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Correlations of endothelin-1 gene polymorphisms and hypertensive intracerebral hemorrhage

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Abstract. – OBJECTIVE: The aim of this study was to investigate the correlations of endothelin-1 (ET-1) gene polymorphisms with the occurrence of hypertensive intracerebral hemorrhage (HICH).

PATIENTS AND METHODS: In this case-control study, 100 HICH patients and 100 controls with matched race, age and gender were enrolled as research subjects. Single nucleotide polymorphisms (rs1920453, rs1022436 and rs1035627) in the promoter region of ET-1 gene were typed *via* conformational difference gel electrophoresis. Whether the distribution frequency of ET-1 genotypes conformed to Hardy-Weinberg equilibrium was evaluated by chi-square test. The correlations of different gene polymorphisms and alleles in the promoter region of ET-1 gene with the occurrence of HICH were analyzed. Furthermore, the associations of rs1920453 polymorphism in the promoter region of ET-1 gene with neurological deficit scores and laboratory parameters of HICH patients were explored.

RESULTS: It was found that ET-1 gene polymorphisms (rs1920453, rs1022436 and rs1035627) conformed to Hardy-Weinberg equilibrium ($p>0.05$). Gene-based association analysis indicated that only rs1920453 polymorphism and alleles were correlated with the occurrence of HICH ($p<0.05$). However, rs1022436 and rs1035627 polymorphisms and alleles had no association with HICH ($p>0.05$). Additionally, NIHSS score and high-density lipoprotein cholesterol level were prominently higher in HICH patients with CG and GG genotypes of ET-1 gene polymorphism rs1920453 than those in patients with CC genotype ($p<0.05$).

CONCLUSIONS: Rs1920453 in the promoter region of ET-1 gene is correlated with the occurrence of HICH.

Key Words:
Endothelin-1, Polymorphisms, Hypertensive intracerebral hemorrhage (HICH).

Introduction

In recent years, with increased incidence of hypertension and serious aging of population, hypertensive intracerebral hemorrhage (HICH) has become a fairly common neurological disease[1]. Due to high medical level and advanced imaging technology, the diagnosis rate of HICH has been greatly improved. This may enhance the efficiency of early intervention for HICH patients. However, its high mortality and disability rates still bring a heavy burden on families and the society[2,3]. HICH is caused by a combination of various factors, among which genetic factors have become a research hotspot. HICH is not a monogenic disease, but the result of complex multi-gene interactions, including lipoprotein-related genes, vascular remodeling-related genes, oxidative stress-related genes and inflammation-related genes[4,5].

Endothelin (ET), composed of 21 amino acid residues, is characterized by three forms (including ET-l, ET-2 and ET-3). So far, ET is the strongest vasoconstrictor, and widely exists in the body[6]. Multiple studies have shown that ET is a bioactive polypeptide converted from big ET by endothelin converting enzyme (ECE). It exerts biological effects *in vivo* through the two G protein-coupled receptors ET-A and ET-B. Generally, ET, ET-A and ET-B receptors, and ECE are collectively known as the endothelin system[7]. It has been reported that ET-1 plays a cruial role in cardiovascular and cerebrovascular diseases. Previous studies have also demonstrated that ET-1 gene polymorphisms are significantly associated with hypertension among overweight people and preeclampsia in pregnant women[8,9]. However, the potential relationship between ET-1 polymorphisms and HICH has not been fully elucidated.

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In the present study, the correlations of single nucleotide polymorphisms (rs1920453, rs1022436 and rs1035627) in the promoter region of ET-1 gene with the occurrence of HICH were analyzed. All our findings might help to provide a certain reference for further exploring the genetic pathogenesis of HICH.

Patients and Methods

Research Subjects

A total of 100 HICH patients (HICH group) aged (60.13±1.48) years old who received treatment in our hospital from September 2016 to September 2019 were selected as research subjects. Inclusion criteria were as follows: 1) patients who met the diagnostic criteria of *Chinese Guidelines for Diagnosis and Treatment of Intracerebral Hemorrhage 2015*: a) a history of hypertension, b) typical bleeding sites, such as basal ganglia, ventricle, thalamus, brain stem or cerebellar hemisphere, c) typical clinical symptoms, such as sudden onset, significant headache, vomiting and neurological dysfunction, and 4) Han Chinese. Exclusion criteria were as follows: 1) patients who did not meet the diagnostic criteria, 2) those with secondary cerebrovascular diseases, including cerebrovascular malformations, aneurysms, arteriovenous fistulas, brain tumors or cavernous vascular malformations, 3) those with coagulopathy disorders, or 4) those with other serious chronic diseases, such as severe malnutrition, untreated hyperthyroidism or hypothyroidism. Meanwhile, 100 normal healthy people aged (59.02±2.82) years old receiving physical examinations during the same period were selected as normal controls (Control group). Inclusion criteria were as follows: 1) people with corresponding age and gender, 2) Han Chinese, 3) those who did not meet the diagnostic criteria for cerebrovascular disease, or 4) those without cardiovascular or cerebrovascular diseases, blood system diseases, autoimmune diseases, severe liver and kidney diseases, or pregnancy. General clinical data of enrolled subjects were collected and recorded, including gender, age, history of hypertension and diabetes, history of smoking, as well as systolic and diastolic blood pressure on admission. Following 8 h of fasting, 4 mL of venous blood was extracted from each subject, anticoagulated with ethylenediaminetetraacetic acid (EDTA) and cryopreserved in a refrigerator at -20°C for use. This study was approved by the Ethics Committee of The Second Hospital of Dalian Medical University. Informed consent was obtained from each subject before the study.

Determination of Blood Biochemical Indicators

After 8 h of fasting, 4 mL of venous blood was extracted from each subject in the morning to determine the peripheral fasting blood-glucose (FBG). Next, the plasma levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) plasma were measured using a fully automatic biochemical analyzer.

Neurological Deficit Scores

Patients with HICH were evaluated by National Institute of Health Stroke Scale (NIHSS) (0-42 points) within 3 days after admission.

Deoxyribonucleic Acid (DNA) Extraction and Polymerase Chain Reaction (PCR) Amplification

A total of 4 mL of EDTA-anticoagulated blood was collected from patients, and genomic DNA was extracted according to the instructions of DNA Extraction Kit (Wuhan Service Biotechnology Co., Ltd., Wuhan, China). Next, the mass of 2 μL of sample was measured in 1.5% agarose gel electrophoresis. The concentration of extracted DNA was determined using an ultraviolet spectrophotometer. Subsequently, primers were designed to amplify ET-1 gene polymorphisms (rs1920453, rs1022436 and rs1035627) (Table I). Glyceralde-

Table I. Primer sequences and product sizes of different polymorphisms in the promoter region of the ET-1 gene.

Polymorphism	Primer sequence (5'-3')	Product (bp)
rs1920453	Forward: AGCTGTTAGTCGTAGTGCTG Reverse: AGCTGATGTTTAGTTAACCC	273
rs1022436	Forward: GTCGTAGTCGTAAGTCCTAGT Reverse: CTGTAGTCGTAGTGTCGTGTAG	322
rs1035627	Forward: CGATGTCTCGTAGTCGTAGTC Reverse: AACGATCGTAGTGCTGATGTCG	302

hyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC -3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'. PCR was performed in a 20 μL system composed of 2.0 μL of DNA template, 10.0 μL of 2 × Mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 7.2 μL of ddH_2O. Specific PCR procedure was as follows: 95°C for 120 s, 35 cycles of 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, and 72°C for 10 min. Finally, the amplification of gene fragments was detected by agarose gel electrophoresis.

Ligase Detection Reaction

The upstream and downstream probes utilized in this reaction were designed and synthesized by BGI Group. After modification by 5'-terminal phosphorylation, all forward probes were prepared into the probe mixture at a concentration of 12.5 pmol/μL. Ligase detection reaction system (3.05 μL) composed of 0.05 μL of ligase, 1 μL of buffer, 1 μL of PCR product, and 1 μL of probe mixture. PCR amplification was performed under the following conditions: 95°C for 120 s, 94°C for 15 s and 50°C for 25 s, for a total of 30 cycles. After that, the concentration was determined using the ultraviolet spectrophotometer. BIG Group was commissioned to sequence and analyze the target gene. All data were analyzed by GeneMapper (Table II).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Enumeration data were expressed by frequency and percentage, and measument data were presented as mean ± standard deviation ($\bar{x}\pm s$). Chi-square test was used for multiple comparisons of enumeration data, while *t*-test and analysis of variance were utilized for measurement data. $p<0.05$ was considered statistically significant.

Results

Comparisons of Clinical Baseline Data Between HICH Group and Control Group

The proportions of smoking and drinking were notably higher in HICH group than those in Control group ($p<0.05$). However, HDL-C level declined remarkably in HICH group compared with Control group ($p<0.05$). Besides, the levels of TG, TC and HDL-C in HICH group were distinctly lower than Control group ($p<0.05$), (Table III).

Analysis Results of ET-1 Gene Polymorphisms (rs1920453, rs1022436 and rs1035627)

ET-1 gene polymorphisms rs1920453, rs1022436 and rs1035627 in HICH group and Control group were cleaved by restriction enzyme BSTU I. These results exhibited that the polymorphism rs1920453 had two alleles (C and G) and three genotypes (CC, CG and GG), rs1022436 had two alleles (A and G) and three genotypes (AA, AG and GG), and rs1035627 had two alleles (A and T) and three genotypes (AA, AT and TT).

Results of Hardy-Weinberg Equilibrium Test

Hardy-Weinberg equilibrium formula was used to detect the linkage disequilibrium of different ET-1 gene polymorphisms. As shown in Table IV, the polymorphisms conformed to Hardy-Weinberg equilibrium in each group ($r^2<0.33$).

Correlations of ET-1 Gene Polymorphisms with HICH

The genotype frequencies of each gene polymorphism in the two groups were shown in Table V. Polymorphism rs1920453 was significantly correlated with the occurrence of HICH ($p<0.05$), while rs1022436 and rs1035627 had no association with HICH ($p>0.05$).

Table II. Ligase detection reaction probe sequences and product sizes of different ET-1 gene polymorphisms.

Polymorphism	Probe	Probe sequence (5'-3')	Product (bp)
rs1920453	rs1920453	P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTT-FAM	131
	rs1920453-C	TTTTTTTTTTTTTTTACCCATTTTTTTTTAT	
	rs1920453-G	TTTTTTTTTTTTTTTGCGACGAGCATTTTTTTTTAAA	
rs1022436	rs1022436	P-AGCCATGCACCCAATTTTTTTTTTTTTTTTTTT-FAM	158
	rs1022436-A	TTTTTTTTTTTTTTTTTTTTTTTTTCGTAGCTAAAC	
	rs1022436-G	TTTTTTTTTTTTTTTTTTTTTTTTTTACGATCGATG	
rs1035627	rs1035627	P-ACGGGATGCCATTTTTTTTTTTTTTTTTT-FAM	126
	rs1035627-A	TTTTTTTTTTTTTTTTTTTTTTTTTTGCGGACGAG	
	rs1035627-T	TTTTTTTTTTTTTTTTTTTTTTTTGCGGCCAAA	

Table III. Comparisons of general clinical data and biochemical indicators between HICH group and Control group ($\bar{x}$±s).

Parameter	HICH group (n=100)	Control group (n=100)	p
Age (years old)	60.13±1.48	59.02±2.82	0.272
Gender (male/female)	70/30	66/34	0.258
Smoking (%)	44%	15%	0.000*
Drinking (%)	38%	12%	0.000*
Diabetes (%)	39%	18%	0.000*
FBG (mmol/L)	5.45±2.34	5.21±2.03	0.023
TG (mmol/L)	0.23±0.06	0.55±0.13	0.000*
TC (mmol/L)	4.68±0.29	5.72±0.12	0.000*
HDL-C (mmol/L)	1.02±0.32	1.33±0.18	0.001*
LDL-C (mmol/L)	2.67±1.11	2.58±0.82	0.287

*p<0.05 *vs.* Control group.

Table IV. Results of linkage equilibrium test for ET-1 gene polymorphisms between groups.

Polymorphism	r2		
	rs1920453	rs1022436	rs1035627
rs1920453	–	0.011	0.232
rs1022436	0.011	–	0.201
rs1035627	0.232	0.202	–

Correlations of ET-1 Alleles with HICH

According to the distribution of different genotypes of each polymorphism in the two groups (Table VI), rs1920453 allele polymorphism was remarkably correlated with the occurrence of HICH (p<0.05), while rs1022436 and rs1035627 allele polymorphisms had no association with HICH (p>0.05).

Associations of ET-1 Gene Polymorphisms with NIHSS Score

Further studies revealed that NIHSS score was distinctly higher in HICH patients with CG and GG genotypes of ET-1 gene polymorphism rs1920453 than that in patients with CC genotype (p<0.05). Meanwhile, NIHSS score was higher in patients with GG genotype in comparison with that in patients with CG genotype (p<0.05) (Figure 1A). In contrast, gene polymorphisms rs1022436 and rs1035627 had no significant correlation with NIHSS score in HICH patients (p>0.05) (Figure 1B, C).

Relations of ET-1 Gene Polymorphism rs1920453 with Blood Glucose and Blood Lipids

The relationship of ET-1 gene polymorphism rs1920453 with blood glucose and blood lipids in HICH patients was finally analyzed in the present study. The results manifested that HDL-C level was prominently higher in HICH patients with CG and GG genotypes of rs1920453 polymorphism than that in patients with CC genotype (p<0.05). Moreover, HDL-C level was notably higher in patients with GG genotype in comparison with that in patients with CG genotype (p<0.05), (Table VII).

Discussion

In China, intracerebral hemorrhage is the second largest type of stroke. Its incidence is second only to ischemic stroke, accounting for 10-20% of total cases. Evidence-based medicine has proved that

Table V. Distribution of different genotypes of ET-1 gene polymorphisms among HICH patients.

Group	rs1920453			rs1022436			rs1035627		
	CC	CG	GG	AA	AG	GG	AA	AT	TT
HICH group	12%	34%	54%	20%	48%	32%	20%	52%	28%
Control group	26%	50%	24%	24%	46%	30%	20%	51%	29%
χ^2	2.341			0.429			0.671		
p	0.002			0.341			0.923		

Table VI. Distribution of alleles of TLR3 gene polymorphisms in cataract patients.

Group	rs1920453		rs1022436		rs1035627	
	C	G	A	G	A	T
HICH group	29%	71%	44%	56%	46%	54%
Control group	51%	49%	47%	53%	45.5%	54.5%
χ^2	1.432		0.782		0.644	
p	0.001		0.114		0.412	

there are few effective treatments for intracerebral hemorrhage[10]. HICH refers to intracerebral hemorrhage that is caused by hypertension, accompanied by changes in the structure and hemodynamics of small blood vessels in the brain when blood pressure suddenly fluctuates. HICH occurs more frequently among people aged 50-70 years old. However, the age of onset tends to be younger at present[11]. HICH, mainly characterized by aphasia, unconsciousness and hemiplegia, has high mortality and disability rates. Meanwhile, its mortality rate is up to 30-50% within a month after the onset. Even if patients survive, most of them will have various degrees of sequelae, such as aphasia and hemiplegia. This may eventually life quality of patients and bring a serious financial burden to families and the society[12].

At present, the management and control of lifestyle and risk factors are the main ways to prevent HICH. However, there are still few relevant studies on identifying risk factors through molecular genetics. If high-risk genes of HICH can be screened *via* genetic testing, it will be more likely to find high-risk populations. Therefore, more targeted prevention and treatment measures can be utilized to prevent the occurrence and control the progression of HICH more effectively[13]. Meanwhile, the top priority task at present is to find genes related to the pathogenesis of intracerebral hemorrhage. Hypertension is the most common and main risk factor for intracerebral hemorrhage and hypertension susceptibility genes are associated with increased risk of stroke[14]. Many domestic and foreign studies have reported that HICH is correlated with genetic factors, and its main susceptibility genes include apolipoprotein E, vascular endothelial growth factor, myeloperoxidase, angiotensin II type 1 receptor and Hind III gene[15-18]. HICH has also been observed related to environmental factors and multi-gene inheritance. Given that a single environmental factor or single gene polymorphism may have no obvious effect on increasing HICH susceptibility, it is necessary to further search for more susceptible genes and analyze the interaction of these genes in the pathogenesis of HICH.

Through molecular biological technology and pharmacological inhibition of the endothelin system, it has been found that ET-1 participates in and maintains the balance of the body's cardiovascular system. Additionally, ET-1 is of great significance in the functional and structural changes such as arteries and essential hypertension, glomerulosclerosis, atherosclerosis, and heart failure[19,20]. ET-1 gene polymorphism Lysl98Asn is associated with hypertension, height and body

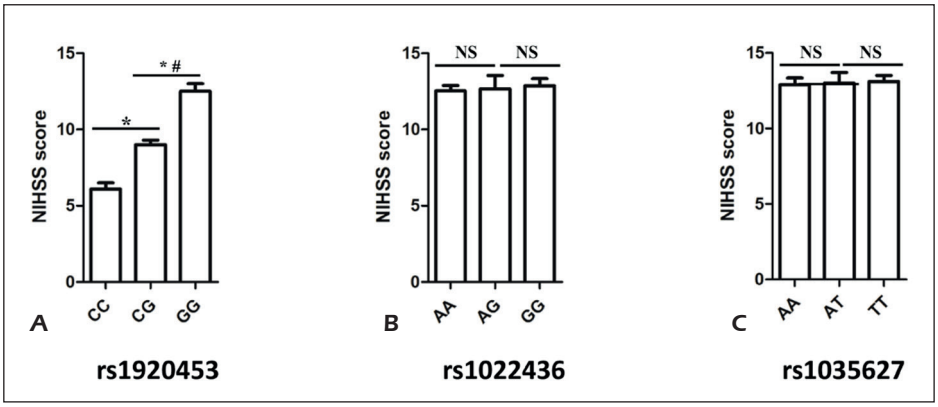

Figure 1. Associations of ET-1 gene polymorphisms with NIHSS score. **A**, *p<0.05 *vs.* CC genotype, $^{\#}p$<0.05 *vs.* CG genotype. **B-C**, NS: No significance, p>0.05.

Table VII. Relations of ET-1 gene polymorphism rs1920453 with blood glucose and blood lipids in HICH patients.

Parameter	CC	CG	GG	*p*
FBG (mmol/L)	5.67±1.66	5.42±1.29	5.52±1.92	0.823
TG (mmol/L)	1.63±0.21	1.65±0.33	1.66±0.28	0.662
TC (mmol/L)	4.73±0.42	4.71±0.82	4.55±0.42	0.734
HDL-C (mmol/L)	1.11±0.02	1.35±0.21*	1.45±0.28*#	0.034
LDL-C (mmol/L)	2.34±0.67	2.28±0.29	2.31±0.42	0.242

*$p<0.05$ *vs.* CC genotype, #$p<0.05$ *vs.* CG genotype.

mass index, HDL-C level in patients with coronary heart disease, and the occurrence of ischemic stroke[21]. Besides, ET-1 gene polymorphism rs1800541 has been shown correlated with asthma in British and Norwegian population[22]. Panoulas et al[23] have indicated that among patients with rheumatoid arthritis, those carrying rsl800541-rs5370 T-T haplotype are more likely to suffer hypertension, showing increased systolic and pulse pressure. In the present study, our findings illuminated that ET-1 gene polymorphism rs1920453 and alleles were correlated with the occurrence of HICH. Patients with GG genotype and allele G had a higher risk of HICH. Moreover, the prognosis of patients with GG was worse, manifested by significantly increased NIHSS score 3 days after surgery. In contrast, the other two polymorphisms (rs1022436 and rs1035627) and alleles had no association with the onset of HICH.

Conclusions

The present study revealed for the first time that GG genotype and allele G of rs1920453 in the promoter region of ET-1 gene are susceptible factors for HICH. Furthermore, rs1920453 polymorphism can serve as a genetic marker to evaluate the risk of HICH.

Conflict of Interests

The authors declare that they have no conflict of interest.

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