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Original Research

Common genetic variants on chromosome 9p21 confers risk of ischemic stroke: A large-scale genetic association study

Rongyao Li[1], Xinyue Zhang[1], Wei Yin[1], Yuzhuo Wang[2], Ying Liu[1*]

[1] Cangzhou Medical College, Guangzhou Road, Yunhe District, Cangzhou, Hebei Province, China
[2] Cangzhou People's Hospital, Guangzhou Road, Yunhe District, Cangzhou, Hebei Province, China

*Correspondence to: liuyinglak@163.com

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Abstract: The objective of this experiment was to observe the allele frequency and genotype distribution of some 9p21 SNPs in the Anhui Han population, and to study its relationship with the susceptibility to ischemic stroke. For this purpose, a collection of 992 patients with ischemic stroke confirmed and hospitalized in our hospital from October 2017 to October 2020 were used as the IS case group, and 951 normal people who had a healthy physical examination in the physical examination center of our hospital during the same period were selected as the control group. After informed consent, cubital venous blood of all subjects was collected, and epidemiological data of the subjects were collected; the rs2383206, rs2383207, rs10757274, and rs1333049 on chromosome 9p21 as the sites to be tested, using Sequenom Mass Array system for genotyping, using Haploview4.2 software to calculate whether the genotype distribution meets Hardy-Weinberg equilibrium. Results showed that there were no significant differences between the two groups in gender, age, and smoking history. There are significant differences in the levels of hypertension, diabetes and hyperlipidemia, total cholesterol, triglycerides, HDL-C and apolipoprotein A1 between the two groups of study subjects. The genotype frequencies of the participating populations were in a balanced state. The results of the association analysis between SNPs and IS susceptibility showed that rs2383207, rs10757274, rs1333049 and rs2383206 are the susceptibility sites of ischemic stroke. It concluded that in Anhui, China, the inheritance of chromosome 9p21 region is associated with ischemic stroke.

Key words:

Introduction

As a group of acute cerebrovascular diseases with high mortality and high disability characterized by focal neurological function loss, stroke has now become a major disease seriously threatening human health, which can be divided into hemorrhagic disease and ischemic disease. Ischemic stroke is a leading cause of death and disability worldwide (1-3). With the rapid development of genetics, the genetics of stroke has gradually become a research hotspot in recent years. Studies have shown that genetic and environmental factors play an important role in the occurrence and development of stroke (4-9). Genome-Wide Association Study (GWAS) based on single nucleotide polymorphisms (SNPs) is a powerful tool for studying complex diseases (10). A large number of susceptible loci and genes have been found, among which 9p21.3 loci have been confirmed to have some diseases. Cardiovascular disease (11), ischemic stroke (12), aortic aneurysm (13), intracranial aneurysm (14), primary open-angle glaucoma (15), type 2 diabetes mellitus (16), glioma (17) and tumor susceptibility (18-21), suggesting the existence of important unstable loci on chromosome 9p21. Therefore, this study aimed to observe the allele frequency and genotype distribution of some SNPs of 9p21 in the Anhui Han population and to investigate their relationship with the susceptibility to ischemic stroke.

Materials and Methods

Case group: All subjects were admitted from October 2017 to October 2020. All selected objects in the diagnosis of reference standard version 9 international classification of diseases and with reference to our country in 1995 of the classification and diagnostic criteria of cerebrovascular disease, and guidelines for the diagnosis and treatment of acute ischemic stroke in China (January 2010), including two TOAST classification, divided into one or two subtypes: artery atherosclerotic stroke, stroke diagnosed with: [1] Multiple transient ischemic attacks (TIA), mostly in the same arterial area;[2] aphasia, neglect, impaired motor function, or cerebellar and brainstem damage; [3] Carotid artery auscultation murmur, decreased pulse, bilateral blood pressure asymmetry, etc.; [4] Cerebral cortex or cerebellar lesions with a brainstem lesion diameter of > 1.5cm may be ischemic stroke caused by latent atherosclerosis of the great arteries. [5] Color ultrasound, transcranial Doppler ultrasound, or digital subtraction angiography can show the associated intracranial or extracranial artery stenosis or occlusion rate > 50%; In addition, the diagnosis of small artery stroke (symptomatic lacunar infarction) meets only one of its diagnostic criteria: typical clinical manifestations of lacunar infarction, and imaging findings showing stroke lesions

<1.5cm are consistent with clinical symptoms.

Exclusion criteria :[1] other types of stroke, including cerebral hemorrhage, subarachnoid hemorrhage, cerebral embolism, stroke caused by the tumor, cerebral infarction caused by definite vascular inflammation, cerebral infarction caused by unknown cause; [2] Concomitant craniocerebral injury and/or vascular dissection, various systemic tumors, autoimmune diseases, history of heart disease (coronary heart disease, atrial fibrillation), severe infections, metabolic diseases (except diabetes), autoimmune diseases, hematological diseases, and severe hepatic and renal insufficiency. Finally enrolled in 992, with a response rate of 87.9% (992/1129)

Control group: [1] Physical examination population, hospitalized or outpatient patients or their family members during the same period. [2] Screening of DNA samples from this research group to select samples that match the geographic location, distribution of origin (urban and rural areas), gender, and age (4-5 years old) frequency of cases. All objects have been history analysis, neurological examination, general physical examination, and electrocardiogram (ECG), myocardial enzyme examination, partly accept cardiac ultrasound examination, the CAG, head CT or MRI, but not including coronary heart disease, cerebrovascular disease, congenital heart disease, valvular heart disease, cancer, aneurysm, severe liver and kidney function is not complete, severe infections, A total of 951 cases were enrolled, and the response rate was 92.0% (951/1034). There were no differences between the two groups in gender, age, or origin.

All the subjects were randomly selected and unrelated Han people, mainly from Anhui. There were no differences in gender, age, or origin among the participants and those who declined to participate in the study. The study was conducted with the informed consent of all participants and the study was approved by the local ethics committee.

Specimen Collection

Questionnaire survey: all selected subjects were collected personal information in the form of a questionnaire and sign the consent form, including demographic characteristics, diet, lifestyle habits such as smoking and drinking, history of neurological diseases, history of high blood pressure and history of diabetes. In the case of coma or unconsciousness, family members were given questionnaires.

Risk factor assessment: physical examination and blood pressure measurement. The physical examination included a neurological examination, BMI, hyperlipidemia, hypertension, and smoking status.

Blood sample collection: the next morning after fasting for 12 hours, 8ml elbow venous blood was collected in two tubes for whole-genome DNA extraction and blood lipid level detection. Blood biochemical indicators to be measured included blood glucose, TC, TG, HDL-C and LDL-C, from which the blood sample for extraction was anticoagulated with sodium citrate (ACD). Freeze at -20℃. Blood samples used for testing lipid levels are immediately separated from the serum and frozen at -80 °C without anticoagulant treatment. Peripheral venous blood was extracted with whole blood genomic DNA extraction kit.

Selection of SNP

In combination with literature reports, four SNPs associated with ischemic stroke were selected for genotyping in the GWA study. The two SNP rs10757274 and rs2383206 in the study of McPherson et al. (22), rs2383207 in the study of Helgadottir et al. (23) and rs1333049 in the study of Samani et al. (24).

Data analysis

SPSS13.0 software was used to analyze the association between general data, alleles, genotypes and ischemic stroke. The Haploview4.2 software was used to calculate whether the genotype distribution met the Hardy-Weinberg equilibrium, meaning that they were equal to 0.05.

Results

Comparison of general data of subjects

The comparison results of the general situation between the control group and the IS case group showed that the IS case group accounted for 66.9% males, with an average age of 61.7±12.9 years, while the control group accounted for 63.9% males, with an average age of 60.8±10.3 years. There was no significant difference in gender and age between the two groups (P>0.05). However, there was a significant difference in BMI between the two groups (P<0.05). The results of blood lipid level showed that the proportion of hypertension, diabetes and patients with a history of hyperlipidemia in IS case group was significantly higher than that in the control group (P<0.05). The age of smoking history in IS case group was 13.9% higher than that in the control group, but there was no statistical difference (P>0.05). The levels of total cholesterol, triglyceride, HDL-C and apolipoprotein A1 were significantly different between the two groups (P<0.05). The specific results are shown in Table 1.

SNP genotyping results

Sequenom Massarray was used to genotyping rs10757274, rs2383206, rs2383207 and rs1333049 on chromosome 9p21, and the genotyping map was obtained. The genotyping maps of the 4 loci are shown in Figures 1-4.

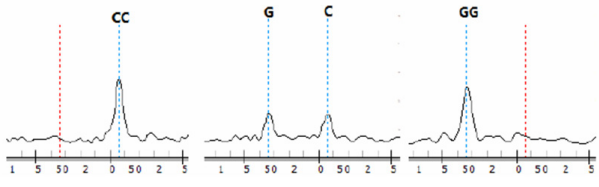

Figure 1. Genotyping of rs1333049 locus.

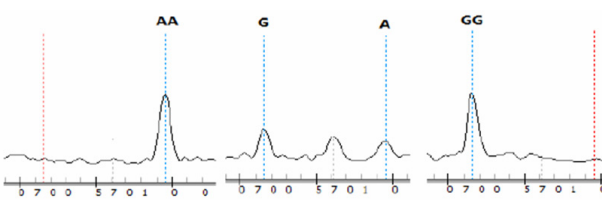

Figure 2. Genotyping of rs2383206.

Table 1. Comparison of general conditions and lipid levels between IS case group and control group.

project	The control group (n=951)	IS case group (n=992)	statistics	*P*
Gender (case)			1.936	0.1641
male	608	664		
female	343	328		
Age (year)	60.8±10.3	61.7±12.9	1.6950	0.0902
BMI index (kg/m²)	22.6±3.4	23.2±4.0	3.5555	0.0000
Smoking history	380	433	3.455	0.0631
diabetes	33	148	73.066	0.0000
hypertension	216	536	136.17	0.0000
hyperlipidemia	402	568	28.408	0.0000
Total cholesterol (mmol/L)	4.25±1.18	4.09±1.02	3.2015	0.0014
triglycerides (mmol/L)	1.01 (0.69)	1.35 (0.93)	9.1214	0.0000
HDL-C (mmol/L)	1.62±0.57	1.28±0.47	14.3702	0.0000
LDL-C (mmol/L)	2.47±0.78	2.49±0.84	0.5433	0.5870
Apolipoprotein A1 (mmol/L)	1.31±0.26	1.08±0.52	12.2507	0.0000
Apolipoprotein B (mmol/L)	0.84±0.19	0.85±0.28	0.9173	0.3591
ApolipoproteinA1/apolipoprotein B	1.61±0.51	1.24±0.58	14.9085	0.0000

Table 2. Frequency distribution of genotypes in Hardy-Weinberg equilibrium test.

SNPs	The control group				IS case group			
	Ho	He	χ^2	*P*	Ho	He	χ^2	*P*
rs2383206	0.456	0.478	0.001	0.9748	0.485	0.485	0	1.000
rs2383207	0.432	0.466	0.001	0.9748	0.421	0.399	0.001	0.9748
rs1333049	0.469	0.489	0	1.000	0.495	0.501	0	1.000
rs10757274	0.471	0.495	0.001	0.9748	0.507	0.499	0	1.000

Table 3. Correlation analysis between different genetic patterns of rs1333049 at 9p21 locus and IS susceptibility.

Genotype/allele	The control group (n=951)	IS case group (n=992)	χ^2	*P*	*OR* (95%CI)
CC	191	224	4.745	0.0933	1
CG	446	475			0.982 (0.899-1.081)
GG	296	265			0.855 (0.735-1.001)
C	858	964	6.747	0.0094	1
G	1037	985			0.912 (0.789-0.977)

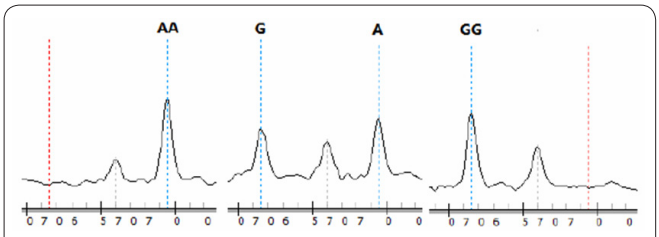

Figure 3. Genotyping of rs2383207.

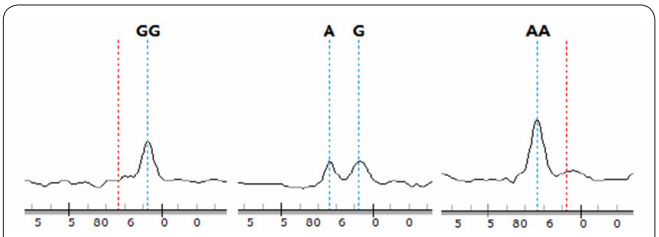

Figure 4. Genotyping of rs10757274 locus.

Hardy-Weinberg genetic balance test

Table 2 showed that the 4 SNP loci in the IS case group and the control group all complied with HWE's law (P>0.05), that IS, there was no deviation from Hardy-Weinberg genetic balance, indicating that the genotype frequency of each locus in the participants was in a state of balance, which was representative of the population.

Association analysis between SNPs and IS susceptibility

Analysis of the genotype and allele of rs1333049 locus and IS susceptibility showed that there was no significant difference in the genotype distribution of this SNP locus between IS case group and control group (P > 0.05), but there was a significant difference in the distribution of allele between the two groups (P < 0.05). G allele carriers are 0.9 times more likely to be infected with IS than C allele carriers, as shown in Table 3.

Analysis of the genotype and allele of rs2383206 site and IS susceptibility showed that the genotype of this SNP site had no significant difference between IS case group and control group (P > 0.05), but there was a significant difference in the distribution of alleles between the two groups (P < 0.05). G allele carriers had a 1.1 times greater risk of IS infection than C allele carriers, as shown in Table 4.

Analysis of the genotype and allele of rs2383207 and

Table 4. Correlation analysis between different genetic patterns of rs2383206 at 9p21 locus and IS susceptibility.

IS case group	The control group (n=951)	IS case group (n=992)	χ^2	*P*	*OR* (95%CI)
AA	305	280	4.111	0.1280	1
AG	447	493			1.009 (0.898-1.125
GG	197	233			1.102 (0.902-1.359
A	1057	1014	7.082	0.0078	1
G	842	959			1.089 (1.001-1.129

Table 5. Correlation analysis between different genetic patterns of rs2383207 at 9p21 locus and IS susceptibility.

Genotype/allele	The control group (n=951)	IS case group (n=992)	χ^2	*P*	*OR* (95%CI)
AA	129	82	15.502	0.0004	1
AG	407	425			1.203 (1.012-1.304)
GG	410	480			1.145 (1.029-1.307)
A	664	589	12.27	0.0005	1
G	1227	1385			1.087(1.035-1.157)

Table 6. Correlation analysis between different genetic patterns of rs10757274 at 9p21 locus and IS susceptibility.

Genotype/allele	The control group (n=951)	IS case group (n=992)	χ^2	*P*	*OR* (95%CI)
AA	317	271	10.657	0.0049	1
AG	454	490			1.086 (0.967-1.195)
GG	173	224			1.304 (1.152-1.603)
A	1088	1031	10.906	0.0009	1
G	800	939			1.125 (1.046-1.238)

IS susceptibility showed that the genotype and allele of this SNP locus were significantly different between the IS case group and the control group ($P < 0.05$). The risk of IS infection in AG genotype and GG genotype carriers was 1.2 and 1.1 times that of AA genotype carriers, respectively. The risk of IS infection in G allele carriers was 1.1 times that of A allele carriers. As shown in Table 5.

Analysis of the genotype and allele of rs10757274 locus and IS susceptibility showed that the genotype and allele of this SNP locus were significantly different between IS case group and control group ($P < 0.05$). The risk of IS infection in AG genotype and GG genotype carriers was 1.1 times and 1.3 times higher than that in AA genotype carriers, respectively. The risk of IS infection in G allele carriers was 1.1 times higher than that in A allele carriers. As shown in Table 6.

Discussion

Stroke is the leading cause of death in China which is still characterized by high morbidity and mortality despite enhanced prevention of stroke and improved diagnosis and treatment. The World Health Organization predicts that the incidence of stroke will increase by 27% by 2025, which will cause great harm to the health of human society and bring a heavy economic burden to patients and society (25, 26). Ischemic stroke accounted for about 80% of the total incidence of cerebral apoplexy, influenced by heredity, environment and the interaction of vascular risk factors and cause a variety of factors of complex diseases (27), about half of the disease can be explained by traditional risk factors, such as ischemic heart disease, hypertension, diabetes, dyslipidemia, smoking, and atrial fibrillation. More and more studies have shown that genetic factors play an important role in the development of ischemic stroke (28). However, scholars at home and abroad have drawn inconsistent conclusions when studying whether 9p21 IS related to IS.

In this study, a total of 992 cases and 951 cases of the control group were selected by the case-control method. There was no statistical difference in the distribution of general information, such as gender and age, between the two groups. Body mass index (BMI) IS an important indicator reflecting the degree of body fat and thinness. The results of this study showed that there was a significant difference in BMI between the two groups. The IS case group was significantly higher than the control group, indicating that obesity may be one of the risk factors for ischemic stroke and plays an important role in the incidence of IS. Hypertension and diabetes mellitus are closely associated with ischemic stroke, and a history of both diseases is an important risk factor for ischemic stroke. The results of blood lipid showed that the IS group had a higher proportion of hypertension and diabetes. The results of studies on the relationship between bad behaviors such as smoking and ischemic stroke are inconsistent. In this study, although there was no significant difference in the proportion of smoking history between the two groups, the proportion of smoking history in the control group was still higher than that in the IS case group. The levels of serum TC, TG, HDL-C and apolipoprotein A1 were significantly different ($P<0.05$). The levels of serum TC and LDL-C in the IS group were lower than those in the control group, suggesting that smoking may be a risk factor for ischemic stroke.

In recent years, GWAS based on SNPs has become the main detection method for the study of complex disease susceptibility sites and genes. The susceptibility sites of ischemic stroke have been found on all chro-

mosomes except chromosome 18, among which genetic variation in chromosome 9p21 region has aroused great concern. The total length of DNA sequence in the 9p21 region is about 175 KB, with high linkage imbalance, mainly including methylthio adenosine phosphorylase, MTAP, cyclin-dependent kinase inhibitive factor (CDKN) 2A, CDKN2B, ANRIL (antisense non-coding RNA) and other genes (29). Matarin et al. (30) used GWAS to detect more than 400,000 specific SNPs in 249 white IS patients and 268 controls, and found that none of the gene loci was closely associated with stroke risk, but this was the first time that CDKN2A on chromosome 9p21 was found to have a weak association with IS. Lovkvist et al. (31) also confirmed that SNP rs4977574 of 9p21 in 3986 IS patients and 2459 healthy controls were associated with IS. Bi et al. (32) pointed out that chromosome 9p21 IS a susceptibility gene of IS, and the influence of its mutation may change with age. This study IS the first to demonstrate that rs10757278 GG genotype, rs1537378-C allele and rs1333047-TT genotype are associated with IS in the Chinese Han population. It was also found that the plasma levels of total cholesterol, triglyceride and low-density lipoprotein of rs10757278-GG in IS carriers were significantly increased, and there were still statistically significant differences after the inclusion of the control group ($P<0.05$). Therefore, it IS speculated that the variation of rs10757278 can affect the level of blood lipid to different degrees. At the same time, people with rs10757278-G allele variation and dyslipidemia may have a higher susceptibility to IS. The influence of genotype on the level of blood lipid will interfere with the process of IS to a certain extent.

In this study, the genotypes of 4 IS risk loci (SNPs) in the reported 9p21 region were detected and analyzed with IS patients and healthy controls in Anhui, China as the subjects. The results of the Hardy-Weinberg genetic balance test showed that the four SNPs in the IS case group and the control group all complied with HWE's law ($P>0.05$), which indicated that the study population was from the natural population of random mating and was representative of the population. The results of unit point analysis showed that the genotypes of rs2383207 and rs10757274 were significantly different between the IS case group and the control group ($P<0.05$). There was no significant difference in the distribution of rs1333049 and rs2383206 genotypes between the IS case group and the control group ($P>0.05$). The results of the analysis of the relationship between alleles and ischemic stroke showed that there were significant differences in the distribution of alleles at four SNPs loci between IS patients and control groups ($P<0.05$). The results of this study indicate that rs2383207, rs10757274, rs1333049 and rs2383206 are the susceptible sites of ischemic stroke in Anhui, China. The C allele at rs1333049 and the G allele at rs2383207, rs10757274 and rs2383206 are risk alleles for ischemic stroke, which can increase the susceptibility to ischemic stroke. The association between chromosomal 9p21 region inheritance and ischemic stroke has been confirmed in Anhui, China.

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