Published in final edited form as:

J Stroke Cerebrovasc Dis. 2018 June ; 27(6): 1683–1691. doi:10.1016/j.jstrokecerebrovasdis.2018.01.029.

Significant Association between *OPG/TNFRSF11B* Variant and Common Complex Ischemic Stroke

Xin Xiong, PhD^{*,1}, Duraid Hamied Naji, PhD^{*,1}, Binbin Wang, PhD^{†,1}, Yuanyuan Zhao, PhD^{*}, Junhan Wang, MD[‡], Dan Wang, PhD^{*}, Yuting Zhang, MS^{*}, Sisi Li, MS^{*}, Shanshan Chen, PhD[§], Yufeng Huang, PhD^{||}, Qin Yang, PhD^{*}, Xiaojing Wang, PhD^{*}, Dan Yin, PhD^{*}, Xin Tu, PhD^{*}, Qiuyun Chen, PhD^{¶,¶}, Xu Ma, PhD[†], Chengqi Xu, PhD^{*}, Qing K. Wang, PhD^{*,¶,¶,††}

^{*}Key Laboratory of Molecular Biophysics of the Ministry of Education, Cardio-X Institute, College of Life Science and Technology, Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, China;

[†]National Research Institute for Family Planning, Beijing, China;

[‡]Department of Clinical Laboratory of University Hospital;

[§]Key Laboratory for Molecular Diagnosis of Hubei Province, The Central Hospital of Wuhan, Tongji Medical College;

^{||}Wuhan Children's Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China;

[¶]Center for Cardiovascular Genetics;

[#]Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio;

^{**}Department of Molecular Medicine, Case Western Reserve University, Cleveland, Ohio;

^{††}Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, Ohio.

Abstract

Background: The serum level of osteoprotegerin (encoded by *OPG* or *TNFRSF11B*) was previously shown to be increased in patients with ischemic stroke. A single nucleotide polymorphism rs3134069 in the *TNFRSF11B* gene was previously associated with ischemic stroke in a population of diabetic patients in Italy. It remains to be determined whether rs3134069 is associated with ischemic stroke in the general population or populations without diabetes.

Materials and Methods: We genotyped rs3134069 and performed a case-control association study to test whether rs3134069 is associated with ischemic stroke in 2 independent Chinese Han

Address correspondence to Chengqi Xu, PhD or Qing K. Wang, PhD, College of Life Science and Technology and Center for Human Genome Research, Huazhong University of Science and Technology, Luoyu Road 1037, Wuhan, 430074, China. cqxu@hust.edu.cn, qkwang@hust.edu.cn, wangq2@ccf.org.

[†]These authors contributed equally to this work.

Appendix: Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.jstrokecerebrovasdis.2018.01.029.

populations, including a China-Central population with 1629 cases and 1504 controls and a China-Northern population with 1206 cases and 720 controls.

Results: rs3134069 showed significant association with ischemic stroke in the China-Central population ($P = 9.24 \times 10^{-3}$, odds ratio [OR] = 1.50). The association was replicated in the independent China-Northern population ($P = 2.45 \times 10^{-4}$, OR = 1.53). The association became more significant in the combined population ($P = 7.09 \times 10^{-6}$, OR = 1.41). The associations remained significant in the male population, female population, and population without type 2 diabetes. Our expression quantitative trait loci analysis found that the minor allele C of rs3134069 was significantly associated with a decreased expression level of *TNFRSF11B* ($P = .002$).

Conclusions: This study demonstrates that rs3134069 in *TNFRSF11B* increases risk of ischemic stroke by decreasing *TNFRSF11B* expression.

Keywords

TNFRSF11B; ischemic stroke; genetics; single nucleotide polymorphism

Introduction

Stroke is a common cause of death worldwide,¹ and accounts for over 20% of total deaths in China.² Ischemia is responsible for about 87% of stroke cases.³ Atherosclerosis in the brain is the major cause for formation of thrombi, and is estimated to cause 70%–80% of ischemic strokes.⁴ Ischemic stroke is known to be affected by genetic risk factors, environmental factors, and their interactions. Genome-wide association studies have identified some loci conferring risk to ischemic stroke, including 12p13 (*NINJ2*), 12q24 (*ALDH2*), 7p21 (*HDAC9*), 9p21, 4q25 (*PITX2*), 16q22 (*ZFHX3*), 9q34 (ABO), and 1p13.2 (*TSPAN2*).^{5–10} Candidate gene approaches also identified *ALOX5AP*, *NOS3*, *PCSK9*, *PDE4D*, *SGK1*, *VKORC1*, and other genes confer risk to ischemic stroke.^{11–16} However, all these genomic variants in aggregate explain only a small proportion of heritability of ischemic stroke, a phenomenon referring to as “missing heritability”.¹⁷ Therefore, many more genomic variants associated with ischemic stroke need to be identified to fully elucidate the genetic architecture of this important disease.

Osteoprotegerin (OPG), which belongs to the tumor necrosis factor receptor superfamily, is encoded by the *TNFRSF11B* gene on chromosome 8q23.¹⁸ Several studies have demonstrated that OPG could promote endothelial cell survival.^{19–21} Moreover, several studies have shown a correlation between increased endogenous serum OPG levels and the presence and severity of clinical coronary artery disease (CAD),^{22,23} stroke,^{24–26} cardiovascular morbidity and mortality²⁷ as well as the progression of atherosclerosis in humans.^{28,29} Further evidence suggests that circulating OPG levels also are associated with the extent of vascular calcification.^{30–32} Moreover, increased OPG serum levels were found in *Ldlr*^{−/−} knockout (KO) mice on a Western diet.³³ Recently, a variant in the *TNFRSF11B* gene was found to be associated with coronary atherosclerosis in patients with rheumatoid arthritis.³⁴ Moreover, Biscetti et al reported that in a small population of 364 diabetic patients with a history of ischemic stroke and 492 diabetic subjects without history of ischemic stroke, single-nucleotide polymorphism (SNP) rs3134069 showed a significant

genotypic association with ischemic stroke under the background of diabetes.³⁴ However, it is unknown whether SNP rs3134069 is associated with ischemic stroke in subjects without type 2 diabetes (T2D). Khoulood et al found that PPAR γ variant C161T was associated with risk to ischemic stroke in populations with T2D, but not in populations without T2D.³⁴

Moreover, only 15% of patients with ischemic stroke have a history of T2D and remaining 85% do not have a history of T2D.³⁵ Therefore, it is necessary to determine whether *TNFRSF11B* variants are associated with ischemic stroke in a more complex population, especially in populations without T2D.

In this study, we analyzed the association between *TNFRSF11B* SNP rs3134069 and ischemic stroke in 2 independent case-control ischemic stroke populations with a total of 5059 Chinese Han subjects. Moreover, we studied whether rs3134069 was a functional variant associated with the expression level of *TNFRSF11B* mRNA by eQTL analysis.

Materials and Methods

Study Subjects

Samples enrolled in this study were selected from the GeneID database, which is an ongoing study in the Chinese Han population and has collected more than 80,000 DNA samples and also all available clinical data. The goal of the GeneID database is to identify susceptibility genes or other risk factors of cardiovascular and cerebrovascular diseases in the Chinese Han population.^{36–43}

In this study, we enrolled a case-control population with a total of 5059 samples, including 2835 patients with ischemic stroke and 2224 comparable controls. The study contained 2 independent populations matched by geographical areas they were enrolled. The China-Central population consisted of 1629 patients with ischemic stroke and 1504 controls, and the cases enrolled were from the patients who were under treatment of ischemic stroke in the hospitals of Wuhan city, whereas the controls of the China-Central population were enrolled from people who subjected physical examinations from the same hospitals of Wuhan city. The China-Northern population included 1206 patients with ischemic stroke enrolled from the patients who were under treatment of ischemic stroke in the hospitals of Beijing, and 720 controls enrolled from other patients who showed no sign of ischemic stroke by related medical examinations in the hospitals of Beijing. All subjects were self-reported to be of Chinese Han origin.

The diagnosis of ischemic stroke was made based on the standard World Health Organization criteria.⁴⁴ The clinical diagnosis was made carefully by at least 2 independent neurologists based on a medical history of stroke, stroke signs by neurological examinations, and cerebral ischemia by computed tomography (CT) or magnetic resonance imaging (MRI) images. In this study, we selected patients with ischemic stroke only, and excluded those patients with subarachnoid hemorrhages, embolic brain infarction, brain tumors, and those with a relevant brain stem or subcritical hemispheric lesions with a diameter of greater than 1.5 cm based on the data from CT or MRI examinations. We selected controls as those which were diagnosed to be stroke-free, had no medical history of stroke, and showed

normal brain CT or MRI examinations in the China-Northern population, or common health individuals in the China-Central population.

Other basic demographical and clinical characteristics such as the age, sex, smoking history, hypertension, and T2D were also obtained from medical records or the data from physical examinations. The diagnostic criteria for hypertension are a systolic blood pressure of 140 mm Hg or higher or a diastolic blood pressure of 90 mm or higher. The diagnostic criteria for T2D are a fasting plasma glucose concentration of 126 mg/dL or higher after at least 8 hours of fasting or a 2-hour plasma glucose level of 200 mg/dL or higher during an oral glucose tolerance test.

This study was approved by the Ethics Committees on human subject research of Huazhong University of Science and Technology and local institutions. Written informed consent was obtained from all subjects. This study followed the guidelines by the Declaration of Helsinki.

SNP Genotyping

The Syto 9 fluorescent dye-based high resolution melt (HRM) method was used to genotype SNP rs3134069 on a Rotor-gene 6200 System (Corbett Life Science, Mortlake, Australia) as described by us.^{13,38,39,45–47} In brief, a total volume of 25 µl of polymerase chain reaction (PCR) reaction mixture contained 2.5 µl of 10 × PCR buffer (containing 15 mM MgCl₂), .5 µl of dNTPs (10 mM), .5 µl of each primer (10 µM), 1 µl of template DNA (25 ng/µl), 5 µmol/L of SYTO 9 fluorescent dye, .15 U of Taq DNA polymerase (TIANGEN, Beijing, China), and ddH₂O. The forward PCR primer is 5'-CCACCATCATCAAAGGGCTAT-3' and the reverse primer is 5'-CAGGGAATTAATGGGGGAGAC-3'. The PCR was performed on an ABI 9700 PCR System (Life Technologies, Gaithersburg, MD) with a profile of 95°C for 3 minutes, 40 cycles of 95°C for 10 seconds, 59°C for 10 seconds and 72°C for 15 seconds, and a final step of 72°C for 10 minutes. PCR products were analyzed and the genotype of each individual was analyzed using the HRM program. During each round of HRM analysis, 3 DNA samples with known genotypes of CC, AC, and AA were included as positive controls, and 1 negative control of ddH₂O without genomic DNA was also included. The total success rate of HRM genotyping was 96.3%.

Direct Sanger sequencing was used to validate the accuracy of HRM genotyping data, and the details were described in the Supplementary Material.

Real-Time Quantitative Reverse Transcription PCR Analysis

The Cq method was used to determine the mRNA expression level of the *TNFRSF11B* gene among study subjects with different genotypes of SNP rs3134069. Quantitative real-time RT-PCR analysis was carried out according to the standard Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines,⁴⁸ and as described previously by us.^{49,50} The details were described in the Supplementary Material.

Statistical Analysis

The Hardy-Weinberg disequilibrium test was performed in each control population using the PLINK program (1.07 version).⁵¹

The case-control association analysis was performed as described previously.^{13,40,52} Chi-square (χ^2) tests were performed using Pearson's 2×2 and 2×3 contingency tables to evaluate the association between allelic frequencies or genotypic frequencies and ischemic stroke using SPSS v17.0 (IBM Corporation, Armonk, NY). Multiple logistic regression analysis was used to adjust covariates, including sex, age, hypertension, smoking history, and T2D using SPSS v17.0 (IBM Corporation). Statistical power analysis of study populations was conducted using program PS (Power and Sample size Calculations, version 3.0.43).⁵³

For statistical analysis of real-time RT-PCR data of the *TNFRSF11B* expression level, a Student's *t*-test was used to compare the differences for the mean RQ values between different genotypes of SNP rs3134069 using SPSS v17.0.

Results

Significant Allelic Association between *TNFRSF11B* SNP rs3134069 and Ischemic Stroke

We performed a case-control association study to test whether SNP rs3134069 in the *TNFRSF11B* gene was associated with ischemic stroke. First, we genotyped SNP rs3134069 in a Central China population (China-Central) and analyzed the potential association between rs3134069 with ischemic stroke. The significant association was then replicated in a Northern China population (China-Northern). Power analysis indicates that the 2 independent populations in this study can provide enough statistics power to test the association. Under the assumptions of the type I error of .05, a minor allele frequency of .13 for SNP rs3134069 in the Chinese Han population (HapMap data), and with an odds ratio (OR) of 1.3, the power of China-Central population was 95% and 80% for the China-Northern population.

The China-Central population consisted of 1629 patients with ischemic stroke and 1504 controls (Table 1). SNP rs3134069 was genotyped in the China-Central population. The genotyping data of the controls in the China-Central population did not deviate from a Hardy-Weinberg equilibrium tests ($P = .76$). In the China-Central population, the frequency of the minor allele C in cases is significant higher in controls (.11 in cases versus .09 in controls). The minor allele C of rs3134069 was significantly associated with an increased risk of ischemic stroke (observed $P_{obs} = 4.26 \times 10^{-3}$ with an OR = 1.27) (Table 2). The data indicate that SNP rs3134069 is a significant genetic risk variant for ischemic stroke. The association remained significant with an OR of 1.50 ($P_{adj} = 9.24 \times 10^{-3}$) after adjusting for covariates, including sex, age, hypertension, smoking history, and T2D (Table 2).

We enrolled another population from northern China (China-Northern) to verify the significant association between SNP rs3134069 and ischemic stroke. The China-Northern population included 1206 patients with ischemic stroke and 720 controls (Table 1). The genotyping data in the China-Northern population did not deviate from HWE test ($P = 1.00$).

The frequency of the minor allele C in the patient group was also significantly higher than in the control group (.13 in cases versus .10 in controls). Significant association was identified between SNP rs3134069 and ischemic stroke in the China-Northern population with a P_{obs} value of 5.60×10^{-3} and an OR of 1.35 (Table 2). After adjusting for covariates of sex, age, hypertension, smoking history, and T2D, the association remained significant ($P_{adj}=2.45 \times 10^{-4}$, OR = 1.53) (Table 2).

We combined the 2 populations and generated a larger population for further association analysis. In the combined population, the association between SNP rs3134069 and ischemic stroke became more significant ($P_{obs}=3.51 \times 10^{-5}$, OR = 1.31; $P_{adj}=7.09 \times 10^{-6}$, OR = 1.41). Together, the data from 2 independent populations strongly suggest that the minor allele C of SNP rs3134069 confers a significant risk of ischemic stroke.

We divided the study subjects by sex, and found that the allelic association between SNP rs3134069 and ischemic stroke was significant in both the female population ($P_{obs}=0.002$, OR = 1.44; $P_{adj}=0.001$, OR = 1.43) and in the male population ($P_{obs}=0.002$, OR = 1.28; $P_{adj}=0.003$, OR = 1.30) (Table 2). The ORs were higher in the female population than in the male population, however, the differences were not significant by a Breslow-Day test ($P > .05$).

As described above, the association between rs3134069 and ischemic stroke remained significant after adjusting T2D and other covariates in the China-Central population (Table 2). However, the rate of T2D in cases was markedly higher than that in controls (Table 1), and may still confound the association analysis. Therefore, to further assess whether T2D affects the association between rs3134069 and ischemic stroke, we excluded the subjects with T2D from both cases and controls and reanalyzed the association. After excluding subjects with T2D, the frequency of the minor allele C in cases was significantly higher than that in controls (.12 in 448 cases versus .09 in 1501 controls; $P_{obs}=0.03$, OR = 1.31; $P_{adj}=0.04$, OR = 1.32) (Table 3). The results were validated in the China-Northern population (1100 cases versus 680 controls, $P_{obs}=0.03$, OR = 1.28; $P_{adj}=0.04$, OR = 1.28) and in the combined population (1548 cases and 2181 controls, $P_{obs}=6.76 \times 10^{-5}$, OR = 1.31; $P_{adj}=6.76 \times 10^{-4}$, OR = 1.30) (Table 3). These data suggest that SNP rs3134069 is a risk factor for ischemic stroke independent of T2D.

Significant Genotypic Association between SNP rs3134069 and Ischemic Stroke

We also analyzed the genotypic association under 3 common genetic models: additive, dominant, or recessive model (Table 4). The data showed that significant genotypic association was identified between SNP rs3134069 and ischemic stroke under an additive model or a dominant model, but not under a recessive model in the China-Central population before and after adjusting for covariates of sex, age, hypertension, smoking history, and T2D ($P_{obs}=1.14 \times 10^{-2}$, $P_{adj}=1.00 \times 10^{-3}$ under an additive model; $P_{obs}=1.13 \times 10^{-2}$, $P_{adj}=1.20 \times 10^{-2}$ under a dominant model) (Table 4). The finding was confirmed in the China-Northern population ($P_{obs}=1.84 \times 10^{-2}$, $P_{adj}=1.54 \times 10^{-4}$ under an additive model; $P_{obs}=4.92 \times 10^{-4}$, $P_{adj}=2.04 \times 10^{-4}$ under a dominant model) (Table 4). The association became more significant in the combined population ($P_{obs}=1.76 \times 10^{-4}$, $P_{adj}=5.91 \times 10^{-6}$ under an additive model; $P_{obs}=8.50 \times 10^{-6}$, $P_{adj}=2.29 \times 10^{-5}$ under a dominant model) (Table 4). When the combined population was divided into the male population and female population,

the genotypic association between SNP rs3134069 and ischemic stroke was significant in both the female population ($P_{obs}=.008$, $P_{adj}=.002$ under an additive model; $P_{obs}=.004$, $P_{adj}=.002$ under a dominant model) or in the male population ($P_{obs}=.008$, $P_{adj}=.003$ under an additive model; $P_{obs}=.003$, $P_{adj}=.004$ under a dominant model) (Table 5).

C allele of SNP rs3134069 is Associated with the Decreased Expression Level of *TNFRSF11B*

SNP rs3134069 is located at a position of 245 bp upstream of the *TNFRSF11B* transcriptional start site. Based on its position, we hypothesized that SNP rs3134069 was a functional variant which may be associated with the expression level of *TNFRSF11B*. We measured the expression levels of *TNFRSF11B* from 89 people randomly selected from the general population undergoing physical examinations by real-time RT-PCR analysis and genotyping of these individuals for SNP rs3134069. The analysis showed that the mean relative expression level of *TNFRSF11B* in the 13 AC genotype carriers was significantly lower than that in the 76 AA genotype carriers ($P=.002$) (Fig 1). These results suggest that the minor allele C of SNP rs3134069 is associated with a decreased expression level of *TNFRSF11B*.

Discussion

In the present study, we identified a novel, highly significant association between SNP rs3134069 in the *TNFRSF11B* gene on chromosome 8q23 and ischemic stroke in the Chinese Han population. First, significant allelic association between SNP rs3134069 and ischemic stroke was found in a Central Chinese population from GeneID with 1629 ischemic stroke cases and 1504 controls before and after adjusting for covariates ($P_{adj}=9.24 \times 10^{-3}$) (Table 2). The minor allele C of SNP rs3134069 confers a risk of ischemic stroke (OR = 1.50). We also found significant genotypic association under an additive or dominant inheritance model (Table 4). Second, both the novel allelic association and genotypic association between SNP rs3134069 and ischemic stroke were verified in the China-Northern population (1206 patients with ischemic stroke and 720 controls) (Tables 2 and 4). Third, after combining the 2 populations, the P values for the association became more significant ($P_{adj}=7.09 \times 10^{-6}$, OR = 1.41 for allelic association; $P_{adj}=5.91 \times 10^{-6}$ under an additive model and $P_{adj}=2.29 \times 10^{-5}$ under a dominant model for genotypic association) (Tables 2 and 4). Finally, we analyzed the association between rs3134069 and ischemic stroke in population without T2D and the association remained significant. These data demonstrated significant association between *TNFRSF11B* SNP rs3134069 and risk of ischemic stroke, especially in population without T2D.

Previously, Biscetti et al found that in a small population of 364 patients with diabetes and a history of ischemic stroke and 492 diabetic subjects without history of ischemic, SNP rs3134069 showed a significant genotypic association with ischemic stroke under the background of diabetes ($P < 10^{-4}$).⁵⁴ It was unknown whether SNP rs3134069 showed a significant allelic association with diabetes-associated ischemic stroke as no data were presented. Moreover, it is unknown whether the *TNFRSF11B* variant is associated with ischemic stroke in population without T2D. Here we show significant association between

TNFRSF11B SNP rs3134069 and ischemic stroke in the general population and in the population without T2D. Therefore, our study significantly expands the spectrum of *TNFRSF11B* association with stroke from a diabetic population to the complex population.

Importantly, we demonstrated significant association between risk allele C of SNP rs3134069 and a decreased expression level of *TNFRSF11B* (Fig 1). The data suggest that rs3134069 is a functional variant for *TNFRSF11B* and that a decreased mRNA expression level of *TNFRSF11B* is a risk factor for the pathogenesis of ischemic stroke. This finding was surprising because several previous studies reported a positive association between increased serum OPG levels and ischemic stroke. In 2001, Browner et al reported that although the serum OPG level was not associated with the risk of incident thrombotic strokes, it was associated with the risk of fatal strokes.²⁶ Guldiken et al later showed that serum OPG levels were significantly higher in 51 patients with ischemic stroke than in 28 control subjects.²⁵ Ustundag et al showed that serum OPG levels were significantly higher in patients with cardioembolic and atherothrombotic strokes than in healthy controls or in patients with the transient ischemic attack.²⁴ Moreover, Jensen et al followed up 244 patients with acute ischemic stroke for 47 months and found that patients with lower serum OPG levels measured at admission had significantly improved survival.⁵⁵ Moreover, Morony et al demonstrated that a treatment with recombinant osteoprotegerin in *Ldlr*^{-/-} KO mice did not affect atherosclerotic lesion sizes or number.²⁴ More importantly, a study with *TNFRSF11B*^{-/-} KO mice clearly demonstrated that the *TNFRSF11B* deficiency accelerated advanced atherosclerotic lesion progression under the *ApoE*^{-/-} KO background.⁵⁵ Our data are consistent with the data from KO mice that decreased *TNFRSF11B* expression increases risk of atherosclerosis-related ischemic stroke, however, this needs to be confirmed in the future. Thus, it is likely that the increased OPG levels in serum samples from patients with stroke may represent an adaptive response of an organism to inflammation associated with atherosclerosis and stroke.

A limitation of the present study is that it enrolled patients with the large vessel subtype of stroke only. It will be interesting to study whether SNP rs3134069 is associated with other subtypes of stroke such as cardioembolic stroke, large vessel disease, small vessel disease, and cryptogenic stroke in the future.

In conclusion, the data in the present study demonstrate significant association between *TNFRSF11B* SNP rs3134069 and ischemic stroke in the complex population, especially in the population without T2D. We also showed that the risk allele C of SNP rs3134069 was associated with decreased *TNFRSF11B* expression. Our results suggest that *TNFRSF11B* is a susceptibility gene for common complex ischemic stroke and that the minor allele C of SNP rs3134069 increases risk of ischemic stroke by decreasing the expression level of *TNFRSF11B*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Grant support:

This study was supported by the China National Natural Science Foundation Program (31430047, 31671302), Hubei Province's Outstanding Medical Academic Leader Program, Hubei Province Natural Science Key Program (2014CFA074), the China National Natural Science Foundation grant (91439129, NSFC-J1103514), NIH/NHLBI grants R01 HL121358 and R01 HL126729, Fundamental Research Funds for the Central Universities (HUST: 2016YXMS254).

References

1. Dichgans M Genetics of ischaemic stroke. *Lancet Neurol* 2007;6:149–161. [PubMed: 17239802]
2. Liu L, Wang D, Wong KSL, et al. Stroke and stroke care in China: huge burden, significant workload, and a national priority. *Stroke* 2011;42:3651–3654. [PubMed: 22052510]
3. Donnan GA, Fisher M, Macleod M, et al. Stroke. *Lancet* 2008;371:1612–1623. [PubMed: 18468545]
4. Markus HS. Stroke genetics. *Hum Mol Genet* 2011; 20:R124–R131. [PubMed: 21831884]
5. Rosand J, Mitchell BD, Ay H, et al. Loci associated with ischaemic stroke and its subtypes (SiGN). *Lancet Neurol* 2016;15:174–184. [PubMed: 26708676]
6. Network NSG, Consortium ISG. Identification of additional risk loci for stroke and small vessel disease: a meta-analysis of genome-wide association studies. *Lancet Neurol* 2016;15:695–707. [PubMed: 27068588]
7. Kilarski LL, Achterberg S, Devan WJ, et al. Meta-analysis in more than 17,900 cases of ischemic stroke reveals a novel association at 12q24. *Neurology* 2014;83:678–685. [PubMed: 25031287]
8. Traylor M, Farrall M, Holliday EG, et al. Genetic risk factors for ischaemic stroke and its subtypes (the METASTROKE collaboration): a meta-analysis of genome-wide association studies. *Lancet Neurol* 2012;11:951–962. [PubMed: 23041239]
9. Bellenguez C, Bevan S, Gschwendtner A, et al. Genome-wide association study identifies a variant in HDAC9 associated with large vessel ischemic stroke. *Nat Genet* 2012;44:328–333. [PubMed: 22306652]
10. Ikram MA, Seshadri S, Bis JC, et al. Genomewide association studies of stroke. *NEJM* 2009;360:1718–1728. [PubMed: 19369658]
11. Löhmußaar E, Gschwendtner A, Mueller JC, et al. ALOX5AP gene and the PDE4D gene in a central European population of stroke patients. *Stroke* 2005;36:731–736. [PubMed: 15731479]
12. Helgadottir A, Gretarsdottir S, Clair DS, et al. Association between the gene encoding 5-lipoxygenase-activating protein and stroke replicated in a Scottish population. *Am J Hum Genet* 2005;76:505–509. [PubMed: 15640973]
13. Xu C, Wang F, Wang B, et al. Minor allele C of chromosome 1p32 single nucleotide polymorphism rs11206510 confers risk of ischemic stroke in the Chinese Han population. *Stroke* 2010;41:1587–1592. [PubMed: 20576952]
14. Bevan S, Dichgans M, Gschwendtner A, et al. Variation in the PDE4D gene and ischemic stroke risk a systematic review and meta-analysis on 5200 cases and 6600 controls. *Stroke* 2008;39:1966–1971. [PubMed: 18420948]
15. Dahlberg J, Smith G, Norrving B, et al. Genetic variants in serum and glucocorticoid regulated kinase 1, a regulator of the epithelial sodium channel, are associated with ischaemic stroke. *J Hypertens* 2011;29:884–889. [PubMed: 21430556]
16. Wang Y, Zhang W, Zhang Y, et al. VKORC1 haplotypes are associated with arterial vascular diseases (stroke, coronary heart disease, and aortic dissection). *Circulation* 2006;113:1615–1621. [PubMed: 16549638]
17. Malik R, Traylor M, Pulit SL, et al. Low-frequency and common genetic variation in ischemic stroke: the METASTROKE collaboration. *Neurology* 2016;86:1217–1226. [PubMed: 26935894]
18. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;89:309–319. [PubMed: 9108485]

19. Cross SS, Yang Z, Brown NJ, et al. Osteoprotegerin (OPG)—a potential new role in the regulation of endothelial cell phenotype and tumour angiogenesis? *Int J Cancer* 2006;118:1901–1908. [PubMed: 16287088]
20. Malyankar UM, Scatena M, Suchland KL, et al. Osteoprotegerin is an alpha vbeta 3-induced, NF-kappa B-dependent survival factor for endothelial cells. *J Biol Chem* 2000;275:20959–20962. [PubMed: 10811631]
21. Pritzker LB, Scatena M, Giachelli CM. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell* 2004;15:2834–2841. [PubMed: 15064358]
22. Tousoulis D, Siasos G, Maniatis K, et al. Serum osteoprotegerin and osteopontin levels are associated with arterial stiffness and the presence and severity of coronary artery disease. *Int J Cardiol* 2013;167:1924–1928. [PubMed: 22640692]
23. Jono S, Ikari Y, Shioi A, et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. *Circulation* 2002;106:1192–1194. [PubMed: 12208791]
24. Ustundag M, Orak M, Guloglu C, et al. The role of serum osteoprotegerin and S-100 protein levels in patients with acute ischaemic stroke: determination of stroke subtype, severity and mortality. *J Int Med Res* 2011;39:780–789. [PubMed: 21819709]
25. Guldiken B, Guldiken S, Turgut B, et al. Serum osteoprotegerin levels in patients with acute atherothrombotic stroke and lacunar infarct. *Thromb Res* 2007;120:511–516. [PubMed: 17258300]
26. Browner WS, Lui LY, Cummings SR. Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. *J Clin Endocrinol Metab* 2001;86:631–637. [PubMed: 11158021]
27. Rasmussen LM, Tarnow L, Hansen TK, et al. Plasma osteoprotegerin levels are associated with glycaemic status, systolic blood pressure, kidney function and cardiovascular morbidity in type 1 diabetic patients. *Eur J Endocrinol* 2006;154:75–81. [PubMed: 16381994]
28. Kiechl S, Werner P, Knoflach M, et al. The osteoprotegerin/RANK/RANKL system: a bone key to vascular disease. *Expert Rev Cardiovasc Ther* 2006;4:801–811. [PubMed: 17173497]
29. Kiechl S, Schett G, Wenning G, et al. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004;109:2175–2180. [PubMed: 15117849]
30. Makarovic S, Makarovic Z, Steiner R, et al. Osteoprotegerin and vascular calcification: clinical and prognostic relevance. *Coll Antropol* 2015;39:461–468. [PubMed: 26753467]
31. Mesquita M, Demulder A, Damry N, et al. Plasma osteoprotegerin is an independent risk factor for mortality and an early biomarker of coronary vascular calcification in chronic kidney disease. *Clin Chem Lab Med* 2009;47:339–346. [PubMed: 19676147]
32. Morony S, Tintut Y, Zhang Z, et al. Osteoprotegerin inhibits vascular calcification without affecting atherosclerosis in *ldlr*($-/-$) mice. *Circulation* 2008;117:411–420. [PubMed: 18172035]
33. Shao JS, Cheng SL, Charlton-Kachigian N, et al. Teriparatide (human parathyroid hormone (1–34)) inhibits osteogenic vascular calcification in diabetic low density lipoprotein receptor-deficient mice. *J Biol Chem* 2003;278:50195–50202. [PubMed: 14504275]
34. Chehaibi K, Noura S, Mahdouani K, et al. Effect of the PPARgamma C161T gene variant on serum lipids in ischemic stroke patients with and without type 2 diabetes mellitus. *J Mol Neurosci* 2014;54:730–738. [PubMed: 24841086]
35. Wang W, Jiang B, Sun H, et al. Prevalence, incidence, and mortality of stroke in China: results from a nationwide population-based survey of 480 687 adults. *Circulation* 2017;135:759–771. [PubMed: 28052979]
36. Chen S, Wang C, Wang X, et al. Significant association between CAV1 variant rs3807989 on 7p31 and atrial fibrillation in a Chinese Han population. *J Am Heart Assoc* 2015;4:pii: e001980. [PubMed: 25953654]
37. Wang F, Xu CQ, He Q, et al. Genome-wide association identifies a susceptibility locus for coronary artery disease in the Chinese Han population. *Nat Genet* 2011;43:345–349. [PubMed: 21378986]

38. Wang P, Yang Q, Wu X, et al. Functional dominantnegative mutation of sodium channel subunit gene SCN3B associated with atrial fibrillation in a Chinese GeneID population. *Biochem Biophys Res Commun* 2010;398:98–104. [PubMed: 20558140]
39. Ren X, Xu C, Zhan C, et al. Identification of NPPA variants associated with atrial fibrillation in a Chinese GeneID population. *Clin Chim Acta* 2010;411:481. [PubMed: 20064500]
40. Chen S, Wang X, Wang J, et al. Genomic variant in CAV1 increases susceptibility to coronary artery disease and myocardial infarction. *Atherosclerosis* 2016;246:148–156. [PubMed: 26775120]
41. Wang P, Xu C, Wang C, et al. Association of SNP Rs9943582 in APLNR with left ventricle systolic dysfunction in patients with coronary artery disease in a Chinese Han GeneID population. *PLoS ONE* 2015;10:e0125926. [PubMed: 25993436]
42. Bai Y, Nie S, Jiang G, et al. Regulation of CARD8 expression by ANRIL and association of CARD8 single nucleotide polymorphism rs2043211 (p.C10X) with ischemic stroke. *Stroke* 2014;45:383–388. [PubMed: 24385277]
43. Tu X, Nie S, Liao Y, et al. The IL-33-ST2L pathway is associated with coronary artery disease in a Chinese Han population. *Am J Hum Genet* 2013;93:652–660. [PubMed: 24075188]
44. Stroke—1989. Recommendations on stroke prevention, diagnosis, and therapy. Report of the WHO Task Force on Stroke and other Cerebrovascular Disorders. *Stroke* 1989;20:1407–1431. [PubMed: 2799873]
45. Shi L, Li C, Wang C, et al. Assessment of association of rs2200733 on chromosome 4q25 with atrial fibrillation and ischemic stroke in a Chinese Han population. *Hum Genet* 2009;126:843–849. [PubMed: 19707791]
46. Cheng X, Shi L, Nie S, et al. The same chromosome 9p21.3 locus is associated with type 2 diabetes and coronary artery disease in a Chinese Han population. *Diabetes* 2011;60:680–684. [PubMed: 21270277]
47. Li C, Wang F, Yang Y, et al. Significant association of SNP rs2106261 in the ZFHX3 gene with atrial fibrillation in a Chinese Han GeneID population. *Hum Genet* 2011;129:239–246. [PubMed: 21107608]
48. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;55:611–622. [PubMed: 19246619]
49. Archacki SR, Angheloiu G, Moravec CS, et al. Comparative gene expression analysis between coronary arteries and internal mammary arteries identifies a role for the TES gene in endothelial cell functions relevant to coronary artery disease. *Hum Mol Genet* 2012;21:1364–1373. [PubMed: 22156939]
50. Xu C, Yang Q, Xiong H, et al. Candidate pathway-based genome-wide association studies identify novel associations of genomic variants in the complement system associated with coronary artery disease. *Circ Cardiovasc Genet* 2014;7:887–894. [PubMed: 25249547]
51. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–575. [PubMed: 17701901]
52. Liu Y, Ni B, Lin Y, et al. The rs3807989 G/A polymorphism in CAV1 is associated with the risk of atrial fibrillation in Chinese Han populations. *Pacing Clin Electrophysiol* 2015;38:164–170. [PubMed: 25196315]
53. Dupont WD, Plummer WD Jr. Power and sample size calculations for studies involving linear regression. *Control Clin Trials* 1998;19:589–601. [PubMed: 9875838]
54. Biscetti F, Straface G, Giovannini S, et al. Association between TNFRSF11B gene polymorphisms and history of ischemic stroke in Italian diabetic patients. *Hum Genet* 2013;132:49–55. [PubMed: 22965192]
55. Jensen JK, Ueland T, Atar D, et al. Osteoprotegerin concentrations and prognosis in acute ischaemic stroke. *J Intern Med* 2010;267:410–417. [PubMed: 19895657]

Figure 1.

Assessment of the relationship between SNP rs3134069 and the expression level of TNFSF11B mRNA by real-time RT-PCR analysis. Total RNA samples were isolated from 89 blood human samples (lymphocytes), converted into cDNA, and used for real time RT-PCR analysis. Genomic DNA samples were isolated from the 89 study subjects and genotyped for SNP rs3134069 by the HRM analysis. A Student's t-test was used to compare the differences for the mean RQ values between different genotypes (AA and AC) of SNP rs3134069. Due to the low minor allele frequency, no homozygous CC genotype was found.

Table 1.

Clinical and demographical characteristic of study subjects

Item	China-Central population		China-Northern population		Combined population	
	Ischemic stroke (n=1629)	Control (n=1504)	Ischemic stroke (n=1206)	Control (n=720)	Ischemic stroke (n=2835)	Control (n=2224)
Male, n (%)	929 (57.03)	994 (66.09)	671 (55.64)	469 (65.14)	1600 (56.44)	1463 (65.78)
Age (y) *	65.88 ± 14.04	45.30 ± 10.09	60.50 ± 10.76	55.73 ± 8.93	63.67 ± 13.07	48.15 ± 10.83
Hypertension, n (%)	395 (24.25)	14 (.93)	366 (30.35)	152 (21.11)	761 (26.84)	166 (7.46)
Smoking, n (%)	327 (20.07)	23 (1.53)	203 (16.83)	64 (8.89)	530 (18.69)	87 (3.91)
T2D, n %	1181 (72.50)	3 (.20)	106 (8.79)	40 (5.56)	1287 (45.40)	43 (1.93)

Abbreviation: T2D, type 2 diabetes.

* Age at the first diagnosis of the disease for CAD cases and age at enrollment for CAD controls.

Table 2.

Allelic association of SNP rs3134069 with stroke in the Chinese Han population *

Study population	Sample size		Without adjustment				Adjustment	
	Case/Control	P_{hwe}	RA	Frequency	P_{obs}	OR (95%CI)	P_{adj}	OR (95%CI)
China-Central	1629/1504	.76	C	.11/.09	4.26×10^{-3}	1.27 (1.08–1.50)	9.24×10^{-3}	1.50 (1.11–2.05)
China-Northern	1206/720	1.00	C	.13/.10	5.60×10^{-3}	1.35 (1.09–1.67)	2.45×10^{-4}	1.53 (1.22–1.92)
Combined	2835/2224	.70	C	.12/.09	3.51×10^{-5}	1.31 (1.15–1.50)	7.09×10^{-6}	1.41 (1.21–1.64)
Female	1170/750	.68	C	.11/.08	.002	1.44 (1.15–1.81)	.001	1.43 (1.20–1.65)
Male	1665/1474	.75	C	.12/.10	.002	1.28 (1.09–1.50)	.003	1.30 (1.10–1.52)

Abbreviations: CI, confidential interval; OR, odds ratio; P_{adj} , P value for association after adjusting for covariates of sex, age, and hypertension by multiple logistic regression analysis using SPSS v17.0; P_{HWE} , P value for Hardy–Weinberg equilibrium (HWE) tests using PLINK version 1.07; P_{obs} , P value for association before adjusting for covariates by 2×2 contingency tables using PLINK version 1.07.

*. Frequency, frequency of the risk allele (RA) in the case group and control group.

Table 3.
Allelic association of SNP rs3134069 with stroke in the population without type 2 diabetes

Study population	Sample size		Without adjustment				Adjustment	
	Case/Control	P_{hwe}	RA	Frequency*	P_{obs}	OR (95%CI)	P_{adj}	OR (95%CI)
China-Central	448/1501	.88	C	.12/.09	.03	1.31 (1.03–1.66)	.04	1.32 (1.03–1.67)
China-Northern	1100/680	.83	C	.13/.10	.03	1.28 (1.03–1.58)	.04	1.28 (1.03–1.56)
Combined	1548/2181	.86	C	.12/.09	6.76×10^{-5}	1.31 (1.16–1.56)	1.17×10^{-4}	1.30(1.16–1.55)

Abbreviations: CI, confidential interval; OR, odds ratio; P_{hwe} , P value for Hardy-Weinberg equilibrium (HWE) tests using PLINK version 1.07; P_{adj} , P value for association after adjusting for covariates of sex, age, and hypertension by multiple logistic regression analysis using SPSS v17.0; P_{obs} , P value for association before adjusting for covariates by 2×2 contingency tables using PLINK version 1.07.

*Frequency, frequency of the risk allele (RA) in the case group and control group.

Table 4.

Genotypic association of SNP rs3134069 with ischemic stroke under 3 different genetic models

Study panels	Model*	Genotypes (CC/CA/AA)			Without adjustment		Adjustment	
		Cases	Controls	P_{obs}	OR (95%CI)	P_{adj}	OR (95%CI)	
China-Central	Additive	25/317/1287	11/251/1242	1.14×10^{-2}	–	1.00×10^{-2}	1.50 (1.10–2.04)	
	Dominant	342/1287	262/1242	1.13×10^{-2}	1.26 (1.05–1.51)	1.20×10^{-2}	1.54 (1.10–2.16)	
	Recessive	25/1604	11/1493	3.51×10^{-2}	2.12 (1.04–4.31)	2.79×10^{-1}	1.97 (.58–6.71)	
China-North	Additive	15/272/919	6/126/588	1.84×10^{-2}	–	1.54×10^{-4}	1.57 (1.24–1.99)	
	Dominant	287/919	132/588	4.92×10^{-3}	1.39 (1.11–1.75)	2.04×10^{-4}	1.61 (1.25–2.07)	
	Recessive	15/1191	6/714	4.01×10^{-1}	1.50 (.58–3.88)	1.47×10^{-1}	2.11 (.77–5.80)	
Combined	Additive	40/589/2206	17/377/1830	1.76×10^{-4}	–	5.91×10^{-6}	1.42 (1.22–1.66)	
	Dominant	629/2206	394/1830	8.50×10^{-5}	1.32 (1.15–1.52)	2.29×10^{-5}	1.43 (1.21–1.68)	
	Recessive	40/2795	17/2207	3.10×10^{-2}	1.86 (1.05–3.29)	1.00×10^{-2}	2.38 (1.23–4.61)	

Abbreviations: OR, odds ratio; CI, confidential interval; P_{adj} , P value for association after adjusting for covariates of sex, age, hypertension, and T2D by multiple logistic regression analysis using SPSS v17.0; P_{obs} , P value for association before adjusting for covariates by 2×2 contingency tables using PLINK version 1.07.

* Additive model = CC/CA/AA; dominant model = CC+CA/AA; recessive model = CC/CA+AA.

Genotypic association of SNP rs3134069 with ischemic stroke under 3 different genetic models in both females and males of the combined population

Table 5.

Study panels	Model*	Genotypes (CC/CA/AA)		Without adjustment		Adjustment	
		Cases	Controls	<i>P_{obs}</i>	OR (95%CI)	<i>P_{adj}</i>	OR (95%CI)
Female	Additive	18/220/932	5/108/637	.008	–	.002	1.40 (1.20–1.65)
	Dominant	238/932	113/637	.004	1.44 (1.23–1.84)	.002	1.45 (1.25–1.77)
	Recessive	18/1152	5/745	.13	2.33 (.86–6.29)	.22	2.40 (.80–6.33)
Male	Additive	22/369/1274	12/269/1193	.008	–	.003	1.44 (1.23–1.74)
	Dominant	391/1274	281/1193	.003	1.30 (1.10–1.55)	.004	1.34 (1.10–1.65)
	Recessive	22/1463	12/1462	.22	1.63 (.85–3.31)	.18	1.88 (.88–3.51)

Abbreviations: CI, confidential interval; OR, odds ratio; *P_{adj}*, *P* value for association after adjusting for covariates of sex, age, hypertension and T2D by multiple logistic regression analysis using SPSS v17.0; *P_{obs}*, *P* value for association before adjusting for covariates by 2×2 contingency tables using PLINK version 1.07.

* Additive model = CC/CA/AA; dominant model = CC+CA/AA; recessive model = CC/CA+AA.