European Review for Medical and Pharmacological Sciences 2019; 23: 4348-4353

Association between ATP2B1 gene polymorphism and the onset of cerebral infarction

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Abstract. – OBJECTIVE: The aim of this study was to investigate the correlation between adenosine triphosphate (ATP) 2B1 gene polymorphism in cerebral infarction (CI) patients and the onset of CI.

PATIENTS AND METHODS: A total of 100 CI patients (CI group) and 88 healthy people who received physical examination (Control group) were enrolled as study subjects. Meanwhile, 4 mL of venous blood was extracted from each subject. The single nucleotide polymorphisms of rs19203, rs13412 and rs28313 in the promoter region of adenosine triphosphate (ATP) 2B1 gene were classified via conformation-difference gel electr[illegible]resis. Chi-square was adopted to test whe[illegible] frequency of ATP2B1 genotype distributio[illegible]n-formed to genetic equilibrium law. Meanwhil[illegible] correlations between ATP2B1 alleles and g[illegible] polymorphism sites and the onset of CI were a[illegible]alyzed. Enzyme-linked immu[illegible]t assa[illegible] (ELISA) was performed to de[illegible]ine level of vascular endothelial growt[illegible]tor (V[illegible]) in the serum of CI patients. Fu[illegible]rmore, [illegible]rela-tion of ATP2B1 gene poly[illegible]his[illegible] pression level of VEG[illegible]as a[illegible]

RESULTS: Hardy[illegible]mberg e[illegible]rium analysis revealed that [illegible]olymorphisi[illegible]hree AT-P2B1 gene loci [illegible]accordance [illegible] genetic equilibriu[illegible]stri[illegible] ($p>0.05$). According to the resul[illegible] of genetic [illegible]ation analysis, the polymor[illegible]ms and allele[illegible]TP2B1 rs19203 and rs1[illegible] were statistically [illegible]related with the onset[illegible]CI ($p<0$[illegible]). However, the rs28313 polymo[illegible]m an[illegible]eles were not correlated with the [illegible]o[illegible] ($p>0.0$[illegible]n addition, a statistically s[illegible]nt cor[illegible]ion between the polymo[illegible]hism [illegible]s19[illegible] and rs13412 and the expr[illegible]on lev[illegible]F was found in CI patients ($p<$[illegible]).

CONCLUSIONS: Rs19203 and rs13412 in the p[illegible]n of the ATP2B1 gene are correlated with the onset of CI. However, rs28313 bears no relationship with CI.

Keywords:
ATP2B1, Polymorphism, Cerebral infarction (CI).

Introduction

Cerebral infarction (CI) or ischemic cerebral stroke, refers to [illegible]erebral tissue necrosis caused by the blockag[illegible]upplying vessels for ce[illegible]ssues, or cere[illegible] ischemia-induced c[illegible]ral stroke[1]. CI is a severe disease with a [illegible] incidence [illegible] mortality and disability rate. [illegible]efore, it has [illegible]come a major public hygienic [illegible] endang[illegible]g the health of people all over the [illegible]een recognized that risk factors for CI [illegible] age, sex, hypertension, blood lipid abnormalities, obesity, diabetes mellitus, smoking, intemperance. However, even with similar environmental factors, the incidence rate of CI varies among different populations worldwide. This suggests that genetic factors may play a certain role in the onset of CI[3-5].

Adenosine triphosphate (ATP) 2B1 can encode plasma membrane calcium ion transporting ATPase 1. Meanwhile, it maintains intracellular calcium homeostasis by regulating the concentration of calcium ions. Therefore, ATP2B1 is critical for depolarization after muscle contraction[6]. Studies[7] have manifested that ATP2B1 is closely correlated with the occurrence and development of hypertension. The possible underlying mechanism may be associated with the effect of ATP2B1 on vascular remodeling in the resting state. In Indian populations, the polymorphism of ATP2B1 rs2681472 is closely related to the occurrence of essential hypertension. The TT genotype and T allele of rs2681472 are risk factors for essential hypertension among Indian women[1]. Lin et al[2] have shown that people with ATP2B1 rs17249754 carrying major alleles, especially those ingesting low calcium and high Na/K, are more vulnerable to hypertension.

Currently, the correlation between ATP2B1 gene polymorphism and the occurrence and prognosis of cerebral infarction has not been report-

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ed yet. In this work, the distribution of ATP2B1 gene polymorphism and allele genotype among CI patients and healthy people was analyzed. Our study might provide certain reference for further exploring the genetic pathogenesis of CI.

Patients and Methods

Patients

A total of 100 CI patients treated in our hospital from January 2015 to November 2017 were selected as study subjects, including 48 males and 52 females aged (57.81±12.74). All CI patients manifested typical neurological deficits. Meanwhile, responsible foci were discovered through imaging examinations, including computed tomography (CT) and (or) magnetic resonance imaging (MRI). The diagnostic standard was based on the China Guidelines for the Diagnosis and Treatment of Acute Ischemic Cerebral Stroke (2014). Exclusion criteria were as follows: 1) patients with coronary heart disease and peripheral arteriovenous thrombosis, 2) patients who suffered from heart, liver, kidney or lung dysfunctions or s re infection, 3) patients who took vitamin B ly, or 4) patients who experienced cardi ic cerebral embolism. During the same perio healthy people who received physical exam tions were enrolled as healthy co includi 40 males and 48 females age 41). Al these healthy controls did n port c o-cerebrovascular diseases pre y. 4 m blood was first extract ac ter that, it was antico ated dium citrate and cryopreserved a refrige -20°C for standby use. Thi was approv he Ethics Committe the ital. Informed consent was obtained om each t before the study.

Main gents

A se (Bio Agarose), primers (BGI), deox ucle id (DNA) extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), GoldViwe (Solarbio, Beijing, China), and 50×TAE (Beijing ComWin Biotech Co., Ltd., Beijing, Chi

DNA Extraction

4 mL of blood sample was fir lected from each subject. Subsequently, it wa oagulated with ethylenediaminetet cetic a DTA; BD Biosciences, Frankli kes, NJ, U sub-packaged in 500 ppend f (EP; burg, Germany) tube media after shaking evenly. Genomic DN cted a ding to the instructio DN ction k angen Biotech, Beiji China). 2 f sample was weighe arose gel el oresis with 1.5% aga ge concentrati of extracted DNA was easure ultraviolet spectrophotomet Optical dens $OD)_{260}/OD_{280}$ ratio of 1.8 icated high pu of DNA, and these s les were applied in subsequent sequencing eriments.

erase C n Reaction (PCR) Am

Prime rs19203, rs13412 and rs28313 in the romoter region of ATP2B1 gene were designed y for amplification. All primer sequences ere own in Table I. Polymerase Chain Reaction (PCR) system was as follows: 2.0 μL of DNA template, 10.0 μL of MIX (2 ×), 0.4 μL of forward primer, 0.4 μL of reverse primer and 7.2 μL of ddH_2O. The PCR amplification conditions were: 95°C for 120 s, 94°C for 30 s, 57°C for 90 s, a total of 30 cycles followed by extension at 72°C for 10 min. Subsequently, the amplification of the gene segment was detected *via* AGE.

Ligase Detection Reaction

Forward and reverse probes used in this reaction were designed and synthesized by BGI. After phosphorylation modification at the 5' end, all forward probes were prepared into 12.5 pmol/μL of probe mixture. The ligase detection reaction

Prime and product size of different sites in the promoter region of ATP2B1 gene.

Sit	Primer sequence	Product (bp)
205	Forward: AGCTGGACCCGGCTGAGGAGG Reverse: TGGGCTAGCTGGCGTAGGGCA	245
3412	Forward: AAGTCGACGTTTTCGATCCCC Reverse: GTGACTTAGGCGATGCTGAT	301
rs2 3	Forward: AGGCAACGATCGTAGCTAG Reverse: ACGGGCTAGTCGTAGCTACG	371

Table II. Ligase reaction probe sequences and product size of different ATP2B1 gene sites.

Site	Probe	Probe sequence 5′-3′	Product (bp)
rs19203	rs19203	PCCGATGCTAGCTTTTTTTTTTTTTTT-FAM	
	rs19203-C	TTTTTTTTTTTTTTACCCATTTTTTTTAT	
	rs19203-T	TTTTTTTTTTTTTTGCGACGAGCATTTTTTTTAAA	
rs13412	rs13412	P-AGTTTCCCAATTTTTTTTTTTTTTTTT-FAM	
	rs13412-A	ACGGGACTTTTTTTTTTTTTTTTTTTTCGTAGCTAAAC	
	rs13412-T	TGCCAAATTTTTTTTTTTTTTTTTTTTTACGATCGA	102
rs28313	rs28313	P-ACGGGATGCCATTTTTTTTTTTTTTTTT-FA	
	rs28313-A	ACGGGACTTTTTTTTTTTTTTTTTTTTGCGACC	
	rs28313-C	GGCTATCGGATTCTTTTTTTTTTTTTTGCGC	98

system (3.05 μL) was as follows: 0.05 μL of ligase, 1 μL of buffer solution, 1 μL of PCR product and 1 μL of probe mixture. PCR amplification conditions were as follows: 95°C for 120 s, 94°C for 15 s, 50°C for 25 s, a total of 30 cycles. After all cycles, the concentration was determined by an ultraviolet spectrophotometer. Then, BIG was entrusted to perform sequencing and segment analysis for target genes. All data were analyzed using GeneMapper (Applied Biosystems, Foster City, CA, USA) (Table II).

Determination of Serum Level of Vascular Endothelial Growth Factor (VEGF) via Enzyme-Linked Immunosorbent Assay (ELISA)

(1) 3 mL of blood samples were collected from each subject. (2) A standard solution was prepared according to the instructions of the kit (R&D Systems, Minneapolis, MN, USA). The standard sample and blood samples were added to each reaction well together. (4) Streptavidin-HRP (Horse Reddish Peroxidase) was added for incubation. (5) Obtained products were washed and developed. (6) After adding the stop solution, absorbance was measured by the ultraviolet spectrophotometer.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 22.0; IBM, Armonk, NY, USA) was used for the statistical analysis. Enumeration data and measurement data were expressed as frequency and percentage and mean ± standard deviation, respectively. After the frequency of genotypes was calculated in each sample, it was tested with Hardy-Weinberg genetic equilibrium formula. The χ^2-test was performed for multiple comparisons of enumeration data. The *t*-test and analysis of variance for measurement data. $p<0.05$ was considered statistically significant.

Results

Basic Clinical Information in CI and Control Groups

The basic clinical information of the two groups was shown in Table III. Compared with the control group, patients in the CI group exhibited significant differences in smoking history, drinking history, diastolic pressure, systolic pressure, and fasting blood glucose ($p<0.05$). However, there were no statistical differences in age, sex composition, body mass index (BMI) and blood lipid level between the two groups ($p>0.05$).

Analysis of rs19203, rs13412 and rs28313 in the Promoter Region of ATP2B1 Gene

Rs19203, rs13412 and rs28313 in CI and Control groups were first cut off using BstUI restriction endonuclease. Rs19203 showed two alleles of C and T and three genotypes of CC, CT and TT. Rs13412 showed two alleles of A and T and three genotypes of AA, AT, and TT. Meanwhile, rs28313 showed two alleles of A and C and three genotypes of AA, AC and CC.

Hardy-Weinberg Equilibrium Test

Hardy-Weinberg equilibrium formula was utilized to detect linkage disequilibrium at different ATP2B1 gene sites. As shown in Table IV, all sites were in accordance with the equilibrium law ($r^2<0.33$).

Table III. Basic clinical information of study subjects.

Variable	Control group (n=100)	CI group (n=88)	χ^2	
Age (years old)	57.81±12.74	57.36±11.41	1.231	0.371
Sex (male/female)	48/52	40/48	2.991	0.459
BMI (No less than 24 kg/m^2, %)	47.00	53.94	1.66	0.249
Smoking history (%)	28.00	13.83	11.241	0.002*
Drinking history (%)	36.00	12.58	.642	.03*
Diastolic pressure (mmHg)	89.14±12.94	78.36±14.52	26	
Systolic pressure (mmHg)	161.34±7.38	124.54±12.57	2.834	0.
Blood lipid level (mmol/L)				
TC	5.00±1.07	5.21±1.61	2.3	0.631
TG	1.54±0.27	1.58±0.44	1	4
LDL	3.82±1.23	3.43±1.92	3	03
HDL	3.25±0.55	3.42±0.81	9	.091
Fasting blood glucose (mmol/L)	7.88±1.21	5.67±1	1	0.001*

Note: TC: total cholesterol, TG: triacylglycerol, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

Correlation Between ATP2B1 Gene Polymorphism and CI

The frequency of polymorphism genotypes of all sites in the two groups was shown in Table V. It was revealed that the polymorphism of rs19203 and rs13412 was markedly associated with the onset of CI (p<0.05). However, there was no significant correlation between rs28313 and the CI (p>0.05).

Correlation Between ATP2B1 Allele Genotypes and the Onset of CI

The frequency of allele genotypes of three sites in CI and control groups was displayed in Table VI. The results revealed that the genotypes of rs19203 and rs13412 alleles exhibited a relationship with the onset of CI. However, the genotypes of rs28313 alleles was not correlated with CI (p>0.05).

Association between Different ATP2B1 Gene Site Genotypes and the Expression Level of VEGF in CI Patients

Based on the average level of VEGF (12.25±2.84) in the serum of CI patients, 100 CI patients were divided into the high expression group and low expression group. The distribution of three sites in the high expression group and low expression group was analyzed. The results indicated that the polymorphisms of rs19203 and rs13412 were significantly correlated with the expression level of VEGF in CI patients (p<0.05) (Table VII).

Table results of age equilibrium among all ATP2B1 ge

Si	r^2		
	rs19203	rs13412	rs28313
9203	-	0.009	0.245
3412	0.009	-	0.189
13	0.245	0.189	-

Note: TC: total cholesterol, TG: triacylglycerol, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

Discussion

Cerebrovascular disease is one of the most common causes of death in China[10]. CI, a kind of irreversible brain tissue injury, is mainly characterized by a high incidence rate, disability and mortality rate. Studies have suggested that CI results from synergistic effects of multiple factors, including environment, heredity and vascular change. However, its specific molecular pathogenesis has not been fully revealed yet[11-13]. Therefore, the identification of genes susceptible to the onset of CI is of great significance for early diagnosis, precision treatment and prognosis of patients.

Current studies have revealed the correlations of numeral genes with the onset of CI, such as VEGF[14], MCP[15], and SNHG 14[16]. All these genes play critical roles in cerebrovascular diseases, either by acting as transcription factors or by expressing important cytokines and structural proteins. Hence, the polymorphism of these genes may have certain effects on the occurrence and development of CI. For example, the polymorphism of the lipoprotein lipase (LPL) gene is correlated with atherosclerosis-induced CI in Japanese patients. The polymorphism of the LPL gene Hind III site is statistically correlated with the

Table V. Distribution of different genotypes of ATP2B1 gene sites in CI.

Group	rs19203			rs13412			rs28313		
	CC	CT	TT	AA	AT	TT	AA	AC	CC
CI group	35.2%	60.0%	4.8%	10.1%	50.9%	39.0%	20.1%	[illegible]%	29.0%
Control group	23.5%	59.4%	17.1%	24.0%	51.2%	24.8%	19.3%	[illegible]%	29.5%
χ^2		2.341			1.642			0.[illegible]	
p		0.018			0.021			0.53[illegible]	

Table VI. Distribution of allele genotypes of ATP2B1 gene sites in CI.

Group	rs19203		rs13412		rs28313	
	C	T	A	T	[illegible]	C
CI	75.21%	24.79%	30.00%	[illegible]%	45.23%	54.77%
Control group	76.22%	23.78%	82.11%	[illegible]89%	42.08%	57.92%
χ^2	2.131		1.829		0.734	
p	0.013		[illegible]		0.721	

Table VII. Association between different genotypes of ATPB21 g[illegible]tes and the ex[illegible]ion level of VEGF in CI patients.

Group	rs19203			[illegible]			rs28313		
	CC	CT	T[illegible]	AA	AT	TT	AA	AC	CC
High expression group	41.8%	52.0%	6.[illegible]	3[illegible]	[illegible]%	46.0%	23.1%	50.9%	26.0%
Low expression group	20.6%	55.4%	24.[illegible]	[illegible]	[illegible]%	23.2%	20.8%	50.2%	29.0%
χ^2		12.811			12.821			0.542	
p		0.[illegible]			0.016			0.738	

occurrence of cerebrova[illegible] dise[illegible] 0.031, 0.234 *vs.* 0.169). Howe[illegible] is[illegible] in PvuII between p[illegible]ts w[illegible]ebrovascular disease and health[illegible]ople who [illegible]d physical examinations[17]. [illegible]mi et al[28] in [illegible]panese population ha[illegible]ou[illegible]at the poly[illegible]orphism of transfor[illegible]ing growth [illegible] (TGF)-β1 T868C gene has [illegible]otential corre[illegible] with the onset of CI [illegible]nts with type 2 dia[illegible]tes. Additionally, th[illegible]sults [illegible]D40 mRNA expression level in [illegible]erip[illegible] blood [illegible]f Chinese people have show[illegible]e poly[illegible]phism of CD40-1C/T [illegible]losely [illegible]siate[illegible]th the occurrence of CI. [illegible]hile, [illegible]f this site can not only increa[illegible]he risk [illegible] occurrence, but also regulate the [illegible] expression of CD40[19]. In the present [illegible]iation between the polymorphisms [illegible]rs19203, rs13412 and rs28313 in the ATP2B1 [illegible]oter region among Chinese populations and C[illegible]rrence was analyzed. A peripheral blood samp[illegible]e was collected from healthy people receiving physical examinations and CI patients, and whole genome DNA was extracted. Genotyping was first performed for target gene sites, and statistical analysis was conducted for the distribution of genotype frequency of all sites and their alleles. The results demonstrated that the genotype and gene polymorphisms of ATPB21 rs19203 and rs13412 had significant correlations with the occurrence of CI ($p<0.05$). People with rs19203 genotype CC were more likely to suffer from CI than those with genotype TT. However, people with rs13412 genotype TT were more vulnerable to CI than those with genotype AA ($p<0.05$). However, rs28313 genotype and gene polymorphisms were not substantially correlated with the occurrence of CI ($p>0.05$). In addition, there was a correlation between the polymorphisms of rs19203 and rs13412 and the expression level of VEGF in CI patients ($p<0.05$). Nevertheless, there were some limitations in this study: 1) the clinical sample size was relatively small, 2) this study lacked prognostic information of CI patients because of the loss of follow-up information, and 3) only the

Chinese population was taken as study subjects, so the results might vary among Europeans and Americans.

Conclusions

The present work found that there were correlations of rs19203 and rs13412 in ATP2B1 gene promoter region with the occurrence of CI. Both Han people with rs19203 genotype CC and those with rs13412 genotype TT were more likely to be attacked by CI. However, there was no correlation between gene polymorphism of rs13412 in the promoter region and CI occurrence.

Conflict of interest

The authors declare no conflicts of interest.

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