Impact of Tag Single Nucleotide Polymorphisms (SNPs) in CCL11 Gene on Risk of Subtypes of Ischemic Stroke in Xinjiang Han Populations

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1,2   **Chen Liang***
ABCD 3   **Guihua Ni***
BC 1   **Jianhua Ma**
BF 2   **Hai Liu**
D 2   **Zhifeng Mao**
B 2   **Honggang Sun**
ADG 1,4   **Xiaoning Zhang**

1 Department of Neurology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P.R. China
2 Department of Neurology, The 7th Division Hospital of Xinjiang Production and Construction Corps, Kuitun, Xinjiang, P.R. China
3 Department of Neurology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, P.R. China
4 Department of Neurology, Traditional Chinese Medicine Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, P.R. China

Corresponding Author:
Source of support:

* These authors contributed equally to this work
Xiaoning Zhang, e-mail: qsyy_sjnk@163.com
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Background: CCL11 is an important inflammatory cytokine associated with inflammation-related diseases such as atherosclerosis and stroke. The aim of this study was to investigate the relationship between CCL11 gene polymorphism with subtypes of ischemic stroke in Xinjiang Han populations.

Material/Methods: The improved multiple ligase detection reaction (iMLDR) method was used to analyze the genotypes of 6 tag SNPs in the CCL11 gene (rs1129844, rs17809012, rs1860183, rs1860184, rs4795898, and rs4795895) in a case-control study of 406 lacunar stroke patients, 214 large-artery atherosclerotic (LAA) stroke patients, and 425 controls.

Results: We found the GG genotype of rs4795895 was significantly associated with increased risk of lacunar stroke (adjusted OR=1.676, 95%CI=1.117–2.515), and the GA genotype of rs17809012 was associated with a significant increase in risk of LAA stroke (adjusted OR=1.337, 95%CI=1.127–1.585). Hypertension stratification analyses showed that the GA genotype of rs17809012 was significantly associated with LAA stroke in the hypertensive group (adjusted OR=1.274, 95%CI=1.015–1.601). In the non-hypertensive group, the GA genotype of rs17809012 was significantly associated with LAA stroke (adjusted OR=1.361, 95%CI=1.041–1.780). The GG genotype of rs4795895 (adjusted OR=1.147, 95%CI=1.115–4.134) and the TT genotype of rs1860184 were significantly associated with lacunar stroke (adjusted OR=2.440, 95%CI=1.550-3.840).

Conclusions: This study demonstrates that the CCL11 gene could play an important role in the pathogenesis of lacunar stroke and LAA stroke in the Han population of China.

MeSH Keywords: **Cerebral Infarction • Chemokine CCL11 • Polymorphism, Genetic**

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Background

Ischemic stroke accounts for approximately 70–80% of all stroke, which is a leading cause of mortality and disability in China [1,2]. In addition to common risk factors such as hypertension, diabetes, and smoking, recent studies have shown that genetic factors have important roles in the pathogenesis of ischemic stroke [3,4].

It is common knowledge that the main cause of ischemic stroke is atherosclerosis. Recent studies showed that inflammatory reaction is an essential component in the appearance and development of atherosclerosis [5]. The inflammatory gene CCL11 maps to chromosome 17q21.1, spans approximately 8 kb, and contains 3 exons [6]. CCL11 is a chemokine exotoxin that promotes migration and activation of eosinophils, atheroma mast cells, and macrophages, participating in formation of atherosclerosis [7]. Recent studies indicate that CCL11 may be associated with inflammatory-related diseases such as asthma [8], atherosclerosis, and myocardial infarction [9], and even stroke. However, few studies have explored the impact of tag SNPs in the CCL11 gene on subtypes of ischemic stroke. In the present study, we selected lacunar stroke and large-artery atherosclerotic (LAA) stroke patients and excluded cardiogenic embolism and non-atherosclerotic stroke patients. The aim of the study was to investigate the association of tag SNPs of the CCL11 gene with 2 subtypes of ischemic stroke in the Xinjiang Han population.

Material and Methods

Ethics approval of the study protocol

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, Urumqi, China (NO. 20160512). Written informed consent was obtained from all participants.

Subjects

Between Mar 2016 and Apr 2017, 406 lacunar stroke patients, 214 LAA stroke patients, and 425 controls were enrolled from the First Affiliated Hospital of Xinjiang Medical University and the 7th Division Hospital of Xinjiang. All participants were of Han ethnicity and were longtime residents of the Xinjiang region of China. Inclusion criteria of the case group were: (1) Stroke subtyping was performed according to the TOAST classification [10]. Large-artery atherosclerosis (LAA) stroke was diagnosed when the patients had significant (>50%) stenosis or occlusion of a major cerebral artery or branch cortical artery with infarct size >1.5 cm on brain imaging [11]. Lacunar stroke was defined as a clinical lacunar

syndrome [12], with an anatomically compatible lesion on MRI (≤1.5 cm in diameter). (2) Onset of stroke within 7 days. (3) Age 18–80 years old. Exclusion criteria were: (1) Coronary heart disease, atrial fibrillation, arteritis, or blood system disease. (2) Severe dysfunction of heart, lung, liver, or kidney. (3) Malignancy. (4) Cerebral hemorrhage, brain trauma, or intracranial tumor. (5) Immune system disease, Parkinson's disease, or hereditary diseases.

Controls consisted of persons who had participated in a health checkup without history of stroke and hospital patients without acute cerebrovascular disease, and cranial MRI or CT scan was normal. Exclusion criteria were the same as for the cases.

Clinical data collection

The general information was recorded for all participants at admission: age, sex, smoking and drinking, and history of hypertension, diabetes mellitus, cardiac disease, and stroke. During the visit to the medical center, an emergency head CT was taken, and the next day an overnight fasting venous blood sample was taken to determine glucose and lipid levels. Locations and volume of infarcts were detected by brain MRI or CT.

Hypertension was defined as history of hypertension or using anti-hypertension drugs or if the mean of 3 measurements of systolic blood pressure (SBP) was >140 mmHg or if diastolic blood pressure (DBP) was >90 mmHg, respectively. Based on the criteria of the American Diabetes Association [13], diabetes mellitus was defined as history of diabetes or taking an anti-hypoglycemic drug, or daytime random blood glucose ≥11.1 mmol/l or after fasting glucose ≥7.0 mmol/l or glucose in line 2 h ≥11.1mmol/l. Hyperlipidemia was defined as history of hyperlipidemia or TC >5.72 mmol/L and (or) TG >1.7 mmol/L.

Tag SNPS selection and genotyping

Genomic DNA was extracted from peripheral blood of all participants based on the technique described elsewhere [14]. We used Haploview 4.2 software to select the 5 tag SNPs in the CCL11 gene, including rs1129844, rs17809012, rs1860183, rs1860184, and rs4795898, based on minor allele frequency (MAF) >0.1 and linkage disequilibrium patterns with r^2> 0.8 as a cut-off. In addition, we selected a tag SNP, rs4795895 (MAF>0.05), which is a positive polymorphism reported in a previous study [15]. These 6 tag SNPs captured the information of the 15 known CCL11 SNPs (Figure 1B). Genotyping was determined by improved multiple ligase detection reaction (iMLDR) [16], with technical support from the Center for Human Genetic Research, Shanghai Genesky Biotech Company. The technicians performing genotyping were blinded to the cases and controls. About 10% of samples were randomly selected for further verification, and the results were 100% concordant.
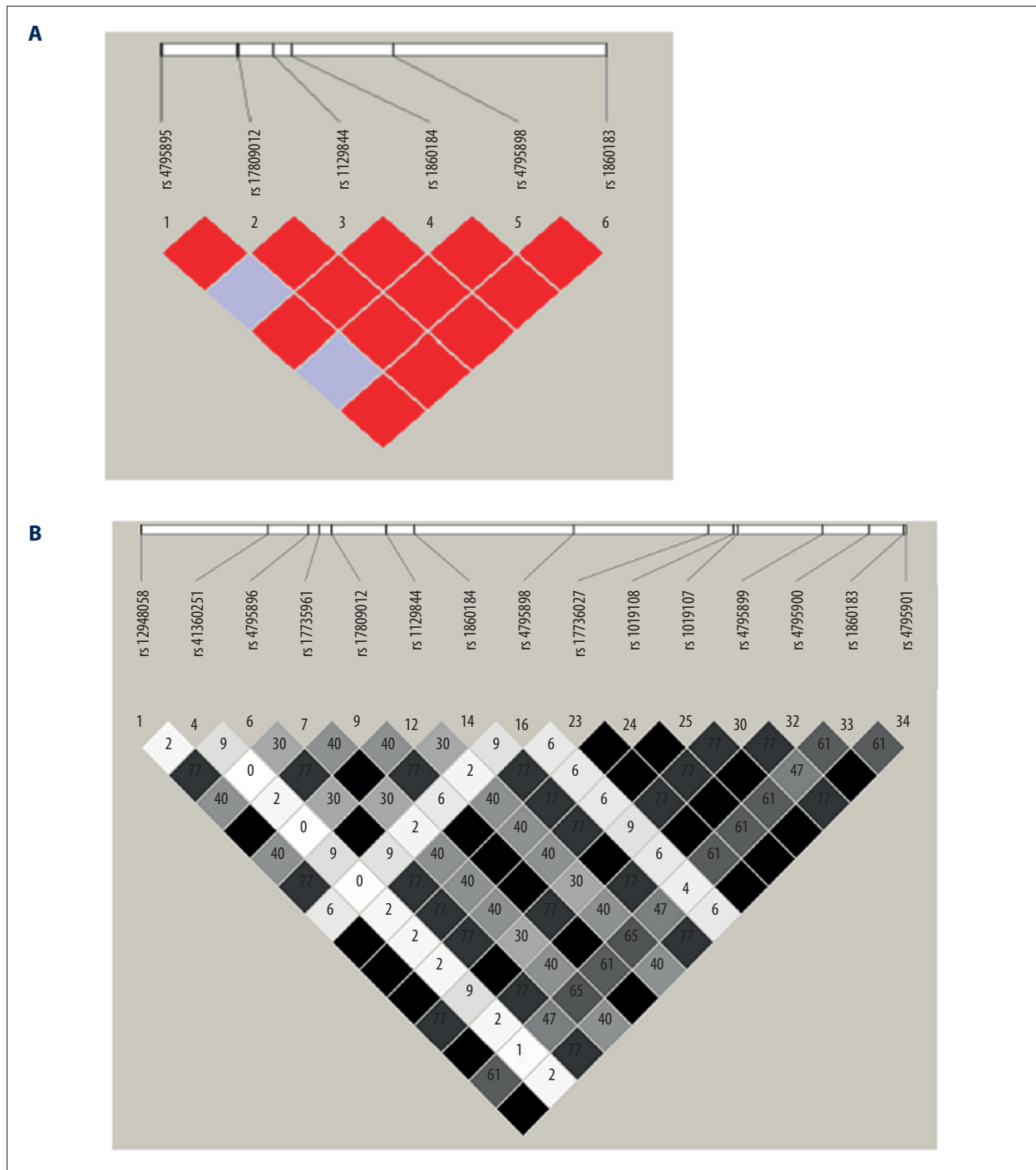
Figure 1. Schematic of pairwise LD between the six tagSNPs with in CCL11 gene. All *D'* values are 1 among the sixtag SNPs (**A**). Details of fifteen respective SNPs captured by the six tagSNPs are indicated. The r² values are plotted as a graph to show the linkage disequlibrium among the fifteen SNPs captured by these six tagSNPs (**B**).

Statistical analysis

Categorical data are presented as numbers (percentage). Chi-square analyses were used for comparison. Univariate and multivariate logistic regression were used to obtain the crude and adjusted odds ratios (ORs) for assessment of CCL11 gene polymorphisms with risk of subtypes of ischemic stroke. Hardy-Weinberg equilibrium was tested by chi-square analysis. Bilateral *P*<0.05 represented a significant difference. We used SPSS17.0 for analysis.

Table 1. Baseline characteristics of lacunar, LAA stroke patients and control subjects.

Characteristics	Lacunar (n=406)		LAA (n=214)		Control (n=425)	
Age (years)[1]						
<60	178	(43.8)	93	(43.5)	192	(45.2)
60–70	165	(40.6)	89	(41.6)	173	(40.7)
>70	63	(15.5)	32	(14.9)	60	(14.1)
Male, n (%)[1]	245	(60.3)	136	(63.6)	238	(56.0)
Hypertension, n (%)[1]	258	(63.5)*	134	(62.6)*	196	(46.1)
Diabetesmellitus, n (%)[1]	131	(32.3)*	60	(28.0)*	52	(12.2)
Hyperlipidemia, n (%)[1]	122	(30.0)	68	(31.8)	119	(28.0)
Smoking, n (%)[1]	129	(31.8)	63	(29.4)	114	(26.8)
Drinking, n (%)[1]	52	(12.8)	24	(11.2)	45	(10.6)

[1] Chi-square between the group of LI, LAA and controls; * $P<0.05$.

Results

The study included 406 lacunar patients, 214 LAA patients, and 425 controls. Baseline characteristics of cases and controls are presented in Table 1. There were no significant differences between the lacunar or LAA group and control group in age, sex, hyperlipidemia, smoking, or drinking ($p>0.05$). The prevalence of hypertension and diabetes was significantly higher in the lacunar and LAA stroke patients than in the control subjects ($p<0.05$).

Figure 1 shows a schematic of pairwise LD between SNPs in the CCL11 gene. All D' values were 1 among the 6 tag SNPs, showing strong linkage disequilibrium. Pairwise LD of the 15 SNPs captured by the 6 tag SNPs are also indicated by r^2 values.

Genotype and allele distributions of the 6 tag SNPs in patients with subtypes of ischemic stroke and control subjects are presented in Table 2. All genotype distribution of cases and controls were consistent with the Hardy-Weinberg equilibrium (data not shown). For the rs4795895 polymorphism, the frequency of GG genotype and G allele was significantly higher in lacunar patients compared with control subjects ($p<0.05$). For the rs17809012 polymorphism, the frequency of GA genotype and G allele was significantly higher in LAA patients compared with control subjects ($p<0.05$). The results from logistic regression analyses are presented in Table 3. Logistic regression analysis showed that the GG genotype of rs4795895 had a significant increase in the risk of lacunar stroke (adjusted OR=1.676, 95%CI=1.117–2.515), and the allele G was significantly associated with an increased risk of lacunar stroke (OR=1.617, 95%CI=1.136–2.302). The GA genotype of rs17809012 had a significant increase in the risk of LAA stroke (adjusted OR=1.337, 95%CI=1.127–1.585), and the allele G was significantly associated with an increased risk of LAA stroke (OR=1.287, 95%CI=1.009–1.641).

Hypertension stratification analyses between the 6 tag SNPs and risk of subtypes of ischemic stroke were carried out, and only meaningful polymorphism sites are presented in Table 4. After stratification by hypertension and adjustment for age, sex, diabetes mellitus, hyperlipidemia, smoking, and drinking, the GA genotype of rs17809012 was significantly associated with LAA stroke in the hypertensive group (adjusted OR=1.274, 95%CI=1.015–1.601). In the non-hypertensive group, the GA genotype of rs17809012 was significantly associated with LAA stroke (adjusted OR=1.361, 95%CI=1.041–1.780). The GG genotype of rs4795895 (adjusted OR=1.147, 95%CI=1.115–4.134) and the TT genotype of rs1860184 were significantly associated with lacunar stroke (adjusted OR=2.440, 95%CI=1.550–3.840).

Discussion

CCL11 (Eotaxin1) is a member of the CC chemokine family and operates through the receptor for eotaxin (CCR3) [17]. CCR3 receptor acts on Th2 lymphocytes, basophils, mast cells, and eosinophils [18], which participate in inflammation. CCL11 is an eosinophil chemotactic factor, and its roles are to direct eosinophils towards the sites of inflammation, promote degranulation, and release eosinophil cationic proteins. Eotaxin plays an important role in the formation of atherosclerosis, and evidence suggests it is overexpressed in endothelium and vascular smooth muscle cells in human atheroma [19]. The mechanism may be that eotaxin promotes endothelial cell migration *in vitro,* as well as angiogenesis and endothelial cell sprouting from the artery [20–22].

Table 2. Genotype and allele distributions of the six SNPS in patients with subtypes of ischemic stroke and control subjects.

Genotypes	Lacunar		Control		P value	LAA		P value
rs1129844								
GG	301	(74.1%)	325	(76.5%)	0.585	158	(73.8%)	0.547
GA	100	(24.6%)	93	(21.9%)		50	(23.4%)	
AA	5	(1.2%)	7	(1.6%)		6	(2.8%)	
Allele G	702	(86.5%)	743	(87.4%)	0.562	366	(85.5%)	0.345
Allele A	110	(13.5%)	107	(12.6%)		62	(14.5%)	
rs17809012								
GG	36	(8.9%)	38	(8.9%)	0.856	17	(7.9%)	0.006*
GA	178	(43.8%)	194	(45.6%)		126	(58.9%)	
AA	192	(47.3%)	193	(45.4%)		71	(33.2%)	
Allele G	250	(30.8%)	270	(31.8%)	0.668	160	(37.4%)	0.045*
Allele A	562	(69.2%)	580	(68.2%)		268	(62.6%)	
rs1860183								
CC	248	(61.1%)	255	(60.0%)	0.799	115	(53.7%)	0.152
CT	140	(34.5%)	154	(36.2%)		85	(39.7%)	
TT	18	(4.4%)	16	(3.8%)		14	(6.6%)	
Allele C	636	(78.3%)	664	(78.1%)	0.918	315	(73.6%)	0.072
Allele T	176	(21.7%)	186	(21.9%)		113	(26.4%)	
rs1860184								
TT	157	(38.7%)	149	(35.1%)	0.432	66	(30.8%)	0.391
TA	196	(48.3%)	210	(49.4%)		118	(55.2%)	
AA	53	(13.0%)	66	(15.5%)		30	(14.0%)	
Allele T	510	(62.8%)	508	(59.8%)	0.203	250	(58.4%)	0.642
Allele A	302	(37.2%)	342	(40.2%)		178	(41.6%)	
rs4795895								
GG	357	(87.9%)	345	(81.2%)	0.024*	181	(84.6%)	0.426
GA	44	(10.8%)	74	(17.4%)		29	(13.5%)	
AA	5	(1.2%)	6	(1.4%)		4	(1.9%)	
Allele G	758	(93.3%)	764	(89.9%)	0.011*	391	(91.4%)	0.399
Allele A	54	(6.7%)	86	(10.1%)		37	(8.6%)	
rs4795898								
CC	18	(4.4%)	7	(1.7%)	0.059	6	(2.8%)	0.317
CT	103	(25.4%)	106	(24.9%)		44	(20.6%)	
TT	285	(70.2%)	312	(79.4%)		164	(76.6%)	
Allele C	139	(17.1%)	120	(14.1%)	0.092	56	(13.1%)	0.613
Allele T	673	(82.9%)	730	(85.9%)		372	(86.9%)	

Table 3. Association of rs17809012, rs4795895 polymorphisms with risk of subtypes of ischemic stroke.

	Crude OR (95%CI)	Crude *P*	Adjust OR (95%CI)[a]	Adjust *P*[a]
Lacunar				
rs4795895				
GG	1.685 (1.146–2.476)	0.008*	1.676 (1.117–2.515)	0.013*
GA	0.760 (0.622–0.929)	0.007*	0.775 (0.627–0.957)	0.018*
AA	0.956 (0.642–1.423)	0.823	0.867 (0.575–1.308)	0.497
G allele	1.617 (1.136–2.302)	0.008*		
A allele	0.626 (0.440–0.893)	0.010*		
LAA				
rs17809012				
GG	0.881 (0.485–1.601)	0.678	0.898 (0.484–1.667)	0.733
GA	1.348 (1.144–1.590)	0.000*	1.337 (1.127–1.585)	0.001*
AA	0.848 (0.757–0.950)	0.005*	0.853 (0.758–0.960)	0.008*
G allele	1.287 (1.009–1.641)	0.042*		
A allele	0.793 (0.622–1.012)	0.062		

[a] Adjust for age, sex, hypertension, diabetes, hyperlipidemia, smoking, drinking.

Recent studies showed that lacunar stroke is a highly heritable and complex disease [12]. In this study, we investigated the relationship between 6 tag SNPs of CCL11 with the risk of lacunar stroke in 406 cases and 425 controls. The results showed that rs4795895 was significantly associated with lacunar stroke, but was not found in LAA stroke. After adjusting for age, sex, hypertension, diabetes mellitus, hyperlipidemia, smoking, and drinking, multiple logistic regression analysis showed that the odds of lacunar stroke were 1.676 times greater in patients with GG genotype than in those without GG genotype, indicating that the GG genotype of rs4795895 may be a potential risk for lacunar stroke. Zhao et al. [15] found that CCL11 rs4795895 was significantly associated with ischemic stroke in a Chinese Han population. However, in contrast to our choice of subtypes of ischemic stroke, they selected all types of stroke patients to observe. Sitara Roy et al. [23] found that CCL11 rs4795895 was significantly associated with lacunar stroke or LAA stroke in a southern Indian population. However, we found no correlation between rs4795895 and LAA stroke. These studies were partly consistent with our study results. The differences may be due to characteristics of participants, as well as regional and ethnic factors.

A previous study reported that stroke patients with or without hypertension may have different pathogenesis [24]. To explore the influence of CCL11 genes on subtypes of ischemic stroke in the hypertension and non-hypertension groups, hypertension stratification analyses were performed. After stratification by hypertension, SNP rs4795895 was significantly associated with lacunar stroke in the non-hypertensive groups. We found a novel polymorphism (rs1860184) associated with lacunar stroke in non-hypertensive populations. The TT genotype of rs1860184 was significantly associated with lacunar stroke in the non-hypertensive groups, indicating that the TT genotype may be a potential risk for lacunar stroke in people without hypertension. In clinical practice, there are some stroke patients without hypertension, diabetes, and other risk factors, but with higher levels of arteriosclerosis; this may be due to genetics. In future studies, patients with fewer risk factors should be selected, which may better identify the pathogenic gene *loci* for ischemic stroke. Although microatheroma constitutes the main etiology, lacunar infarcts may be caused in less than 5% of cases, by various etiologies, mainly hematological diseases and infectious or inflammatory arteritis [25]. An interesting area of future research could be to investigate the association of tag SNPs of the CCL11 gene in this unusual etiological lacunar infarct subgroup of patients.

In the present study, we found a novel polymorphism (rs17809012) associated with LAA stroke in the Chinese Han population. The odds of LAA stroke were 1.337 times greater in patients with GA genotype than in those without the GA genotype, indicating that the GA genotype of rs17809012 may be a potential risk factor for LAA stroke. Hypertension stratification analyses showed that rs17809012 remained significant in hypertensive and non-hypertensive groups.

Table 4. Hypertension stratification analyses between SNP and risk of subtypes of ischemic stroke.

Variables	N (cases/controls)	Percentage (cases/controls)	Adjusted OR (95%CI)[a]		p Value
Hypertension					
rs4795895 (Lacunar)	258/196				
GG	226/170	87.6/86.7	1.161	(0.660–2.044)	0.604
GA	29/22	11.2/11.2	0.959	(0.711–1.293)	0.782
AA	3/4	1.2/2.1	0.835	(0.500–1.397)	0.493
rs1860184 (Lacunar)					
TT	82/72	31.8/36.7	0.770	(0.516–1.149)	0.201
TA	140/95	54.3/48.5	1.134	(0.937–1.372)	0.196
AA	36/29	13.9/14.8	1.016	(0.848–1.216)	0.867
rs17809012 (LAA)	134/196				
GG	12/20	8.9/10.2	0.978	(0.451–2.121)	0.954
GA	75/87	56.0/44.4	1.274	(1.015–1.601)	0.037*
AA	47/89	35.1/45.4	0.878	(0.752–1.026)	0.101
Non-hypertension					
rs4795895 (Lacunar)	148/229				
GG	132/186	89.2/81.2	1.147	(1.115–4.134)	0.022*
GA	14/41	9.5/18.0	0.689	(0.491–0.968)	0.032*
AA	2/2	1.3/0.8	0.809	(0.424–1.544)	0.520
rs1860184 (Lacunar)					
TT	75/77	50.7/33.6	2.440	(1.550–3.840)	0.000*
TA	56/115	37.8/50.2	0.739	(0.591–0.924)	0.008*
AA	17/37	11.5/16.2	0.857	(0.686–1.069)	0.170
rs17809012 (LAA)	80/229				
GG	5/18	6.3/7.9	0.887	(0.310–2.537)	0.822
GA	51/107	63.7/46.7	1.361	(1.041–1.780)	0.024*
AA	24/104	30.0/45.4	0.790	(0.655–0.954)	0.014*

[a] Adjust for age, sex, diabetes, hyperlipidemia, smoking, drinking.

There are limitations to our study. Firstly, the procedures of selecting and grouping patients could not exclude possible selection bias. Secondly, the clinical data were gathered by self-reported questionnaires, which could lead to information bias. Thirdly, because the percentage of diabetes in the control group was too low, diabetes stratification analyses were not carried out. Prospective studies with larger sample sizes are needed.

Conclusions

This study demonstrates that rs4795895 of the CCL11 gene might be associated with lacunar stroke, particularly in the non-hypertensive group. rs1860184 was associated with a higher risk of lacunar stroke in the non-hypertensive group. rs17809012 was associated with LAA stroke in the Chinese Han population.

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