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Original Article

Evaluation of the Obesity Genes *FTO* and *MC4R* for Contribution to the Risk of Large Artery Atherosclerotic Stroke in a Chinese Population

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Key Words

FTO · *MC4R* · Obesity prone gene · Large artery atherosclerotic (LAA) stroke · Genetic risk factor · Synergistic effect

Abstract

Background: Obesity is a well-established risk factor for large artery atherosclerotic (LAA) stroke. The aim of the study was to explore whether obesity genes, such as *MC4R* and *FTO*, contribute to LAA stroke risk in the Chinese Han population. ***Methods:*** 322 LAA stroke patients and 473 controls were recruited. Gene polymorphism of *MC4R* (rs17782313) and *FTO* (rs8050136 and rs9939609) were genotyped. ***Results:*** No differences were observed in genotype frequencies of variants of *FTO* (rs8050136 and rs9939609) or *MC4R* (rs17782313) between LAA stroke patients and control subjects. However, rs17782313 of the *MC4R* gene was associated with LAA stroke susceptibility in smokers (rs17782313: $p = 0.020$, OR (95% CI) = 1.55 (1.07–2.23)) in the stratified analysis. Furthermore, multifactor dimensionality reduction analysis revealed that the combination of *MC4R* variant (rs17782313), hypertension and smoking habit was significantly associated with increased risk of LAA stroke ($p < 0.0001$, OR (95% CI) = 6.57 (4.79–9.01)). ***Conclusion:*** Our study indicated that the synergistic effects of *MC4R* variants, hypertension, and smoking habit contribute significantly to the risk of LAA stroke in the Chinese Han population. The finding revealed that obesity gene *MC4R* contribute to the risk of LAA stroke via a synergistic mechanism, which will provide new insight into the genetic architecture of LAA stroke.

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Introduction

Cerebrovascular disease is one of the three main causes of death and disability worldwide. Cerebral infarction is the most common type of CVD, which is recognized as a complex disease with genetic predisposition and environmental risk factors [1, 2]. There was increasing evidence to indicate that overweight/obesity is an independent risk factor for cardiovascular diseases, especially for cerebral infarction [3, 4]. Furthermore, a recent MRI-based body fat quantification study discovered the association between different patterns of fat distribution and atherosclerosis of the brain-feeding arteries, indicating the importance of visceral adiposity as a novel risk factor for large artery atherosclerotic (LAA) stroke [5]. It seems that overweight/obesity is regarded as a pivotal risk factor for LAA stroke. However, the underlying mechanism for the association between overweight/obesity and LAA stroke is not fully elucidated. The remarkable influences of obesity on metabolic syndrome and adipocytokines may take part in the pathogenesis [6–8]. Additionally, genetic risk factors may also play an important role in the disease (atherosclerosis) process. Furthermore, it is postulated that genetic predisposition is not only involved in the pathogenesis of vascular diseases but also represents an underlying link between obesity, metabolic syndrome, inflammation and atherosclerosis.

The heritability of obesity has long been appreciated, and the genetics of obesity has been the focus of intensive study for decades. Many obesity-prone genes, such as *FTO, MC4R, TBX15-WARS2, GRB14, ADAMTS9, LY86, RSPO3* and *ITPR2-SSPN*, have been found in genome-wide association studies (GWASs) on subjects of European ancestry [9–11]. Especially, *FTO* and *MC4R* have been found to be significantly associated with BMI in also GWASs including subjects of African descent, indicating that the *FTO* and *MC4R* were associated with BMI across populations of diverse ethnicities [12]. Furthermore, two recent studies revealed that polymorphisms of *FTO* and *MC4R* were associated with the risk for childhood obesity in a Chinese population [13, 14], providing evidence that *FTO* and *MC4R* are obesity-prone genes in the Chinese Han population. Though obesity is a well-established risk factor for stroke, the association between obesity-prone genes and stroke is not entirely convinced in previous studies [15]. Since it had been reported in previous studies [9–14] that rs17782313 (mapped 188 kb downstream of *MC4R*-coding sequence), rs8050136, and rs9939609 (within *FTO*) were associated with obesity, we choose these three single-nucleotide polymorphisms (SNPs) as genetic markers to explore the association between obesity-prone genes and LAA stroke. The aim of the study was to explore whether the variants of the two well confirmed obesity-prone genes (rs17782313 near *MC4R*, rs8050136 and rs9939609 within *FTO*) contribute to the risk for LAA stroke in the Chinese Han population, and to evaluate the contribution of the interplay of genetic and conventional risk factors to the LAA stroke risk in order to elucidate the underlying genetic architecture of LAA stroke.

Material and Methods

Study Population

A total of 795 unrelated individuals were recruited in this study: 322 LAA stroke patients and 473 controls. All of them were Chinese Han population from the Changsha area. The 322 patients with LAA stroke were prospectively recruited from those admitted to the Stroke Unit of the Department of Neurology, Third Xiangya Hospital of Central South University, between June 2011 and March 2014. Each diagnosis of a LAA stroke was made based on the results of a neurological examination using MRI according to the International Classification of Diseases, 9th Revision. The subtypes of ischemic stroke (IS) were classified based on the TOAST criteria [16]. Patients with IS related to LAA stroke were included. Patients with IS caused by lacunar stroke, cardioembolism, stroke caused by other determined causes, or stroke of undetermined cause were

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excluded. The following baseline characteristics and risk factors for cerebrovascular disease were recorded: age, gender, hypertension (treated or systolic blood pressure (SBP) ≥ 140 mm Hg or diastolic blood pressure (DBP) ≥ 90 mm Hg), and diabetes (treated or fasting plasma glucose ≥ 7.0 mmol/l or 2-hour postprandial plasma glucose or random plasma glucose ≥ 11.1 mmol/l). Quantitative traits, including the levels of BMI, blood pressure, fasting blood sugar and blood lipids (including total cholesterol (TC), triglycerides (TGs), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C)) were also recorded. Furthermore, lifestyle-based risk factors, including smoking and drinking status, were recorded. A total of 473 controls that had never experienced any stroke, were matched with the LAA stroke cases with respect to age, sex, and ethnic background. The control subjects reported no history of stroke, hematological disease, other cerebrovascular disease, liver ailment, nephrosis, autoimmune disease, or pregnancy. The protocols of this study were approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, and all participants signed informed consent.

SNP Genotyping

Peripheral venous blood (5 ml) was collected from all participants, and genomic DNA was extracted from peripheral blood leukocytes using phenol-chloroform methods. The genotypes were determined using Sequenom iPLEX assays with allele detection by mass spectroscopy using Sequenom MassARRAY technology (Sequenom, San Diego, CA, USA) according to the manufacturer's protocol. The SNP genotyping was performed by laboratory technicians who had no prior knowledge of the subjects' disease status. The primers for PCR amplification and extension reactions were designed using MassARRAY Assay Design Software (Sequenom) (supplementary table 1, available at *http://www.karger.com/ProdukteDB/produkte.asp?doi=448588*). The single-base extension reaction products were separated and evaluated using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [17].

Statistical Analysis

Statistical analyses were performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). To identify potential confounders, univariate analyses were performed comparing demographic, clinical and lifestyle risk factors in LAA stroke patients and controls. An unpaired Student's t-test was used to compare the quantitative data and to determine the differences between the cases and the controls, whereas the chi-square test or Fisher's exact test was used to compare categorical data. The chi-square test was also applied to identify deviations from Hardy-Weinberg equilibrium and the differences of genotype frequency distribution between LAA stroke patients and control subjects. Additionally, the differences of genotype frequency distribution in LAA stroke patients and control subjects were analyzed in a stratified analysis according to the smoking status. Then, logistic regression analyses were done to verify the effect of the variant on LAA stroke risk after adjustment for the covariates (status of diabetes mellitus, hypertension, and overweight). The odds ratio (OR) and 95% confidence intervals (95% CIs) were calculated using the additive genetic model.

To identify higher-order epistatic or synergistic interactions in our samples, we used the multifactor dimensionality reduction method (MDR 3.0.2, *www.epistasis.org*), which is a nonparametric and genetic model-free machine that outperforms logistic regression for detecting and characterizing nonlinear interactions among discrete genetic and environmental attributes. The MDR method combines attribute selection, attribute construction, and classification with cross-validation and permutation testing to provide a comprehensive and powerful approach to detecting epistasis or synergistic effects. This method is regarded as a useful and efficient statistical tool for detecting synergistic interactions while avoiding the 'dimension curse', especially for detecting synergistic interactions in case-control studies using relatively small sample sizes [18]. Furthermore, MDR has been successfully applied to numerous different complex multifactorial diseases and is currently being adapted to GWASs [19].

We examined synergistic interactions using the MDR method to identify higher-order gene-conventional risk factor interactions in our samples; the MDR method was performed to identify whether interactions between genetic variants (rs8050136, rs9939609 and rs17782313) and conventional risk factors (status of diabetes mellitus, hypertension, overweight, and smoking habit) increase the susceptibility of LAA stroke. This result was confirmed by a permutation test of 1,000 implemented in the MDR software. P values (two-tailed) < 0.05 were considered statistically significant.

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Table 1. Demographic and clinical characteristics of recruited individuals

Characteristic	Cases (n = 322) mean ± SD	Controls (n = 473) mean ± SD	p value
Age	61.92 ± 9.75	60.73 ± 9.21	0.081[a]
TC, mmol/l	4.75 ± 1.27	4.53 ± 1.03	0.006 [a]
LDL-C, mmol/l	2.64 ± 0.89	2.48 ± 1.56	0.101 [a]
HDL-C, mmol/l	1.31 ± 0.35	1.35 ± 0.73	0.381 [a]
TG, mmol/l	1.79 ± 1.59	1.62 ± 1.49	0.147 [a]
BMI, kg/m^2	23.18 ± 2.96	22.73 ± 3.09	0.046 [a]
FBS, mol/l	6.21 ± 2.84	5.43 ± 1.98	2.106×10^{-5} [a]
Characteristic	**Cases (n = 322) n (%) yes**	**Controls (n = 473) n (%) yes**	**p value**
Males	175 (54.3)	252 (53.2)	0.766 [b]
DM	112 (34.8)	79(16.7)	1.497×10^{-8} [b]
HBP	185 (57.5)	77 (16.3)	7.858×10^{-34} [b]
Smoking habit	89 (27.6)	75 (15.9)	5.561×10^{-5} [b]
Drinking status	81 (25.2)	103 (21.8)	0.267 [b]

[a]The p value was calculated by the unpaired Student's t-test.
[b]The p value was calculated by the chi square test.

Results

Baseline data of the cases and controls are shown in table 1. The levels of TC, BMI, and fasting blood sugar were significantly higher in cases than in controls (all $p < 0.05$). Notably, cases had a higher prevalence of diabetes mellitus, hypertension, and smoking habit (all $p < 0.05$). In our sample, rs8050136 and rs9939609 are in tight linkage disequilibrium: the D' value between the two SNPs is 0.99, and the r^2 is 0.95. Genotype frequency distribution and association with LAA stroke susceptibility for rs17782313 were summarized in table 2. Genotype frequency distribution and association with LAA stroke susceptibility for rs8050136 and rs9939609 was summarized in supplementary table 2 and 3 (available at *http://www.karger.com/ProdukteDB/produkte.asp?doi=448588*), respectively. No differences were observed in genotype frequencies of polymorphisms of *MC4R* (rs17782313) or *FTO* (rs8050136 or rs9939609) between LAA stroke patients and control subjects (all $p > 0.05$).

In a stratified analysis according to the smoking status, no differences were observed in genotype frequencies of variants (rs8050136 or rs9939609) of *FTO* between LAA stroke patients and control subjects in the smoker or nonsmoker subgroups (all $p > 0.05$); Though rs17782313 of the *MC4R* was not associated with LAA stroke in nonsmokers ($p > 0.05$), rs17782313 of the *MC4R* was significantly associated with LAA stroke susceptibility in smokers ($p < 0.05$). The association between *MC4R* locus (rs17782313) and LAA stroke susceptibility in the smoker subgroup remained significant after adjustment for the covariates (status of diabetes mellitus, hypertension, and overweight). (rs17782313: $p = 0.020$, OR (95% CI) = 1.55 (1.07–2.23)).

The results of the MDR analysis are summarized in table 3. The best model was the model composed of *MC4R* locus (rs17782313), hypertension, and smoking habit, suggesting that the genetic and conventional risk factors together contribute to the etiology of LAA stroke. This model displayed the highest level of testing balance accuracy (0.7151) and a cross-validation consistency of 10/10. The results of MDR analysis revealed that the synergistic effects model of *MC4R* locus (rs17782313), hypertension, and smoking habit may increase the risk of LAA stroke statistically, and the results of the permutation test were consistent (with 1,000 permu-

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Table 2. Genotype frequency distribution and association with LAA stroke susceptibility for the SNP (rs17782313)

Genotype	Genotype frequencies, %		OR (95% CI)[a]	p value[a]
	case, n (%)	control, n (%)		
Total participants				
TT	223 (0.693)	305 (0.645)	1.154 (0.968–1.376)	0.111
TC	87 (0.270)	154 (0.325)		
CC	12 (0.037)	14 (0.030)		
Non-smoker subgroup				
TT	160 (68.7%)	264 (66.3%)	1.060 (0.866–1.297)	0.571
TC	64 (27.5%)	12 (30.2%		
CC	9 (3.8%)	14 (3.5%)		
Smoker subgroup				
TT	63 (70.8%)	41 (54.7%)	1.55 (1.07–2.23)	0.020
TC	23 (25.8%)	34 (45.3%)		
CC	3 (3.4%)	0 (0%		

LAA stroke = Large artery atherosclerotic stroke; CI =confidence interval; OR = odds ratio; SNPs = single-nucleotide polymorphisms (only associated SNPs are shown).

[a]OR (95% CI) and p value for the additive genetic model after adjustment for covariates (hypertension, overweight, diabetes).

Table 3. MDR models of loci and conventional risk factors for LAA stroke susceptibility

Model	Training Bal.Acc	Testing Bal.Acc	CV consistency	p value	p value for permutation test
Hypertension	0.7059	0.7059	10/10	<0.0001	0.001
Rs17782313 hypertension	0.7059	0.7044	7/10	<0.0001	0.001
Rs17782313 hypertension smoking habit	0.7151	0.7151	10/10	<0.0001	0.001

MDR = Multifactor dimensionality reduction; LAA stroke = large artery atherosclerotic stroke; Training Bal.Acc = training balanced accuracy; Testing Bal. Acc = testing balanced accuracy; CV consistency = cross-validation consistency.

tations p value is 0.001). In MDR analysis, the 3D combinations of *MC4R* locus (rs17782313), hypertension, and smoking habit were classified into high- or low-risk groups. Background shading within each cell indicated the risk to LAA stroke of each given combination. High-risk groups are indicated by cells filled with dark shading, low-risk groups by cells filled with light shading (as shown in fig. 1). Based on the chi-square test, the OR of the high-risk combinations of the synergistic effects model indicated a 6.57-fold increased risk of LAA stroke ($p < 0.0001$, OR (95% CI) = 6.57 (4.79–9.01)). Then, we used the software 'PS: Power and Sample Size Calculation (PS: version: 3.0.43)' to calculate the statistical power for our study. In assumption that OR is 1.582, a threshold power value of 80% will be detected for our sample size. As the OR value is 6.57 in our MDR analysis, the statistical power according to our sample size is nearly 1.0. These results confirmed that MDR method is an efficient and powerful statistical tool for detecting synergistic interactions while avoiding the 'dimension curse', especially for detecting synergistic interactions in case-control studies using relatively small sample sizes.

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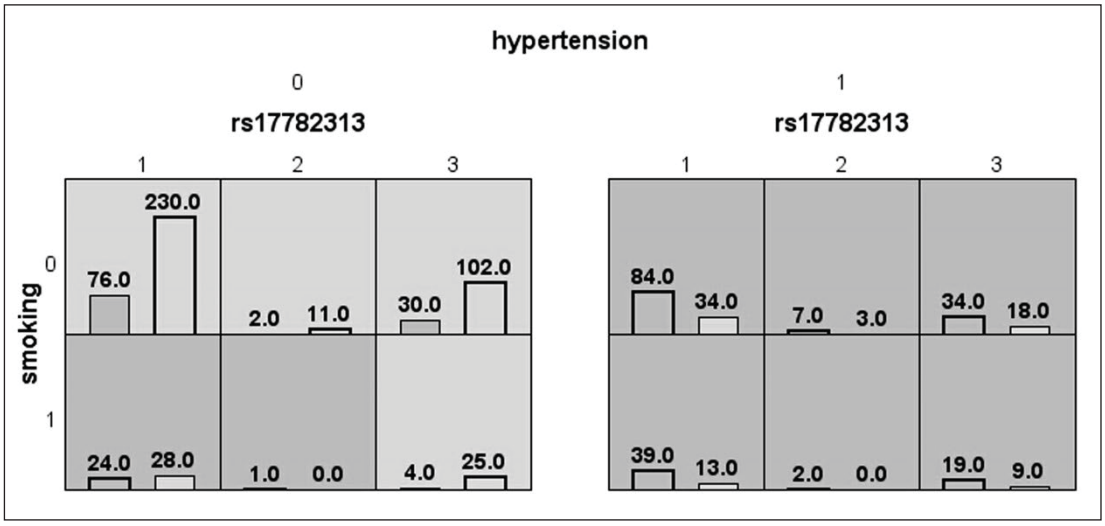

Fig. 1. The best model of factors contributing to LAA stroke identified by MDR. Notation: rs17782313 1/2/3 = TT/CC/TC. Smoking 0/1 = non-smoker/smoker. Hypertension 0/1 = non-hypertension/hypertension. The best model which is composed of MC4R locus (rs17782313), hypertension, and smoking habit may increase the risk of LAA stroke statistically. The number in the left bar of each cell represents the number of LAA stroke patients with the given combination, which is indicated with bar filled with dark shading. While the number in the right bar of each cell represents the number of control subjects with the given combination, which is indicated with bar filled with light shading. Background shading within each cell indicated the risk to LAA stroke of each given combination. In MDR analysis, the 3D combinations of *MC4R* locus (rs17782313), hypertension, and smoking habit were classified into high- or low-risk groups. High-risk cells are indicated by dark shading, low-risk cells by light shading. Based on the chi-square test, the OR value of the high-risk combinations of the synergistic effects model indicated a 6.57-fold increased risk of LAA stroke ($p < 0.0001$, OR (95% CI) = 6.57 (4.79–9.01)).

Discussion

Identification of the Contribution of Synergistic Effect between Obesity Gene and Conventional Risk Factors to LAA Stroke Risk in a Chinese Population

China is a country with a high incidence of stroke, and intracranial atherosclerosis is more prevalent in the Chinese Han population than in Western populations [20–22]. Meanwhile, obesity is a well-established risk factor for LAA stroke, and many obesity-prone genes have been found in GWASs. Intriguingly, the associations between obesity-prone genes and LAA stroke in the Chinese Han population have not been studied previously. It was of interest to investigate the relationship between obesity-prone genes (such as *FTO* and *MC4R*) and LAA stroke in the Chinese Han population.

Our study revealed no evidence for association between single variants of *FTO* or *MC4R* and LAA stroke, which is in accordance with the report of the Mannheim-Heidelberg stroke study in Germany [15]. However, our stratified analysis also showed evidence that rs17782313 of *MC4R* was associated with LAA stroke susceptibility in a smoker subgroup based on a stratified analysis according to the smoking status. The association between *MC4R* locus (rs17782313) and LAA stroke susceptibility in the smoker subgroup remained significant after adjusting for covariates. Thus, the result of stratified analysis indicated a possible synergistic effect between *MC4R* polymorphism and smoking habit. To further identify higher-order genetic-conventional risk factor interactions in our samples, we applied the MDR

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method, which is regarded as an advanced and promising strategy to assess gene-environment and gene-gene interactions in the etiology analysis of complex diseases [18, 23], such as cardiovascular disease and cerebrovascular disease. Interestingly, our MDR analysis revealed that the combination of *MC4R* locus (rs17782313), hypertension, and smoking habit significantly increased the risk of LAA stroke ($p < 0.0001$, OR (95% CI) = 6.57 (4.79–9.01)). The results of MDR analysis indicated a prominent synergistic interplay of gene and conventional risk factors with regard to LAA stroke risk.

The Finding of Synergistic Effects between MC4R and Conventional Risk Factors on Stroke Risk Provide New Insight into the Genetic Architecture of LAA Stroke

MC4R is located on chromosome 18q22. The protein encoded by this gene is a G-protein coupled receptor that is expressed in the central nervous system and has a crucial role in regulating the balance between feeding behavior and energy expenditure. The alteration of MC4R function or expression may result in imbalance of the energy homeostasis, body fat accumulation, and eventually obesity. Loos RJ et al. [11] analyzed genome-wide association data from 16,876 individuals of European descent; the study revealed the strongest association signal (rs17782313, $p = 2.9 \times 10^{-6}$) mapped 188 kb downstream of *MC4R* coding sequence, which established that common variants near *MC4R* influence fat mass, weight, and obesity risk at the population level. The SNP location and patterns of phenotypic associations are consistent with effects mediated through altered MC4R function, although there is no research to prove that this SNP has a functional effect on MC4R at present. Further studies should be conducted to identify the causal variant and the underlying mechanisms of the identified association in the future. Furthermore, the result of a recent study focusing on the Chinese Han population indicated that rs17782313 of *MC4R* was associated with childhood obesity [14], which is consistent with the results of GWASs of European descent. Additionally, previous studies revealed evidence that rs17782313 affects not only the levels of plasma lipids including TC and LDL-C [24], but also glycemic traits and susceptibility of type 2 diabetes in the Chinese Han population [25]. A recent study also revealed that rs17782313 variants of *MC4R* may be associated with nocturnal blood pressure levels in the Chinese Han population, indicating a possible role of *MC4R* on blood pressure regulation [26].

The remarkable influences of *MC4R* on obesity and related metabolic syndromes (ranging from disturbance of blood glucose and lipids to elevated blood pressure levels) may increase the susceptibility of atherosclerosis, the underlying pathologic change of LAA stroke. A previous report [15] has analyzed the contribution of *MC4R* to stroke, but the association between *MC4R* polymorphisms and stroke has not been convincing. Moreover, the result of our single-locus findings concerning the association between *MC4R* gene polymorphisms and LAA stroke was also negative. Although we did not find any significant main effect, we did report an association between LAA stroke and the SNP (rs17782313) in smoker subgroups in the stratified analysis according to smoking status. It is the first study indicating that rs17782313 may contribute to stroke risk. Furthermore, we attribute the difference between our results and previous studies to either racial difference or a synergistic effect between rs17782313 and smoking on LAA stroke risk. It is well known that many genetic and environmental risk factors may take part in the disease pathogenesis of complex diseases such as LAA stroke. And it is believed that the more risk factors are present, the higher is the susceptibility to come down with this complex disease. Thus, we postulated that the *MC4R* gene is a low-penetrance susceptibility marker that exerts a minor marginal effect on LAA stroke, and this gene may impact stroke risk via a synergistic mechanism. Our MDR analysis confirmed that the three-way combination of *MC4R* polymorphism (rs17782313), hypertension, and smoking habit contributes significantly to the risk of LAA stroke in the Chinese Han population, which is consistent with previous reports demonstrating the synergistic effect of gene and conven-

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tional risk factors by increasing the risk to suffer from complex disease [27, 28]. The revealing finding of MDR analysis will provide new insights into the genetic architecture of LAA stroke.

The Obesity-Prone Gene MC4R Contributes to the Risk of LAA Stroke via a Synergistic Mechanism, Which May Provide Enlightenment for the Prevention of LAA Stroke

From the perspective of stroke prevention, the risk factors can be divided into various categories: congenital or acquired, modifiable or not, classical or non-classical [29, 30]. It is well known that interventions of acquired and modifiable risk factors, such as managements of hypertension and smoking cessation, are efficient strategies for stroke prevention [31–33]. Our finding revealed that obesity-prone genes such as *MC4R* contribute to the risk of LAA stroke via a synergistic mechanism, indicating that congenital risk factors, such as *MC4R* polymorphism, may also play an important role in the pathogenesis of LAA stroke. Although the *MC4R* polymorphism is not modifiable for clinic practice at present, several treatment options have been investigated in subjects with *MC4R* mutation-related obesities. A study showed that an intensive lifestyle intervention induces similar weight reduction in *MC4R* mutation carriers in comparison to *MC4R* mutation non-carriers [34]. However, long-term body weight maintenance is hardly ever achieved in *MC4R* mutation carriers [35]. Meanwhile, a variety of in vitro experimental studies on the management of *MC4R* disruption have been conducted. In vitro studies with melanocortin agonists showed that mutated human *MC4R* with impaired endogenous agonist functional response can be activated by some of these agonists and may represent a valuable therapeutic target [36]. Furthermore, another study indicated that pharmacological chaperones that recover cell surface expression of MC4R may represent a candidate for the development of a targeted therapy suitable for patients with MC4R-deficient obesity [37]. It is postulated that chemical chaperones and pharmacological agonists efficiently restore cell surface expression and that endogenous agonist response of mutated *MC4R* may be effective in the treatment of adiposity. However, further research in the development of drugs for *MC4R* variations is needed. Although pharmacotherapy targeting obesity-prone genes such as *MC4R* has not clinically been tested in the prevention of cerebrovascular disease, identification of pertinent genes may unravel new therapeutic strategies to counter the influence of gene polymorphism on obesity and hence minimize its notable vascular complications, such as LAA stroke.

Conclusion

In conclusion, our results revealed no evidence of association between *FTO* polymorphism and LAA stroke. However, our study indicated that a synergistic effect of *MC4R* polymorphism (rs17782313), hypertension, and smoking habit contribute significantly to the risk of LAA stroke in the Chinese Han population. The finding revealed that the obesity-prone gene *MC4R* contributes to the risk of LAA stroke via a synergistic mechanism, which will provide new insights into the genetic architecture of LAA stroke. We believe these findings will eventually guide a promising prevention strategy of LAA stroke in the future.

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Disclosure Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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