


Polymorphisms of Calgranulin Genes and Ischemic Stroke in a Chinese Population

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Background: The S100/calgranulin gene appears to modulate neuroinflammation following cerebral ischemia and could be a valuable biomarker for stroke prognosis, according to growing research. This study aimed at evaluating the correlation between calgranulin gene variants and susceptibility to ischemic stroke (IS) in the Southern Chinese population.

Methods: Using an enhanced multi-temperature ligase detection reaction genotyping, 310 IS patients and 324 age-matched healthy controls were genotyped to identify five calgranulin gene variants.

Results: According to the obtained results, the S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variants were linked to a higher risk of IS, while the S100A9 rs3014866 variant was associated with a lower risk of IS. Moreover, the T-T-C-A-T, T-T-C-G-T, or C-C-C-G-C haplotypes have been linked to a greater risk of developing IS, according to haplotype analysis. The occurrence of the variant C allele there in S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variants may impart a greater risk of stroke in the LAA subtype, according to further stratification by IS subtypes, while the T allele of the S100A9 rs3014866 variant may be linked to a reduced risk of stroke of all subtypes. Furthermore, patients with the variant C allele of the S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variants presented with increased circulating S100A8 and S100A12 levels and larger infarct volumes relative to those with the major TT genotype.

Conclusion: Our findings suggest that calgranulin gene variants are linked to IS susceptibility, implying that the calgranulin gene may be a potential biomarker for IS prevention and personalized treatment.

Keywords: ischemic stroke, calgranulin, single-nucleotide polymorphism, risk

Introduction

Stroke is a complex disorder that is one of the most common causes of adult disability across the globe. In China, the rate of stroke has risen considerably in recent decades, and around 80% of strokes are ischemic. Ischemic stroke (IS) occurs when blood flow within the area of an occluded blood vessel is disrupted, depriving local brain tissue of oxygen and resulting in malacia and necrosis.¹ A growing body of evidence supports that both genetic and environmental factors play pivotal roles in the pathophysiology of IS.² Atherosclerosis, chronic inflammation of blood vessel walls, has been recognized as an important pathogenic factor for IS. Furthermore, S100, a calcium-binding protein belonging to the EF-Hand family, has been considerably expressed in the human brain, particularly in astrocytes. The underlined protein family has been shown to play a significant role in various pathological activities, including proliferation, differentiation, cytoskeletal assembly, protein phosphorylation, and intracellular calcium homeostasis.^{3,4} Some S100 proteins regulate

cellular function in an autocrine or paracrine manner by activating surface receptors such as RAGE receptors to promote NF- κ B signaling, recruit and activate cellular pro-inflammatory effectors, thereby facilitating inflammation.⁵ Up to now, more than 25 S100 proteins have been discovered, among which S100A1, S100B, S100A6, S100A7, S100A8, S100A9 and S100A12 been reported to be involved in the pathogenesis of multiple neurological disorders including Alzheimer's disease,⁶ Parkinson's disease⁷ and stroke.^{8,9,10}

The S100/calgranulins family comprising S100A8, S100A9, and S100A12 proteins, are primarily expressed in myeloid-derived cells including monocytes, neutrophils, and dendritic cells.^{11,12} Numerous investigations have shown that calgranulins play a role in a variety of inflammatory disorders including inflammatory bowel disease,¹³ rheumatoid arthritis,¹⁴ myocardial infarction¹⁵ and IS.¹⁶ Furthermore, S100A8 and S100A9 typically exist as S100A8/A9 heterodimers, and they usually modulate inflammatory processes through both intracellular and extracellular pathways. S100A8/A9, an innate immune mediator with an inflammatory component, is elevated in the plasma of patients with acute IS that led to accelerating atherosclerotic plaque rupture and, as a result, stroke progression.¹⁷ In recent decades, it has been revealed that elevated S100A8/A9 levels are also linked to a higher probability of bad prognosis in IS.¹⁶ When compared to patients with TIA or healthy controls, S100A12 was considerably higher in patients with acute IS.¹⁸ According to prospective research, S100A12 levels in the blood have been linked to poor functional outcomes in patients with acute IS.¹⁹ In vitro evidence also suggests that S100A12 promotes inflammation, oxidative stress, and apoptosis induced by oxygen-glucose deprivation/reperfusion through activating ERK signaling.²⁰ These findings have led us to hypothesize that the calgranulin genes may have a role in IS pathogenesis.

The calgranulin genes encoding S100A8, S100A9, and S100A12 are located on chromosome 1q21. Previous studies have reported the associations between calgranulin genes polymorphisms and the risk of numerous diseases. Sun et al indicated that the S100A8 gene polymorphisms may influence the susceptibility and severity of aggressive periodontitis.²¹ Blanco-Rojo et al found that decreased T2D risk is linked with minor allele (T) of the S100A9 variant rs3014866 in three populations of varied ancestry including CORDIOPREV, GOLDN, and BPRHS.²² Salminen et al revealed that genetic polymorphism in the S100A9-S100A12-S100A8 locus influences serum and plasma MMP-8 levels and may be linked to the risk of cardiovascular disease in a genome-wide association study.²³ The calgranulin family, as a key pro-inflammatory mediator, has a variety of regulatory activities in IS and other inflammatory disorders. Hence, the calgranulin genes appear to be good candidate genes for transmitting hereditary susceptibility to IS. The purpose of the present study was to investigate the association of S100A8, S100A9, and S100A12 polymorphisms with IS in a case-control study of the southern Chinese population.

Materials and Methods

Participant's Recruitment

Between 2018 and 2020, we recruited 310 patients with IS (209 males, 101 females) from the Department of Neurology at Guangdong Medical University's Affiliated Hospital. The clinical signs and symptoms were recorded to diagnose IS. All of the patients had regular blood tests as well as computed tomography (CT) scans and/or magnetic resonance imaging (MRI) studies. Two neurologists used the TOAST¹⁸ criteria to categorize IS into five subgroups based on clinical signs and neuroimaging findings. The study excluded patients with a history of transient IS, cerebral hemorrhage, coronary artery disease, atrial fibrillation, haematologic illnesses, subarachnoid hemorrhage, systemic inflammatory diseases, autoimmune diseases, malignant tumors, and chronic infectious diseases. The control subjects were 324 participants (153 male and 171 female) who were selected during the same time from the Health Examination Center of Guangdong Medical University's Affiliated Hospital and were found similar in age and race to the IS subjects. Subjects with a recent history of the cerebrovascular disorder or myocardial infarction were excluded from the study. All of the registered participants were Han Chinese adults from Guangdong Province who were genetically unrelated. Each participant gave their informed consent in the written form. The present study was conducted in compliance with the Declaration of Helsinki and its approval was provided by the Guangdong Medical University Affiliated Hospital's Ethics Committee (No: YJYS2018053).

SNP Selection and Genotyping

Based on prior published results, five SNPs were chosen: S100A8 rs3795391 and rs3806232, S100A9 rs3014866 and rs1560833, and S100A12 rs2916191.^{21–24} The TIANamp Blood DNA kit (Tiangen Biotech, Beijing, China) was employed to extract genomic DNA from each person's peripheral leukocytes based on guidelines provided by the manufacturer. Following that, a DNA spectrophotometer (ND-1000, NanoDrop, Wilmington USA) was employed to evaluate the purity and concentration of DNA. The primers were utilized to genotype the chosen calgranulin SNPs using an iMLDR-TM method. The primers are indicated in [Table S1](#). The iMLDR-TM reactions and PCR methods were carried out as stated previously.²⁵

Enzyme-Linked Immunosorbent Assay

As the diagnosis was confirmed, participants' peripheral venous blood samples were taken. S100A9, S100A8, and S100A12 levels in serum were measured (in duplicate) employing ELISA kits (R&D Systems, Minneapolis, MN, USA), as suggested by the manufacturer.

Quantification of Infarct Volume

MIPAV software (Medical Image Processing, Analysis, and Visualization, version 3.0; NIH, Bethesda, MD) was used to calculate infarct volumes based on DWI.²⁶ To discriminate acute from non-acute diffusion variation, a semiautomatic segmentation approach was used to characterize acute diffusion lesions on a slice-by-slice basis, relying on apparent diffusion coefficient and fluid-attenuated inversion recovery imaging sequences. On multiplying the slice thickness by the entire lesion area, DWI infarct volumes may be determined.

Statistical Evaluation

SPSS version 19.0 (IBM, NY, USA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA) were used to perform statistical evaluations. The Chi-squared test or Fisher's exact test was used to evaluate allele frequencies and genotype distribution among IS patients and controls. The link between the S100A8, S100A9, and S100A12 genotypes and IS were assessed using the OR and 95% CI. HWE software was used to assess the Hardy-Weinberg equilibrium (HWE). Haploview was used to examine linkage disequilibrium (LD) and haplotypes. Student's *t*-test for normally distributed data or the Mann-Whitney *U*-test for nonparametric data were used to compare levels of S100A8, S100A9, and S100A12 with different genotypes in the patient and control groups. A multiple linear regression model was used to examine the correlations between the genotypes of the S100A8, S100A9, and S100A12 polymorphisms with the DWI infarct volume. Linear regressions were adjusted for age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. Bonferroni correction was applied for multiple comparisons with control type 1 error. For all the evaluations, two-tailed *P* value under 0.05 was regarded as statistically considerable.

Results

Demographic Characteristics

In total, 634 participants (310 IS patients and 324 healthy controls) were enrolled in the study, and their demographics and medical conditions were detailed in [Table 1](#). In terms of age ($P = 0.93$), no considerable differences were observed in the levels of serum uric acid ($P = 0.59$), triglyceride ($P = 0.61$), total cholesterol ($P = 0.22$), low-density lipoprotein (LDL) ($P = 0.71$), high-density lipoprotein (HDL) ($P = 0.26$), and homocysteine ($P = 0.78$) between patients and controls. However, substantial variations in sex, smoking status, hypertension, and diabetes were recorded between the control and SI groups ($P < 0.001$).

Association Between the Calgranulin Gene Variants and the Risk of IS

[Table 2](#) lists the allele and genotype frequencies for the calgranulin gene variants in the healthy (control) as well as IS patients. The HWE test revealed no selection bias in either group ($P > 0.05$). [Figure S1](#) depicts the linkage pattern of polymorphisms within the calgranulin gene. The X^2 test was used to compare the genotype distributions of IS patients and healthy individuals.

Table 1 Characteristics of Ischemic Stroke Cases and Controls

Variables	IS (n=310)	Control (n=324)	P value	P value ^a
Mean age (years)	62.2±9.9	62.3±9.2	0.89	0.93
Male/female	209/101	153/171	<0.001	<0.001
Smokers, n (%)	84(27.1)	34(10.5)	<0.001	<0.001
Hypertension, n (%)	237(76.5)	112(34.6)	0.003	<0.001
Diabetes, n (%)	112(36.1)	23(7.1)	<0.001	<0.001
SUA (mmol/L)	323.3±93.1	319.3±92.8	0.59	0.59
Total cholesterol (mg/dL)	5.00±1.23	5.11±1.15	0.25	0.22
Triglycerides (mmol/L)	1.52±1.08	1.47±1.04	0.55	0.61
HDL (mmol/L)	1.35±0.82	1.41±0.45	0.25	0.26
LDL (mmol/L)	3.01±1.08	3.04±0.95	0.71	0.71
HCY (mmol/L)	10.9±6.1	10.8±3.2	0.79	0.78

Notes: Continuous data are presented as mean ± SD, median (range) or n (%). P value under 0.05 were indicated in bold font. ^aAdjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia.

Abbreviations: IS, ischemic stroke; SUA, serum Uric acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HCY, Homocysteine.

Table 2 Genotype and Allele Frequencies of *S100A8/A9* and *S100A12* Polymorphisms Between IS Patients and Controls, and Corresponding ORs for IS

Genotype & Allele	IS Patients (n=310)	Controls (n=324)	OR (95% CI)	P value	P value ^a
<i>S100A8</i> rs3795391					
TT	247(79.7)	287(88.6)		0.0030	0.0040
TC	57(18.4)	36(11.1)			
CC	6(1.9)	1(0.3)			
TT vs TC +CC	63(20.3)	37(11.4)	1.98(1.27–3.07)	0.0020	0.0040
TT+TC vs CC	304(98.1)	323(99.7)	6.36(0.76–53.26)	0.063	0.063
T allele	551(88.9)	610(94.1)	1.00		
C allele	69(11.1)	38(5.9)	2.01(1.33–3.04)	0.0010	0.0040
<i>S100A8</i> rs3806232					
TT	248(80.0)	288(88.9)		0.0030	0.0040
TC	56(18.1)	35(10.8)			
CC	6(1.9)	1(0.3)			
TT vs TC +CC	62(20.0)	36(11.1)	2.00(1.28–3.12)	0.0020	0.0040
TT+TC vs CC	304(98.1)	323(99.7)	6.38(0.76–53.26)	0.063	0.063
T allele	552(89.0)	611(94.3)	1.000		
C allele	68(11.0)	37(5.7)	2.03(1.34–3.09)	0.0010	0.0040
<i>S100A9</i> rs3014866					
CC	126(40.7)	100(30.9)		<0.001	<0.001
CT	147(47.4)	149(46.0)			
TT	37(11.9)	75(23.1)			
CC vs CT+TT	184(59.3)	224(69.1)	0.65(0.47–0.90)	0.010	0.010
CC+CT vs TT	273(88.1)	249(76.9)	0.45(0.29–0.69)	<0.001	<0.001
C allele	399(64.4)	349(53.9)	1.000		
T allele	221(35.6)	299(46.1)	0.65(0.52–0.81)	<0.001	<0.001
<i>S100A9</i> rs1560833					
GG	115(37.1)	116(35.8)		0.74	0.97
GA	153(49.4)	169(52.2)			
AA	42(13.5)	39(12.0)			
GG vs GA+AA	195(62.9)	208(64.2)	1.06(0.77–1.46)	0.74	0.97
GG+GA vs AA	268(86.5)	285(88.0)	1.15(0.72–1.83)	0.57	0.97
G allele	383(61.8)	401(61.9)	1.000		
A allele	237(38.2)	247(38.1)	1.01(0.80–1.26)	0.97	0.97

(Continued)

Table 2 (Continued).

Genotype & Allele	IS Patients (n=310)	Controls (n=324)	OR (95% CI)	P value	P value ^a
S100A12 rs2916191					
TT	246(79.4)	283(87.4)		<0.001	<0.001
TC	58(18.7)	40(12.3)			
CC	6(1.9)	1(0.3)			
TT vs TC+CC	64(20.6)	41(12.7)	1.80 (1.17–2.75)	0.0070	0.0090
TT+TC vs CC	304(98.1)	323(99.7)	6.38(0.76–53.26)	0.050	0.050
T allele	550(88.7)	606(93.5)	1.000		
C allele	70(11.3)	42(6.5)	1.84(1.23–2.74)	0.0030	0.0060

Notes: Data are presented as number (%). ^aAdjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

The obtained results revealed a significant statistical relationship between the S100A8 rs3795391 ($P = 0.0040$) and rs3806232 ($P = 0.0040$), S100A9 rs3014866 ($P < 0.001$), and S100A12 rs2916191 ($P < 0.0010$) variants and the risk of IS. In a dominant model (TT vs TC/CC, TT vs TC/CC, CC vs CT/TT and TT vs TC/CC, respectively), considerable differences in the frequencies were seen between the IS group and the controls for rs3795391 (OR = 1.98, 95% CI: 1.27–3.07), rs3806232 (OR = 2.0, 95% CI: 1.28–3.12), rs3014866 (OR = 0.65, 95% CI: 0.47–0.90), and rs2916191 (OR = 1.80, 95% CI: 1.17–2.75), with P values of 0.0040, 0.0040, <0.001 , and 0.0090, respectively. The recessive model's rs3795391, rs3806232, and rs2916191 variants showed no considerable differences between the IS group and controls ($P > 0.05$). The recessive model for S100A9 rs3014866 frequency (CC+CT vs TT) in the IS group was significantly different from that in the controls (OR = 0.45, 95% CI: 0.29–0.69, $P < 0.0010$). The IS group had a considerably higher frequency of the variant C allele at rs3795391 (OR = 2.01, 95% CI: 1.33–3.04, $P = 0.0040$), rs3806232 (OR = 2.03, 95% CI: 1.34–3.09, $P = 0.0040$), and rs2916191 (OR = 1.84, 95% CI: 1.23–2.74, $P = 0.0060$), as well as the variant T allele at rs3014866 (OR = 0.65, 95% CI: 0.52–0.81, $P < 0.001$). On the other hand, the genotype and allele distributions for the S100A9 rs1560833 variant were found to be similar between IS patients and controls ($P > 0.05$).

Haplotype Analysis

The frequencies of the T-T-C-A-T (OR = 1.38, 95% CI: 1.03–1.84, $P = 0.040$), T-T-C-G-T (OR = 1.54, 95% CI: 1.14–2.07, $P = 0.010$), and C-C-C-G-C (OR = 2.82, 95% CI: 1.63–4.86, $P < 0.001$) haplotypes (according to the rs3795391-rs3806232-rs3014866-rs1560833-rs2916191 variants) were found to be considerably elevated in IS patients relative to the control group. Following adjustment for sex, age, smoking, diabetes mellitus, hyperlipidemia, and hypertension, these haplotypes were related to an elevated risk of IS (Table 3).

Table 3 The Frequencies of Haplotypes of S100A8/A9 and S100A12 Gene in Patients and Controls

Haplotypes	Case (n%)	Control (n%)	OR (95%)	P value	P value ^a
Calgranulin gene variants (rs3795391, rs3806232, rs3014866, rs1560833, rs2916191)					
T-T-T-G-T	167(26.9)	225(34.9)	1.000(Reference)		
T-T-C-A-T	179(28.9)	175(27.0)	1.38(1.03–1.84)	0.030	0.040
T-T-C-G-T	163(26.2)	143(22.1)	1.54(1.14–2.07)	0.005	0.010
T-T-T-A-T	41(6.7)	62(9.6)	0.89(0.57–1.39)	0.61	0.61
C-C-C-G-C	46(7.5)	22(3.5)	2.82(1.63–4.86)	<0.001	<0.001

Notes: Adjusted for age, sex, hypertension, diabetes mellitus, hyperlipidemia, and smoking. ^aFalse discovery rate-adjusted P value for multiple hypotheses testing using the Benjamini-Hochberg method. P value under 0.05 were indicated in bold font.

Associations Between the Calgranulin Gene Variants and Demographic Characteristics

Tables 4–8 shows the correlations of S100A8, S100A9, and S100A12 variants with demographic factors. After stratifying participants by age, sex, hypertension, diabetes, and smoking status, we discovered that the S100A8 rs3795391 C allele was linked to an elevated risk of IS in individuals over 70 ($P = 0.010$), men ($P = 0.013$), and non-diabetic patients ($P = 0.010$) (Table 4). The S100A8 rs3806232 C allele was found to be correlated with an elevated risk of IS in non-diabetic patients ($P = 0.010$) (Table 5). The S100A12 rs2916191 C allele was associated with a higher risk of IS in male ($P = 0.030$), non-diabetic ($P = 0.020$) and non-hypertensive patients ($P < 0.01$) (Table 8). In contrast, a decreased risk of IS was correlated with the variant T allele at S100A9 rs3014866 in male ($P = 0.0050$) and nondiabetic

Table 4 A Comparison Between the Baseline Characteristics of the S100A8 rs3795391 Genotypes and Alleles in the IS Patient and Control Groups

Characteristics	IS Patient Group					Control Group					P_G^a value	P_A^a value
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)			
	TT	TC	CC	T	C	TT	TC	CC	T	C		
Age												
≥70 years	61(80.3)	14(18.4)	1(1.3)	136(89.5)	16(10.5)	214(87.0)	31(12.6)	1(0.4)	459(93.3)	33(6.7)	0.22	0.13
<70 years	186(79.5)	43(18.4)	5(2.1)	415(88.7)	53(11.3)	71(93.4)	5(6.6)	0(0)	147(96.7)	5(3.3)	0.047	0.010
Gender												
Male	168(80.4)	37(17.7)	4(1.9)	373(89.2)	45(10.8)	138(90.2)	15(9.8)	0(0)	291(95.1)	15(4.9)	0.043	0.013
Female	79(78.2)	20(19.8)	2(2.0)	178(88.1)	24(11.9)	149(87.1)	21(12.3)	1(0.6)	319(93.3)	23(6.7)	0.18	0.056
Diabetes												
Yes	89(79.5)	22(19.6)	1(0.9)	200(89.3)	24(10.7)	21(91.3)	2(8.7)	0(0)	44(95.6)	2(4.4)	0.48	0.18
No	158(79.8)	35(17.7)	5(2.5)	351(88.6)	45(11.4)	266(88.4)	34(11.3)	1(0.3)	566(94.0)	36(6.0)	0.05	0.01
Hypertension												
Yes	197(83.1)	36(15.2)	4(1.7)	430(90.7)	44(9.3)	102(91.1)	10(8.9)	0(0)	214(95.5)	10(4.5)	0.17	0.043
No	50(68.5)	21(28.8)	2(2.7)	121(82.9)	25(17.1)	185(87.3)	26(12.3)	1(0.4)	396(93.4)	28(6.6)	0.010	<0.010

Notes: P_G : p value of the difference in genotypes between the case and control groups; P_A : p value of the difference in alleles between the case and control groups. ^aAdjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Table 5 A Comparison Between the Baseline Characteristics of the S100A8 rs3806232 Genotypes and Alleles in the IS Patient and Control Groups

Characteristics	IS Patient Group					Control Group					P_G^a value	P_A^a value
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)			
	TT	TC	CC	T	C	TT	TC	CC	T	C		
Age												
≥70 years	61(80.3)	14(18.4)	1(1.3)	136(89.5)	16(10.5)	72(92.3)	6(7.7)	0(0)	150(96.2)	6(3.8)	0.070	0.038
<70 years	187(79.9)	42(18.0)	5(2.1)	416(88.9)	52(11.1)	216(87.8)	29(11.8)	1(0.4)	461(93.7)	31(6.3)	0.070	0.016
Gender												
Male	169(80.9)	36(17.2)	4(1.9)	374(89.5)	44(10.5)	139(90.9)	14(9.1)	0(0)	292(95.4)	14(4.6)	0.040	0.013
Female	79(78.2)	20(19.8)	2(2.0)	178(88.1)	24(11.9)	149(87.1)	21(12.3)	1(0.6)	319(93.3)	23(6.7)	0.15	0.043
Diabetes												
Yes	89(79.5)	22(19.6)	1(0.9)	200(89.3)	24(10.7)	21(91.3)	2(8.7)	0(0)	44(95.6)	2(4.4)	0.48	0.18
No	159(80.3)	34(17.2)	5(2.5)	352(88.9)	44(11.1)	267(88.7)	33(11.0)	1(0.3)	567(94.2)	35(5.8)	0.05	0.01
Hypertension												
Yes	198(83.5)	35(14.8)	4(1.7)	431(90.9)	43(9.1)	102(91.1)	10(8.9)	0(0)	214(95.5)	10(4.5)	0.16	0.045
No	50(68.5)	21(28.8)	2(2.7)	121(82.9)	25(17.1)	186(87.7)	25(11.8)	1(0.5)	397(93.6)	27(6.4)	<0.010	<0.010

Notes: P_G : p value of the difference in genotypes between the case and control groups; P_A : p value of the difference in alleles between the case and control groups. ^aAdjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Table 6 A Comparison Between the Baseline Characteristics of the *S100A9 rs3014866* Genotypes and Alleles in the IS Patient and Control Groups

Characteristics	IS Patient Group					Control Group					P_G^a value	P_A^a value	
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)				
	CC	CT	TT	C	T	CC	CT	TT	C	T			
Age													
≥70 years	30(39.4)	36(47.4)	10(13.2)	96(63.2)	56(36.8)	22(28.2)	35(44.9)	21(26.9)	79(50.6)	77(49.4)	0.086	0.034	
<70 years	96(41.1)	111(47.4)	27(11.5)	303(64.7)	165(35.3)	78(31.7)	114(46.3)	54(22.0)	270(54.9)	222(45.1)	0.013	0.007	
Gender													
Male	85(40.6)	103(49.3)	21(10.1)	273(65.3)	145(34.7)	44(28.8)	73(47.7)	36(23.5)	161(52.6)	145(47.4)	0.010	0.005	
Female	41(40.6)	44(43.6)	16(15.8)	126(62.4)	76(37.6)	56(32.8)	76(44.4)	39(22.8)	188(55.0)	154(45.0)	0.27	0.091	
Diabetes													
Yes	43(38.4)	56(50.0)	13(11.6)	142(63.4)	82(36.6)	8(34.8)	7(30.4)	8(34.8)	23(50.0)	23(50.0)	0.027	0.10	
No	83(41.9)	91(46.0)	24(12.1)	257(64.9)	139(35.1)	92(30.5)	142(47.2)	67(22.3)	326(54.1)	276(45.9)	0.013	0.010	
Hypertension													
Yes	91(38.4)	116(48.9)	30(12.7)	298(62.9)	176(37.1)	31(27.7)	51(45.5)	30(26.8)	113(50.4)	111(49.6)	0.015	0.005	
No	35(47.9)	31(42.5)	7(9.6)	101(69.2)	45(30.8)	69(32.6)	98(46.2)	45(21.2)	236(55.7)	188(44.3)	0.030	0.007	

Table 7 A Comparison Between the Baseline Characteristics of the *S100A9 rs1560833* Genotypes and Alleles in the IS Patient and Control Groups

Characteristics	IS Patient Group					Control Group					P_G^a value	P_A^a value	
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)				
	GG	GA	AA	G	A	GG	GA	AA	G	A			
Age													
≥70 years	27(35.5)	39(51.3)	10(13.2)	93(61.2)	59(38.8)	30(38.5)	37(47.4)	11(14.1)	97(62.2)	59(37.8)	0.89	0.95	
<70 years	88(37.6)	114(48.7)	32(13.7)	290(62.0)	178(38.0)	86(34.9)	132(53.7)	28(11.4)	304(61.8)	188(38.2)	0.52	0.96	
Gender													
Male	83(39.7)	100(47.9)	26(12.4)	266(63.6)	152(36.4)	53(34.6)	83(54.3)	17(11.1)	189(61.8)	117(38.2)	0.48	0.61	
Female	32(31.7)	53(52.5)	16(15.8)	117(57.9)	85(42.1)	63(36.8)	86(50.3)	22(12.9)	212(62.0)	130(38.0)	0.62	0.35	
Diabetes													
Yes	37(33.0)	58(51.8)	17(15.2)	132(58.9)	92(41.1)	7(30.4)	12(52.2)	4(17.4)	26(56.5)	20(43.5)	0.95	0.76	
No	78(39.4)	95(48.0)	25(12.6)	251(63.4)	145(36.6)	109(36.2)	157(52.2)	35(11.6)	375(62.3)	227(37.7)	0.66	0.73	
Hypertension													
Yes	85(35.8)	118(49.8)	34(14.4)	288(60.8)	186(39.2)	46(41.1)	57(50.9)	9(8.0)	149(66.5)	75(33.5)	0.22	0.14	
No	30(41.1)	35(47.9)	8(11.0)	95(65.1)	51(34.9)	70(33.0)	112(52.8)	30(14.2)	252(59.4)	172(40.6)	0.43	0.23	

patients ($P = 0.010$) (Table 6). When stratified by sex, age, hypertension, diabetes, and status of smoking, no considerable variations in *S100A9 rs1560833* variant distributions and IS risk were found ($P > 0.05$) (Table 7).

Associations Between the Calgranulin Gene Variants and Stroke Subtypes

We divided IS patients into stroke subgroups based on TOAST categorization to see if the *S100A8*, *S100A9*, and *S100A12* variants were localized to a certain subset. Carriers with the C allele of the *S100A8 rs3795391* and *rs3806232* and *S100A12 rs2916191* variants ($P < 0.05$) appeared to have an elevated risk of stroke of the LAA or SAO (small-artery occlusion) subtype relative to the controls, as illustrated in Tables 9,10 and 13. In contrast, the *S100A9 rs3014866* T allele appeared to run a lower risk of stroke with all subtypes ($P < 0.05$) (Table 11). However, no statistical correlation was found between the *S100A9 rs1560833* variant and all stroke subtypes when compared with the controls ($P > 0.05$) (Table 12).

Table 8 A Comparison Between the Baseline Characteristics of the *S100A12* rs2916191 Genotypes and Alleles in the IS Patient and Control Groups

Characteristics	IS Patient Group					Control Group					P _G ^a value	P _A ^a value
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)			
	TT	TC	CC	T	C	TT	TC	CC	T	C		
Age												
≥70 years	61(80.3)	14(18.4)	1(1.3)	136(89.5)	16(10.5)	72(92.3)	6(7.7)	0(0)	150(96.2)	6(27.3)	0.11	0.046
<70 years	185(79.1)	44(18.8)	5(2.1)	414(88.5)	54(11.5)	211(85.8)	34(13.8)	1(0.4)	456(92.7)	36(7.3)	0.12	0.042
Gender												
Male	167(79.9)	38(18.2)	4(1.9)	372(89.0)	46(11.0)	137(89.5)	16(10.5)	0(0)	290(94.8)	16(5.2)	0.060	0.030
Female	79(78.2)	20(19.8)	2(2.0)	178(88.1)	24(11.9)	146(85.4)	24(14.0)	1(0.6)	316(92.4)	26(7.6)	0.24	0.12
Diabetes												
Yes	89(79.5)	22(19.6)	1(0.9)	200(89.3)	24(10.7)	21(91.3)	2(8.7)	0(0)	44(95.7)	2(4.3)	0.48	0.18
No	157(79.3)	36(18.2)	5(2.5)	350(88.4)	46(11.6)	262(87.1)	38(12.6)	1(0.3)	562(93.4)	40(6.6)	0.080	0.020
Hypertension												
Yes	197(83.1)	36(15.2)	4(1.7)	430(90.7)	44(9.3)	101(90.2)	11(9.8)	0(0)	213(95.1)	11(4.9)	0.24	0.064
No	49(67.1)	22(30.1)	2(2.8)	120(82.2)	26(17.8)	182(85.9)	29(13.7)	1(0.4)	393(92.7)	31(7.3)	0.010	<0.010

Notes: P_G: p value of the difference in genotypes between the case and control groups; P_A: p value of the difference in alleles between the case and control groups. ^aAdjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Table 9 The Relationship Between *S100A8* rs3795391 Genotypes and is Stratified by TOAST Classification in IS Patients

	Genotypes n (%)			P value ^a	P value ^{a*}	Alleles n (%)		P value ^b	P value ^{b*}	OR (95% CI)
	TT	TC	CC			T	C			
Control (n=324)	287(88.6)	36(11.1)	1(0.3)			610(94.1)	38(5.9)			
LAA (n=186)	148(79.6)	34(18.3)	4(2.2)	0.007	0.028	330(88.7)	42(11.3)	0.0020	0.0080	0.49(0.31~0.77)
SAO (n=105)	84(80.0)	19(18.1)	2(1.9)	0.038	0.076	187(89.0)	23(11.0)	0.013	0.026	0.51(0.29~0.87)
CE (n=8)	7(87.5)	1(12.5)	0(0)	1.000	1.000	15(93.8)	1(6.3)	1.000	1.000	0.93(0.12~7.3)
SOE&SUE (n=11)	8(72.7)	3(27.3)	0(0)	0.154	0.205	19(86.4)	3(13.6)	0.146	0.195	0.40(0.11~1.39)

Notes: ^ap value of the difference in genotype between the case and control groups; ^bp value of the difference in alleles between the case and control groups; ^{*}Adjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Abbreviations: LAA, large-artery atherosclerosis; SAO, small-vessel occlusion; CE, Cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

Table 10 The Relationship Between *S100A8* rs3806232 Genotypes and is Stratified by TOAST Classification in IS Patients

	Genotypes n (%)			P value ^a	P value ^{a*}	Alleles n (%)		P value ^b	P value ^{b*}	OR (95% CI)
	TT	TC	CC			T	C			
Control (n=324)	288(88.9)	35(10.8)	1(0.3)			611(94.3)	37(5.7)			
LAA (n=186)	149(80.1)	33(17.7)	4(2.2)	0.008	0.032	331(89.0)	41(11.0)	0.002	0.008	0.49(0.31~0.78)
SAO (n=105)	84(80.0)	19(18.1)	2(1.9)	0.037	0.074	187(89.0)	23(11.0)	0.010	0.02	0.49(0.29~0.85)
CE (n=8)	7(87.5)	1(12.5)	0(0)	1.000	1.000	15(93.8)	1(6.3)	0.615	0.615	0.91(0.12~7.07)
SOE&SUE (n=11)	8(72.7)	3(27.3)	0(0)	0.147	0.196	19(86.4)	3(13.6)	0.138	0.184	0.38(0.11~1.36)

Notes: ^ap value of the difference in genotype between the case and control groups; ^bp value of the difference in alleles between the case and control groups; ^{*}Adjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Abbreviations: LAA, Large-artery atherosclerosis; SAO, small-vessel occlusion; CE, cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

The Serum Levels of the Calgranulins Based on Different Genotypes

The serum levels of S100A8, S100A9, and S100A12 were detected in 62 IS patients and 66 controls. As indicated in [Figure 1](#), the serum levels of S100A8 ([Figure 1A](#)), S100A9 ([Figure 1D](#)) and S100A12 ([Figure 1F](#)) (with P-values of < 0.001, < 0.001, and < 0.001, accordingly) were considerably elevated in the IS patients when compared with the control. Moreover, we assessed whether there was an association between the serum levels of S100A8, S100A9, and S100A12 in IS patients and the calgranulin

Table 11 The Relationship Between *S100A9* rs3014866 Genotypes and is Stratified by TOAST Classification in IS Patients

	Genotypes n (%)			P value ^a	P value ^{a*}	Alleles n (%)		P value ^b	P value ^{b*}	OR (95% CI)
	CC	CT	TT			C	T			
Control (n=324)	100(30.9)	149(46.0)	75(23.1)			349(53.9)	299(46.1)			
LAA (n=186)	70(37.7)	94(50.5)	22(11.8)	0.006	0.024	234(62.9)	138(37.1)	0.005	0.020	0.69(0.53~0.89)
SAO (n=105)	44(41.9)	46(43.8)	15(14.3)	0.052	0.069	134(63.8)	76(36.2)	0.012	0.016	0.66(0.48~0.91)
CE (n=8)	5(62.5)	3(37.5)	0(0)	0.140	0.140	13(81.2)	3(18.8)	0.030	0.030	0.27(0.08~0.95)
SOE&SUE (n=11)	7(63.6)	4(36.4)	0(0)	0.049	0.098	18(81.8)	4(18.2)	0.010	0.020	0.26(0.09~0.78)

Notes: ^ap value of the difference in genotype between the case and control groups; ^bp value of the difference in alleles between the case and control groups; *Adjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Abbreviations: LAA, Large-artery atherosclerosis; SAO, small-vessel occlusion; CE, cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

Table 12 The Relationship Between *S100A9* rs1560833 Genotypes and is Stratified by TOAST Classification in IS Patients

	Genotypes n (%)			P value ^a	P value ^{a*}	Alleles n (%)		P value ^b	P value ^{b*}	OR (95% CI)
	GG	GA	AA			G	A			
Control (n=324)	116(35.8)	169(52.2)	39(12.0)			401(61.9)	247(38.1)			
LAA (n=186)	66(35.5)	91(48.9)	29(15.6)	0.505	2.020	223(59.9)	149(40.1)	0.541	1.082	0.92(0.71~1.20)
SAO (n=105)	42(40.0)	52(49.5)	11(10.5)	0.722	0.722	136(64.8)	74(35.2)	0.454	1.816	1.13(0.82~1.57)
CE (n=8)	2(25.0)	6(75.0)	0(0)	0.513	1.026	10(62.5)	6(37.5)	0.960	0.960	1.03(0.37~2.86)
SOE&SUE (n=11)	5(45.5)	4(36.4)	2(18.1)	0.513	0.684	14(63.6)	8(36.4)	0.868	1.157	1.08(0.45~2.61)

Notes: ^ap value of the difference in genotype between the case and control groups; ^bp value of the difference in alleles between the case and control groups; *Adjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia.

Abbreviations: LAA, Large-artery atherosclerosis; SAO, small-vessel occlusion; CE, cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

Table 13 The Relationship Between *S100A12* rs2916191 Genotypes and is Stratified by TOAST Classification in IS Patients

	Genotypes n (%)			P value ^a	P value ^{a*}	Alleles n (%)		P value ^b	P value ^{b*}	OR (95% CI)
	TT	TC	CC			T	C			
Control (n=324)	283(87.4)	40(12.3)	1(0.3)			606(93.5)	42(6.5)			
LAA (n=186)	148(79.6)	34(18.3)	4(2.1)	0.020	0.080	330(88.7)	42(11.3)	0.007	0.028	0.55(0.35~0.85)
SAO (n=105)	83(79.0)	20(19.1)	2(1.9)	0.044	0.088	186(88.6)	24(11.4)	0.019	0.038	0.54(0.32~0.91)
CE (n=8)	7(87.5)	1(12.5)	0(0)	1.000	1.000	15(93.8)	1(6.2)	1.000	1.000	1.04(0.13~8.06)
SOE&SUE (n=11)	8(72.7)	3(27.3)	0(0)	0.184	0.245	19(86.4)	3(13.6)	0.178	0.237	0.44(0.13~1.54)

Notes: ^ap value of the difference in genotype between the case and control groups; ^bp value of the difference in alleles between the case and control groups; *Adjusted for age, gender, smoking, hypertension, diabetes mellitus and dyslipidemia. P value under 0.05 were indicated in bold font.

Abbreviations: LAA, large-artery atherosclerosis; SAO, small-vessel occlusion; CE, cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology.

gene genotypes. When the samples were stratified based on *S100A8* genotypes, the serum *S100A8* levels were significantly higher in patients with the TC+CC genotypes in the rs3795391 ($P = 0.031$) (Figure 1B) or rs3806232 ($P = 0.038$) (Figure 1C) variant than those with the TT genotypes. Serum *S100A9* levels in IS patients of various genotypes did not differ significantly when patients were divided according to their *S100A9* genotype (Figure 1E). In addition, a considerable elevation in serum *S100A12* levels was observed in IS patients carrying the mutated rs2916191 TC+CC genotypes ($P = 0.032$) (Figure 1G). However, no considerable correlations were detected in the healthy controls carrying the major common alleles compared with the mutated alleles of *S100A8*, *S100A9*, and *S100A12* variants ($P > 0.05$) (Figure 1B, C, E and G).

Association of the Calgranulin Gene Variants with the Infarct Volume

Associations of the *S100A8*, *S100A9* and *S100A12* variants with the actual volumetric measurements of infarct volume by diffusion-weighted magnetic resonance imaging (DWI) in 58 individuals suffering from IS were evaluated. Their obtained results are presented in Figure 2. The mean infarct volumes in the IS patients with the *S100A8* (rs3795391 and

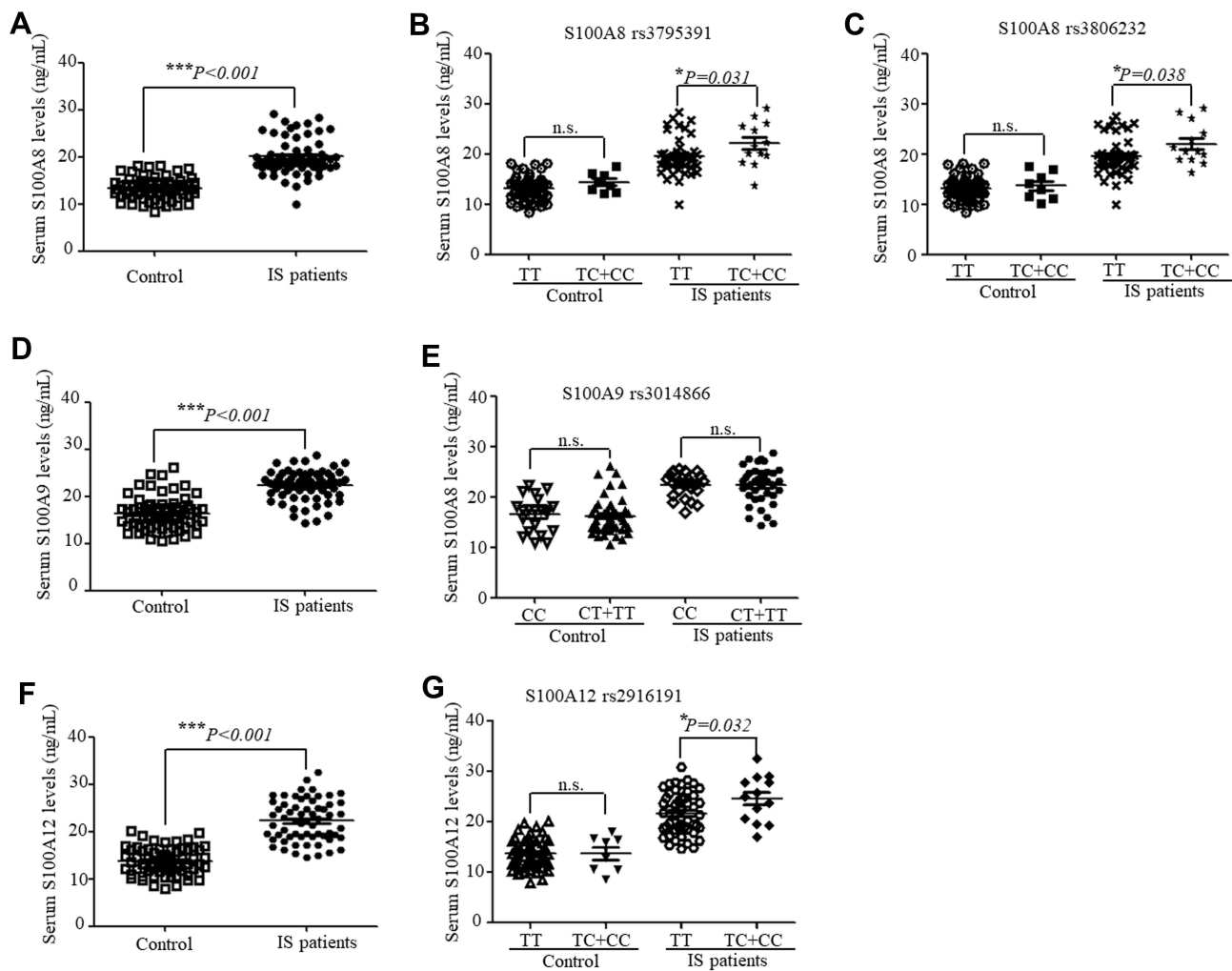


Figure 1 The serum calgranulin levels in IS patients (n=62) and controls (n=66) stratified based on corresponding genotypes of S100A8, S100A9 and S100A12. The serum S100A8 (A), S100A9 (D), and S100A12 (F) levels in IS patients and controls were measured using ELISA. The serum S100A8 levels in IS patients and controls were stratified according to different genotypes of S100A8 rs3795391 (B) and rs3806232 (C). The serum S100A9 levels in IS patients and controls were stratified according to S100A9 rs3014866 genotypes (E). The serum S100A12 levels in IS patients and controls were stratified according to S100A12 rs2916191 genotypes (G). The data are shown as medians with interquartile ranges (* $P < 0.05$, *** $P < 0.001$).

Abbreviation: n.s., not significant.

rs3806232) (Figure 2A and B) and S100A12 (rs2916191) (Figure 2C) variant genotypes (TC+CC) were found to be larger when compared to those patients associated with the major TT genotype ($P = 0.023$, $P = 0.015$ and $P = 0.043$, respectively).

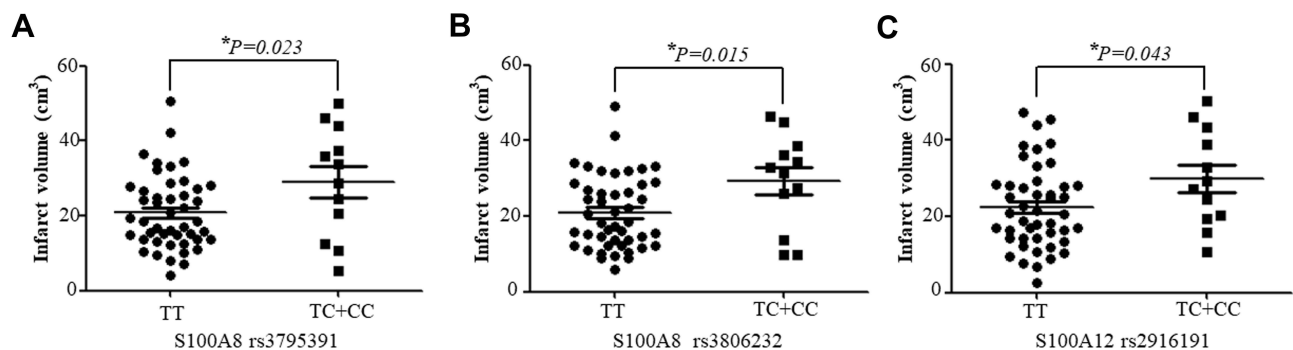


Figure 2 The mean infarct volumes in the IS patients (n=58) stratified based on corresponding genotypes of S100A8 rs3795391 (A), rs3806232 (B) and S100A12 rs2916191 (C). The data are shown as the mean \pm standard error (* $P < 0.05$).

Discussion

In this hospital-based case-control study, we demonstrate for the first time that S100A8 rs3795391, rs3806232, S100A9 rs3014866, and S100A12 rs2916191 variants are associated with susceptibility to IS in a Southern Chinese population. Haplotype analysis indicated that individuals with the T-T-C-A-T, T-T-C-G-T, or C-C-C-G-C haplotype may have an increased risk of IS. Further stratification revealed that the S100A8 rs3795391 and rs3806232 and S100A12 rs2916191 variants may confer an increased risk of LAA or SAO subtype stroke, while the S100A9 rs3014866 variant may associate with a lower risk of stroke of all subtypes. The circulating calgranulins levels were considerably elevated in the IS patients with the variant C alleles of the S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variants than in those carrying the major TT genotypes. Moreover, patients with the S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variant C alleles have greater infarct volumes than those with the main TT genotypes.

The most important pathologic underpinning of IS is atherosclerosis, which is a chronic inflammation of the vascular wall driven by activated T- and B-lymphocytes, macrophages, and smooth muscle cells with high calgranulin expression. Guo et al indicated that higher serum S100A8/9 levels were linked with an increased risk of poor prognosis for IS.¹⁶ According to Wakisaka et al, an elevated plasma S100A12 level at admission has been linked to poor functional outcomes in individuals with acute IS.¹⁹ S100A8/A9 was reported to induce neuroinflammation and enhance the pathophysiology of IS,^{27,28} and inhibiting S100A8/A9 reduced the size, inflammation, and vulnerability of atherosclerotic plaques in mice.²⁹ Moreover, APOE^{-/-} mice overexpressing S100A12 showed a significant increase in atherosclerotic plaque size, accompanied by increased oxidative stress and vascular calcification. In addition, inhibitor ABR-215757 could competitively bind with S100A12 in vitro and reduce atherosclerosis.³⁰ Given these facts, the calgranulin genes may serve a considerable role in the occurrence and development of IS. Calgranulin may be involved in the pathogenesis and prognosis of IS through several possible pathophysiological pathways. First, calgranulins are involved in the accumulation of neutrophils and macrophages, macrophage cytokine production, and smooth muscle cell proliferation in response to vascular inflammatory injury.³¹ Second, calgranulins can enhance activation of the RAGE or TLR4 receptors and promote atherosclerosis.^{30,32} Third, calgranulins stimulate the secretion of proinflammatory cytokines and are associated with inflammatory damage during cerebral ischemia-reperfusion.^{27,30} Despite these developments, the calgranulin alleles that cause IS remain elusive.

S100 calgranulin levels have been linked to a positive relationship between higher IS risk and a bad prognosis. In a previous investigation, elevated plasma S100A8/A9 concentrations were found to be considerably linked to an increased risk of poor clinical outcomes three months following the onset of IS.¹⁶ Another study showed that plasma S100A8/A9 is an independent predictor of 3-month mortality and helps identify patients with poor AIS prognosis.³³ Furthermore, circulating S100A12 levels have been linked to functional outcomes following IS, and short-term mortality, severity, and inflammatory process following spontaneous intracerebral hemorrhage.³⁴ It is highly speculated that the calgranulin gene variants might affect the progression and prognosis of IS by influencing the circulating calgranulins. Hence, we investigated the relationship between calgranulin gene variants and S100A8, S100A9, and S100A12 levels. Our study showed that patients with the variant C allele of the S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variants exhibited higher circulating S100A8 and S100A12 levels in IS, relative to those having the common T allele. Hence, it is believed that individuals with the mutant C allele at the rs3795391, rs3806232, and rs2916191 loci expressing elevated S100A8 or S100A12 levels may have enhanced pro-inflammatory signaling, which could exacerbate the consequent cellular responses to cerebral ischemia injury.

The damage to brain tissue induced by cerebral ischemia activates several pro-inflammatory and apoptotic cascades, resulting in the accumulation of many triggered microglia, infiltrating macrophages, and lymphocytes in the infarct area.³⁵ It is reported that S100A9 downregulation may reduce the infarct volume and brain swelling in a murine MCAO model.³⁶ Additional studies indicated that blocking calprotectin could reduce infarct volume, vascular inflammation, and vulnerability of atherosclerotic plaques.^{29,37,38} Danger-associated molecular patterns, including S100A8/A9 and HMGB1 are warning signals released by apoptotic cells in a variety of situations, including ischemia and hypoxia.³⁹ In the post-ischemic brain, inhibiting HMGB1 signaling with short hairpin RNA reduced infarct size, microglial stimulation, and the production of pro-inflammatory mediators.⁴⁰ Our previous study reported that the HMGB1 rs2249825 variant was

associated with low infarct volume and was a protective factor for IS in the Han Chinese population.⁴¹ Because of the involvement of S100 calgranulins in vascular inflammation, we hypothesized that they may have a role in stroke infarct size. Patients with the variant C alleles of the S100A8 rs3795391, rs3806232, or S100A12 rs2916191 variant have greater infarct sizes than those with the major T alleles, according to the findings. This is also supported by the evidence that the variant C alleles of the S100A8 rs3795391, rs3806232, or S100A12 rs2916191 variant were associated with increased circulating S100A8 and S100A12 levels, suggesting the calgranulin gene variants may induce the different expression of S100A8 and S100A12, which is associated with the infarct size.

There are several limitations to our case-control study. First, the study sample was not sufficiently large, which could have resulted in non-representative results. Second, potential biases such as information, selection, and confounding bias cannot be eliminated. Third, this study only investigated five calgranulin gene polymorphisms, although other variants may have had a role in IS susceptibility. Fourth, numerous environmental risk factors, such as smoking, diabetes, hypertension, hyperlipidemia, and hypercholesterolemia could have obscured the relationship between calgranulin gene variants and IS. Finally, the particular methods by which these calgranulin gene variants affect the expression of S100A8, S100A9, and S100A12 are unknown. Before the conclusions are evaluated for predicting individuals' risk of having IS, more independent validation of these outcomes in larger groups with varied ethnic backgrounds should be undertaken.

Finally, our research is the first to show a correlation between calgranulin gene variants and susceptibility to IS in a Southern Chinese population. The S100A8 rs3795391, rs3806232, and S100A12 rs2916191 variant C allele is linked to elevated circulating S100A8 and S100A12 levels as well as larger infarct volumes in IS patients. Our research could lead to a better understanding of individual susceptibility to IS and the development of effective IS control and preventive strategies. Future research is needed to confirm our findings and give an understanding of how calgranulin variants affect IS pathogenesis.

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Author Contributions

All authors have made significant contributions to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The authors have no actual or potential conflicts of interest related to this manuscript.

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