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TLR4 rs1927911, but Not TLR2 rs5743708, Is Associated With Atherosclerotic Cerebral Infarction in the Southern Han Population

A Case–Control Study

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Abstract: The objective of this study was to explore the association of toll-like receptor (TLR) 4 rs1927911 and TLR2 rs5743708 with atherosclerotic cerebral infarction (ACI) and their effects on blood pressure, fasting blood glucose, and blood lipids in the Han population of Hunan Province.

TLR4 rs1927911 and TLR2 rs5743708 were detected by polymerase chain reaction and restriction fragment length polymorphism in 170 patients with ACI and 149 healthy controls.

Our results indicated that the genotype and allele frequencies of TLR4 rs1927911 were significantly different between ACI patients and controls, whereas those of TLR2 rs5743708 were not significantly different between the 2 groups. For TLR4 rs1927911, blood pressure, fasting blood sugar, and serum lipid levels were not significantly different among different genotypes in the ACI and control groups.

The rs1927911 polymorphism of the TLR4 gene may be a risk factor for ACI in the Southern Han population of Hunan Province; however, it may not be associated with blood pressure, fasting blood sugar, or blood lipids.

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Abbreviations: ACI = atherosclerotic cerebral infarction, ANOVA = analysis of variance, BMI = body mass index, CI = confidence interval, DBP = diastolic blood pressure, FBS = fasting blood sugar, HDL = high-density lipoprotein, LDL = low-density lipoprotein, OR = odds ratio, PCR = polymerase chain reaction, RFLP = restriction fragment length polymorphism, SNP = single-nucleotide polymorphism, SBP = systolic blood pressure, TC = total cholesterol, TG = triglyceride, TLR = toll-like receptor.

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INTRODUCTION

Stroke is one of the leading causes of death in China; with high morbidity, mortality, and disability rates, it is both a serious threat to human health and a substantial burden to society and the patients' family.[1] Atherosclerotic cerebral infarction (ACI) is the most common type of stroke.[2] The development of ACI is a complex process of interactions between environmental factors and genetic backgrounds. Atherosclerosis is considered as one of the major pathological mechanisms of ACI, and inflammation and immune dysfunction may play a crucial role in the pathogenesis of atherosclerosis.[3–5] Therefore, genes associated with inflammation and immunity can be considered important candidate genes for ACI.

Toll-like receptors (TLRs) are a homologous family of receptors that are responsible for innate immunity. TLR activation can induce the expression of proinflammatory cytokine genes.[6–8] Overexpression of TLR2 and TLR4 has been shown in atherosclerotic lesions.[9–11] Studies have found that TLR2 and TLR4 expression levels were associated with the prognosis of ACI.[12,13] Therefore, TLR2 and TLR4 are considered to play a role in the development and progression of atherosclerosis and ACI.

Several studies have shown that Asp299Gly and Thr399Ile variants of the TLR4 gene are associated with stroke risk or poor prognosis.[14,15] However, other studies have drawn the opposite conclusion.[16–18] Hang et al[19] noted that the allele frequencies of these 2 single-nucleotide polymorphisms (SNPs) are low, whereas rs1927911 exhibits a high frequency in the Chinese population. Moreover, Yuan et al[20] confirmed that SNPs Asp299Gly and Thr399Ile of the TLR4 gene are rare in the Hunan population. The rs893629 polymorphism of the TLR2 gene was found to be related to the risk of arterial thrombosis in American systemic lupus erythematosus patients.[21] In recent years, the rs5743708 (Arg753Gln) polymorphism of TLR2 has been shown to be relevant to the risk of undergoing percutaneous transluminal coronary angioplasty and restenosis following coronary artery procedures.[22] To date, association studies focused on polymorphisms of the TLR2 gene and ACI risk have not been reported. In this study, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to detect rs1927911 and rs5743708 polymorphisms in ACI patients and controls from the southern Han population in Hunan province of China.

MATERIALS AND METHODS

Study Population

For the ACI group, 170 ACI patients (114 men and 56 women, average age = 64.1 ± 10.2 years) were recruited from the Department of Neurology in Xiangya Hospital, Central South University (Changsha, Hunan Province) from March to

December 2008. All participants were from the Southern Han population living in Hunan Province. All cases were clinically diagnosed as ACI and confirmed by CT and/or MRI imaging, according to the diagnostic criteria of the Fourth National Academic Conference on Cerebrovascular Disease.[23] ACI patients were excluded for any of the following conditions: trauma, inflammation, blood diseases, tumor or vascular malformation, cerebral embolism, infarction hemorrhage, severe liver and kidney disease, autoimmune disease, coronary heart disease, or treatment with lipid-lowering therapy in the last 6 months.

For the control group, 149 age- and sex-matched healthy Han volunteers (99 men and 50 women, average age = 63 ± 6.7 years) were recruited from the Department of Health Management, Xiangya Hospital. Following clinical and imaging examinations, subjects with the following conditions were excluded: a family history of cerebrovascular disease or coronary heart disease, cerebrovascular disease, liver and kidney disease, severe blood disease, autoimmune diseases, history of thrombosis, or treatment with lipid-lowering therapy in the last 6 months.

This study was approved by the ethics committee of Xiangya Hospital. All participants gave written informed consent.

Biochemical Tests, Clinical Data Acquisition, and Genomic DNA Extraction

Peripheral venous blood (10 mL) was obtained from subjects in the morning after fasting for 12 hours. A volume of 5 mL (no anticoagulant) was used for blood glucose and blood lipid testing. An additional 5 mL (sodium citrate anticoagulation, preserved at −70°C) was used to extract genomic DNA using conventional phenol-chloroform methods. The body mass index (BMI = body weight [kg]/height^2 [m^2]) of each subject was measured. Blood pressure was measured at least 3 different times in 1 day, and the average value was recorded. Hypertension was defined as a blood pressure ≥140/90 mm Hg or taking antihypertensive drugs. Fasting blood sugar (FBS) and blood lipids were measured using an automatic biochemical analyzer. Hyperlipidemia was defined as a total cholesterol (TC) value ≥5.2 mmol/L and/or a triglyceride (TG) value ≥1.7 mmol/L.

Primer Design and PCR

Primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA) and synthesized by Tiangen Biotech Co, Ltd (Beijing, China). The sequences of the primers used to amplify the rsl927911 polymorphism of the TLR4 gene are as follows: 5′-CCTGCATGCTCTGCACATG-3′ (forward primer) and 5′-ACCATGGGAATCCATGCAC-3′ (reverse primer). The length of the amplified fragment was 240 bp. The sequences of the primers used to amplify the rs5743708 polymorphism of the TLR4 gene are as follows: 5′-TATGGTCCAGG AGCTGGAGA-3′ (forward primer) and 5′-TGACATAAAGAT CCCAACTAGACAA-3′ (reverse primer). The amplified product for rs5743708 was 430 bp in length.

Restriction Digestion of the Amplification Product

The restriction endonuclease StyI (MBI Fermentas, Vilnius, Lithuania) was used to genotype rs1927911, and PstI (MBI Fermentas) was used to genotype rs5743708. The digestion reaction was performed at 37°C for 5 hours, and 5 μL of the reaction product was analyzed by agarose gel electrophoresis (0.5 × TBE, 110 V, 45 minutes) using standard DNA molecular weight markers.

Determination of the rs1927911 Genotype

Homozygous CC alleles were cleaved into 170 bp and 70 bp fragments. Heterozygous CT alleles were cleaved into 3 fragments (240 bp, 170 bp, and 70 bp) following digestion. Homozygous TT alleles cannot be digested; therefore, the amplification product remained a 240 bp fragment.

Determination of the rs5743708 Genotype

Homozygous GG alleles cannot be digested and remained a 430 bp fragment. The heterozygous GA allele was cleaved into 3 fragments of 430 bp, 284 bp, and 146 bp, respectively. The homozygous AA allele was cleaved into 2 fragments of 284 bp and 146 bp.

Statistical Analysis

The direct counting method was used to calculate the frequencies of alleles and genotypes in case and control groups. The exact χ^2 goodness-of-fit test was used to examine the Hardy–Weinberg equilibrium. SPSS 18.0 software (SPSS Inc, Chicago, IL) was used for statistical analysis. Genotype and allele frequencies between case and control groups were compared using the χ^2 test. Measurement data are expressed as means ± standard deviation, and a t test was used to determine differences between groups. A χ^2 test and analysis of variance (ANOVA) were used to determine differences in data obtained by the direct counting method. Logistic regression analysis was used to calculate the P values, odds ratios (ORs), and 95% confidence intervals (CIs). A value of $P < 0.05$ was considered significant.

RESULTS

Demographic Data of Patients and the Control Subjects

The overall clinical data of the ACI and control groups are shown in Table 1. Gender, average age, BMI, FBS, TG, smoking history, drinking history, and history of type 2 diabetes did not exhibit significant differences between the 2 groups ($P > 0.05$). History of hypertension, systolic blood pressure (SBP), diastolic blood pressure (DBP), TC, and low-density lipoprotein (LDL) levels in the ACI group were significantly higher than those in the control group ($P < 0.05$). High-density lipoprotein (HDL) levels in the ACI group were significantly lower than those in the control group ($P < 0.05$).

Comparison of Genotype Distribution

To confirm the genetic susceptibility of the SNPs in TLR4 and TLR2 to ACI in the Hunan Han population, 2 polymorphisms (rs1927911 and rs5743708) were genotyped in 319 participants, including 170 ACI patients and 149 control subjects. Genotypic and allelic distributions of TLR4 rs1927911 and TLR2 rs5743708 in the ACI and control groups were consistent with the Hardy–Weinberg equilibrium as determined by the exact χ^2 goodness-of-fit test. As shown in Table 2, χ^2 tests indicated that genotype and allele frequencies of rs1927911 between the ACI and control groups were significantly different ($P < 0.05$).

Comparison Between Subgroups

In this study, we divided ACI patients into 2 subgroups according to diabetes history. We found no significant differences in the genotype frequency of rs1927911 or rs5743708

TABLE 1. Clinical Data of Subjects

Clinical Characters	ACI Group (n = 170)	Control Group (n = 149)	*P*
Age, y	64.1 ± 10.2	63.0 ± 6.7	0.263
Male/female	114/56	99/50	0.907
BMI, kg/m^2	23.24 ± 3.19	23.68 ± 2.44	0.165
Smoking history (Y/N)	82/88	66/83	0.481
Drinking history (Y/N)	131/39	37/112	0.000*
Diabetes history (Y/N)	31/139	20/129	0.242
SBP, mm Hg	148.1 ± 23.5	129.9 ± 17.4	0.000*
DBP, mm Hg	88.1 ± 15.0	79.9 ± 10.3	0.000*
TC, mmol/L	4.49 ± 1.31	3.31 ± 1.14	0.000*
TG, mmol/L	2.13 ± 1.53	2.02 ± 1.32	0.482
LDL, mmol/L	2.70 ± 0.87	2.53 ± 0.70	0.045*
HDL, mmol/L	1.23 ± 0.31	1.35 ± 0.32	0.001*
FBS, mmol/L	5.84 ± 2.25	5.55 ± 1.69	0.191

ACI = atherosclerotic cerebral infarction, BMI = body mass index, DBP = diastolic blood pressure, FBS = fasting blood sugar, HDL = high-density lipoprotein, LDL = low-density lipoprotein, N = no, SBP = systolic blood pressure, TC = total cholesterol, TG = triglyceride, Y = yes.
* $P < 0.05$.

between the diabetic and nondiabetic subgroups ($P > 0.05$) (Table 3).

Nonconditional Logistic Regression Analysis

BMI, smoking history, drinking history, hypertension history, history of type 2 diabetes, FBS, SBP, DBP, TC, TG, LDL, HDL, the genotypes of rs1927911, or rs5743708 were introduced into multivariate logistic regression models using a stepwise regression method to determine the risk factors for ACI (Tables 4 and 5). In the logistic regression analysis, ACI diagnosis (yes/no) was used as the dependent variable. Variable inclusion and exclusion criteria were set as $\alpha_{in} = 0.10$ and $\alpha_{out} = 0.15$. The comprehensive results of Tables 4 and 5 indicated that smoking history, hypertension history, and TC may be independent risk factors, whereas HDL may be a protective factor for ACI. Although BMI and DBP were entered into the model, the result indicated that *P* value was >0.05 for both factors, as shown in Table 4. TLR4 rs1927911 was not entered into the model. Although TLR2 (Arg753Gln) was entered into the model, the *P* value was >0.05. Therefore, TLR4 rsl927911 and TLR2 (Arg753Gln) cannot be inferred as independent risk factors for ACI. Other candidate variables (including drinking history, history of diabetes, FBS, SBP, TG, and LDL) were not entered into the model.

TABLE 2. Genotype and Allele Frequencies of Polymorphisms in ACI and Control Subjects

SNP	ACI Group (n = 170)	Control Group (n = 149)	*P*
rs5743708 Genotypes			0.602
GG	151	135	
AG	19	14	
AA	0	0	
rs5743708 Alleles, %			0.612
G	94.4	95.3	
A	5.6	4.7	
rs1927911 Genotypes			0.000*
CC	100	56	
CT	60	73	
TT	10	20	
rs1927911 Alleles, %			0.000*
C	76.5	62.1	
T	23.5	37.9	

ACI = atherosclerotic cerebral infarction, SNP = single-nucleotide polymorphism.
* $P < 0.001$.

Relationship Between rs1927911 Polymorphism and ACI Risk Factors

By means of studying the relationship between 3 genotypes of rs1927911 and ACI risk factors, we found both in the ACI and control groups, there were no significant difference in the FBS, SBP, DBP, TC, TG, LDL, or HDL among 3 different genotypes (all $P > 0.05$, Table 6).

DISCUSSION

TLR2 and TLR4 have been shown to play a pivotal role in foam cell differentiation and macrophage activation, which are important pathogeneses in atherosclerosis development. TLR2 and TLR4 can mediate the immune response to exogenous or endogenous ligands, and promote cytokine production and subsequent inflammatory damages.[24] TLR4 can promote oxidized LDL-induced foam cell formation.[25,26] TLR2 gene knockout mouse model or pharmacological inhibition of TLR2 activity can show the inhibitive effect to atherosclerosis progression.[27–29] A TLR4 antagonist reduces early-stage atherosclerosis in diabetic apolipoprotein E-deficient mice.[30] Accumulating evidence has indicated a link between *Chlamydia pneumoniae* infection and atherosclerosis.[31] TLR2 and TLR4 may play a role in *C pneumoniae*-induced foam cell formation and VSMC migration.[32–34] Therefore, TLR4 and TLR2 are important candidate genes for ACI.

In this study, we utilized the PCR-RFLP technique to genotype rs1927911 and rs5743708 in 170 cases of ACI and 149 controls. We found that the rs1927911 genotype distribution of the ACI group was significantly different ($P < 0.05$) compared with that of the control group. No significant

TABLE 3. Genotype and Allele Frequencies Between Subgroups According to History of Type 2 Diabetes

Subgroups (n)	rs5743708 Genotypes, n (%)			rs5743708 Alleles, %			rs1927911 Genotypes, n (%)			rs1927911 Alleles, %		
	GG	GA +AA	*P1*	G	A	*P2*	CC	CT+TT	*P3*	C	T	*P4*
T2D (−) (139)	122 (87.8)	17 (12.2)	0.356	93.9	6.1	0.370	79 (56.8)	60 (43.2)	0.265	75.5	24.5	0.391
T2D (+)(31)	29 (93.5)	2 (6.5)		96.8	3.2		21 (67.7)	10 (32.3)		80.6	19.4	

T2D(−) = without history of type 2 diabetes, T2D(+) = with history of type 2 diabetes.

TABLE 4. Logistic Regression Analysis for Major Risk Factors of ACI (TLR4 rs1927911)

Variables	Coeffective	*P*	OR	95% CI
BMI	−0.598	0.040*	0.550	0.310–0.974
Smoking history	0.630	0.028*	1.877	1.069–3.296
Hypertension history	2.141	0.000*	8.504	4.600–15.722
DBP	0.582	0.070	1.790	0.954–3.360
TC	1.033	0.008	2.810	1.309–6.029
HDL	−0.826	0.013*	0.438	0.228–0.841
Constant	−5.154	0.000	0.000	

ACI = atherosclerotic cerebral infarction, BMI = body mass index, CI = confidence interval, DBP = diastolic blood pressure, HDL = high-density lipoprotein, OR = odds ratio, TC = total cholesterol, TLR = toll-like receptor.
* $P < 0.05$.

difference was found in genotype distribution of rs5743708 between 2 groups ($P > 0.05$). The rs5743708 polymorphism showed no significant association upon ACI susceptibilities in the Southern Han population. Because the incidence of ACI is the result of interactions between multiple genetic and environmental factors, this study was performed to build a multivariate logistic regression model. The results indicated that TLR4 rs1927911 did not enter the logistic regression model. Therefore, we concluded that the TLR4 rs1927911 polymorphism may be related to ACI; however, it may not be an independent risk factor for the Southern Han population. This finding is similar to that of Xu et al.[35] This study demonstrated that hypertension history and hyperlipidemia were significantly different between ACI and control groups, and additional logistic regression analysis confirmed that hypertension history and TC are risk factors of ACI; however, HDL is a protective factor. Additionally, this study confirmed that ACI results from interactions of genetic and environmental factors.

The TLR family plays an important role in inflammation, immunity, and atherosclerosis. Polymorphisms of the TLR4 and TLR2 genes have been reported to be associated with the risk of type 2 diabetes.[36–39] Therefore, we divided ACI patients into 2 subgroups according to diabetes history. No significant differences were found in the genotype frequencies of rs1927911 or rs5743708 between the diabetic and nondiabetic subgroups ($P > 0.05$). As shown in the Table 6, different genotypes of rs1927911 may not affect the levels of blood glucose, blood pressure, or blood lipids in both the ACI and control groups. These results indicated that the rs1927911 genotypes may not affect the blood pressure, blood sugar, and blood lipids in the Southern Han population.

TABLE 5. Logistic Regression Model for Major Risk Factors of ACI (TLR2 rs5743708)

Variables	Coefficient	*P*	OR	95% CI
BMI	−0.564	0.055	0.569	0.320–1.013
Smoking history	0.637	0.028*	1.891	1.073–3.333
Hypertension history	2.241	0.000*	9.406	4.990–17.727
DBP	0.589	0.068	1.801	0.957–3.392
TC	0.958	0.014*	2.606	1.217–5.580
HDL	−0.870	0.010*	0.419	0.216–0.812
TLR2 rs	0.810	0.074	2.248	0.925–5.462
Constant	−6.718	0.000	0.001	

ACI = atherosclerotic cerebral infarction, BMI = body mass index, CI = confidence interval, DBP = diastolic blood pressure, HDL = high-density lipoprotein, OR = odds ratio, TC = total cholesterol, TLR = toll-like receptor.
* $P < 0.05$.

TABLE 6. Comparison of Clinical Data Between Different Genotypes of TLR4 rs1927911

Clinic Data	rs1927911 Genotypes in ACI Group			*P1*	rs1927911 Genotypes in Control Group			*P2*
	CC (n = 100)	CT (n = 60)	TT (n = 10)		CC (n = 56)	CT (n = 73)	TT (n = 20)	
SBP, mm Hg	148.52 ± 25.68	146.72 ± 19.78	152.00 ± 23.63	0.776	132.23 ± 18.17	129.67 ± 18.02	124.50 ± 10.94	0.230
DBP, mm Hg	88.58 ± 16.53	87.22 ± 11.93	88.80 ± 16.73	0.849	81.86 ± 9.93	78.68 ± 10.61	78.70 ± 9.45	0.188
FBS, mmol/L	6.11 ± 2.60	5.40 ± 1.56	5.89 ± 1.53	0.154	5.46 ± 1.72	5.56 ± 1.63	5.80 ± 1.85	0.743
TC, mmol/L	4.60 ± 1.48	4.32 ± 1.07	4.49 ± 1.31	0.443	3.29 ± 1.20	3.58 ± 1.07	3.40 ± 0.80	0.322
TG, mmol/L	2.25 ± 1.70	2.02 ± 1.33	1.62 ± 0.45	0.365	2.02 ± 1.14	2.02 ± 1.32	2.01 ± 1.79	1.000
HDL, mmol/L	1.20 ± 0.25	1.28 ± 0.40	1.14 ± 0.30	0.204	1.40 ± 0.39	1.32 ± 0.25	1.34 ± 0.31	0.361
LDL, mmol/L	2.78 ± 0.93	2.59 ± 0.79	2.57 ± 0.48	0.360	2.68 ± 0.73	2.46 ± 0.70	2.43 ± 0.56	0.158

ACI = atherosclerotic cerebral infarction, DBP = diastolic blood pressure, FBS = fasting blood sugar, HDL = high-density lipoprotein, LDL = low-density lipoprotein, SBP = systolic blood pressure, TC = total cholesterol, TG = triglyceride, TLR = toll-like receptor.

It is unclear about the underlying mechanism how polymorphism rs1927911 affects ACI risk. The rs1927911 polymorphism is located in an intron of the TLR4 gene. It is not yet known whether this SNP is located at a functional site. The disease-causing mutation is most likely closely associated with the SNP. To further characterize this mutation, tagSNPs should be used to cover the entire TLR4 gene (including coding and regulatory regions). Limitation of this study lies in the size of the sample which is relatively smaller. It is necessary to enlarge sample sizes in the Southern Han population or confirm this association in other populations.

CONCLUSION

In conclusion, the TLR4 rs1927911 polymorphism may be associated with ACI risk in the Southern Han population. Studies in larger sample sizes or other populations are necessary to verify this finding.

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