Genetic Association Analysis Between RNF213 Common Variants and Symptomatic Intracranial Atherosclerotic Stenosis in a Chinese Population

Xi Li
Xiangya Hospital Central South University https://orcid.org/0000-0002-9601-5076
Junyan Wang
Xiangya Hospital Central South University
Xiaoqing Zhou
Xiangya Hospital Central South University
Fang Yu
Xiangya Hospital Central South University
Xianjing Feng
Xiangya Hospital Central South University
Zeyu Liu
Xiangya Hospital Central South University
Qin Huang
Xiangya Hospital Central South University
Di Liao
Xiangya Hospital Central South University
Xin Jin
Central South University Third Xiangya Hospital
Jian Xia (xjian1216@csu.edu.cn)
Central South University https://orcid.org/0000-0001-9154-131X

Research

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Abstract

Background: RNF213 is the gene involved in symptomatic intracranial atherosclerotic stenosis (sICAS). The rare variants of this gene have a significant association with the clinical phenotype of the disease in the East Asian populations. However, the association between the common variants of RNF213 and sICAS has remained unclear. This study investigated the possible association between the common variants of RNF213 and sICAS in the Chinese population.

Result: A total of 39 common variants of the RNF213 gene were detected. The chi-squared test revealed two sites (rs8082521 and rs55996424) at which the genotype frequency and the allele frequency were significantly different between the sICAS group and the healthy control group (*P*-value < 0.05). The haplotype analysis demonstrated that rs55996424 does not have haploids with the other nine SNPs, including rs8082521.

Conclusion: The T allele of the RNF213 common mutation rs55996424 increases the risk of sICAS. Compared to females, males are more likely to carry the pathogenic allele.

Background

Symptomatic intracranial atherosclerotic stenosis (sICAS) is the stenosis of the intracranial artery that occurs due to atherosclerosis. In this, the blood supply area of the same stenotic artery encounters an ischemic stroke or a transient ischemic attack (TIA). Intracranial atherosclerosis is the most common cause of sICAS throughout the world. However, there exists a racial difference in the incidence of sICAS. Asian populations, for example, exhibit a significantly higher incidence of sICAS compared to the Caucasians in Europe and the United States.[1]China has the largest number of sICAS patients in the world.[2, 3] The occurrence and development of sICAS is the consequence of the combination of genetic and environmental factors.[4–7] Traditional risk factors for sICAS include smoking, alcohol, age, hypertension, diabetes, dyslipidemia, obesity, and metabolic syndrome. Although it was demonstrated in the WASID (Warfarin-Aspirin Symptomatic Intracranial Disease) Trial that controlling the risk factors may reduce the risk of ischemic events in the sICAS patients, the recurrence rate of stroke within two years nonetheless remains as high as 18.6%, while the patients with severe vascular stenosis or a recent onset exhibit a higher recurrence rate.[8] In summary, genetic factors play an important role in the occurrence and development of sICAS. Therefore, the study on the genetic background and pathogenesis of sICAS holds a great significance.

The RNF213 gene, located at position 17q25.3, encodes a 591-kDa cytoplasmic protein, which contains a loop finger structure (C3HC4 type) that binds to two zinc atoms and mediates the protein-protein interactions with E3 ubiquitin ligase, HMG-CoA reductase, and AAA-ATPase.[9] Numerous studies have identified an association between the RNF213 gene and Moyamoya Disease (MMD). In addition to that, the RNF213 gene also shows a significant association with the onset of MMD in the East Asian populations. The proportion of RNF213 gene mutations in the non-smog ICAS/O (Intracranial

Atherosclerotic Stenosis/Occlusion) patients was significantly higher than the proportion of these mutations in the normal population;[10–12] this suggests that sICAS/O and MMD may have similar genetic backgrounds.

Currently, the studies on the genetic variations associated with the sICAS and RNF213 genes are limited to one or several candidate sites. In consideration of the lack of studies conducted on the common sites of RNF213, including all the exons and regulatory regions, all common variants in the potentially functional regions, such as exons and regulatory regions, of the RNF213 gene were screened in the present study. It is anticipated that by comparing the differences in the genotype and allele frequencies of each variant between the sICAS group and the control group, the relationship between these variations and sICAS in the Chinese population can be identified. The present study delineated the molecular mechanism associated with sICAS and search for novel molecular therapeutic targets.

Results

Patient Characteristics

Among the total 400 patients with Symptomatic Intracranial atherosclerotic Stenosis (sICAS), 265 (66.2%) were male, and the average age of the group was 59.1 ± 12.3 years (age range: 26–87 years). The characteristics of these patients with sICAS are presented in Table 1.

Table 1
Characteristics of the patients with sICAS

Clinical data	sICAS
Male	265
Age (year)	26–87
Average age (year)	59.1±12.3
History of hypertension	276(69.0%)
History of diabetes	114(28.5%)
History of lipid metabolism disorder	128 (32.0%)
History of Coronary heart disease	49 (12.3%)
History of Atrial fibrillation	1 (0.3%)
History of Stroke	58 (14.5%)
Systolic blood pressure(mmHg)	143 (128–155)
Diastolic blood pressure(mmHg)	83 (74–93)
Blood sugar(mmol/l)	5.0 (4.28–6.22)
TG*(mmol/l)	1.37 (0.8–1.92)
TC†(mmol/l)	4.02 (2.67–4.87)
HDL‡(mmol/l)	1.07(0.1–1.30)
LDL§(mmol/l)	2.6(2.12–3.29)
HCY‖(mmol/l)	10.52(7.02–14.07)
Table cited in page 4, line 15; *: triglyceride (TG); †: total cholesterol (TC); ‡: high-density lipoprotein (HDL);	
§: low-density lipoprotein (LDL); ‖: homocysteine (HCY)	

Distribution of common variants of gene RNF213

A total of 39 common variants of the gene RNF213 were detected in the sICAS group. The SNP database of NCBI (National Center for Biotechnology Information) was queried in order to analyze the localization and functionality of these common variants. All the MAFs of the common variants were greater than 5% in the HapMAP–HCB database (http://hapmap.ncbi.nlm.nih.gov/). Plink software was utilized to perform

the Hardy-Weinberg Equilibrium (HWE) test. The resulting types, distribution, and HWE values of the common variants of the RNF213 gene are listed in Table 2.

Table 2
Thirty-nine common variant types and distribution of RNF213

SNP*	Rare	Common	Region and Predicted	sICAS	Control	HWE**
	allele†	allele‡	Protein Variants§	(11/01/00) ‖	(11/01/00) ¶	
rs76269241	T	C	intronic	3/72/315	16/218/766	1.000
rs17853714	A	G	exon4: c.378G > A: p. P126P	0/51/348	3/126/878	0.271
rs7215243	G	A	exon4: c.453A > G: p. P151P	4/91/304	16/250/741	0.211
rs17857135	C	T	exon4: c.809T > C: p.M270T	0/50/344	3/126/878	0.271
rs62076476	G	C	intronic	0/50/349	3/126/876	0.271
rs17853989	C	T	exon6: c.962T > C: p.M321T	0/51/348	3/126/878	0.271
rs17853713	A	G	exon6: c.990G > A: p. K330K	0/50/349	3/126/877	0.271
rs59209118	G	T	intronic	2/44/354	4/145/858	0.840
rs7503557	C	T	intronic	19/118/263	29/316/662	0.794
rs9890495	G	C	intronic	0/50/350	5/121/880	1.000
rs72849841	T	C	exon11: c.2186C > T: p. P729L	0/8/392	1/25/981	0.193
rs8066993	T	C	intronic	31/183/186	79/426/501	0.124
rs78857404	C	G	intronic	13/157/230	42/339/626	0.115
rs55996424	T	A	exon17: c.3101A > T: p. K1034M	42/196/162	161/475/371	0.778
rs61359568	A	G	exon18: c.3121G > A: p. A1041T	0/11/389	2/29/976	0.043
rs8082521	A	C	exon20: c.3397C > A: p. Q1133K	9/122/269	51/368/588	0.388

Table cited in page 4, line 22; *: SNP, the number of locus in the dbsnp of the NCBI database; †: Rare allele, the lower frequency allele at the locus; ‡: Common allele, the higher frequency allele at the locus; §: Region and Predicted Protein Variants, gene region and predicted protein variation; ‖: sICAS (11/01/00), sample number of sICAS group rare allele homozygous (11), heterozygous (01), common allele homozygous (00); ¶: Control (11/01/00), the number of samples of the control group rare allele homozygous (11), heterozygous (01), and common allele homozygous (00); **: HWE, *P*-value of the Hardy-Weinberg equilibrium test of all test subjects;

SNP*	Rare	Common	Region and Predicted	sICAS	Control	HWE**
rs10782008	A	G	exon20: c.3397C > A: p. Q1133K	49/193/158	146/487/374	0.366
rs9913636	C	G	exon21: c.3814G > C: p. E1272Q	47/188/165	134/475/398	0.527
rs8074015	G	A	exon21: c.3992A > G: p. D1331G	48/196/156	144/492/371	0.175
rs9908287	G	C	exon21: c.4020C > G: p. V1340V	46/193/161	137/484/386	0.253
rs7211876	T	C	intronic	37/181/182	102/442/463	0.541
rs4890009	A	G	exon24: c.4650G > A: p. A1550A	40/191/169	119/473/415	0.159
rs9674961	A	G	exon29: c.7001G > A: p. S2334N	39/191/170	119/474/414	0.142
rs4890012	C	G	exon29: c.7245G > C: p. P2415P	45/193/162	132/488/387	0.136
rs7223115	T	C	intronic	82/212/105	201/533/273	0.018
rs9944443	A	T	intronic	44/182/170	119/469/413	0.378
rs12051723	T	G	intronic	22/155/212	60/378/565	0.476
rs7216493	G	A	exon34: c.10470G > A: p. E3490E	87/223/89	233/521/253	0.033
rs8067292	C	T	ncRNA intronic	40/195/165	233/521/253	0.178
rs61741791	A	G	exon34: c.10470G > A: p. E3490E	0/11/389	2/28/977	0.039
rs35332090	C	G	exon40: c.11512G > C: p. V3838L	0/13/387	2/34/971	0.073
rs61740658	G	A	exon41: c.11744A > G: p. E391G	0/11/388	2/30/973	0.047
rs77506504	A	G	exon44: c.11988G > A: p. P3996P	5/102/293	13/260/733	0.028

Table cited in page 4, line 22; *: SNP, the number of locus in the dbsnp of the NCBI database; †: Rare allele, the lower frequency allele at the locus; ‡: Common allele, the higher frequency allele at the locus; §: Region and Predicted Protein Variants, gene region and predicted protein variation; ||: sICAS (11/01/00), sample number of sICAS group rare allele homozygous (11), heterozygous (01), common allele homozygous (00); ¶: Control (11/01/00), the number of samples of the control group rare allele homozygous (11), heterozygous (01), and common allele homozygous (00); **: HWE, *P*-value of the Hardy-Weinberg equilibrium test of all test subjects;

SNP*	Rare	Common	Region and Predicted	sICAS	Control	HWE**
rs116948489	A	G	exon49: c.12807G > A: p. R426R	0/11/388	2/28/977	0.039
rs113236556	C	T	exon52: c.13186–13T > C	0/11/389	2/28/977	0.039
rs148731719	A	G	exon52: c.13195G > A: p. A439T	1/42/357	3/105/899	1.000
rs112535386	C	T	ncRNA intronic	0/11/389	2/28/977	0.039
rs4889848	C	T	exon56: c.13671C > T: p.H4557H	80/208/112	185/512/310	0.178
rs3185057	A	G	exon67: c.15321G > A: p. A510A	0/11/389	2/30/975	0.047

Table cited in page 4, line 22; *: SNP, the number of locus in the dbsnp of the NCBI database; †: Rare allele, the lower frequency allele at the locus; ‡: Common allele, the higher frequency allele at the locus; §: Region and Predicted Protein Variants, gene region and predicted protein variation; ||: sICAS (11/01/00), sample number of sICAS group rare allele homozygous (11), heterozygous (01), common allele homozygous (00); ¶: Control (11/01/00), the number of samples of the control group rare allele homozygous (11), heterozygous (01), and common allele homozygous (00); **: HWE, *P*-value of the Hardy-Weinberg equilibrium test of all test subjects;

Correlation Analysis of RNF213 common variants between sICAS group and control group

The common sites in the control group were further screened to identify the sites that met the Hardy–Weinberg equilibrium criteria ($P > 0.05$). This ensured that the sample met the genetic balance of the population. The frequencies of the genotypes and alleles in the sICAS group and the control group were compared using the chi-squared test. Two common sites (rs8082521 and rs55996424), which fulfilled the criterion of P-value < 0.05, were identified (Table 3). The gene frequencies of these two common sites of RNF213 (rs55996423 and rs8082521) in both sICAS and control groups were analyzed under three genetic models (dominant model, recessive model, and additive model). Although the proportion of allele T was higher in the sICAS and control groups, the allele T of rs55996424 was a mutant type, while allele A was the wild type. The results demonstrated that rs55996424 correlated significantly with sICAS under the additive and recessive models ($P = 0.0239$; $P = 0.0087$), while no significant correlation with sICAS was observed under the dominant model ($P = 0.2022$). This observation implied that people carrying the T allele might have a greater risk of developing sICAS. In regard to rs8082521, allele C was identified as the wild type. The results (Table 4) demonstrated that rs8082521 correlated significantly with sICAS under additive, dominant, and recessive models ($P = 0.0006$; $P = 0.0022$; $P = 0.0218$).

Table 3
RNF213 rs55996424 and rs8082521's distribution in sICAS and Control

SNP	Genotype/	sICAS	Control	X2	*P*-value
	Allele				
rs55996424	Genotype			7.189	0.0275
	TT	162 (40.5%)	371 (36.8%)		
	TA	196 (49.0%)	475 (47.2%)		
	AA	42 (10.5%)	161 (16.0%)		
	Allele			5.068	0.0244
	T	520(65.0%)	1217(60.4%)		
	A	280(35.0%)	797(39.6%)		
rs8082521	Genotype			12.010	0.0025
	CC	269 (67.3%)	588 (58.4%)		
	CA	122 (30.5%)	368 (36.5%)		
	AA	9 (2.2%)	51 (5.1%)		
	Allele			11.490	0.0007
	C	660(82.5)	1544(76.7%)		
	A	140(17.5%)	470(23.3%)		

Table 4

Logistic regression analysis of rs55996424 and rs8082521 between sICAS and control groups

SNP	Hypothetical model	Gene type	*P*-value	OR (95%CI)
rs55996424	Dominant	AA + AT vs. TT	0.2022	0.8570
	Recessive	AA vs. TT + AT	0.0087	0.6165
	Additive	AA vs. TT	0.0239	0.8204
rs8082521	Dominant	AA + AC vs. CC	0.0022	0.6834
	Recessive	AA vs. CC + AC	0.0218	0.4315
	Additive	AA vs. CC	0.0006	0.6896

Linkage disequilibrium blocks of common SNPs of RNF213

The results of the analysis of the linkage disequilibrium blocks of 39 SNPs of RNF213 in 400 patients with sICAS are presented in Fig. 1A. Similar LD blocks were observed in the general control population (Fig. 1B). The LD was significant between rs55996424 and three other SNPs (rs8082521, rs77506504, and rs148731719) in both case and control groups, while they were observed between rs55996424 and 9 other SNPs (rs72849841, rs61359568, rs61741791, rs35332090, rs61740658, rs116948489, rs113236556, rs112535386, and rs3185057) only in the control group and not in the case group. A high level of LD was identified between rs8082521 and 19 other SNPs (rs72849841, rs55996424, rs61359568, rs10782008, rs9913636, rs8074015, rs9908287, rs4890009, rs9674961, rs4890012, rs7223115, rs7216493, rs6171991, rs61740658, rs77506504, rs116948489, rs113236556, rs112535386, and rs4889848) in the case group; among these 19 SNPs, non-significant LD was observed between rs8082521 and 2 other SNPs (rs72849841 and rs77506504) and the LD was also strong between rs8082521 and the remaining 17 SNPs in the control group. LD was found to be significant between rs8082521 and three other SNPs (rs35332090, rs148731719, and rs3185057) only in the control group and not in the case group.

Haplotype analysis

Haplotype analysis was performed in three blocks (block1: rs17857135 and rs17853989; block 2: rs55996424; and block 3: rs8082521, rs10782008, rs9913636, rs8074015, rs9674961, rs35332090, and rs61740658), the results of which are presented in Fig. 2A and 2B. No significant LD was seen among these three blocks in both case and control groups.

Clinical characteristics of patients with and without allele T of rs55996424

Among 400 sICAS patients, 358 carried allele T. Further analysis revealed males are more likely to carry the pathogenic allele T than females(Table 5). Among female patients, there is no significant difference in smoking and drinking with or without T allele (*P*-value:0.387; 0.629). Majority of female patients carry the T allele (85.33%).

Table 5
Clinical Characteristics of Symptomatic Intracranial Atherosclerotic Stenosis Patients with and without T Allele of rs55996424

Characteristics	without T (n = 42)	with T (n = 358)	*P**
Female, n (%)	22(52.4%)	128(35.8%)	0.043
Age, years (Mean ± SD)	62.6 ± 11.9	59.0 ± 11.3	0.070
Hypertension, n (%)	32(76.2%)	272(76.0%)	0.999
History of stroke, n (%)	8(19.1%)	61(17.0%)	0.672
Hyperlipemia, n (%)	25(59.5%)	240(67.0%)	0.389
Type 2 diabetes, n (%)	13(31.0%)	112(31.3%)	0.999
Smoking, n (%)	10(23.8%)	154(43.0%)	0.020
Alcohol drinking, n (%)	3(7.1%)	114(31.8%)	0.001
FBG	6.38	6.19	0.708
TG	1.79	1.97	0.383
TC	4.59	4.61	0.952
HDL	1.13	1.2	0.145
LDL	2.93	2.88	0.799

*: Continuous variable was analyzed through t-test, and categorical variable was analyzed by chi-square test. Threshold of statistical significance was set at $P < 0.05$.

Discussion

This is the first study on the frequency distribution of all common sites of RNF213, including all exons and regulatory regions, in a Chinese cohort. The two SNPs, namely, rs8082521 and rs55996424, were identified to be associated with sICAS.

Protein RNF213 has a hexameric structure, which includes two functional domains: AAA + ATPase and E3 ligase; the protein dynamically changes its formation through adenosine-triphosphate (ATP)/adenosine diphosphate (ADP) binding and hydrolysis cycles.[3, 13] Gene RNF213 was initially identified as a susceptibility gene for MMD.[1, 3] Miyawaki et al. investigated the prevalence of p.R4810K (c.14576G > A) variant of RNF213 in a Japanese non-MMD ICAS patient population, and they found an association with anterior circulation ICAS.[14] Recently, Kamimura et al. found the RNF213 p.R4810K variant is correlated with early-onset ischemic stroke with anterior circulation stenosis in Japan.[15] According to statistics in 2012, approximately 16 million people in East Asian countries carry this genetic polymorphism.[2] In the process of study on MMD disease, it was found that RNF213 genetic variants may result in arterial

fragility and susceptibility to hemodynamic stress,[16] and this mechanism can also lead to the pathogenesis of sICAS. In addition, a previous functional study suggested that Protein RNF213 may cause low angiogenic activity and high mitotic abnormalities.[1, 17] This finding has also been confirmed in animal experiments.[18, 19] Choi et al. used high-resolution magnetic resonance to analyze consecutive stroke patients, and draw the conclusion that RNF213 variant may be associated with vasculogenesis and smaller intracranial arteries which predisposing hemodynamic compromise in the presence of intracranial atherosclerosis.[20] On the basis of the above-stated studies, it may be stated that gene RNF213 and its transcriptional product may affect the morphogenesis and the morphological change in the intracranial artery,[21] thereby affecting the pathogenesis of sICAS.

The RNF213 common mutation, namely, rs8082521, is the conversion of cytosine (C) to adenine (A). The corresponding amino acid conversion is of glutamine (Q) to lysine (K). Limited studies have been conducted previously on the RNF213 rs8082521 site, and this site has not been reported in Caucasians. A study conducted with Japanese MMD patients in 2014 demonstrated a significant negative correlation between the RNF213 rs8082521 allele A and MMD, although no significant correlation was observed after performing adjustment for the p.R4810K genotype.[22] In the present study, it was observed that the rs8082521 locus genotype frequency and the allele frequency were significantly different between the sICAS group and the healthy control group. It was indicated that allele A decreases the risk of sICAS. Furthermore, the results of the analysis of the rs8082521 locus genotype frequencies revealed that rs8082521 correlated significantly with sICAS under the additive (AA vs. CC), dominant (AA + AC vs. CC), and recessive (AA vs. CC + AC) models ($P = 0.0006$; $P = 0.0022$; $P = 0.0218$). The results obtained were similar to the findings of previous research conducted in Japan on a related topic.

The RNF213 common mutation, namely, rs55996424, in which the base thymine (T) becomes adenine (A), is located in exon 17. Rs55996424 is involved in the coding of E3 ubiquitin-protein ligase RNF213 isoform 2, and the corresponding amino acid conversion is of lysine (K) to methionine (M). Only one previous study mentioned this mutation. A study conducted on Japanese hypertensive patients in 2015 demonstrated that there was no significant association between the rs55996424 and hypertension.[23] In our study, the rs55996424 locus genotype frequency and allele frequency were significantly different between the sICAS group and the healthy control group. It was indicated that the T allele increased the risk of sICAS. Furthermore, the results of the analysis of the rs55996424 locus genotype frequencies demonstrated that rs55996424 correlated significantly with sICAS under the additive (AA vs. TT) and recessive (AA vs. TT + AT) models ($P = 0.0239$; $P = 0.0087$). Although no statistically significant difference was observed under both additive and recessive models between the control and sICAS groups ($P > 0.05/39$ or $P = 0.00128$) after the Bonferroni correction. However, there remained a possibility of overcorrection, which could have resulted in false-negative results. The next step for future research could be increasing the sample size.

According to previous research, the reported pathogenic mutations, such as p.R4810K and p.R4859*k*, are located in the region between the exons 42 and 68, [1, 24] which corresponds to the region from the RING

finger domain to the C-terminus of the RNF213 protein. The mutations in the C-terminus region of RNF213 could, therefore, be predicted to cause functional alterations in the protein.[25] We observed significant LD between rs55996424 and three other SNPs (rs8082521, rs77506504, and rs148731719) in both case and control groups; and significant LD between rs55996424 and nine other SNPs (rs72849841, rs61359568, rs61741791, rs35332090, rs61740658, rs116948489, rs113236556, rs112535386, and rs3185057) only in the control group. Among the aforementioned nine SNPs, rs116948489, rs113236556, rs112535386, and rs318505 are located in the 42–68 exon region. Since no significant LD was observed between rs55996424 and the four SNPs located in the 42–68 exon region, it may be stated with a certain degree of confidence that the pathogenicity of rs55996424 is not associated with the pathogenic genes that have been reported in previous studies.

A previous study conducted in Japan considered that nine SNPs (rs17857135, rs17853989, rs8082521, rs10782008, rs9913636, rs8074015, rs9674961, rs35332090, and rs61740658) located on the same haplotypes could be regarded as tag markers for p.R4810K susceptibility.[22] In our study's haplotype analysis part, these nine SNPs were set as block 1 and block 3, and rs55996424 was set as block 2. The haplotype analysis was conducted in these three blocks, the result of which demonstrated no significant LD among these three blocks in both case and control groups. Therefore, the common mutation rs55996424 might be a novel independent risk factor for sICAS in the Chinese population.

The present study had certain limitations. Firstly, data of healthy control subjects was obtained from the GeneSky in-house database, and their clinical characteristic information was not available. Therefore, the impact of environmental risk factors could not be completely eliminated. Secondly, common mutations exhibit only minimal pathogenic effects and can explain only a small proportion (approximately 10%) of genetic risk, and sICAS is a polygenic disease. The third limitation was that this study was a single-center study, larger population-based multicenter studies are required in order to verify the findings of the present study.

Conclusions

In summary, this study detected 39 common variants of the gene RNF213., and After Bonferroni correction, only rs55996424 remained significantly associated with sICAS. The T allele of rs55996424 increased the risk of sICAS. Compared to females, males are more likely to carry this pathogenic allele.

Methods

Study aim

This study investigated the possible association between the common variants of RNF213 and sICAS in the Chinese population.

Study Population

The study subjects were patients with cerebral infarction or TIA who were admitted to the Department of Neurology, Xiangya Hospital, in the period between July 2015 and December 2017.[9] The diagnosis of CI and TIA was based on the diagnostic criteria of the Chinese Guidelines for the Diagnosis and Treatment of Acute Ischemic Stroke 2014.[26] The patients whose classification of TOAST was the large arterial atherosclerosis type were selected to be included in the case group.[27] Subsequent to the completion of magnetic resonance angiography (MRA), arch vascular imaging (CE-MRA), CTA, or DSA screening, 400 Chinese sICAS patients were selected to finally constitute the case group. All enrolled patients have informed consent. Procedures were performed in accordance with the ethical standards. Allelic frequencies of controls were obtained from the GeneSky in-house database (1007 samples).

Inclusion criteria were as follows: (1) Age > 18 years, all Chinese descendants without any blood relationship; (2) On the basis of the results of MRA, CE-MRA, CTA, or DSA, the sICAS patients had severe stenosis or occlusion in the intracranial arterial lumen (moderate 50–69%, severe 70–99%, and occlusion 100%). The calculation method of ICAS stenosis was based on the standards established by the Warfarin-Aspirin Symptomatic Intracranial Disease (WASID) trial;[28] (3) All the patients included in this study provided informed consent.

Exclusion criteria were as follows: (1) Cardiac, small arterial occlusion, and CI due to unknown reasons; (2) Other causes such as moyamoya disease, arthritis, intracranial infection, blood disease, cerebral amyloid angiopathy, muscle fiber dysplasia, arterial dissection, etc.; (3) Other criteria combined with extracranial artery stenosis; and (4) Combined severe heart, liver, and kidney diseases, serious infections, tumors, etc.

Clinical and laboratory data collection

The data related to age, sex, previous medical history (hypertension, diabetes, lipid metabolism disorder, coronary heart disease, atrial fibrillation, stroke history, etc.), history of smoking and drinking, family history, etc., were collected and recorded for the 400 patients. Hypertension, diabetes, lipid metabolism disorder, coronary atherosclerotic heart disease, and atrial fibrillation were defined as published earlier.[29–31]

DNA Isolation and Genotyping

8–10 mL fasting peripheral blood samples were collected in an EDTA anticoagulant tube and stored in a refrigerator at − 20°C until used for the extraction of genomic DNA. The DNA extraction was performed using the kit obtained from Kangwei Century Biotechnology Co. Ltd., in accordance with the manufacturer's instructions. The quality and quantity of DNA samples were assessed using Nanodrop 2000. A custom capture array was designed to capture target regions containing exonic and regulatory regions (i.e., introns, splice sites, 3 - and 5 -untranslated regions, and promotors) of gene RNF213. Target sequencing was performed on an Illumina Hiseq high-throughput sequencing platform using 150-bp double-end sequencing mode. Target-gene targeted capture sequencing was performed by the Shanghai Tianzhu Biotechnology Company.

Raw FastQ data were aligned to the human genome (hg19, University of California-Santa Cruz Genome Browser) using the Burrows-Wheeler aligner. Variant calling was performed with VarScan and Haplotype-Caller from the Genome Analysis Toolkit. After sequencing, variants were filtered on the following terms: (a) common in-reference databases (allele frequency was > 0.05 in 1000 Genome Project Chinese population); (b) the recall rates of single nucleotide variant typing of case and control samples were > 0.8. Phen-2 and SIFT were used to predict the effect of functional variants.

Statistical analysis

All the clinical data were analyzed statistically using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Results presented as means standard deviations or number of subjects (percentage). The frequencies of the genotypes and alleles were compared using the chi-squared test. Hardy–Weinberg equilibrium (HWE) test for SNP was conducted, considering $P > 0.05$ following the HWE equilibrium law. Logistic regression analysis was performed on the basis of three hypothetical models (Dominant, Recessive, and Additive) for correlation analysis. Bonferroni's correction with $P < 0.05/39$ ($P = 0.00128$) was considered statistically significant. Haploview software (version 4.2) was utilized for linkage disequilibrium and haplotype analyses. A *P*-value of < 0.05 was considered the threshold of statistical significance.

List Of Abbreviations

Abbreviation	Full name
sICAS	Symptomatic intracranial atherosclerotic stenosis
TIA	Transient ischemic attack
ICAS/O	Intracranial Atherosclerotic Stenosis/Occlusion
MMD	Moyamoya Disease

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Xiangya Hospital. All enrolled patients have informed consent. Procedures were performed in accordance with the ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conceptualization, JX; Methodology, XL, JW. XZ and FY; Data acquisition, XL, JW, XF, ZL, QH, DL, XJ; Formal analysis, XL; writing-original draft preparation, XL; writing-review and editing, JX; All authors approved the final version to be published.

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Figures

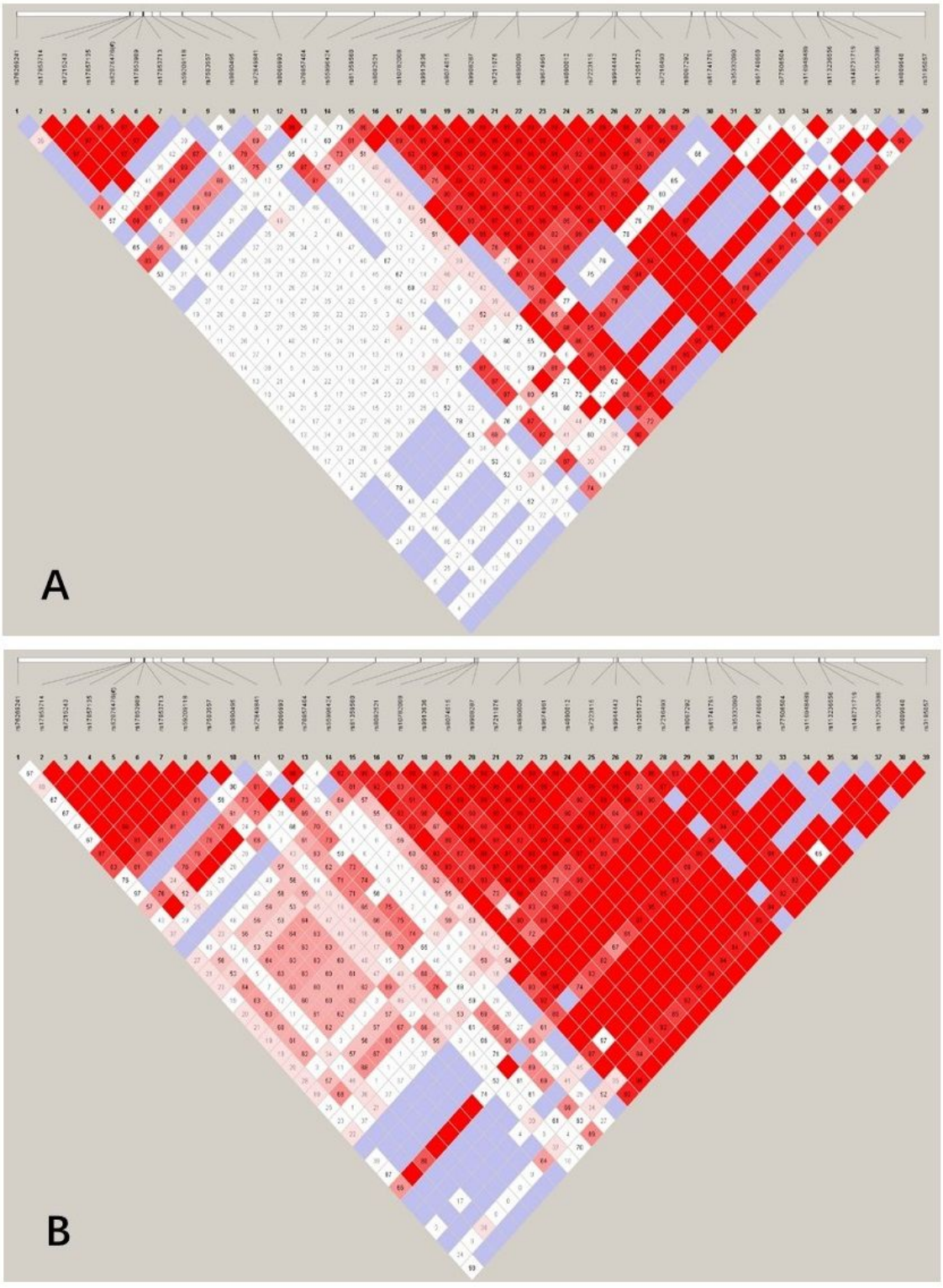

Figure 1

Linkage disequilibrium (LD) blocks from a region from rs76269241 to rs3185057 in two independent populations. Fig cited in page 7, line 2; Fig 1A. LD blocks for 400 unrelated sICAS patients.; Fig 1B. LD blocks for 1000 unrelated controls. White diamond: complete Linkage equilibrium; shades of pink: 0<D'<1; red diamond: represents a D' of 100%, complete LD. Numbers in diamonds are D'-value (%).

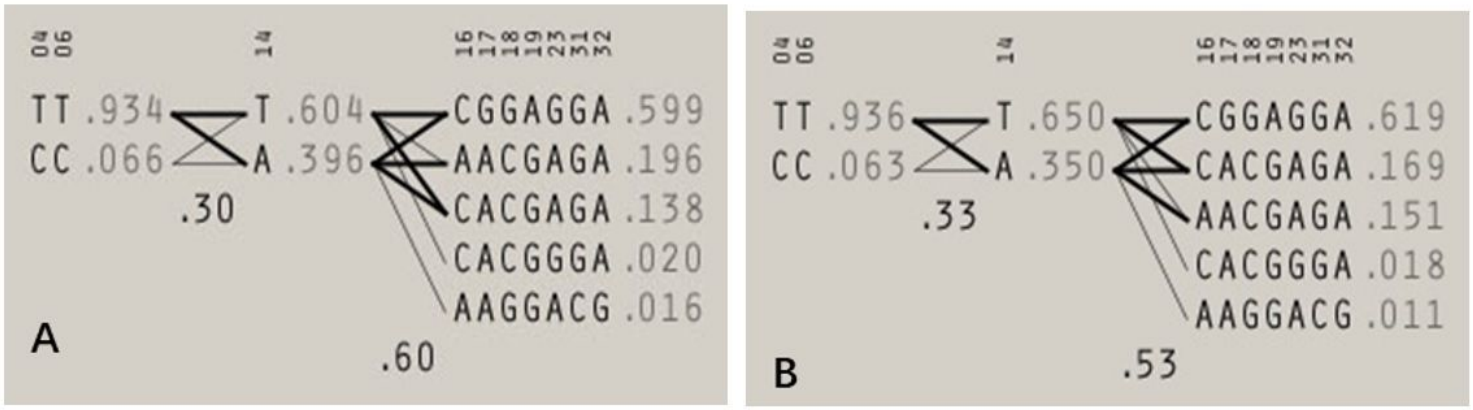

Figure 2

Haplotype analyses of 3 LD blocks. Grey numbers are genotype frequency. Fig cited in page 7, line 18; Fig 2A. Haplotype analyses for 400 unrelated sICAS patients; Fig 2B. Haplotype analyses for 1000 unrelated controls. Black numbers are LD in adjacent blocks. Examine haplotypes above 1.0%. Connect with thin lines if >1.0%, connect with thick lines if >5.0%.