Association studies of candidate single-nucleotide polymorphisms with Symptomatic Intracranial Atherosclerotic Stenosis in a Chinese Han population

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Abstract

Background: Genetic factors underlying predisposition to Symptomatic Intracranial Atherosclerotic Stenosis (sICAS) remain unknown, the purpose of the present study was to identify genetic variants that confer susceptibility to sICAS in a Chinese Han population.

Methods: The study population comprised of 379 Chinese individuals, including 193 patients with sICAS and 186 unrelated healthy controls. A total of 96 polymorphisms selected by genome-wide association or candidate-gene association studies of atherosclerosis and atherosclerotic diseases were examined in the present study with the use of Illumina VeraCode technology. Statistical analyses were performed with PLINK and SPSS software, and each genotype was assessed according to dominant, recessive, and additive genetic models.

Results: Comparisons between subjects with sICAS and controls revealed that rs3798220 of the lipoprotein(a) gene (LPA) and rs9818870 of the muscle RAS oncogene homolog gene (MRAS) were significantly ($P<0.0005$, Bonferroni correction) associated with the prevalence of sICAS. Comparisons of genotypes in three genetic models (dominant, recessive and additive models) between groups showed that rs3798220 (LPA) was associated with sICAS in the dominant model ($P=0.000005$, OR=2.629, 95% CI=1.735-3.985) and additive model ($P=0.000005$, OR=2.293, 95% CI=1.605-3.276),while rs9818870 (MRAS) was associated with sICAS in the additive model ($P=0.000464$, OR=3.245, 95% CI=1.679-6.272). Furthermore, logistic regression analysis showed that the genotype distribution of rs3798220 (LPA) was significantly associated with sICAS, with its minor C allele elevating sICAS risk ($P=0.00002$, dominant model, OR=3.951, 95% CI=2.100-7.434 and $P=0.000053$, additive model, OR=2.916, 95% CI=1.736-4.899). While rs9818870 (MRAS) showed no such significance ($P>0.0005$).

Conclusion: LPA rs3798220 might be susceptibility loci for sICAS in Chinese Han individuals.

Keywords: Symptomatic Intracranial Atherosclerotic Stenosis; risk factors; candidate gene; SNP.

Introduction

Symptomatic intracranial atherosclerotic stenosis (sICAS) is an important cause of ischemic stroke worldwide. The atherosclerotic lesion distribution of cerebrovascular varies with ethnicity. For example, sICAS is more prevalent in Asians than in Westerners[1], and inherited susceptibility of intracranial vessels to atherosclerosis is thought to be one reason for this racial difference[2]. The Chinese Intracranial Atherosclerosis (CICAS) Study showed that sICAS is the most common vascular lesion in patients with cerebrovascular disease in China (46.6%)[3]. Despite substantial progress in secondary prevention measure and treatment, sICAS is still burdened with a high recurrent stroke rate[3]. Stroke is a complex syndrome rather than one disease[4], many factors contribute to disease outcome. Thus far, the etiology and pathophysiology of sICAS have not been completely illuminated. A number of studies have shown the contribution of conventional risk factors such as smoking, hypertension, diabetes mellitus, and metabolic syndrome in the development of sICAS[5–7]. Yet, unlike other vascular diseases, little is known

regarding associations of sICAS and genetic polymorphisms. Recent genetic studies have suggested the importance of genetic factors in predisposition to sICAS such as genotypes or polymorphisms of *APOE, CRP, Renalase, RNF213, LPL* and *ALDH2* [8–16], but few candidate genes have been replicated. Genetic research of subjects who had sICAS can be a promising approach for identification of novel biological mechanisms that underlie the development of sICAS.

Atherosclerosis is a common etiology and pathological change in sICAS. In recent years, genome-wide association studies (GWAS) and their meta-analyses have identified many new single nucleotide polymorphisms (SNPs) that are closely associated with atherosclerosis and atherosclerotic diseases such as ischemic stroke (IS), coronary heart disease (CAD) and peripheral arterial disease. CAD and IS share common vascular risk factors like hypertension, hypercholesterolemia, and diabetes mellitus[17]. The atherosclerotic pathogenesis applies to CAD can also contribute to the development of IS especially to the development of large artery stroke (LAS)[18]. Some genetic variants that were originally detected to affect a risk of coronary artery disease were also associated with ischemic stroke (particularly atherothrombotic cerebral infarction), for example, variant in *HDAC9,* initially associated with coronary artery disease, was associated with large vessel ischemic stroke, suggesting a shared genetic architecture for the two conditions[19]. Candidate-gene association studies or meta-analyses showed that the previously described sICAS associated genes like *LPL* and *ALDH2* were also related to atherosclerosis or CAD[20, 21]. Other studies also suggested a close relationship between CAD and sICAS [22, 23]. We can infer that ICAS may share some common genetic risk factors with coronary artery disease.

The aim of the present study was to examine the possible association of symptomatic intracranial atherosclerotic stenosis disease in Han Chinese populations with 96 single-nucleotide polymorphisms (SNPs) previously identified as susceptibility loci for ischemic stroke, atherosclerosis and coronary atherosclerotic heart disease in GWAS studies or candidate-gene association reports.

Materials And Methods

Study population

This study included 193 unrelated patients with symptomatic intracranial atherosclerotic stenosis (sICAS group) who were recruited from the Department of Neurology, Xiangya Hospital between October 2011 and March 2015. At the same time, 186 healthy control subjects (control group) were randomly selected from volunteers of XiangYa Hospital Health Examination Center. All patients were of Han nationality and from Hunan province. Data were collected through case report forms. Ischemic stroke was confirmed by CT and/or MRI as we described before[24–26]. All patients had undergone the evaluation of cerebral vascular via MRA/CTA/DSA/ CE-MRA or transcranial Doppler (TCD) or carotid color duplex ultrasonography. Intracranial stenosis was defined as a narrowing ≥50% in arterial column reduction affecting the main cerebral large arteries. The degree of ICAS was calculated by the published method in the Warfarin-Aspirin Symptomatic Intracranial Disease Study[27]. Intracranial arteries included intracranial segment of internal carotid artery and vertebral artery, basilar artery, anterior cerebral artery,

middle cerebral artery and posterior cerebral artery. Patients with extracranial stenosis and cardioembolism, stroke of other determined etiology, stroke of undetermined etiology according to the TOAST classification were excluded in this study[28]. Control individuals had no history of ischemic or hemorrhagic stroke, other cerebral diseases, coronary artery disease, aortic aneurysm, peripheral arterial occlusive disease, or of other thrombotic, embolic or hemorrhagic disorders. Two independent reviewers evaluated the vascular images.

At enrollment, patients were interviewed by investigators to obtain the basic information. The clinical information included age, sex, ethnic, vascular risk factors like hypertension, diabetes mellitus, dyslipidemia, smoking, alcohol intaking. Concentrations of glucose, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), blood urea nitrogen(BUN), creatinine(Cr), uric acid(UA) and glycated hemoglobin HbA1C were measured according to our laboratory protocols. Hypertension, diabetes mellitus, and dyslipidemia were defined as we published before[24–26].

This study was approved by the Ethics Committee of Xiangya Hospital of the Central South University in China and carried out in accordance with approved guidelines.

Selection and genotyping of polymorphisms

The genetic variants analyzed in this study included 96 SNPs. SNPs in the entire coding area, together with the areas<2 kb upstream and< 1 kb downstream of each gene and some tag-SNPs within intron areas proven to be associated with ischemic stroke, atherosclerosis and coronary atherosclerotic heart disease in previous GWAS studies or candidate-gene association reports were included in this study. These SNPs were checked with the National Center for Biotechnology Information SNP Database (www.ncbi.nlm.nih.gov/SNP/), based on functional location and validation status. The minor allele frequency (MAF) of most SNPs was >5% in the HapMAP-HCB databank (www.hapmap.org/). Details of all SNPs are in Table S1 in Supplementary material online.

Genomic DNA was extracted from venous blood samples (peripheral blood leukocytes) using a standard phenol-chloroform method. The quality and quantity of DNA were assessed with a fluorometer. Then genomic DNA was stored at −80℃ for use. All DNA samples were normalized to 50 ng/L. The selected 96 SNPs with Illumina design score >0.6 were genotyped by the Illumina VeraCode technology (Illumina, Inc., San Diego, CA, USA) in accordance with the manufacturer's protocol for the SNP Golden Gateassay on the BeadXpress Genotyping Platform. Briefly, 250 ng of genomic DNA was amplified at 37℃for 20 hours, and then the amplified DNA was fragmented and precipitated. The dried pellet was resuspended and hybridized to beadchips and were then incubated at 48℃ for 20 hours, washed, and underwent another single-base extension step. After that, beadchips were stained, washed, coated, and dried. Finally, signal-intensity data was generated by an Illumina BeadArray Reader. We randomly selected 20% of the total samples and genotyped them in duplicate. As a result, 99.8% concordance was observed, and the inconsistent data were excluded from the final analysis.

The genotypes for each SNP were calling with the BeadStudio software version 3.3 (Illumina, Inc., San Diego, CA, USA). SNPs with poor Illumina design scores were genotyped by sequencing technology on an ABI 3730 DNA analyzer (Applied Biosystems, Inc., Foster City, CA, USA).

Statistical analyses

Hardy-Weinberg equilibrium (HWE) test was performed in both case and control groups with PLINK (version 1.05, http://pngu.mgh.harvard.edu/purcell/plink/). P values below 0.05 were considered to represent significant violation of Hardy-Weinberg equilibrium. Other statistical analysis was performed with the SPSS 22.0(IBM SPSS, Chicago, Ill, USA). Kolmogorov-Smirnov test was used to assess the normality of the distribution of measurement data. Normally distributed data are expressed as mean±SD, and non-normally distributed data as medians and interquartile range. Categorical variables were presented as percentages. Independent samples t-test for the comparison of normally distributed parameters, and Wilcoxon test for the comparison of non-normally distributed variables. Allele frequencies were estimated by the gene counting method, qualitative variables, and genotype or allele frequencies were tested by the χ^2 test and Fisher exact test. Associations of the polymorphisms with sICAS were further adjusted by binary logistics regression analysis with a forward model including several factors that may influence sICAS, in which age, gender, smoking status, drinking status, the prevalence of hypertension, diabetes mellitus, dyslipidemia, and the serum concentration of creatinine were included in the equation. The P-value, odds ratio and 95% confidence interval were calculated. Each genotype was assessed according to dominant, recessive, and additive genetic models, in which an additive effect of the variant allele was assumed. When a dominant effect was assumed, genotype W/W was coded as 0 and W/V and V/V combined were coded as 1. Accordingly, scores of 0 for W/W and W/V combined and 1 for V/V were used in a model that assumed a recessive effect. It is possible that some of our controls which are younger in age will develop diabetes since the prevalence of sICAS increases with age. In order to solve this problem age was adjusted for in the logistic analysis. As for multiple comparisons, the significance level was adjusted to 0.05/96≈0.0005 with the Bonferroni method.

Results

Subject characteristics

Baseline characteristics of the subjects with sICAS and controls are shown in Table1. A total of 379 individuals were included in our study. Age and gender were matched in the two groups. The age of the study population ranged from 33 to 86 years old, and 236 (62.3%) patients were men, 132 (34.8%) were smokers, 106 (28.5%) were drinkers, 162 (42.7%) had a history of hypertension, 95 (25.1%) dyslipidemia and 64 (16.9%) diabetes mellitus. The prevalence of hypertension, diabetes mellitus, dyslipidemia and smoking were higher in sICAS group. Systolic and diastolic blood pressure, serum concentrations of LDL-cholesterol and creatinine, and plasma glucose level were greater in subjects with sICAS when compared with the healthy controls.

Comparisons of genotype distributions and allele frequencies

Comparisons between subjects with sICAS and controls revealed that rs3798220 of the lipoprotein(a) gene (LPA) and rs9818870 of the muscle RAS oncogene homolog gene (MRAS) were significantly (P<0.0005, Bonferroni correction) associated with the prevalence of sICAS. The TT, TC and CC genotype frequencies of LPA SNP rs3798220 were 0.415/0.497/0.088 and 0.651/0.317/0.032 in the sICAS patients and controls respectively; T/C allele frequencies were 0.663/0.337 and 0.809/0.191 respectively; CC genotype and C allele frequency of sICAS were significantly higher than those in the control group (P = 0.000014, P = 6.00E−06). The GG, AG and AA genotype frequencies of rs9818870 were 0.803/0.192/0.005 and 0.930/0.070/0.000 in the sICAS patients and controls respectively; G/A allele frequencies were 0.899/0.101 and 0.965/0.035 respectively; An allele frequency of sICAS were significantly higher than those in the control group (P = 0.000289) while genotype between groups showed no such significance as determined by the Bonferroni correction (P = 0.001241). (as shown in Table 2).

Comparisons of genotypes in three genetic models (dominant, recessive and additive models) between groups showed that rs3798220 (LPA) was associated with sICAS in the dominant model (P = 0.000005, OR = 2.629, 95%CI = 1.735–3.985) and additive model (P = 0.000005，OR = 2.293, 95% CI = 1.605–3.276). While rs9818870 (MRAS) was associated with sICAS in the additive model (P = 0.000464，OR = 3.245, 95% CI = 1.679–6.272). (as shown in Table 3).

Multivariable logistic regression analysis

The logistic analysis revealed that the rs3798220 of *LPA* (dominant and additive models), but not rs9818870 of *MRAS,* was significantly (P<0.0005) associated with sICAS groups, with the minor C allele, being harmful in sICAS (as shown in Table 4).

Discussion

Intracranial atherosclerosis is a complex disease and its etiology and pathophysiology have not been fully elucidated. Intracranial atherosclerosis can be considered as a multifactorial process in which atherosclerotic risk factors like hypertension, dyslipidemia, diabetes, were involved in the disease development process[29–32]. Hereditary factors are also believed to play important roles in sICAS as associations of intracranial atherosclerosis and genetic polymorphisms are described in several studies[8–15]. Thus, 96 SNPs were examined in the present study, which were all proved to be related to ischemic stroke, atherosclerosis and coronary atherosclerotic heart diseases. To our knowledge, the study, for the first time, evaluated the relationship between sICAS and ischemic stroke, atherosclerosis and CAD associated SNPs in Chinese Han individuals and reported that C-allele carriers of rs3798220 in *LPA* gene were at an increased risk of sICAS.

Many pathophysiological mechanisms such as endothelial injury, lipid deposition, inflammation, angiogenesis and fibrinolytic system damage play important roles in the development of intracranial atherosclerosis[29–31]. Lipoprotein(a) [Lp(a)] is a circulating low-density lipoprotein (LDL)-like particle which composed of apolipoprotein (a) covalently bound to apoB100[33]. Lp(a) is highly structural homologous to plasminogen, suggests that Lp(a) may contribute to the process of thrombosis through competitive inhibition of plasmin activation and interference with endogenous fibrinolysis[34, 35]. The atherogenic potential of Lp(a) may also be conferred by cholesterol deposition in the arterial wall, inflammatory cell recruitment, the binding of pro-inflammatory-oxidized phospholipids and the inhibition of endogenous endothelial fibrinolysis, and the inhibition of the expression of tissue factor[36–40]. Relationship between Lp(a) and endothelial dysfunction, proinflammatory state and defective fibrinolysis may be considered as plausible mechanisms to the connection with sICAS[41, 42]. The role of Lp(a) in sICAS were revealed in several researches. One study showed that high Lp(a) level was independent markers of a greater extent of intracranial large artery occlusive disease and another suggested that patients with more advanced intracranial and extracranial carotid stenosis tended to have higher Lp(a) levels[43, 44]. It was also showed that Lp(a) and other inflammatory molecules and endogenous fibrinolysis inhibitors could predict intracranial large artery atherosclerosis progression[41].

The apolipoprotein (a) gene (*LPA),*, located at chromosome 6q25.3-q26, as it encodes apo(a) of the Lp(a) lipoprotein particle, is associated with increased risk of atherosclerosis[45, 46]. Lp(a) is recognized as a lipoprotein with atherogenic and thrombogenic characteristics and is highly heritable because apo(a) gene polymorphism has a large influence on Lp(a) levels[47]. It was reported that the minor allele carriers of missense SNP rs3798220 in *LPA* gene were associated with elevated plasma lipoprotein(a) levels and had been implicated in coronary artery disease (CAD)[47, 48]. Lp(a)-related rs3798220 is also proved to be related to other vascular diseases like aortic stenosis (AS), peripheral arterial disease (PAD), large-artery atherosclerosis (LAA) and abdominal aortic aneurysm (AAA)[49, 50]. However, up to now, there is no report on the genetic relationship between LPA gene mutation and sICAS. To our knowledge, the present study is the first time to report that *LPA* rs3798220 is a susceptibility locus for sICAS in Chinese Han population. In this study, we found that the genotype distribution and allele frequency of *LPA* gene rs3798220 were significantly different between sICAS patients and normal individuals. Further Logistic regression analysis results showed that the *LPA* gene rs3798220 was independently associated with the prevalence of sICAS, with the C allele increased the risk of sICAS. Previous study had found that *LPA* gene polymorphisms are related to the circulating plasma lipoprotein(a) levels[45, 50]. Another study showed that C allele of *LPA* rs3798220 was associated with high plasma Lp(a) levels[51]. Therefore, we hypothesize that the C allele of the *LPA* gene rs3798220 may increase the Lp(a) levels and increase the risk of sICAS. The potential mechanism remains to be further studied.

The *MRAS (*muscle RAS oncogene homolog) gene resides on chromosome 3 at the band 3q22.3 and includes 10 exons. *MRAS* encodes a Ras (M-ras) protein which belongs to the Ras protein family member of the GTP-binding protein and is involved in many biological processes and has a broad distribution, especially in cardiovascular system [52]. Animal experiments show that M-ras can activate tumor necrosis factor-α (TNF-α) and lymphocyte-associated antigen (LFA-l) activity and participates in the

formation of atherosclerosis through cell adhesion molecule signaling pathways[53, 54]. Previous studies found that four SNPs of *MRAS* (rs9818870, rs2306374, rs1720819 and rs1199337) were associated with the risk of coronary heart disease in European populations[52, 55, 56]. Rs9818870, located at the microRNA binding site of *MRAS,* was most closely associated with cardiovascular disease[57]. A study from China showed that rs40593 of *MRAS* was associated with susceptibility to atherosclerotic cerebral infarction in Chinese Han population[58]. However, up to now, no relationship between rs9818870 and ischemic stroke has been reported. In our study, allele frequency of rs9818870 was significantly different between sICAS patients and normal population, and the genotype frequency was different in additive model, but further Logistic regression analysis in three genetic model analysis showed no such significance, suggesting that rs9818870 polymorphism of *MRAS* may not be the genetic risk factor of sICAS in Chinese Han population.

Limitations of the present study are: (i) The measurements of Lp(a) levels were not available, rendering it impossible to show directly that *LPA*variants raised sICAS risk through Lp(a) levels; (ii) It is possible that one or more polymorphisms associated with sICAS in the present study are in linkage disequilibrium with other polymorphisms in the same gene or in other nearby genes that are actually responsible for the development of this condition; (iii) Given that the results of the present study were not replicated, further researches in independent subject panels were required.

In conclusion, our present study suggests the *LPA* rs3798220 might be susceptibility locus for sICAS in Chinese Han individuals. Evaluating this risk SNP may therefore contribute to more effective primary and secondary prevention of sICAS in such individuals.

Declarations

Declaration of Interest

The authors declared no conflict of interest.

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Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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Tables

Table 1. Baseline characteristics of the subjects with ICAS and controls.

	ICAS (n=193)	Controls (n=186)	*P* value
Age(years)	59.1±10.9	57.2±10.6	0.091
Gender(male,N,%)	127 (65.8%)	109 (58.6%)	0.168
Smoking(N,%)	77 (39.9%)	55 (29.6%)	0.040
Drinking(N,%)	55 (28.5%)	53 (28.5%)	1.000
Hypertension(N,%)	138 (71.5%)	24 (12.9%)	<0.0001
SBP (mmHg)	142 (128-160)	119 (110-129)	<0.0001
DBP (mmHg)	85 (78-95)	75 (68-81)	<0.0001
Diabetes mellitus (N,%)	58 (30.1%)	6 (3.2%)	<0.0001
Plasma glucose (mmol/L)	5.49 (4.91-6.89)	4.51 (4.23-4.77)	<0.0001
Dyslipidemia(N,%)	70 (36.3%)	25 (13.4%)	<0.0001
TC (mmol/L)	4.54 (3.88-5.37)	4.52 (3.98-5.18)	0.893
TG (mmol/L)	1.46 (1.02-1.96)	1.26 (0.88-2.02)	0.059
HDL(mmol/L)	1.12 (0.98-1.35)	1.18 (1.03-1.39)	0.161
LDL (mmol/L)	2.84 (2.20-3.40)	2.12 (1.77-2.55)	<0.0001
BUN (mmol/L)	5.13 (3.92-6.27)	4.70 (4.01-5.47)	0.067
Cr (mmol/L)	81.0 (67.7-99.0)	61.1 (51.7-70.3)	<0.0001
UA (mmol/L)	304.24 ± 97.79	304.27± 74.83	0.998

sICAS: Intracranial atherosclerotic stenosis;SBP: systolic blood pressure; DBP:diastolic blood pressure; TC: Total cholesterol; TG: Triglyceride; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; BUN: blood urea nitrogen; Cr: serum creatinine; UA: uric acid.

Table 2. Comparisons of genotype distributions and allele frequencies in sICAS and control groups

dbSNP	Gene	Genotype/Allele	sICAS	Control	P value(genotype)	P value(allele)
rs3798220	*LPA*	Genotype				
		TT	80(41.5%)	121(65.1%)	0.000014	6.00E-06
		TC	96(49.7%)	59(31.7%)		
		CC	17(8.8%)	6(3.2%)		
		Allele				
		T	256(66.3%)	301(80.9%)		
		C	130(33.7%)	71(19.1%)		
rs9818870	*MRAS*	Genotype				
		GG	155(80.3%)	173(93.0%)	0.001241	0.000289
		AG	37(19.2%)	13(7.0%)		
		AA	1(0.5%)	0(0.0%)		
		Allele				
		G	347(89.9%)	359(96.5%)		
		A	39(10.1%)	13(3.5%)		

LPA: lipoprotein(a); *MRAS*: muscle RAS oncogene homolog.

dbSNP: single nucleotide polymorphism database.

Table 3. Comparisons of genotypes of rs3798220(*LPA*) and rs9818870(*MRAS*) according to three genetic models.

dbSNP	Gene	Dominant		Recessive		Additive	
		P value	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)
rs3798220	*LPA*	0.000005	2.629 (1.735-3.985)	0.029	2.898 (1.117-7.520)	0.000005	2.293 (1.605-3.276)
rs9818870	*MRAS*	0.001	3.263 (1.676-6.350)	N	N	0.000464	3.245 (1.679-6.272)

LPA: lipoprotein(a); *MRAS*: muscle RAS oncogene homolog.

dbSNP: single nucleotide polymorphism database.

Table 4. Multivariable logistic regression analysis of the polymorphisms associated with sICAS.

dbSNP	Gene	Dominant		Recessive		Additive	
		P value	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)
rs3798220	*LPA*	0.000020	3.951 (2.100-7.434)	0.105	2.768 (0.808-9.477)	0.000053	2.916 (1.736-4.899)
rs9818870	*MRAS*	0.339	1.529 (0.640-3.651)	N	N	0.338	1.530 (0.641-3.650)

LPA: lipoprotein(a); *MRAS*: muscle RAS oncogene homolog. dbSNP: single nucleotide polymorphism database.

Multivariable logistic regression analysis was performed with adjustment for age, gender, smoking status, drinking status, the prevalence of hypertension, diabetes mellitus and dyslipidemia and the serum concentration of creatinine. Due to the low frequency of the A allele of rs9818870, the analysis was unstable in a recessive model.