Regulation of CARD8 Expression by ANRIL and Association of CARD8 Single Nucleotide Polymorphism rs2043211 (p.C10X) With Ischemic Stroke

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Background and Purpose—ANRIL has long been considered as the strongest candidate gene at the 9p21 locus, robustly associated with stroke and coronary artery disease. However, the underlying molecular mechanism remains unknown. The present study works to elucidate such a mechanism.

Methods—Using expression quantitative loci analysis, we identified potential genes whose expression may be influenced by genetic variation in ANRIL. To verify the identified gene(s), knockdown and overexpression of ANRIL were evaluated in human umbilical vein endothelial cells and HepG2 cells. Ischemic stroke and coronary artery disease risk were then evaluated in the gene(s) demonstrated to be mediated by ANRIL in 3 populations of Chinese Han ancestry: 2 ischemic stroke populations consisting of 903 cases and 873 controls and the Northern China cohort (816 cases and 879 controls) and 1 coronary artery disease cohort consisting of 772 patients and 873 controls.

Results—Expression quantitative loci analysis identified CARD8 among others, with knockdown of ANRIL expression decreasing CARD8 expression and overexpression of ANRIL increasing CARD8 expression. The minor T allele of a previously identified CARD8 variant (rs2043211) was found to be significantly associated with a protective effect of ischemic stroke under the recessive model in 2 independent stroke cohorts. No significant association was found between rs2043211 and coronary artery disease.

Conclusions—CARD8 is a downstream target gene regulated by ANRIL. Single nucleotide polymorphism rs2043211 in CARD8 is significantly associated with ischemic stroke. ANRIL may increase the risk of ischemic stroke through regulation of the CARD8 pathway. (Stroke. 2014;45:00-00.)

Key Words: CARD8 gene | case–control studies | coronary artery disease

Atherosclerosis is one of the most common causes of both ischemic stroke and coronary artery diseases (CADs). Stroke is a leading cause of morbidity and mortality in China and other parts of the world. Ischemic stroke accounts for ~87% of all strokes and is caused by both genetic and environmental factors. To date, large-scale genome-wide association studies have identified several risk loci for ischemic stroke, including HDAC9, PITX2, ZFHX3, 9p21, PRKCH, and NINJ2. However, most loci have small effects and may explain a small proportion of the
heritability of ischemic stroke. Nearly 50 risk loci for CAD have been identified by genome-wide association study but explain <10% of the heritability of CAD only. Therefore, more genetic factors for ischemic stroke and CAD remain to be discovered.

Single nucleotide polymorphisms (SNPs) at the chromosome 9p21 locus were found to be associated with both ischemic stroke and CAD by genome-wide association study. The 9p21 locus contains an annotated noncoding RNA, termed ANRIL (antisense noncoding RNA in the INK4a locus). ANRIL is considered a prime candidate gene for atherosclerosis at the 9p21 locus. First, SNPs associated with ischemic stroke and CAD by genome-wide association study. The direction of the effects is still in dispute. Moreover, the expression with atherosclerosis severity, even though the 9p21 SNP genotypes and showed a correlation of several studies investigated the association of types and tissues that are involved in atherosclerosis. Third, risk alleles of rs10811656 and rs10757278 disrupted a binding site for transcriptional factor STAT1, and STAT1 in turn regulated ANRIL expression. The STAT1 signaling pathway mediates responses to inflammation on stimulation of the proinflammatory cytokine interferon γ. These results supported the notion that ANRIL might play a role in the inflammatory response and atherosclerosis. The molecular mechanism by which ANRIL mediates atherosclerosis is unknown. However, as a long noncoding RNA, ANRIL may play its role in atherosclerotic processes by influencing the expression of other genes.

In this study, we identified CARD8 as a downstream gene of ANRIL and assessed the association between CARD8 SNP rs2043211 and ischemic stroke or CAD in Chinese Han populations. 

Materials and Methods

Analysis of Expression Quantitative Loci for ANRIL SNPs

To identify potential downstream genes regulated by ANRIL, we analyzed ischemic stroke- and CAD-associated SNPs rs10116277, rs7865618, rs564398, rs496892, and rs7044859. These SNPs were shown to influence the mRNA level of ANRIL.7 We performed expression quantitative loci analysis for these SNPs by searching the database at University of Michigan Center for Statistical Genetics (http://www.sph.umich.edu/csg/liang/imputation/). These studies identified several genes whose expression may be associated with SNPs evaluated in our study. We chose to evaluate CARD8 for the other identified genes because of its increased expression in atherosclerotic lesions.

Cell Transfection and Quantitative Real-Time Polymerase Chain Reaction Analysis

Details of cell transfection and quantitative real-time polymerase chain reaction are described in the Materials in the online-only Data Supplement. The sequence of ANRIL siRNA was as follows: 5′-GGAAATGAGAGGACACGTGA-3′. Plasmid pcDNA3.1-ANRIL (NR_003529.3) was synthesized by GENEWIZ (Beijing, China). The sequences of primers used for quantitative real-time polymerase chain reaction are listed in Table I in the online-only Data Supplement.

Study Subjects

All study participants were selected from the GeneID database. Diagnostic criteria for ischemic stroke, CAD, and related factors are described in detail in the Materials in the online-only Data Supplement. This study followed the principles outlined in the Declaration of Helsinki and has been approved by local institutional review boards on human subject research. Written informed consent was obtained from all participants.

Genotyping and Statistical Analysis

Details of isolation of genomic DNA, SNP genotyping, and statistical analysis are described in the Materials in the online-only Data Supplement.

Results

ANRIL Regulates Expression of CARD8

Five 9p21 SNPs rs10116277, rs7865618, rs564398, rs496892, and rs7044859 are located within ANRIL and affect the expression level of ANRIL mRNA.7 By searching a public expression quantitative loci database (http://www.sph.umich.edu/csg/liang/imputation/), we identified 87 genes whose expression may be associated with 1 of the 5 9p21 SNPs (Table II in the online-only Data Supplement). One of the 87 genes, CARD8, became a strong candidate gene downstream of ANRIL because it also showed differential expression in a preliminary microarray analysis comparing HepG2 cells treated with ANRIL siRNA to those transfected with control siRNA (data not shown).

To verify that CARD8 is a downstream gene regulated by ANRIL, HepG2 cells were transfected with ANRIL-specific siRNA to knockdown the ANRIL expression (NC siRNA as negative control) and used for quantitative real-time polymerase chain reaction analysis. Compared with NC siRNA, ANRIL siRNA successfully reduced its own expression by >83% (P<2.0×10−4) and the expression of CARD8 by ≈55% (P=2.4×10−4; Figure [A]). Similarly, human umbilical vein endothelial cells transfected with ANRIL-specific siRNA showed significant reduction of ANRIL by 70% (P<1.5×10−7) and CARD8 by 48% (P=6.7×10−6) when compared with cells with NC siRNA (Figure [B]). These data suggest that ANRIL regulates the expression of CARD8. Consistent with the siRNA studies, HepG2 cells transfected with pcDNA3.1-ANRIL for 48 hours showed a 57-fold increase in ANRIL mRNA expression (P=5.84×10−5) and 1.6-fold increase in CARD8 mRNA expression (P<3.1×10−3; Figure [A]). Because of a difficulty in transfection of the specific line of human umbilical vein endothelial cells under this study with plasmid DNA, we did not obtain any data on the effect of ANRIL overexpression on CARD8 in human umbilical vein endothelial cells.

Characteristics of Study Subjects

Two independent cohorts were used to assess whether CARD8 SNP rs2043211 is associated with ischemic stroke. The discovery cohort for the ischemic stroke study consisted of 903 cases and 873 controls enrolled from Hubei Province in Central China. The replication cohort for the ischemic stroke study consisted of 816 cases and 879 controls enrolled from hospitals in Northern China (Table 1). The case–control cohort for the CAD study consisted of 772 patients with CAD and 873 controls from Hubei Province in Central China.
Patients with ischemic stroke or CAD had a higher prevalence of conventional risk factors, including smoking, history of hypertension, diabetes mellitus, and a lower level of high-density lipoprotein cholesterol (Table 1).

Statistical power analysis was performed for all 3 cohorts before each study. Each cohort had >90% of power to detect an association between rs2043211 and ischemic stroke or CAD with an odds ratio (OR) of ≥1.20 at the nominal type I error rate of >0.05 and a minor allelic frequency of >0.43 for rs2043211 in the Chinese population (HapMap HCB data).10

### Significant Genotypic Association Between SNP rs2043211 and Ischemic Stroke in 2 Independent Chinese Populations

The genotyping data for rs2043211 did not deviate from the Hardy–Weinberg equilibrium in the control group (P>0.05).

In the discovery population for the ischemic stroke study, no significant allelic association was detected between rs2043211 and ischemic stroke (P-obs=0.077, P-adj=0.092; Table III in the online-only Data Supplement). Similarly, no significant association between rs2043211 and ischemic stroke was detected in the replication cohort or the combined discovery/replication population (P>0.05; Table III in the online-only Data Supplement).

Genotypic association analysis was then conducted because this type of study can provide genetic insights into the association under different inheritance models (additive, dominant, or recessive). Interestingly, the minor allele T of SNP rs2043211 showed significant association with a protective effect of ischemic stroke under either a recessive model (P-obs=3.0×10−4) or an additive mode (P-obs=2.88×10−4; Table 2). After multivariate logistic regression analysis by adjusting for covariates...
of the age, sex, body mass index, smoking history, hypertension, diabetes mellitus, and lipid concentrations, the genotypic association between rs2043211 and ischemic stroke remained significant only under the recessive model (P-adj=0.028; OR, 0.68; Table 2).

To confirm the initial finding of genotypic association between rs2043211 and ischemic stroke in the discovery population, we validated the finding in an independent replication cohort. The results showed that rs2043211 was also significantly associated with a protective effect of ischemic stroke under either a recessive model (P-obs=9.00×10⁻³; Table 4) or an additive mode (P-obs=4.26x10⁻⁵; Table 2). The genotypic association between rs2043211 and ischemic stroke remained significant only under the recessive model in the replication population after multivariate logistic regression analysis (P-adj=0.017; OR, 0.70; Table 2). For the combined population of the discovery and replication cohorts, the P value for the genotypic association between rs2043211 and ischemic stroke under the recessive model became much more significant (P-obs=9.78×10⁻⁶; P-adj=5.83x10⁻⁴; OR, 0.70; Table 2; Figure I in the online-only Data Supplement). These data suggest that SNP rs2043211 confers a protective effect of ischemic stroke under a recessive model of inheritance.

The genotypic association between rs2043211 and ischemic stroke under the recessive model was more significant in the female group (P-adj=0.007; OR, 0.65) than in the male group (P-adj=0.024; OR, 0.73; Table 2; Figure I in the online-only Data Supplement). The genotypic association was significant in the early onset (<60 years) ischemic stroke group under the recessive model (P-adj=0.001; OR, 0.61; Table 2; Figure I in the online-only Data Supplement). The genotypic association was stronger under the recessive model for the female early onset ischemic stroke group (P-adj=0.004; OR, 0.51; Table 2; Figure I in the online-only Data Supplement).

### Lack of Significant Association Between SNP rs2043211 and CAD

We also analyzed SNP rs2043211 for its association with CAD. In a case–control study with 772 CAD cases and 873 controls, SNP rs2043211 did not show any significant association with CAD in the standard allelic association analysis (P-obs=0.235; P-adj=0.300) or in the genotypic association analysis under 3 different genetic models (all P>0.05). The association remained nonsignificant in either male or female CAD groups (Table IV in the online-only Data Supplement).

### Discussion

In the present study, we identified CARD8 as a downstream gene regulated by ANRIL and demonstrated an association between the CARD8 SNP rs2043211 and ischemic stroke in 2 independent Chinese Han populations. CARD8 encodes a member of the caspase recruitment domain (CARD)–containing family and is also known as TUCAN/CARDINAL. Previous population-based studies found that the functional SNP rs2043211 (p.C10X) located in exon 5 of CARD8 may be a genetic risk factor for chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. Our finding that rs2043211 is associated with ischemic stroke could indicate a shared inflammatory response or pathway similar to inflammatory bowel disease and rheumatoid arthritis. SNP rs2043211 has been previously associated with other diseases. Roberts et al. observed a significant genotypic association in New Zealand between rs2043211 and abdominal aortic aneurysm (P=0.047; OR, 0.83), which is a disease.

Table 2. Genotypic Association Analysis Between rs2043211 and Ischemic Stroke

<table>
<thead>
<tr>
<th>Cohorts</th>
<th>Model</th>
<th>Case (n)</th>
<th>Control (n)</th>
<th>P-obs</th>
<th>P-adj</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneID-Central IS (903/873)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>173/521/209</td>
<td>230/430/213</td>
<td>2.88E-04</td>
<td>0.082</td>
<td>0.83</td>
<td>(0.67–1.02)</td>
</tr>
<tr>
<td>Dominant</td>
<td>694/209</td>
<td>660/213</td>
<td>0.535</td>
<td>0.520</td>
<td>1.12</td>
<td>(0.80–1.56)</td>
</tr>
<tr>
<td>Recessive</td>
<td>173/730</td>
<td>230/643</td>
<td>3.00E-04</td>
<td>0.028</td>
<td>0.68</td>
<td>(0.48–0.96)</td>
</tr>
<tr>
<td>GeneID-North IS (816/879)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive</td>
<td>174/464/178</td>
<td>235/404/240</td>
<td>4.26E-05</td>
<td>0.692</td>
<td>0.97</td>
<td>(0.81–1.15)</td>
</tr>
<tr>
<td>Dominant</td>
<td>638/178</td>
<td>639/240</td>
<td>0.009</td>
<td>0.085</td>
<td>1.28</td>
<td>(0.97–1.69)</td>
</tr>
<tr>
<td>Recessive</td>
<td>174/642</td>
<td>235/644</td>
<td>0.009</td>
<td>0.017</td>
<td>0.70</td>
<td>(0.53–0.94)</td>
</tr>
<tr>
<td>Combined IS (1719/1752)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire cohort</td>
<td>Additive</td>
<td>347/985/387</td>
<td>465/834/453</td>
<td>3.13E-08</td>
<td>0.324</td>
<td>0.94 (0.83–1.06)</td>
</tr>
<tr>
<td>Entire cohort</td>
<td>Dominant</td>
<td>1332/387</td>
<td>1299/453</td>
<td>0.021</td>
<td>0.075</td>
<td>1.19 (0.98–1.45)</td>
</tr>
<tr>
<td>Entire cohort</td>
<td>Recessive</td>
<td>347/1372</td>
<td>465/1287</td>
<td>9.78E-06</td>
<td>5.83E-04</td>
<td>0.70 (0.57–0.86)</td>
</tr>
<tr>
<td>Women</td>
<td>Recessive</td>
<td>127/561</td>
<td>203/563</td>
<td>2.57E-04</td>
<td>0.007</td>
<td>0.65 (0.48–0.89)</td>
</tr>
<tr>
<td>Men</td>
<td>Recessive</td>
<td>220/811</td>
<td>262/724</td>
<td>0.006</td>
<td>0.024</td>
<td>0.73 (0.56–0.96)</td>
</tr>
<tr>
<td>Age &lt;60 y</td>
<td>Recessive</td>
<td>104/442</td>
<td>360/965</td>
<td>2.17E-04</td>
<td>0.001</td>
<td>0.61 (0.45–0.83)</td>
</tr>
<tr>
<td>Age &gt;60 y</td>
<td>Recessive</td>
<td>243/930</td>
<td>105/322</td>
<td>0.100</td>
<td>0.107</td>
<td>0.79 (0.59–1.05)</td>
</tr>
<tr>
<td>Women and age &lt;60 y</td>
<td>Recessive</td>
<td>43/206</td>
<td>155/421</td>
<td>0.003</td>
<td>0.004</td>
<td>0.51 (0.32–0.80)</td>
</tr>
<tr>
<td>Men and age &lt;60 y</td>
<td>Recessive</td>
<td>61/236</td>
<td>205/544</td>
<td>0.022</td>
<td>0.104</td>
<td>0.71 (0.48–1.08)</td>
</tr>
</tbody>
</table>

Model: additive (TT/AT/AA); dominant (TT/AT/AA); recessive (TT/AT/AA). P-obs, P value using 2×2 contingency table χ² tests before adjustment for covariates. P-adj, P-value adjusted by multivariate logistic regression analysis for traditional risk factors, including age, sex, body mass index, smoking history, hypertension, diabetes mellitus, total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. CI indicates confidence interval; IS, ischemic stroke; and OR, odds ratio.
that shared some similar pathological characteristics and risk factors with atherosclerosis, such as inflammation and angiogenesis. However, Paramel et al10 found that there was no significant association between rs2043211 and myocardial infarction (FIA [First Myocardial Infarction in Northern Sweden] cohort: P=0.10; OR, 1.1 and SCARF [Stockholm Coronary Atherosclerosis Risk Factor]: P=0.66; OR, 1.0). García-Bermúdez et al13 found that there was no evidence for the role of rs2043211 in the development of cardiovascular events in Spanish patients with rheumatoid arthritis (P=0.67; OR, 0.96). Meanwhile, there were still some conflicting results in association studies between rs2043211 and some chronic inflammatory diseases. Roberts et al14 found that the minor allele T of rs2043211 conferred a potential protective effect against early disease onset of Crohn disease in New Zealand whites. But other studies reported that the minor allele T of rs2043211 was associated with increased severity of inflammatory bowel disease15 (P=0.001; OR, 1.50) and rheumatoid arthritis,16 as well as increased risk of Alzheimer disease17 in women (P=0.01; OR, 2.39). Intriguingly, SNP rs2043211 was associated with ischemic stroke but not with CAD in this study. These results are consistent with the findings for myocardial infarction and cardiovascular events by Paramel et al10 and García-Bermúdez et al.13 Although the underlying molecular mechanism for the positive association with ischemic stroke and negative association with CAD by rs2043211 is not known, it is possible that the role of ANRIL-regulated CARD8 pathway may be limited to cerebral infarction but not to CAD.

CARD8 acts as an adaptor molecule that negatively regulates nuclear factor κB activation, caspase 1-dependent interleukin-1β secretion, and apoptosis and reduces the inflammatory response.18 SNP rs2043211 results in an A to T transition that changes codon 10 into a stop codon in CARD8 mRNA (Cys10Stop). Previous studies showed that homozygotes for the stop codon allele T can reduce the expression of CARD8 mRNA and a functional SNP in CARD8 was significantly associated with ischemic stroke; thus, it may be interesting to investigate whether CARD8 variant is associated with a specific stroke subtype in the future. Several schemes were reported to classify stroke subtypes, including the Trial of ORG 10172 in Acute Stroke Treatment classification system, the Causative Classification System, or the phenotypic System A-S-C-O (A for atherosclerosis, S for small vessel disease, C for cardiac source, O for other cause).22 In the present study, many patients with specific subtype were excluded, thus we were unable to perform association studies with specific stroke subtypes. In the future, we can collect more clinical information, including the data from the carotid study, classify the cases into different subtypes, and assess the association between CARD8 SNP rs2043211 and individual stroke subtype.

Conclusions
We show that ANRIL can regulate the expression level of CARD8 mRNA and a functional SNP in CARD8, rs2043211 located in exon 5 of CARD8 was significantly associated with ischemic stroke but not with CAD in the Chinese Han population. Although the detailed mechanism of CARD8 in the pathogenesis of ischemic stroke remains unclear, this study indeed links the ANRIL-regulated CARD8 pathway to the development of ischemic stroke.

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Disclosures
None.

References
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Regulation of \textit{CARD8} Expression by ANRIL and Association of \textit{CARD8} SNP rs2043211 (p.C10X) with Ischemic Stroke

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Supplemental Materials and Methods

Cell Transfection and Quantitative Real-Time PCR (qRT-PCR) Analysis
HepG2 cells were purchased from ATCC (American Type Culture Collection, USA) and maintained in the Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Pricells (Wuhan, China) and maintained in the human endothelial basal growth medium supplemented with 10% FBS, 10 ng/ml of EGF and 1 µg/ml of hydrocortisone. Cells were cultured at 37°C in a humidified incubator with 5% CO2.

Specific small interfering RNA (siRNA) for human ANRIL (ANRIL siRNA) and negative control siRNA (NC siRNA) were purchased from Ribobio (Guangzhou, China). The sequence of ANRIL siRNA was as follows: 5’-GGAATGAGGAGCACAGTGA-3’. HepG2 cells and HUVECs were transfected with siRNA and incubated for 48 hours. Transfection of siRNA was performed using Lipofectamine 2000 with a final concentration of 100 nM according to the manufacturer’s protocol.

Plasmid pcDNA3.1-ANRIL (NR_003529.3) was purchased from GENEWIZ (Beijing, China). For transfection of pcDNA3.1-ANRIL into HepG2 cells, we used a DNA (µg) to Lipofectamine 2000 (µl) ratio of 1:2. Cells were incubated for 6 h at 37°C in the presence of the transfection mixture and grown in fresh media for 48 hrs. Treated and untreated cells were used for preparation of RNA samples for qRT-PCR analysis.

Cells were harvested 48 hrs after transfection and total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. Total RNA was converted into cDNA by reverse transcription using the First-Strand cDNA Synthesis kit and random primers (Promega, USA). The qRT-PCR analysis was performed with a FastStart Universal SYBR Green Master kit (Roche Applied Science, Germany) and analyzed on an ABI 7900-HT Genetic Analyzer. The PCR profile included 94°C for 5 min and 40 amplification cycles of 94°C for 20 sec and 60°C for 15 sec. Melting curve analysis was performed at the end of each PCR reaction to verify the specificity of PCR products. β-actin (ACTB) was used as internal control. The sequences of primers used are listed in Supplement Table I. Results were representative of three independent experiments and each PCR reaction was run in triplicate. Data analysis was performed using the 2-△△Ct method as described.1

Study Subjects
All participants in our study were selected from the GeneID database.2 We performed case-control association studies for ischemic stroke in two independent cohorts involving a total of 1,719 ischemic stroke cases and 1,752 controls. The first discovery population of 903 cases and 873 controls was enrolled from Hubei Province in Central China, whereas the second replication population of 816 cases and 879 controls was enrolled from hospitals in Northern China.

Diagnosis of ischemic stroke was made by following the World Health Organization criteria.3 We classified subjects in the patient case group based on a medical history of ischemic stroke or a stroke diagnosis by magnetic resonance imaging (MRI)/computed tomography (CT). Subjects were excluded if they had a known single-gene stroke disorder, central nervous system vasculitis, intracerebral hemorrhage, subarachnoid hemorrhage, brain tumors, embolic brain infarction,
transient ischemic attack, cardioembolic stroke and a relevant brain stem or subcortical hemispheric lesion with a diameter of <1.5 cm.

The study subjects for an association study for CAD included 772 cases and 873 controls (the same controls used for ischemic stroke studies). CAD was diagnosed based on the American College of Cardiology/American Heart Association criteria. Subjects were classified as CAD cases if more than 70% of luminal stenosis was detected by coronary angiography in at least one main branch of the coronary artery, a procedure of either percutaneous coronary intervention or coronary artery bypass graft was performed, and/or myocardial infarction (MI) was diagnosed. Subjects were excluded if they had a myocardial bridge, congenital heart disease, or childhood hypertension.

Individuals without ischemic stroke and CAD were used as control subjects in the present study.

Other clinical information including the age, sex, smoking history, hypertension, diabetes mellitus, and lipid concentrations were collected from the patients’ medical records. Hypertension was defined as a systolic blood pressure of higher than 140 mmHg or a diastolic blood pressure of higher than 90 mmHg. Diabetes was diagnosed on ongoing therapy of diabetes or a fasting plasma glucose level of higher than 7.0 mmol/L. Fasting concentrations of the total cholesterol (Tch), triglyceride (TG), LDL cholesterol (LDL-c), and HDL cholesterol (HDL-c) were measured according to standard methods.

This study followed the principals outlined in the Declaration of Helsinki and has been approved by the local institutional review boards on human subject research. Written informed consents were provided from all participants.

**Genotyping and Statistical Analysis**

Genomic DNA was isolated from peripheral blood leukocytes with Wizard Genomic DNA Purification Kit (Promega). Genotyping of rs2043211 was carried out using TaqMan SNP Genotyping Assay (c_11708080_1) and analyzed with the ABI 7900-HT Genetic Analyzer according to the manufacturer’s instructions (Applied Biosystems). To verify the results by TaqMan assays, we randomly selected 96 DNA samples and genotyped rs2043211 by direct DNA sequencing analysis. The results showed that the accuracy rate of TaqMan assays was 100% in the study. All cluster plots were manually inspected, and ambiguous results were excluded.

The data are reported as mean ± SEM for qRT-PCR analysis and analyzed using a Student t test (SPSS 17.0). The means were considered significantly different when \( P \) was <0.05. A statistical power analysis was carried out using PS software version 3.0.2 for a case-control study. \(^{4}\) Hardy-Weinberg disequilibrium tests were carried out in the control group using PLINK version 1.05. \(^{5}\) Genotyping data were analyzed for allelic or genotypic association using Pearson’s 2 × 2 and 2 × 3 contingency table Chi-squared tests, respectively (SPSS version 17.0). Odds ratios (OR), 95% confidence intervals (CIs) and \( P \) values were computed by SPSS, version 17.0. Multivariate logistic regression analysis was performed using SPSS version 17.0 by adjusting for traditional risk factors for ischemic stroke or CAD, including the age, sex, body mass index (BMI), smoking history, hypertension, diabetes mellitus and lipid concentrations.
Supplemental Figure I. Comparison of odds ratios (ORs). Horizontal axis shows OR after adjustment for covariates under a recessive model. Vertical axis indicates different groups of patients with ischemic stroke subdivided based on sex and age. Solid rhombus centered on the OR estimate and scaled in proportion to sample size with 95% CI (horizontal bar) are shown for each subgroup.
Supplemental Tables

Supplemental Table I. Primers used in real time PCR analysis for \textit{CARD8}, \textit{ANRIL} and \textit{\(\beta\)-actin}

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Bold and italic: genes with possible involvement in atherosclerosis;
*Reported phenotypic association.
**Supplemental Table III. Allelic association analysis between SNP rs2043211 and ischemic stroke.**

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<thead>
<tr>
<th>Cohort (n, case/control)</th>
<th>MAF Case/Control</th>
<th>without adjustment</th>
<th>with adjustment</th>
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<tr>
<td><strong>GeneID-Central IS(903/873)</strong></td>
<td>(0.48/0.51)</td>
<td>0.077</td>
<td>0.89(0.78-1.01)</td>
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<td><strong>GeneID-North IS(816/879)</strong></td>
<td>(0.50/0.50)</td>
<td>0.982</td>
<td>1.00(0.88-1.15)</td>
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<td><strong>Combined IS</strong></td>
<td><strong>Entire cohort</strong> (1719/1752)</td>
<td>(0.49/0.50)</td>
<td>0.210</td>
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MAF: Minor allele (T) frequency; OR, odds ratio; \( P-obs \), \( P \) value using 2 × 2 contingency table \( \chi^2 \) tests before adjustment for covariates; \( P-adj \), \( P \) value adjusted by multivariate logistic regression analysis for traditional risk factors, including age, sex, BMI, smoking history, hypertension, DM, TC, TG, HDL- and LDL-C.
## Supplement Table IV. Association analysis between SNP rs2043211 and CAD

<table>
<thead>
<tr>
<th>Model</th>
<th>Case (MAF or num)</th>
<th>Control (MAF or num)</th>
<th>P-obs</th>
<th>P-adj</th>
<th>OR(95%CI)</th>
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<tr>
<td>entire cohort</td>
<td>0.49(772)</td>
<td>0.51(873)</td>
<td>0.235</td>
<td>0.300</td>
<td>0.90(0.74-1.10)</td>
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<td>Male</td>
<td>0.49(538)</td>
<td>0.51(495)</td>
<td>0.430</td>
<td>0.721</td>
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<tr>
<td>Female</td>
<td>0.48(234)</td>
<td>0.51(378)</td>
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<td>0.372</td>
<td>0.85(0.60-1.22)</td>
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<td><strong>Additive association</strong></td>
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<td>entire cohort</td>
<td>174/407/191</td>
<td>230/430/213</td>
<td>0.182</td>
<td>0.296</td>
<td>0.90(0.90-1.11)</td>
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<tr>
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<td>0.718</td>
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<tr>
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<td>105/177/96</td>
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<td>0.359</td>
<td>0.84(0.58-1.22)</td>
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<td><strong>Dominant association</strong></td>
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<td>entire cohort</td>
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Model: Additive (TT/AT/AA), Dominant (TT+AT/AA), Recessive (TT/AT+AA); MAF: Minor allele (T) frequency; OR, odds ratio; P-obs, P value using 2 × 2 contingency table χ² tests before adjustment for covariates; P-adj, P value adjusted by multivariate logistic regression analysis for traditional risk factors, including age, sex, BMI, smoking history, hypertension, DM, TC, TG, HDL-C and LDL-C)
Supplemental References