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Angiotensin-converting enzyme gene polymorphism and middle cerebral artery stenosis in a Chinese Han population★

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Abstract

The angiotensin-converting enzyme gene is a candidate gene of stroke. The present study involved 62 healthy volunteers and 148 patients with middle cerebral artery stenosis as confirmed by brain color ultrasound from a Han population in North China, and determined the peripheral blood angiotensin-converting enzyme genotype using PCR-restriction fragment length polymorphism analysis. The results showed that the frequencies of the DD genotype and D allele were increased in patients with middle cerebral artery stenosis, but the difference was not statistically significant compared with healthy controls. The findings of this study on the relationship between stroke genes and middle cerebral artery stenosis indicate no significant correlation between the frequencies of the DD genotype and D allele of angiotensin-converting enzyme and middle cerebral artery stenosis in this Han population from North China. In the future, studies will be carried out to investigate correlations between multiple stroke candidate gene synergy and middle cerebral artery stenosis to provide a foundation for the development of gene therapy.

Key Words

neural regeneration; brain injury; stroke; angiotensin-converting enzyme; gene; polymorphism; middle cerebral artery; angiostenosis; North China; Han population; neuroregeneration

Research Highlights

(1) PCR-restriction fragment length polymorphism analysis was used to determine angiotensin-converting enzyme genotypes in normal volunteers and patients with middle cerebral artery stenosis as confirmed by brain color ultrasound in a Han population from North China.
(2) The results indicate no significant correlation between the frequencies of the DD genotype and D allele of angiotensin-converting enzyme and middle cerebral artery stenosis in the Han population from North China.

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INTRODUCTION

The renin-angiotensin system is associated with cardiovascular system regulation, water-salt balance, and maintenance of internal environmental homeostasis[1], and plays an important role in ischemic cerebrovascular disease[2]. The renin-angiotensin system mainly includes prehypertension and angiotensin-converting enzyme[3-4]. This system plays a critical role in the development of hypertension and arteriosclerosis, and angiotensin-converting

enzyme participates in vascular remodeling and the development of atherosclerosis[5-8]. The angiotensin-converting enzyme gene comprises 26 exons and 25 introns[9] localized at the long arm of chromosome 17 (17q23), and intron 16 has or deletes a 287-bp Alu repetitive sequence, presenting insertion/deletion (I/D) polymorphism[10]. Deletion, but not insertion, is the cause of angiotensin-converting enzyme gene polymorphism[10]. Recent evidence indicates that the angiotensin-converting enzyme gene is a main candidate gene for hereditary susceptibility to various cardio-cerebrovascular diseases[11]. Intracranial atherosclerotic stenosis accounts for 8% to 10% of ischemic cerebrovascular disease and is higher in Asian, African, and Spanish individuals[12]. Approximately 10% of cases of ischemic stroke and 8% of cases of transient ischemic attack are caused by intracranial arteriostenosis[13], and the incidence of middle cerebral artery stenosis is the highest[14]. One study demonstrated that a high plasma concentration of angiotensin-converting enzyme can induce vessel wall thickening and vasculopathy and that the angiotensin-converting enzyme gene participates in the progression of vessel wall atherosclerosis and induces atherosclerotic cerebral infarction[15]. To date, there are few studies regarding the correlation between the angiotensin-converting enzyme gene and intracranial arteriostenosis, such as middle cerebral artery stenosis. No study has been conducted to investigate the correlation between the angiotensin-converting enzyme gene polymorphism and middle cerebral artery stenosis in the Han population of North China. Thus, the present study compared the angiotensin-converting enzyme gene in Han patients from North China with middle cerebral artery stenosis with that in normal controls to analyze whether there is a correlation between angiotensin-converting enzyme gene polymorphism and middle cerebral artery stenosis.

RESULTS

Quantitative analysis of participants

One hundred and forty-eight patients with middle cerebral artery stenosis from North China, as confirmed by brain color ultrasound, were selected as the case group, and 62 healthy volunteers with normal brain color ultrasound results were selected as controls. No participant withdrew from the study. There were no significant differences between the two groups in terms of mean age and gender. The number of patients with a history of hypertension, diabetes, smoking, and increased carotid intima-media thickness (≥1.1 mm) was significantly greater in the case group than in the control group ($P < 0.05$), but patients with a history of hyperlipidemia and drinking were similar between the two groups ($P > 0.05$; Table 1).

Table 1 Clinical data of patients with middle cerebral artery stenosis and normal controls

Item	Case group	Control group
n	148	62
Gender [*n*(%)]		
Male	110(74.3)	45(72.6)
Female	38(25.7)	17(27.4)
Age (year)	52.8	48.9
Hypertension [*n*(%)]	84(56.8)[a]	16(25.8)
Hypertension grade[16] [*n*(%)]		
1	19(12.4)	10(16.1)
2	20(13.5)[a]	4(6.4)
3	45(30.4)[a]	2(3.2)
Diabetes [*n*(%)]	36(24.3)[a]	7(11.3)
Hyperlipemia [*n*(%)]	39(26.4)	10(16.1)
Smoking [*n*(%)]	89(60.1)[a]	17(27.4)
Drinking [*n*(%)]	3(21.0)	11(14.7)
Thickened carotid intima-media thickness [*n*(%)]		
< 1.1 mm	66(44.6)	42(67.7)
≥ 1.1 mm	82(55.4)[b]	20(32.3)

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. control group (chi-square test).

Angiotensin-converting enzyme gene polymorphism in patients with middle cerebral artery stenosis

The angiotensin-converting enzyme gene is associated with three genotypes due to Alu insertion/deletion, insertion (II), deletion (DD), and heterozygote (ID)[9]. The angiotensin-converting enzyme gene electrophoresis results are shown in Figure 1.

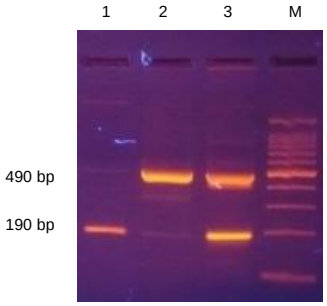

Figure 1 Three genotypes of angiotensin-converting enzyme gene due to Alu insertion/deletion

M: Molecular weight standard; 1: DD genotype, 190 bp, homozygous deletion; 2: 490 bp, homozygous insertion; 3: ID genotype, 490 and 190 bp, heterozygous.

The frequencies of the DD genotype and D allele were increased in patients with middle cerebral artery stenosis, but the difference was not statistically significant compared with healthy controls ($P > 0.05$). There was no significant difference in the frequencies of genotypes and

alleles between patients with a history of hypertension, diabetes, smoking, and increased carotid intima-media thickness ($P > 0.05$; Tables 2–4).

Table 2 Angiotensin-converting enzyme genotype and allele distribution [*n* (%)] in patients with middle cerebral artery stenosis and normal controls

Group	Genotype		
	II	ID	DD
Case	47(34.80)	40(27.00)	61(41.20)
Control	24(38.70)	10(16.10)	28(45.20)

Group	Allele	
	I	D
Case	155(52.30)	141(47.70)
Control	76(61.30)	48(38.70)

Table 3 Frequency of angiotensin-converting enzyme genotype [*n* (%)] in patients with middle cerebral artery stenosis and normal controls

Clinical condition	Case group			
	II	ID	DD	
Hypertension	25(25.76)	34(40.48)	25(25.76)	
Diabetes	6(16.67)	18(50.00)	12(33.33)	
Smoking	28(31.46)	35(39.33)	26(29.21)	
IMT ≥ 1.1 mm	24(28.92)	33(42.17)	26(28.91)	
Clinical condition	Control group			*P*
	II	ID	DD	
Hypertension	5(31.25)	6(37.50)	5(31.25)	>0.05
Diabetes	2(28.57)	2(28.57)	3(42.86)	>0.05
Smoking	7(38.89)	8(44.44)	3(16.67)	>0.05
IMT ≥ 1.1 mm	5(25.00)	10(75.00)	5(25.00)	>0.05

IMT: Carotid intima-media thickness.

Table 4 Angiotensin-converting enzyme allele frequency distribution [*n* (%)] in patients with middle cerebral artery stenosis and normal controls

Clinical condition	Case group		
	I	D	
Hypertension	84(50.00)	84(50.00)	
Diabetes	30(41.67)	42(58.33)	
Smoking	91(51.12)	87(48.88)	
IMT ≥ 1.1 mm	81(48.80)	85(51.20)	
Clinical condition	Control group		*P*
	I	D	
Hypertension	16(50.00)	16(50.00)	>0.05
Diabetes	6(42.86)	8(57.14)	>0.05
Smoking	22(61.11)	14(38.89)	>0.05
IMT ≥ 1.1 mm	20(50.00)	20(50.00)	>0.05

IMT: Carotid intima-media thickness.

Risk factors of middle cerebral artery stenosis

Multiple-factor logistic regression analysis including angiotensin-converting enzyme genotype and allele evaluation showed that the angiotensin-converting enzyme genotype and allele were not risk factors for middle cerebral artery stenosis, further confirming that hypertension, smoking, and increased carotid intima-media thickness are independent risk factors for middle cerebral artery stenosis (independent-variable quantization standard in Table 5, results in Table 6).

Table 5 Quantization standard of risk factors for middle cerebral artery stenosis

Factor	Quantization value
Age (year)	≤ 50 years: 0; > 50 years: 1
Gender	Female: 0; male: 1
Hypertension	No: 0; yes: 1
Hypertension grade	No: 0; grade 1: 1, grade 2: 2; grade 3: 3
Diabetes	No: 0; yes: 1
Coronary artery disease	No: 0; yes: 1
Hyperlipemia	No: 0; yes: 1
Smoking	No: 0; yes: 1
Drinking	No: 0; yes: 1
IMT	< 1.1 mm : 0; ≥ 1.1 mm: 1
ACE genotype	DD: X1 1; non-DD: X1 0; DI: X2 1, non-DI: X2 0; II: X3 1; non-II: X3 0
ACE allele	D: X1 1; non-D: X1 0; I: X1 1, non-I: X1 0

IMT: Carotid intima-media thickness; ACE: angiotensin-converting enzyme.

Table 6 Multivariate logistic regression analysis of patients with middle cerebral artery stenosis

Factor	Regression coefficient	Standard error	Wald value
IMT	1.518 5	0.745 4	4.150 4
Hypertension grade	0.762 8	0.205 4	13.788 9
Smoking	1.509 6	0.365 5	17.059 0

Factor	*P*	95% confidence interval
IMT	0.041 6	1.059–19.676
Hypertension grade	0.000 2	1.434–3.207
Smoking	< 0.000 1	2.211–9.262

IMT: Carotid intima-media thickness.

DISCUSSION

Increasing numbers of studies have demonstrated that the distribution of vessel damage differs among various ethnicities. For example, intracranial large artery stenosis is the main cause of ischemic cerebrovascular disease, especially middle cerebral artery stenosis[17-18]. Poor compensation of other vessels or absence of effective establishment of collateral circulation following middle cerebral artery stenosis may lead to cerebral vessel ischemic events. Kern *et al* [19] reported that symptomatic middle cerebral artery stenosis is an independent factor for entire and ipsilateral cerebral

vessel events (entire, $P < 0.01$; ipsilateral, $P = 0.02$), and the recurrence rate of cerebral ischemia was higher in patients with symptomatic middle cerebral artery stenosis compared with those with asymptomatic stenosis, and was even higher than the incidence of extracranial internal carotid artery stenosis. Studies of the correlation between the condition of the middle cerebral artery and mortality and the recurrence rate of ischemic cerebrovascular disease indicate that middle cerebral artery occlusion has the highest risk of death and relapse (21.4%), followed by middle cerebral artery stenosis (16.6%) and a normal middle cerebral artery (12.2%)[13]. In addition, the preclinical phase of arterial stenosis is long prior to clinical symptoms of stroke[20]. Therefore, it is clinically important to investigate risk factors for middle cerebral artery stenosis. With developments in molecular biology, growing evidence has indicated that gene polymorphism plays a role in susceptibility to cerebrovascular disease, especially ischemic cerebrovascular disease. The human angiotensin-converting enzyme gene has aroused the most attention as a candidate stroke gene. In 1992, Cambin *et al* [21] first reported that I/D polymorphism is a potential risk factor for myocardial infarction. Subsequent studies suggested that this polymorphic site could increase the risk of cerebrovascular disease[22-23]. Studies of patients with symptomatic cerebral infarction and normal controls showed that the D/I allele frequency (0.59/0.41) in the patients was significantly different from that in the controls (0.48/0.52) and that the DD genotype was significantly greater than that in the controls, who showed a relative risk of 1.98 for cerebrovascular disease. These results indicate that the risk of cerebrovascular disease with the DD genotype is higher than that with the II and ID genotypes[24].

In the present study, the frequency of the I and D alleles in the control group was 61.30% and 38.70%, respectively, indicating that the percentage of the I allele was relatively higher, similar to previous results of the angiotensin- converting enzyme gene polymorphism distribution in a normal Han population[25]. However, the D allele frequency was relatively higher in Europeans, and the I/D frequency was about 0.44/0.55[26-28]. These results indicate significant race differences in the frequency of the I and D alleles in normal people. Results of the present study indicate no obvious correlation between I/D polymorphism and middle cerebral artery stenosis, consistent with previous results[29]. However, there was statistical significance between the case and control groups in terms of history of hypertension, diabetes, smoking, and increased carotid intima-media thickness. Multivariate logistic regression analysis further demonstrated that angiotensin-converting enzyme gene I/D polymorphism may not be an independent risk factor for middle cerebral artery stenosis and that a history of hypertension, diabetes, smoking, and increased carotid intima-media thickness play important roles in the development of middle cerebral artery stenosis. In addition, carotid intima-media thickness is a reliable index for early atherosclerosis detection[30]. Thus, carotid intima-media thickness of ≥ 1.1 mm can be used as a marker of middle cerebral artery stenosis.

The association between angiotensin-converting enzyme polymorphism and ischemic cerebrovascular disease remains controversial. This may result from: (1) different study methods: some retrospective studies of a case meta-analysis indicated a significant correlation between the angiotensin-converting enzyme gene D allele and ischemic cerebrovascular disease, but no correlation was found in prospective paired studies; (2) different case selection conditions: for example, the patients with cerebral infarction included those with lacunar infarction, large-sized infarction, or carotid artery blood supply region infarction, which led to different results; (3) sample sizes: investigations of gene interactions and environments require large sample sizes, and insufficient samples may result in different and even contrary results; (4) distribution of angiotensin-converting enzyme gene polymorphism: this is influenced by ethnic differences, and environmental and geographical variations. For example, the frequency of the D allele of the angiotensin-converting enzyme gene is significantly higher in Europeans than in Asians. No large-sample statistical data are available regarding the gene distributions in related populations. The number of cases in the present study was small, and the results could be regarded as a predication of one tendency; however, larger samples are needed for further validation.

A large number of epidemiologic studies and animal experiments have demonstrated that most ischemic cerebrovascular disease is controlled by genetic factors[31-32]. Synergistic effects of assortments of some genes on ischemic cerebrovascular disease have been found in addition to correlations between candidate gene polymorphism and ischemic cerebrovascular disease. Angiotensin-converting enzyme gene polymorphisms have synergistic effects with some other genes, such as the prehypertension[33-34] gene, methylene

tetrahydrofolate reductase gene[35-36], apolipoprotein E gene[37-38], and 4G/5G polymorphism of the plasminogen activator inhibitor-1[39] during induction of ischemic cerebrovascular disease. Different genes have different clinical phenotypes. The risk for disease is increased when many predisposing genes function at the same time.

Studies of gene factors can further elucidate the pathogenesis of middle cerebral artery stenosis-induced ischemic cerebrovascular disease and screen susceptible populations, which can allow for primary and secondary prevention and be used in treatment. Studies have been conducted to investigate gene expression after middle cerebral artery occlusion in humans[40], regulation of gene expression in a rat model of brief middle cerebral artery occlusion[41], and accommodation of signal transducer and transcription factor activation pathways during regional cerebral ischemia using gene expression microarray[42-43].

In conclusion, angiotensin-converting enzyme gene I/D polymorphisms are not an independent risk factor for middle cerebral artery stenosis in the investigated Han population from North China. The present results further demonstrate that a history of hypertension, diabetes, smoking, and increased carotid intima-media thickness are risk factors for middle cerebral artery stenosis, indicating that primary and secondary prevention is important for cerebral vessel stenosis or cerebrovascular disease. Future studies on the association of angiotensin-converting enzyme gene polymorphisms with intracranial main vessel stenosis should focus on sample size, ethnicities of participants, and the synergistic effect of multiple stroke candidate genes to further elucidate the pathogenesis of stroke gene polymorphisms and intracranial main vessel stenosis-induced ischemic cerebrovascular disease, and to provide target sites for gene techniques in the treatment of ischemic cerebrovascular disease. With the development of life science and technology, research on the analysis and detection of disease-related genes has advanced to clinical levels and provides a scientific basis for diagnosis and differential diagnosis of diseases. Genetic testing is a means to confirm monogenic disease. For multigenic disease, gene mutation analysis helps to identify the etiology. The present study aimed to elucidate the correlation between stroke genes and middle cerebral artery stenosis to provide a reference for studies on the correlation between genetic synergy and middle cerebral artery stenosis.

SUBJECTS AND METHODS

Design

A case-control study of gene polymorphism.

Time and setting

Subject screening, clinical data collection, and blood sampling were performed in the Department of Neurology, Jilin University First Hospital, and DNA extraction, PCR amplification, and electrophoresis were conducted in the Laboratory of Biochemistry, Jilin University Basic Medicine College, China from January 2006 to April 2008.

Subjects

Participants were enrolled from the Out-patient Clinic and Admission Department, First Hospital of Jilin University from January 2006 to April 2008. Middle cerebral artery stenosis was diagnosed if the peak systolic velocity (Vs) was ≥ 160 cm/s or the mean blood flow rate (Vm) was ≥ 120 cm/s; if low-frequency components were increased; in the presence of a vortex and vascular murmur; and with reduction of blood flow in distal and proximal stenosis[44] as confirmed by a transcranial and peripheral vessel Doppler/monitor system (SciMed Ltd., Bristol, UK). The degree of vessel stenosis was classified as follows[45]: mild stenosis, 160 ≤ Vs ≤ 180 cm/s; moderate stenosis, 180 < Vs ≤ 220 cm/s; and severe stenosis, Vs > 220 cm/s.

Inclusion criteria for the (1) case group (asymptomatic Han population in North China) were the presence of middle cerebral artery stenosis as confirmed by transcranial Doppler ultrasound through a temporal window for evaluation of the blood flow of the bilateral middle cerebral artery; complete conduction of the questionnaire, genotyping, and biochemical detection; and acquisition of informed consent from patients and their families. Inclusion criteria for the (2) normal control group (healthy Han population in North China) were the absence of middle cerebral artery or other vessel stenosis as confirmed by transcranial Doppler ultrasound through a temporal window for evaluation of the blood flow of the bilateral middle cerebral artery; complete conduction of the questionnaire, genotyping, and biochemical detection; and acquisition of informed consent from patients and their families. Exclusion criteria were trauma, tumor, or other non-gene factors. Finally, 148 patients with middle cerebral artery stenosis and 62 individuals with no abnormalities as confirmed by transcranial Doppler ultrasound were included. The study

was performed in accordance with the *Declaration of Helsinki*.

Methods

Collection of case histories

Case histories were inquired in detail, especially the risk factors of cerebrovascular disease, including hypertension, diabetes, coronary artery disease, hyperlipidemia, smoking, and drinking. In addition, patient age, gender, symptoms, and transcranial Doppler ultrasound results were recorded.

Carotid artery ultrasonography

Carotid artery ultrasonography was conducted to record intima thickness and carotid artery plaque formation.

PCR-restriction fragment length polymorphism analysis for angiotensin-converting enzyme gene polymorphism

After addition of ethylenediaminetetraacetic acid, 2 to 3 mL of blood was harvested from the antecubital vein and stored at –20 °C. DNA was extracted using a guanidinium isothiocyanate method and stored at –70°C. The angiotensin-converting enzyme forward primer was 5′-CTG GAG ACC ACT CCC CAT CCT TTC T-3′, and the reverse primer was 5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′. PCR was performed in a 50-μL solution containing 5 μL of 10 × PCR buffer, 4 μL of dNTP, 1 μL each of the forward and reverse primer, 0.25 μL of ExTaq enzyme (TaKaRa, Dalian, Liaoning Province, China), 3 to 5 μL of template, and sterilized double-distilled water. PCR was conducted for 35 cycles with predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 56 °C for 2 minutes, and extension at 72°C for 1 minute. Another extension at 72°C was performed for 10 minutes. Sterile distilled water replacing the template was used as a negative control. The appropriate amount of PCR products were mixed with corresponding amounts of 6 × loading buffer and electrophoresed in 3% agarose gel at 100 V for 20 minutes. Electrophoresis results were observed under an ultraviolet lamp. PCR product purification and DNA sequencing were performed by Dalian Takara, China.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 software (SPSS, Chicago, IL, USA). Count data were analyzed using the chi-square test. Multivariate logistic regression analysis of risk factors was performed. The logistic regression model used middle cerebral artery stenosis as the dependent variable and gender, age, history of hypertension, hypertension grade, diabetes, coronary artery disease, hyperlipidemia, carotid intima-media thickness, smoking, drinking, angiotensin-converting enzyme genotype, and allele frequency as independent variables.

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Author contributions: Chunshu Rong conceived and designed the study, collected data, and wrote the manuscript. Chunshu Rong, Juan Wang, and Baoshan Gao conducted the statistical analysis. Yingqi Xing was in charge of brain color ultrasound and screening of subjects. Xinmei Jiang and Jianjun Zhao provided technical and data support. Kangding Liu guided the study and revised the manuscript. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Medical Ethics Committee of First Hospital, Jilin University, China.

Author statements: The manuscript is original; has not been submitted to and is not under consideration by another publication; has not been previously published in any language or any form, including electronic; and contains no disclosure of confidential information or authorship/patent application disputations.

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