ONLINE SUPPLEMENT MATERIAL

Regulation of *CARD8* Expression by *ANRIL* and Association of *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% CO_2.

Specific small interfering RNA (siRNA) for human *ANRIL* (*ANRIL* siRNA) and negative control siRNA (NC siRNA) were purchased from RioboBio (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℃ for 5 min and 40 amplification cycles of 94℃ for 20 sec and 60℃ 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 $2^{-\triangle\triangle Ct}$ 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 preformed, 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.

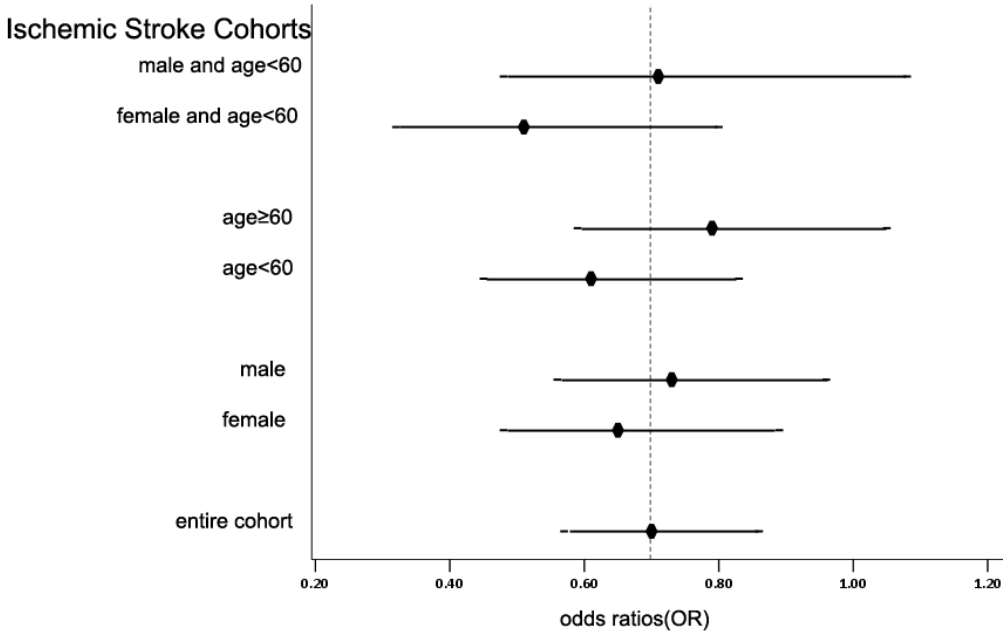
This study followed the principals outlined in the Declaration of Helsinki and has been approved by the local institutional review boards on human subject research. Written informed consents were provided from all participants.

Genotyping and Statistical Analysis

Genomic DNA was isolated from peripheral blood leucocytes 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



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 *CARD8*, *ANRIL* and *β-actin*

Primer	Sequence
CARD8-Forword	5' ctg aag gaa atg tgg atg ttg agt 3'
CARD8-reverse	5' cca cag ata cca gcc agc agt 3'
ANRIL-Forword	5' tgg aga cac aca gat gcc taa cg 3'
ANRIL-reverse	5' act cgg gaa gtg cta gca gca atg 3'
β-actin-Forword	5' gga ctt cga gca gga gat gg 3'
β-actin-reverse	5' gca ccg tgt tgg cgt aga gg 3'

Supplemental Table Ⅱ. Identification of potential eQTLs for 9p21 SNPs (rs10116277, rs7865618, rs564398, rs496892, rs7044859)

Gene	Probe	effect	SE	h2	LOD	pvalue
ABLIM1	200965_s_at	0.275	0.079	3.64	2.652	0.00047
AJAP1	215789_s_at	-0.273	0.078	3.55	2.623	0.00051
ALDOB	211357_s_at	0.284	0.079	4.04	2.772	0.00035
ANKRD50	225731_at	0.263	0.079	3.36	2.444	0.00079
ARL17	229028_s_at	0.283	0.078	3.81	2.873	0.00028
BCAT1	214452_at	0.291	0.077	4.36	3.101	0.00016
BTF3L4	238675_x_at	0.274	0.078	3.58	2.647	0.00048
C10orf88	222852_at	0.271	0.078	3.52	2.627	0.00051
C11orf32	212560_at	0.27	0.079	3.49	2.556	0.0006
C11orf58	200084_at	0.271	0.078	3.67	2.616	0.00052
C18orf30	1563584_at	0.276	0.078	3.83	2.689	0.00043
C1orf108	222459_at	0.265	0.079	3.35	2.458	0.00077
C6orf120	221787_at	0.264	0.079	3.31	2.413	0.00086
CARD8*	***1554479_a_at***	***0.263***	***0.078***	***3.45***	***2.456***	***0.00077***
CD44	***212063_at***	***0.271***	***0.079***	***3.67***	***2.577***	***0.00057***
CHCHD3	217972_at	0.256	0.077	3.42	2.413	0.00086
CLCN3	201733_at	0.259	0.078	3.39	2.413	0.00086
CSF3	207442_at	-0.282	0.079	3.81	2.792	0.00034
CYP2S1	223385_at	0.266	0.078	3.56	2.497	0.0007
DDX58	***242961_x_at***	***0.272***	***0.079***	***3.52***	***2.591***	***0.00055***
DFNA5	203695_s_at	0.272	0.077	3.67	2.698	0.00042
DIP2B	224872_at	0.33	0.08	5.13	3.722	3.50E-05
DKFZP564O0823	225809_at	0.261	0.079	3.4	2.384	0.00092
DMKN	226926_at	0.286	0.079	3.94	2.87	0.00028
DYNC2H1	1565149_at	-0.268	0.078	3.44	2.587	0.00056
EIF2S3	224936_at	0.244	0.071	3.72	2.606	0.00053
FAM129C	230983_at	0.278	0.078	3.7	2.776	0.00035
FKBP5	224856_at	0.26	0.078	3.21	2.38	0.00093
FLJ35348	212547_at	0.263	0.078	3.33	2.482	0.00072
FOXN3	218031_s_at	0.263	0.079	3.28	2.434	0.00081
GLOD4	209092_s_at	0.267	0.08	3.35	2.432	0.00082
GLUL	***200648_s_at***	***0.26***	***0.078***	***3.42***	***2.436***	***0.00081***
GOLM1	217771_at	0.259	0.078	3.44	2.419	0.00084
HECW2	232080_at	0.282	0.078	4.07	2.862	0.00028
HLA-DQA1*	***212671_s_at***	***0.257***	***0.078***	***3.32***	***2.366***	***0.00096***
HOXC4	206194_at	-0.26	0.078	3.48	2.39	0.00091
IAH1	230621_at	0.257	0.078	3.32	2.367	0.00096
IGHV1-69	240915_at	-0.265	0.079	3.36	2.448	0.00079
INADL	223681_s_at	0.298	0.079	4.18	3.06	0.00017
JARID2	203298_s_at	0.261	0.079	3.22	2.377	0.00094
KIF1B	209234_at	0.269	0.079	3.45	2.521	0.00066
KLHL6	1555275_a_at	0.268	0.078	3.59	2.534	0.00064
LARGE	215543_s_at	0.289	0.079	4.16	2.914	0.00025

LAT2	221581_s_at	0.267	0.078	3.53	2.566	0.00059
LCP2	205270_s_at	0.26	0.077	3.47	2.453	0.00078
LIX1L	225793_at	0.276	0.079	3.65	2.665	0.00046
LOC153546	236124_at	-0.275	0.078	3.83	2.723	0.0004
LOC200830	1556898_at	-0.268	0.079	3.39	2.494	0.0007
LOC389831	225046_at	0.26	0.078	3.24	2.383	0.00092
NPM1	200063_s_at	0.292	0.078	4.35	3.05	0.00018
LRPPRC	211971_s_at	0.325	0.078	5.33	3.784	3.00E-05
MAPK8IP1	213013_at	-0.264	0.079	3.29	2.403	0.00088
MIAT	240607_at	-0.264	0.078	3.32	2.478	0.00073
MPEG1	226818_at	0.271	0.078	3.74	2.625	0.00051
MPZL3	227747_at	0.289	0.078	3.99	2.973	0.00022
NARG2	228960_at	0.331	0.079	5.19	3.836	2.60E-05
NCBP2	201517_at	0.279	0.078	3.91	2.751	0.00037
NEXN	1552309_a_at	-0.265	0.078	3.63	2.48	0.00073
NID1	202007_at	0.267	0.078	3.66	2.579	0.00057
NR0B2	206410_at	-0.285	0.079	3.83	2.832	0.0003
PAQR8	227626_at	0.29	0.078	4.2	2.988	0.00021
PCMTD1	235507_at	0.258	0.078	3.34	2.362	0.00097
PIGN	219048_at	0.274	0.077	3.63	2.717	0.0004
PIK3CB	***212688_at***	***0.269***	***0.079***	***3.44***	***2.517***	***0.00066***
PNMA6A	235758_at	-0.262	0.078	3.28	2.445	0.00079
PPP1CB	201409_s_at	0.268	0.079	3.6	2.536	0.00063
R3HDM2	203831_at	0.276	0.078	3.65	2.734	0.00039
RAB6IP1	212561_at	0.27	0.08	3.45	2.508	0.00068
RABEP1	225064_at	0.273	0.079	3.73	2.627	0.00051
RIPK3	228139_at	-0.262	0.077	3.31	2.492	0.0007
RPRM	219370_at	-0.264	0.079	3.3	2.438	0.00081
SCRN1	201462_at	0.259	0.078	3.19	2.387	0.00092
SIX1	205817_at	-0.262	0.079	3.25	2.385	0.00092
SLC16A9	227506_at	0.288	0.078	4.01	2.942	0.00023
SLC34A3	1569926_s_at	-0.286	0.079	3.87	2.85	0.00029
SLC7A11	217678_at	0.263	0.079	3.28	2.42	0.00084
SMU1	222618_at	0.267	0.078	3.56	2.511	0.00067
SPG20	236600_at	-0.267	0.078	3.6	2.515	0.00067
ST3GAL6	213355_at	0.272	0.08	3.49	2.533	0.00064
TGFB1I1	209651_at	0.256	0.078	3.21	2.352	0.001
TMEM185A	227880_s_at	0.266	0.079	3.37	2.451	0.00078
TMPRSS3	220177_s_at	0.27	0.077	3.82	2.689	0.00043
TNFRSF11A	238846_at	0.267	0.079	3.43	2.514	0.00067
TRIM6	223599_at	0.283	0.076	4.05	2.968	0.00022
UBE1L2	222602_at	0.284	0.077	4.09	2.912	0.00025
USH1C	211184_s_at	-0.268	0.079	3.41	2.508	0.00068
ZMYM5	206744_s_at	-0.263	0.079	3.48	2.434	0.00081

Bold and italic: genes with possible involvement in atherosclerosis;

*Reported phenotypic association.

Supplemental Table III. Allelic association analysis between SNP rs2043211 and ischemic stroke.

Cohort	MAF	without adjustment		with adjustment	
(n, case/control)	Case/Control	*P-obs*	*OR(95%CI)*	*P-adj*	OR(95%CI)
GeneID-Central IS(903/873)	(0.48/0.51)	0.077	0.89(0.78-1.01)	0.092	0.84(0.68-1.03)
GeneID-North IS(816/879)	(0.50/0.50)	0.982	1.00(0.88-1.15)	0.697	0.97(0.82-1.15)
Combined IS Entire cohort (1719/1752)	(0.49/0.50)	0.210	0.94(0.86-1.03)	0.337	0.94(0.84-1.06)

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

Model	Case (MAF or num)	Control (MAF or num)	*P-obs*	*P-adj*	OR(95%CI)
allelic association					
entire cohort	0.49(772)	0.51(873)	0.235	0.300	0.90(0.74-1.10)
Male	0.49(538)	0.51(495)	0.430	0.721	0.95(0.75-1.22)
Female	0.48(234)	0.51(378)	0.361	0.372	0.85(0.60-1.22)
Additive association					
entire cohort	174/407/191	230/430/213	0.182	0.296	0.90(0.90-1.11)
Male	124/280/134	125/253/117	0.693	0.718	0.95(0.75-1.22)
Female	50/127/57	105/177/96	0.134	0.359	0.84(0.58-1.22)
Dominant association					
entire cohort	581/191	660/213	0.872	0.408	0.87(0.63-1.20)
Male	404/134	378/117	0.634	0.386	0.83(0.56-1.25)
Female	177/57	282/96	0.773	0.668	1.14(0.63-2.04)
Recessive association					
entire cohort	174/598	230/643	0.073	0.381	0.86(0.62-1.20)
Male	124/414	125/370	0.408	0.782	1.05(0.71-1.59)
Female	50/184	105/273	0.076	0.053	0.55(0.30-1.01)

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 $\chi 2$ 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)

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