	(28%)	5	(42%)	1	(14%)	14	(29%)
headache	4	(14%)	3	(25%)	2	(29%)	9	(19%)
edema (other)	5	(17%)	4	(33%)	0	(0%)	9	(19%)
gingival hyperplastica	6	(21%)	0	(0%)	0	(0%)	6	(13%)
palpitation	1	(3%)	2	(17%)	2	(29%)	5	(10%)
dizziness	2	(7%)	0	(0%)	1	(14%)	3	(6%)
infirmity	0	(0%)	1	(8%)	1	(14%)	2	(4%)
pruritus	1	(3%)	0	(0%)	0	(0%)	1	(2%)
chest discomfort	1	(3%)	0	(0%)	0	(0%)	1	(2%)
abdominal distention	1	(3%)	0	(0%)	0	(0%)	1	(2%)

of ADRs (Aellig, 1998).

Sequencing Results

The overall genotype distribution of the CACNA1C gene was in Hardy-weinberg equilibrium. Because common ADRs such as edema and flush induced by CCBs could be resulted from the excessive vessel dilation, we investigated the L-type calcium channel containing the binding site of 1,4-DHP.

L-Type calcium channel is a complex of the main, pore-forming subunit alpha-1 and four additional subunits: alpha-2, delta, beta and gamma (Uneyama *et al*., 1997). Among the subtype of alpha-1, 1,4-DHP binds to the alpha 1C subunits (CACNA1C) with high affinity (Birnbaumer *et al.*, 1994; Hofmann *et al*., 1994). Thus, we expected that some SNPs in this gene might be involving ADRs. However, There was no SNP within exons encoding the binding regions of 1,4-DHPs in this study. In stead, we found four novel SNPs in the flanking regions of Exon 24, 25, and 36 and we analyzed their association with the presence of ADRs in each groups as shown in Table IV. And we couldn't find out any SNPs in exon 8. These results suggest that there was no relationship between the SNP encoding the binding site of 1,4-DHPs and ADRs from 1,4-DHPs in Korean.

DISCUSSION

The person-to-person variability of drug response is a major problem in clinical practice (Meyer, 2000). It can lead to adverse drug reactions in individual or subpopulations of patients (Meyer, 2000). Recently, it has become clear that the genetic polymorphism may significantly modify drug responses or increase the risk for ADRs (Pirmohamed and Park, 2001; Schmitz *et al.*, 2001). Many researchers are trying to find valid genetic biomarkers to predict the efficacy or toxicity of drug. Therefore, SNPs can be very useful genetic biomarkers to identify the association of ADR with relevant or susceptible genes (Lee *et al.*, 2003). Actually, the studies on pharmacogenomics have become an essential work frame and have significant influences on drug development (Ginsburg *et al.*, 2005; Guo *et al.*, 2005).

Through retrospective pharmacogenomic studies, many patients can be protected from ADRs. For examples, drugs metabolized by CYP2D6 such as haloperidol and codeine vary from increased risk of ADRs at recommended doses in poor metabolizers (Linder *et al*., 1997). Genetic variants of CYP2C19 enzyme may cause differences in metabolism of omeprazole. Polymorphisms of thiopurine S-methyltransferase

Table IV. The detected SNPs in the CACNA1C gene (N= 96)

SNP location	Group	Control (N= 49)			Case (N= 47)		
	Genotypes	w/w	w/m	m/m	w/w	w/m	m/m
	Variation	N (%)			N (%)		
IVS24+85C/T	C>T	48 (98)	1 (2)	0 (0)	47 (100)	0 (0)	0 (0)
IVS25+139T/C	T>C	20 (41)	19 (39)	10 (20)	17 (36)	23 (49)	7 (15)
IVS35-80C/A	C>A	5 (10)	13 (27)	31 (63)	5 (11)	12 (25)	30 (64)
IVS36+47G/A	G>A	47 (96)	2 (4)	0 (0)	46 (98)	1 (2)	0 (0)

reduce biotransformation of thiopurine drugs such as azathioprine, and 6-mercaptopurin can lead to potentially fetal hematopoietic toxicity in some patients (Relling *et al.*, 1999).

Until now, pharmacogenetic study is somewhat limited in the research for the relationship of pharmacokinetic parameter and genetic polymorphism. Although many researchers have tried to elucidate the correlation between SNPs and ADRs, there is a little evidence less than we expected. It is assumed that adverse drug events are not caused by simple biological mechanism which involves abnormal gene expression failure (Licinio and Wong, 2002). In this study, we wanted to find useful information for hypertensive patients who have experienced adverse drug reaction with CCBs, since CCBs were one of the most commonly prescribed antihypertensive agents in Korea (Kim *et al*., 2000). It also has been reported that the incidences of ADRs associated with antihypertensive agents were different by race, age and drug class (Aellig, 1998). Thus, we evaluated baseline characteristics of study subjects to exclude any possibility of differences coming from their characteristics (Table II). And we confirmed that their age or the proportion of prescribing drugs class were not different between the case and the control group. Here we investigated a correlation between SNPs of CACNA1C, calcium channel encoding gene and CCB's adverse reactions.

CYP3A4, a metabolizing enzyme for most calcium channel antagonist can be a potential candidate gene. Because genetic variants of metabolic enzymes such as CYP2D6, CYP2C19 and CYP2C9 have been known to affect the activity of these enzymes, and to be responsible for most ADRs demonstrated (Nelson *et al*., 1996), metabolic enzymes can be proper targets to identify the cause of adverse reactions (Dalen *et al*., 2003; Koytchev *et al*., 1998). However, few functional polymorphisms are known for the CYP3A4, and CYP3A4 activity is highly variable among patients even in the absence of known mutations (Nakagawa and Ishizaki, 2000; Phillips *et al*., 2001; Severino and Del Zompo, 2004; Torpet *et al*., 2004). Therefore, we focused on the CACNA1C gene encoding the binding site of cacium channel antagonist.

Although we couldn't find SNPs in the investigated exons, we found four novel SNPs in the exon-intron junction region. It is known that SNPs which associate directly with phenotype usually exist within the exon. However, SNPs to exist intronic gene regions may have a significant role because these SNPs can provide an important clue through linkage disequilibrium (LD) and they also have functional consequences. In addition, it also has been known that SNPs that lie close to intron-exon boundaries can affect the gene splicing or the gene expression. Actually, splicing-site mutations in the SURF1 gene result in loss of SURF1 protein and develop Lei syndrome (Pequignot *et al*., 2001). And intronic sequences in BRACA1 gene have been demonstrated to have transcriptional repressor activity (Suen and Goss, 2001). Herein, we couldn't demonstrate any association between SNPs we found and the occurrence of ADRs. However, it still needs to be further investigated whether novel SNPs has any functional change of gene activity by in vitro transcriptional study.

Although several studies have reported that genes encoding calcium channel have diverse genetic polymorphism, there have been no reports regarding associations between SNP in gene encoding calcium channel and ADRs from CCBs. One study reported that several SNPs in exonal regions has been associated with the efficacy of antihypertensive agents. Thus, ADRs or efficacy from antihypertensive agents might be clearly associated with several known SNPs on exons based on previous studies. And in this study, we couldn't find previously known SNPs on exons in Korean and this might be the reason we couldn't find any association. In conclusion, our results suggest that other novel SNPs on flanking regions in Korean were not associated with adverse reaction caused by CCBs, even if it still need to be required to explore in more depth by further detailed study.

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