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Calcium/calmodulin-dependent protein kinase IV gene polymorphisms in Korean alcohol-dependent patients

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ABSTRACT

Introduction: A relationship between alcohol dependence (AD) and calcium/calmodulin-dependent protein kinase IV (CAMKIV) has been reported in a whole genome study of Korean AD patients. The purpose of the present study is to compare the frequency of CAMKIV genotypes and alleles between AD and control subjects in Korea.
Methods: The present study includes 281 AD patients and 139 control subjects. Seven single nucleotide polymorphism of CAMKIV gene known to show significant separation ratio in Asians were searched in SNP database and previous studies related to CAMKIV gene. Polymerase chain reaction and restriction fragment length polymorphism techniques were used to analyze genotype of CAMKIV gene SNPs.
Results: Major TT genotype and T allele frequencies of rs 25917 in AD patients were significantly higher than those of control subjects (genotype frequency, $p = 0.002$; allele frequency, $p = 0.001$). Major CC genotype and C allele frequencies of rs 117590959 in AD patients were also significantly higher than those of control subjects (genotype frequency, $p < 0.001$; allele frequency, $p = 0.001$). Major genotypes of rs25917 ($p = 0.002$, odd ratio: 3.13, 95% CI: 1.54–6.38) and rs11790959 ($p = 0.002$, odd ratio: 3.22, 95% CI: 1.52–6.81) showed significantly higher odds ratios associated with AD than minor genotypes in logistic regression.
Discussion: These results suggest that CAMKIV might be a candidate AD gene. Further research is needed to determine the precise relationship between CAMKIV and AD and the function of each SNP.

1. Introduction

Much effort has been focused on identifying the mechanisms by which alcohol affects the brain to cause a change in behaviors (Valenzuela, 1997). An important finding is that alcohol can influence the function of specific neurotransmitters. However, the function of neurotransmitters and their receptors cannot fully explain this complex illness (Valenzuela, 1997). Although behavioral and psychological effects of alcohol intake have been determined, the molecular mechanisms by which alcohol influences cellular function and behavior remain unclear (Balino et al., 2014). Neuroadaptational changes induced by the intake of alcohol might be associated with dysregulation of cell signaling and changes in gene transcription and expression (Moonat et al., 2010). For this reason, research studies have been conducted to identify the cell signaling pathways associated with alcohol.

Calcium/calmodulin-dependent protein kinase IV (CAMKIV) is one of the factors. CAMKIV is the only CREB-phosphorylating protein kinase that is found predominantly in the nuclei of neurons (Bilbao et al., 2008). It regulates the function of CREB and the transcription of CREB targets, such as neuropeptide Y, brain-derived neurotrophic factor, corticotropin-releasing factor, and activity-regulated cytoskeleton- associated protein, which might play important roles in alcohol-induced molecular changes and genetic predisposition to AD (Moonat et al., 2010). Some previous animal studies suggest that CAMKIV might be associated with the effects of alcohol and AD. For example, alteration of exploratory behavior in rats caused by chronic alcohol intake might entail the downregulation of CAMKIV in nucleus accumbens. Moreover, naloxone may reduce alcohol intake by antagonizing the downregulation of CAMKIV in the nucleus accumbens (Li et al., 2008).

Meanwhile, genetic linkage and association studies have investigated the causes and risk factors of AD. Some linkage studies have shown a risk locus on chromosomes 4q, 4p, 1q, 2q35, 13q, 22q, 2q, 9q, 19p and so on (Kimura and Higuchi, 2011; Rietschel and Treutlein, 2013; Samochowiec et al., 2014). Most of the risk genes found in candidate gene association studies have been from the dopaminergic, serotonergic, GABAergic, cholinergic, ethanol metabolic pathway and opioidergic pathway (Zuo et al., 2014). In Korea, several studies have been conducted to clarify the relationship between Korean alcohol-

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dependent subjects and polymorphism of mu opioid receptor gene, aldehyde dehydrogenase gene, dopamine transporter gene, dopamine D2 receptor gene, ankyrin repeat and kinase domain containing 1 gene etc (Choi et al., 1999; Kang et al., 2009; Kim et al., 2003; Lee et al., 2013). As can be seen from polymorphism of ADH, ALDH, OPRM, 5-HTTLPR genes which showed different allele frequencies among Asians, Caucasians, and Africans (Enoch, 2014), ethnic differences are also important variables in genetic studies. Interestingly, relevance between AD and CAMKIV has also been mentioned in a whole genome study of Korean AD patients. A previous genetic study (Park, 2005) in Korea carried out whole-genome sequencing for alcohol-dependent subjects and control subjects, and it suggested six high-ranking candidate loci. In that study, CAMKIV gene and CREB binding protein gene were located in close proximity to the second and fifth candidate locus, respectively. A relationship between AD and cell signaling cascades involving CAMKIV or CREB has been suggested.

As mentioned above, CAMKIV might be associated with the risk of AD. However, no human genetic study of CAMKIV for AD, particularly in Korean AD patients, has been reported. Therefore, the objective of this study was to compare the frequencies of **single nucleotide polymorphism** (SNP) genotypes and alleles of the CAMKIV gene between Korean AD patients and control subjects.

2. Materials and methods

2.1. Subjects

Subjects who were diagnosed as AD by a psychiatrist according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), were enrolled as patients. All patients were inpatients in a closed psychiatric ward. Among them, patients with other substance use problem (except nicotine and caffeine) or major psychiatric problems such as schizophrenia or major mood disorder were excluded. The total number of patients in the AD group was 281 (218 males and 63 females).

The control group comprised individuals attending hospital for regular medical check-ups. These subjects had not drunk more than five standard drinks per month on average. They were > 50 years in age. Such age and alcohol drinking history were chosen based on the fact that they would have enough chance and exposure to alcohol. The total number of subjects in the control group was 139 (80 males and 59 females). The present study was approved by the Institutional Review Board (IRB) of Pusan National University Yangsan Hospital.

2.2. Analysis of CAMKIV gene SNPs

2.2.1. SNPs of the CAMKIV gene

SNPs of CAMKIV with significant separation ratio in Asians were searched against the SNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) and previous studies of the CAMKIV gene. Among these, seven SNPs (rs553763462, rs25917, rs544431829, rs3797740, rs3733995, rs10491334 and rs117590959) that could be analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) were selected. Locations of these SNPs in the CAMKIV gene are summarized in Table 1. All seven SNPs were located in introns.

2.2.2. Analysis of CAMKIV gene SNP genotype and allele frequency

After separation and purification of genomic DNA, PCR was performed to identify CAMKIV genotypes. The PCR reaction comprised of 1.5 mM $MgCl_2$, 200 μM dNTP (dATP, dCTP, dGTP, and dTTP), 0.4 μM forward and reverse primers for each SNP, 25 ng of genomic DNA, and 1 unit of Taq polymerase (Bionics, Canada) in 20 μl of reaction mixture. PCR program included an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. For amplification of rs117590959, rs10491334, rs553763462, rs3797740, and rs544431829, the annealing temperature was set at 54 °C. For PCR amplification of rs25917 and rs3733995, annealing temperature was set at 51 °C and 48 °C, respectively. PCR products (2 μl) were subjected to 2% agarose gel electrophoresis. PCR amplified products of rs117590959 and rs10491334 were of 499 bp in size. Amplification products of rs553763462, rs3797740, and 544431829 were of 383 bp in size. The size of rs25917 was 323 bp. The amplification product of rs3733995 was 209 bp in size.

Table 1
Nucleotide variation and positions of the CAMKIV gene.

SNP/InsDel ID	Nucleotide variation	Position on chromosome 5 (NCBI mapviewer)	Localization
rs117590959	C to T	111,436,459	Intron
rs10491334	C to T	111,436,706	Intron
rs553763462	A to C	111,446,445	Intron
rs3797740	G to A	111,446,468	Intron
rs544431829	C to T	111,446,634	Intron
rs25917	C to T	111,473,151	Intron
rs3733995	A to G	111,482,723	Intron

InsDel: insertion/deletion, NCBI: National Center for Biotechnology Information.

2.2.3. Digestion of PCR products with specific restriction enzyme

Amplified CAMKIV genes of AD and control subjects were analyzed with restriction fragment length polymorphism (RFLP) technique. Some data could not be analyzed exactly. However, the number of such cases was small. Their effects on the main results could be ignored. The amplified product of SNP rs117590959 (499 bp) was digested with 2.5 units of *Bsr*I endonuclease at 65 °C for 30 min. The band pattern was then assessed after performing 3% agarose gel electrophoresis at 200 V for 2 h. Rs117590959 products of 248, 145, and 106 bp were interpreted as C/C genotype. Products of 248, 177, 145, 106, and 41 bp were interpreted as C/T genotype. Products of 177, 145, 106, and 41 bp were interpreted as TT genotype. The same technique was used to analyze other SNPs.

3. Statistics

Patients' baseline and clinical characteristics were summarized as descriptive statistics. Chi-squared test was used to compare categorical variables between groups while independent *t*-test was used to compare continuous variables between groups. Chi-squared test was also used to determine whether genotype distributions were in Hardy-Weinberg equilibrium (HWE).

SNP genotype and allele frequency (rs553763462, rs544431829, rs10491334, rs3733995, rs3797740, rs25917 and rs117590959) distributions were compared between AD patients and controls using chi-squared test or Fisher's exact test. Significant variables were subjected to logistic regression analysis. All p values of less than 0.05 were considered statistically significant. Bonferroni's correction (*Pc*) of significance level was applied for multiple testing. *Pc*-values were counted by dividing p-value by the number of SNPs tested. All statistical analyses were carried out using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp, Armonk, NY, USA).

4. Results

4.1. Demographic and clinical characteristics of AD patients

The demographic and clinical characteristics of AD patients are summarized in Table 2. The mean age of AD patients was 46.8 ± 9.5 years. The mean age of starting drinking was 21.3 ± 6.7 years. Female AD patients started drinking significantly later than male AD patients (female AD = 26.3 ± 9.9 vs male AD = 19 ± 4.6, $p < 0.001$). The mean number of admissions due to

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