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Original Research

Linkage Disequilibrium between LDLR *rs688* and *AvaII* Genes and its Significant Association with Ischemic Stroke

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Abstract

Background: To analyze the polymorphism distribution of low density lipoprotein receptor *rs688*, *AvaII*, *NcoI* gene in ischemic stroke, and explore the linkage disequilibrium among them. The correlation between the linkage disequilibrium and ischemic stroke was further analyzed. **Methods**: The levels of serum lipid (triglyceride, cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, apolipoprotein A1, apolipoprotein B) and *rs688*, *AvaII*, *NcoI* polymorphism of low density lipoprotein receptor gene were tested in patients with ischemic stroke (n = 140), healthy control (n = 129) and patients with other cerebrovascular diseases (n = 122). Chi-square test was used to compare the gene frequency and allele frequency of each group. Both the linkage disequilibrium of the three genes and the alleles correlated with ischemic stroke were analyzed. The correlation of linkage disequilibrium gene and ischemic stroke was analyzed with logistic binary regression. **Results**: In the ischemic stroke group, significant difference was observed in frequencies and allelic frequencies of low density lipoprotein receptor (LDLR) *rs688* and *AvaII*. No difference of *NcoI* was found. Linkage disequilibrium was found for *rs688* and *AvaII* (D' = 0.927, $R^2 = 0.509$). Allelic genes correlate with ischemic stroke included *T* of *rs688* ($X^2 = 46.105$, $p < 0.001$) and *C* of *AvaII* ($X^2 = 20.436$, $p < 0.001$). **Conclusions**: Linkage disequilibrium existed between LDLR *rs688* and *AvaII* genes. With the wild type gene (WT) (*rs688/AvaII*: *CC/TT*) as reference, *rs688/AvaII*: *CT/TC*, *CT/CC* and *TT/CC* increased the risk of ischemic stroke, which might be a genetic marker used for the screen of high-risk population contributing to the prevention of the disease.

Keywords: *rs688*; *AvaII*; *NcoI*; genetic polymorphism; ischemic stroke

1. Introduction

Stroke is a disease with high incidence, disability rate, mortality and recurrence rate, which has been the leading cause of death and disability for adults in China. In the data from Global Burden of Disease (GBD) 2016, stroke had been the leading cause of life loss in China for many years [1–3]. The main types of stroke include ischemic stroke and hemorrhage stroke, and in 2016, the prevalence rates of ischemic and hemorrhage stroke were 1762.77 and 406.16 cases per 100,000 people, respectively [4]. The prevention and management of stroke, especially ischemic stroke has been a great public health concern in China.

There are ten manageable risk factors of ischemic stroke including hypertension, diabetes, dyslipidemia, heart disease, smoking, alcohol intake, unhealthy diet, abdominal obesity, physical inactivity and psychological factors [5]. In addition, genetic factors including genetic polymorphism have been widely investigated and proven to contribute to the occurrence of ischemic stroke [6–9]. In various polymorphism studies, many have focused on the correlation between ischemic stroke and single nucleotide polymorphisms (SNPs) of low density lipoprotein receptor (LDLR) genes including *rs11669576*, *rs5925* (*AvaII*), *rs688*, *rs1122608* [10–14]. However, most studies investigated the frequencies and correlation of different SNP loci independently, and further studies are needed for the correlation between SNP loci as the linked gene and ischemic stroke [15–17].

Base on the correlation of *rs688*, *AvaII* and *rs5742911* (*NcoI*) with ischemic stroke, we conducted this study to further investigate the linkage disequilibrium among the three genes and the combination of genes correlated with ischemic stroke.

2. Materials and Methods

2.1 Subjects

We screened the patients with ischemic stroke who visited Quanzhou First Hospital between January 2019 and November 2019. Eligible subjects were those diagnosed as ischemic stroke for clinical manifestations including progressive dizziness, limb weakness, sudden headache, obnubilation and imaging findings on Magnetic Resonance Imaging (MRI) of intracranial or extracranial arterial stenosis or occlusion, new lesion of ischemic cerebral infarction, and etiology of large artery stenosis or small vessel occlusion. All the subjects did not receive standardized hypolipidemic therapy. Patients with obvious inducement for ischemic stroke such as trauma, infection, heart disease, and those with other cardiovascular and cerebrovascular diseases including coronary heart disease, heart failure and

Table 1. Primer sequences and PCR products.

Gene		Prime sequence	Allelic gene (WT/MT)	Polymerase chain reaction (PCR) product
rs688	F	*CCCTCTGGGACTGGCATCA*	*C/T*	304 bp
	R	*AAGACCTCCTCCTAGTCACAAC*		
AvaII (*rs5925*)	F	*GTCATCTTCCTTGCTGCCTGTTTAG*	*T/C*	219 bp
	R	*GGTTCCACAAGGAGGTTTCAAGGTT*		
NcoI (*rs5742911*)	F	*GTCGTCTTTATGTCCGCCCA*	*A/G*	972 bp
	R	*CAGTGCAACAGTAACACGGC*		

WT/MT, wild-type/mutant.

pulmonary vascular disease were excluded. Subjects with single comorbidity such as hypertension and head and neck atherosclerosis were included.

During the same period, patients with similar clinical manifestations but no intracranial or extracranial arterial stenosis or occlusion on MRI and diagnosed as cerebral hemorrhage, subarachnoid hemorrhage, intracranial aneurysm or other cerebrovascular diseases were included in the control group.

Healthy controls were matched from physical examination center synchronously for similar age and gender.

Clinical characteristics including age, gender, smoking history, alcohol consumption, underlying diseases including diabetes and hypertension were collected.

Height, weight and body mass index were not collected, for most of the patients were admitted in emergency and needed to rest in bed for the whole treatment. The protocol was approved by the Ethics Committee of Quanzhou First Hospital (approval number: [2018]213).

2.2 Instruments and Methods

We tested serum lipid level and genetic polymorphism of peripheral venous blood samples which were obtained before treatment in the stroke group and other cerebrovascular disease group, and during physical examination in the control group.

2.2.1 Lipid Level Test

Chemistry Analyzer (AU5800, Beckman Coulter Inc., Brea, CA, USA) was used for the test of triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB). Calibration and quality control for instruments and reagents were applied before the test to ensure the accuracy of results.

2.2.2 Detection of Genetic Polymorphism

DNA extraction Kit (Tiangen Biotech, Beijing, China) was used for the extraction of DNA from whole blood specimens. Primer was designed synthesized by Sangon Biotech (Shanghai, China), and the primer sequences and PCR products for *rs688*, *AvaII* and *NcoI* are listed in Table 1.

(1) PCR reaction system: 10 $\times$ buffer 2.0 µL, dNTP 1.6 µL, forward primer S1 0.4 µL, reverse primer S2 0.4 µL, template DNA 1.5 µL, Taq enzyme 0.1 µL, with the addition of dd$H_2$0 to 20 µL; (2) the condition of PCR circulation: 0.5 mL-Eppendorf tube was placed in PCR amplification instrument (22331, Eppendorf, Hamburg, German), pre-denaturation for 5 min at 95 °C,denaturation for 30 sec at 94 °C, annealing for 30 sec at 58 °C and extension for 1 min at 72 °C for a total of 35 cycles, extension for 5 min at 72 °C ultimately, subsequently the amplification products were preserved at 4 °C; (3) detection and sequencing of PCR products: 3% agarose gel was disposed, 5 µL of PCR product and 1 µL of 6 $\times$ loading buffer were blended and loaded, 2000 bp DNA Marker was added as marker, and after electrophoresis for 30 min under 120 V, the results were observed with gel imager. Sequencing was performed after obtaining the target band. The Tag enzyme (R001A, TaKaRa, Osaka, Japan), and PCR amplification instrument was provided by Eppendorf German.

2.3 Statistical Analysis

Clinical features and lipid level were described by $\bar{x} \pm$ s, *t* test or single factor analysis was used for normal data, and Kruskal-wallis rank sum test was used for non-normal data for the comparison between groups. The distribution of genotypes was tested by Hardy-Weinberg equation. Chi-square test was used for the comparison of percentages, the frequency of genotypes and allele frequencies between groups. Linkage disequilibrium was analyzed for the three LDLR genes, and case-control study (Haploview 4.2) (Broad Institute Inc., Cambridge, MA, USA) was used to test the correlation of case-control of ischemic stroke. Logistic binary regression was used for the correlation between the indexes collected in this study and ischemic stroke. Differences were considered to be statistically significant when $p < 0.05$. Statistical analysis was performed with SPSS 21.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Clinical Characteristics of Study Population

A total of 140 patients with ischemic stroke were included, 81 males and 59 females, with a median age of 64 years (range, 46–75 years). Another 122 patients with

Table 2. Clinical characteristics of the three groups.

	Healthy control	Patients with other cerebrovascular disease	Patients with ischemic stroke
	(n = 129)	(n = 122)	(n = 140)
Age (years)	61.87 ± 5.65	61.89 ± 9.39	62.40 ± 7.74
Male (%)	50.77	48.36	57.86
Smoking (%)	13.85	9.02	27.86*▲
Alcohol intake (%)	6.92	4.92	10.00
Hypertesion (%)	4.62	73.77*	75.71*
Diabetes (%)	7.69	14.75	34.29*▲
Systolic pressure (mmHg)	125.87 ± 16.75	155.74 ± 25.23*	149.04 ± 23.11*
Diastolic pressure (mmHg)	77.78 ± 10.95	89.61 ± 14.69*	84.73 ± 13.08*▲
TG (mmol/L)	1.07 ± 0.35	1.49 ± 1.62	1.39 ± 0.88*
TC (mmol/L)	4.69 ± 0.57	4.86 ± 0.94	4.70 ± 1.07
HDL-C (mmol/L)	1.38 ± 0.28	1.20 ± 0.33*	1.14 ± 0.28*
LDL-C (mmol/L)	2.84 ± 0.50	3.01 ± 0.77	2.93 ± 0.98
ApoA1 (g/L)	1.53 ± 0.24	1.33 ± 0.26*	1.29 ± 0.21*
ApoB (g/L)	0.95 ± 0.20	1.04 ± 0.25*	1.00 ± 0.25

* $p < 0.05$ compared with healthy control; ▲ $p < 0.05$ compared with disease control. TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B.

other cerebrovascular diseases were included, 59 males and 63 females, whose median age was 61 years (range, 46–83 years). 129 healthy controls consisted of 65 males and 64 females, with their median age being 61 years (range, 54–74 years). For clinical characteristics of the three groups, there were significant differences in smoking, hypertension, diabetes, systolic pressure, diastolic pressure, TG, HDL-C, ApoA1 between ischemic stroke group and healthy control group, while smoking, diabetes and diastolic pressure were also different between ischemic stroke group and other cerebrovascular disease group (Table 2).

3.2 Genotype Frequency

The genetic polymorphism of LDLR *rs688*, *AvaII*, *NcoI* conformed to Hardy-Weinberg equilibrium, and all the alleles reached genetic equilibrium with group representativeness. The gene distribution and allele frequency of *rs688* and *AvaII* in the ischemic stroke group were significantly different from those in the healthy control group and other cerebrovascular disease group, and there was no significant difference found in *NcoI* among the three groups (Table 3).

3.3 Linkage Disequilibrium

In comparison with healthy controls and a linkage disequilibrium between *rs688* and *AvaII* was observed in the patients with ischemic stroke (Fig. 1 and Table 4). In case-control correlation analysis, the alleles found correlating ischemic stroke included *T* of *rs688* ($X^2 = 46.105$, $p < 0.001$) and *C* of *AvaII* ($X^2 = 20.436$, $p < 0.001$).

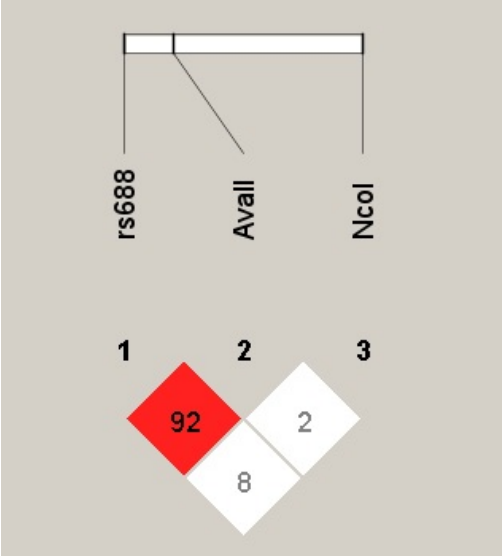

Fig. 1. LDLR *rs688*, *AvaII*, *NcoI* linkage disequilibrium. LDLR, low density lipoprotein receptor.

3.4 Analysis of Risk Factors for Ischemic Stroke

3.4.1 Single Factor Analysis by Logistic Binary Regression

Due to the linkage disequilibrium existing between LDLR *rs688* and *AvaII*, *rs688/AvaII* as a whole was included in the analysis of risk factors of ischemic stroke. In comparison with healthy controls and patients with other cerebrovascular diseases, single factor analysis of patients with ischemic stroke showed that smoking, hypertension, diabetes, systolic pressure, HDL-C, ApoA1, *rs688/AvaII* were significantly correlated with the incidence of ischemic stroke (Table 5).

Table 3. Distribution of genetic polymorphism LDLR *rs688*, *AvaII* and *NcoI*.

LDLR gene	Genotype/ allele [n (%)]	Healthy control (n = 129)	Patients with other cerebrovascular disease (n = 122)	Patients with ischemic stroke (n = 140)
rs688	*CC*	112 (86.82)	86 (70.49)	62 (44.29)
	CT	15 (11.63)	30 (24.59)	66 (47.14)
	TT	2 (1.55)	6 (4.92)	12 (8.57)
HW (*p*-value)*		0.093		
X^2		53.261	18.241	
p		<0.001	<0.001	
	C	239 (92.64)	202 (82.79)	190 (67.86)
	T	19 (7.36)	42 (17.21)	90 (32.14)
X^2		51.030	15.421	
p		<0.001	<0.001	
AvaII	*TT*	73 (56.59)	68 (55.74)	53 (37.86)
	TC	53 (41.09)	50 (40.98)	66 (47.14)
	CC	3 (2.33)	4 (3.28)	21 (15.00)
HW (*p*-value)*		0.061		
X^2		17.675	14.458	
p		<0.001	0.001	
	T	199 (77.13)	186 (76.23)	172 (61.43)
	C	59 (22.87)	58 (23.77)	108 (38.57)
X^2		15.468	13.197	
p		<0.001	<0.001	
NcoI	*AA*	74 (57.36)	56 (45.90)	80 (57.14)
	AG	48 (37.21)	61 (50.00)	54 (38.57)
	GG	7 (5.43)	5 (4.10)	6 (4.29)
HW (*p*-value)*		0.828		
X^2		0.224	3.532	
p		0.898	0.171	
	A	196 (75.97)	173 (70.90)	214 (76.43)
	G	62 (24.03)	71 (29.10)	66 (23.57)
X^2		0.016*	2.063	
p		0.920*	0.151	

* HW (*p*-value): *p* value tested with Hardy-Weinberg law by taking healthy control group; X^2 and *p* value: obtained by comparing different genotypes or allele frequencies of *rs688*, *AvaII* and *NcoI* with those of ischemic stroke group. LDLR, low density lipoprotein receptor.

Table 4. Linkage disequilibrium of LDLR *rs688*, *AvaII* and *NcoI*.

Gene	D'	LOD	R^2
rs688/AvaII	0.927	56.4	0.509
rs688/NcoI	0.083	0.39	0.005
AvaII/NcoI	0.028	0.01	0.001

D', degree of linkage disequilibrium; LOD, logarithm of odds.

3.4.2 Multivariate Analysis by Logistic Binary Regression

Multivariate analysis was performed on the above covariants which were significantly related to the incidence of ischemic stroke. With the wild type gene (WT) of *rs688/AvaII*: *CC/TT* as reference, the logistic binary regression analysis showed that the incidence of ischemic stroke increased in *rs688/AvaII*: *CT/TC*, *CT/CC* and *TT/CC*, while decreased in *rs688/AvaII*: *CC/TC* (Table 6).

4. Discussion

LDLR is a cell surface glycoprotein with a length of 839 amino acids which distributes widely in tissue cells. LDLR mediates the endocytosis of LDL and regulates serum LDL level via the recognition of apoprotein B-100 and chylomicron residues on LDL particles and apoprotein E on intermediate density lipoprotein [18,19]. The coding gene of LDLR locates across 45 kb on chromosome 19p13.1-13.1, including 18 exons and 17 introns. Mutations of LDLR gene are common including point mutations, fragment deletions, insertions and rearrangements, and until now there have been more than 800 mutation types found worldwide [20]. The correlations between SNP site mutations of LDLR gene and atherosclerotic diseases such as coronary heart disease, carotid atherosclerosis, and ischemic stroke have been investigated extensively. Sinha and Salazar *et al.* [21,22] proposed that *AvaII* may be a

Table 5. Single risk factor analysis for ischemic stroke.

Co-variants	B	S.E	Wals	*p*	OR (95% CI)
Age (years)	0.025	0.014	3.299	0.069	1.025 (0.998∼1.053)
Male (%)	–0.341	0.213	2.568	0.109	0.711 (0.469∼1.079)
Smoking (%)	1.084	0.273	15.761	<0.001	2.956 (1.731∼5.048)
Alcohol intake (%)	0.559	0.388	2.076	0.150	1.748 (0.818∼3.737)
Hypertesion (%)	1.616	0.236	46.882	<0.001	5.034 (3.169∼7.995)
Diabetes (%)	1.414	0.268	28.217	<0.001	4.155 (2.457∼7.028)
Systolic pressure (mmHg)	0.010	0.004	5.863	0.015	1.011 (1.002∼1.019)
Diastolic pressure (mmHg)	0.002	0.008	0.079	0.779	1.002 (0.987∼1.018)
TG (mmol/L)	0.107	0.101	1.115	0.291	1.113 (0.913∼1.356)
TC (mmol/L)	–0.088	0.120	0.537	0.464	0.916 (0.723∼1.159)
HDL-C (mmol/L)	–1.782	0.383	21.630	<0.001	0.168 (0.079∼0.357)
LDL-C (mmol/L)	0.018	0.136	0.017	0.895	1.018 (0.780∼1.328)
ApoA1 (g/L)	–2.430	0.476	26.104	<0.001	0.098 (0.035∼0.224)
ApoB (g/L)	0.038	0.450	0.007	0.932	1.039 (0.431∼2.508)
rs688/AvaII	0.362	0.064	31.974	<0.001	1.436 (1.267∼1.628)
NcoI	–0.173	0.183	0.896	0.344	0.841 (0.588∼1.203)

B, regression coefficient; S.E, standard error.

Table 6. *rs688/AvaII* genetic polymorphism combination and ischemic stroke.

Co-variants	B	S.E	Wals	*p*	OR (95% CI)
Smoking (%)	0.962	0.351	7.510	0.006	2.617 (1.315∼5.208)
Hypertesion (%)	0.544	0.369	2.177	0.140	1.723 (0.836∼3.550)
Diabetes (%)	1.023	0.327	9.792	0.002	2.781 (1.466∼5.279)
Systolic pressure (mmHg)	0.003	0.007	0.145	0.703	1.003 (0.990∼1.016)
HDL-C (mmol/L)	–0.862	0.864	0.994	0.319	0.423 (0.078∼2.298)
ApoA1 (g/L)	–1.662	1.065	2.438	0.118	0.190 (0.024∼1.529)
*rs688/AvaII**					
CC/TC	–1.032	0.506	4.153	0.042	0.356 (0.132∼0.961)
CC/CC	22.669	23205.422	0.000	0.999	0.000 (/)
CT/TC	1.482	0.322	21.129	<0.001	4.400 (2.342∼8.265)
CT/CC	3.026	0.839	13.005	<0.001	20.615 (3.980∼106.766)
TT/TT	–20.174	28420.722	0.000	0.999	0.000 (/)
TT/TC	–21.007	28420.722	0.000	0.999	0.000 (/)
TT/CC	1.980	0.673	8.664	0.003	7.240 (1.938∼27.056)

* With the WT genotype of *rs688/AvaII*: *CC/TT* as reference, we calculated the relative incidence rate of each *rs688/AvaII* gene polymorphism combination. There was no *rs688/AvaII*: *CT/TT* found in this study.

risk factor for coronary heart disease through the regulation of serum lipid level. The research of Jha suggested that *TT* genotype and allele *T* of *rs688* might be susceptibility genes of coronary heart disease [23]. Meng reported that the efficacy of rosuvastatin in carotid atherosclerosis was closely related to the genetic polymorphism of *rs688*, and patients with *CT* type had limited relief of plaque [24]. Besides, *rs11669576*, *AvaII*, *rs688*, *rs1122608*, *rs1433099* were also found to be correlated with atherosclerotic disease [10–14,16,25].

The above studies are mainly on the correlation of disease with genetic polymorphism of single SNP loci and the combination of different SNP loci, which fail to cover the analysis for linkage disequilibrium of genes. The correlation between the occurrence of disease and certain combination of genotypes needs to be further illustrated. In this study, we chose three common LDLR SNP loci, analyzed their frequency distribution in ischemic stroke, and further investigated the linkage disequilibrium between genes to find the specific genotype combination with linkage disequilibrium which may be important for the occurrence of ischemic stroke.

In our study, patients with ischemic stroke had a higher rate of *C*>*T* mutation in *rs688*, and the differences were significant compared with those in both healthy control and disease control, which were consistent with the results from Yue and Li [17,26]. The frequency of *AvaII* genetic polymorphism in ischemic stroke group was also significantly different from that in the two control groups. No significant difference of the frequency distribution of *NcoI*

gene was found in the three groups. The comparison of *AvaII* and *NcoI* between ischemic stroke group and healthy control was consistent with the results from Guo *et al*. [16], while the added disease control group in our study provided more reliable evidence for the function of these genes in ischemic stroke. Case-control correlation analysis indicated that the allelic genes correlated with ischemic stroke were *T* of *rs688* ($X^2 = 46.105$, $p < 0.001$) and *C* of *AvaII* ($X^2 = 20.436$, $p < 0.001$). These results indicate that both *C*>*T* mutation of *rs688* and *T*>*C* mutation of *AvaII* may increase the incidence of ischemic stroke.

Results of our analysis showed that linkage disequilibrium existed between *rs688* and *AvaII* genes (D' = 0.927, $R^2 = 0.509$), but not between *AvaII* and *NcoI* genes, *rs688* and *NcoI* genes. This conclusion is different from what Ekata reported in a study of coronary heart disease where linkage disequilibrium was found between *AvaII* and *NcoI* (D' = 0.6027, $R^2 = 0.2032$) [22]. Given the lack of previous report on linkage disequilibrium between *rs688* and *AvaII* [27] providing us with no reference, we further tested several types of gene polymorphism combination of *rs688* and *AvaII*. In the 391 specimens, the proportions of WT genotypes (*rs688/AvaII*: *CC/TT*), *rs688/AvaII*: *CT/TC* and *CC/TC* were 49.10%, 26.09% and 16.88% respectively, which may indicate that the incidence of the latter two genotypes is higher than others during inheritance, which we will further analyze with extended sample size.

Accordingly, it was indicated that there was a significant correlation between *rs688/AvaII* and ischemic stroke. Further in the multivariate analysis for ischemic stroke, with the WT genotype (*rs688/AvaII*: *CC/TT*) as reference, *rs688/AvaII*: *CT/TC*, *CT/CC* and *TT/CC* were found increasing the incidence of ischemic stroke, whereas *rs688/AvaII*: *CC/TC* reducing the incidence.

Patients selected in the ischemic stroke group in this study were all atherosclerotic, a type whose occlusions have already been proved in some works to be with a worse outcome compared to thromboembolic disease [28]. Therefore, the detection of *rs688/AvaII* genotype polymorphism combination may help to screen out the high-risk population of ischemic stroke. Early monitoring and predicting for these population will help prevent them from severe ischemic stroke with poor prognosis in the future.

5. Conclusions

Compared with control groups, the frequency distributions of LDLR *rs688*, *AvaII* gene polymorphism were significantly different in patients with ischemic stroke. Linkage disequilibrium was found between *rs688* and *AvaII* genes. The genotype polymorphism combination of *rs688/AvaII*: *CT/TC*, *CT/CC*, *TT/CC* increased the incidence of ischemic stroke, which might be a genetic marker to be used for screening high-risk population, contributing to the prevention of the disease.

Consent for Publication

All authors consent to submit the manuscript for publication.

Availability of Data and Materials

The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

Author Contributions

YC—contributed to the study design and drafted of the manuscript; HC, YS—performed the experiment; JZ, ZZ—acquired the data and revised the manuscript; YW, ZL—performed data analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee at Quanzhou First Hospital (NO. [2018]213), and subjects provided the informed consent authorizing the use of their clinical information and blood samples.

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Conflict of Interest

The authors declare no conflict of interest.

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