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Original Article

Genetic Variants of *RAMP2* and *CLR* are Associated with Stroke

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***Aim*: Stroke is associated closely with vascular homeostasis, and several complex processes and interacting pathways, which involve various genetic and environmental factors, contribute to the risk of stroke. Although adrenomedullin (ADM) has a number of physiological and vasoprotective functions, there are few studies of the ADM receptor system in humans. The ADM receptor comprises a calcitonin-receptor-like receptor (CLR) and receptor activity-modifying proteins (RAMPs). We analyzed single nucleotide polymorphisms (SNPs) in the *RAMP2* and *CLR* genes to determine their association with stroke in the light of gene-environment interactions.**

***Methods*: Using cross-sectional data from the Japan Multi-Institutional Collaborative Cohort Study in the baseline surveys, 14,087 participants from 12 research areas were genotyped. We conducted a hypothesis-based association between stroke prevalence and SNPs in the *RAMP2* and *CLR* genes based on data abstracted from two SNPs in *RAMP2* and 369 SNPs in *CLR*. We selected five SNPs from among the *CLR* variants (rs77035639, rs3815524, rs75380157, rs574603859, and rs147565266) and one *RAMP2* SNP (rs753152), which were associated with stroke, for analysis.**

***Results*: Five of the SNPs (rs77035639, rs3815524, rs75380157, rs147565266, and rs753152) showed no significant association with obesity, ischemic heart disease, hypertension, dyslipidemia, and diabetes. In the logistic regression analysis, rs574603859 had a lower odds ratio (0.238; 95% confidence interval, 0.076−0.745, adjusted for age, sex, and research area) and the other SNPs had higher odds ratios for association with stroke.**

***Conclusions*: This was the first study to investigate the relationships between ADM receptor genes (*RAMP2* and *CLR*) and stroke in the light of gene-environment interactions in human.**

Key words: Adrenomedullin, Receptor activity-modifying protein 2, Calcitonin-receptor-like receptor, Stroke

Introduction

The vascular system plays a crucial role in organ homeostasis, being essential for organ and tissue construction, the supply of oxygen and nutrients, and mobilization of inflammatory cells to regions of injury[1, 2]. Current and novel therapeutic approaches aimed at improving vascular function provide real benefits with respect to reducing cerebrovascular disease[3]. In addition, the vascular system can be considered the largest system in the body, given its length and area, and via its active secretion of bioactive molecules, plays a central role in vascular homeostasis[4-6]. Revealing the mechanisms underlying the functional integrity of the vascular system could lead to novel approaches to therapy and preventive medicine.

Strokes are associated closely with vascular homeostasis, and disruption of vascular function can also cause a stroke. A stroke is the clinical culmination of several complex processes and interacting pathways that involve various genetic and environmental factors[7]. Genetic contributions to strokes may result from common variants with small effect sizes, rare variants with large effect sizes, or their combination[8]. However, environmental risk factors are associated with the pathogenesis of stroke, and considerable evidence suggests that gene-environment interactions are important[9].

Adrenomedullin (ADM) is a vasoactive peptide first identified in human pheochromocytoma[10]. Although ADM is secreted by various organs and tissues, it is produced mainly by vascular endothelial cells and serves a number of physiological functions[11-14]. The ADM receptor is a seven transmembrane domain G protein-coupled receptor, called the calcitonin-receptor-like receptor (CLR)[15, 16]. The specificity of CLR for ADM is thought to be regulated by receptor activity-modifying proteins (RAMPs), which are membrane proteins having a single membrane-spanning domain. Analysis of genetically engineered knockout mice revealed that the ADM signal is indispensable for CLR and RAMP2 function[17-20]. By contrast, there have been few studies of the ADM receptor system in humans.

The present study aimed to analyze *RAMP2* and *CLR* single nucleotide polymorphisms (SNPs) and evaluate their association with stroke in the light of gene-environment interactions. This analysis was conducted as a cross-sectional study using a large-scale pooled analysis of the Japanese general population.

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Methods

Study Participants

In the present study, we evaluated participant data collected during the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study from the baseline surveys using the cross-sectional data. That cohort study evaluated the general Japanese population in 12 research areas, using genetic and clinical data to detect and confirm gene-environment interactions related to lifestyle-associated diseases[21]. The study participants were 35−69 years old, and were enrolled after responding to study announcements in their specific research areas, attending health check-up examinations that were commissioned by their local governments, visiting local health check-up centers, or visiting a cancer hospital. A total of 14,539 participants were selected. We analyzed the data while minimizing the number of deleted participants. Each parameter was separated in the analysis because of missing data.

The J-MICC study participants included citizens, health check examiners, and first-visit patients to a cancer hospital. All participants in this study gave written informed consent. The study protocol was approved by the Ethics Committees at Aichi Cancer Center, the Nagoya University Graduate School of Medicine, and other institutions participating in the J-MICC study. The present study was conducted according to the principles expressed in the World Medical Association Declaration of Helsinki.

Lifestyle and Blood Biochemistry Data

In the present study, we evaluated the lifestyle and medical information obtained through self-administered questionnaires (alcohol consumption status, smoking habits, and physical exercise). The body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2). Obesity was defined as a BMI ≥ 25.0 kg/m^2. Alcohol consumption of each type of beverage was determined by the average number of drinks per day, and then converted into the Japanese sake unit, 'gou' (180 ml), which is equivalent to 23 g of ethanol (0, 0.1−22.9, 23.0−45.9, or ≥46.0 g ethanol/day). Regular physical activity was defined as three times a week and lasting over 30 minutes. Anamnesis and medication history were also assessed using a questionnaire. Information on stroke (n=248) and ischemic heart disease (n=403) was available from the self-administered questionnaires. Hypertension was defined as a systolic/diastolic blood pressure ≥ 140/90 mm Hg and/or current use of medication for hypertension. Dyslipidemia was defined as non-high density lipoprotein-C

(HDL-C) ≥ 170 mg dl^{-1} and/or HDL-C < 40 mg dl^{-1} and/or current use of medication for dyslipidemia. Diabetes was defined as a glycated hemoglobin (HbA1c) level ≥ 6.5% and/or current use of medication for diabetes. The participants who have the absence of laboratory data and/or insufficient data were excluded in each analytic criterion.

In addition, blood chemistry data (serum levels of triglycerides, total cholesterol, HDL-C, non-HDL-C, creatinine, and HbA1c) and anthropometric data were obtained from health check-ups performed in the research areas. The estimated glomerular filtration rate (eGFR) was calculated using the following equation: eGFR (mL/min/1.73 m^2) = 194 × $creatinine^{-1.094}$ × $age^{-0.287}$ (for men) and eGFR (mL/min/1.73 m^2) = 194 × $creatinine^{-1.094}$ × $age^{-0.287}$ × 0.739 (for women)[22]. Each blood sample was centrifuged and the plasma was separated and stored at −80℃ until analysis. Laboratories in each research area analyzed the serum samples.

Genotyping and Quality Control Filtering

In the study, buffy coat fractions and DNA were prepared from blood samples and stored at −80℃ at the central J-MICC study office. DNA was extracted from all buffy coat fractions using a BioRobot M48 Workstation (Qiagen Group, Tokyo, Japan) at the central study office. For the samples from two areas (Fukuoka and Kyushu-KOPS), DNA was extracted locally from samples of whole blood, using an automatic nucleic acid isolation system (NA-3000, Kurabo, Co., Ltd, Osaka, Japan). The 14,539 study participants from the 12 areas of the J-MICC study were genotyped at RIKEN Center for Integrative Medicine using a HumanOmniExpressExome-8 v1.2 BeadChip array (Illumina Inc., San Diego, CA, USA). Twenty-six samples with inconsistent sex information between the questionnaire and the estimate from the genotyping results were excluded. The identity-by-descent method implemented in the PLINK 1.9 software[23, 24] identified 388 closely related pairs (pi-hat > 0.1875) and one sample of each pair was excluded. Principal component analysis (PCA)[25, 26] with the 1000 Genomes reference panel (phase 3)[27] detected 34 subjects whose estimated ancestries were outside of the Japanese population[28]. These 34 samples were excluded. All the remaining 14,091 samples met a sample-wise genotype call rate criterion (≥ 0.99). SNPs with a genotype call rate < 0.98 and/or a Hardy-Weinberg equilibrium exact test *P*-value < 1×10^{-6} were removed, resulting in 873,254 autosomal variants. Among these, 298,644 variants with a low minor allele frequency (MAF) < 0.01 were excluded. This quality control filtering resulted in 14,091 participants and 570,162 SNPs. After genotyping, data from four participants who withdrew from follow-up were excluded from further analysis, resulting in 14,087 participants were included in the analyses.

Genotype Imputation

Genotype imputation was performed using SHAPIT[29] and Minimac3[30] software based on the 1000 Genomes Project cosmopolitan reference panel (phase 3)[27]. After the genotype imputation, variants with an R^2 value < 0.3 were excluded, resulting in 12,617,547 variants. We identified variants in the *RAMP2* and *CLR* loci, which identified two and 369 SNPs, respectively.

Statistical Analyses

We compared the associations between various genotypes and stroke, after combining the heterozygous and minor homozygous alleles because of the small number of minor homozygotes. We selected SNPs from the *RAMP2* and *CLR* genes that exhibited a statistically significant association with stroke. Continuous variables are expressed as mean ± standard deviation (SD) and categorical data are expressed as sums and percentages. Inter-group comparisons were performed using Welch's *t*-tests for continuous variables, and the chi-square or Fisher's exact tests for categorical variables (sex, alcohol consumption, regular physical activity, smoking, stroke, obesity, ischemic heart disease, hypertension, dyslipidemia, and diabetes). The chi-square test was performed to examine the Hardy−Weinberg equilibrium for each locus studied. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using logistic regression analyses, in which stroke was defined as the dependent variable, and age, sex, research area, alcohol intake, current smoking, regular physical activity, obesity, hypertension, diabetes, dyslipidemia, and ischemic heart disease, were included as independent variables. The haplotype frequency and linkage disequilibrium of the SNPs were estimated using Haploview[31]. All statistical tests were two-tailed, and differences with a *p*-value < 0.05 were considered statistically significant. SPSS software (version 18.0, SPSS, Japan, Inc.) was used for all statistical analyses.

Results

Among these 14,087 participants, the mean age of the included 6337 men was 55.4 years, compared to 54.3 years for the 7750 women.

We identified two and 369 SNPs among the genetic variants of *RAMP2* and *CLR*, respectively (**Supplementary Table 1**, Chromosomal locations were described based on hg19/GRCh37 coordinates). **Supplementary Fig. 1** shows the linkage disequilibrium analyses of 13 *CLR* SNPs associated with stroke identified using the chi-square test. The position of the

Table 1. Genotype and allele distributions in stroke.

SNPs Chromosome: position		Genotype		*p* value
		Major Homo	Hetero + Minor Homo	
rs753152 (T/G) chr17: 40913505	control (n)	12139	881	**0.003**
	(%)	93.2%	6.8%	
	Stroke (n)	219	29	
	(%)	88.3%	11.7%	
rs77035639 (A/G) chr2: 188220301	control (n)	12579	441	**0.020**
	(%)	96.6%	3.4%	
	Stroke (n)	232	16	
	(%)	93.5%	6.5%	
rs3815524 (G/C) chr2: 188224322	control (n)	11519	1501	**0.041**
	(%)	88.5%	11.5%	
	Stroke (n)	209	39	
	(%)	84.3%	15.7%	
rs75380157 (A/T) chr2: 188271085	control (n)	12005	1015	**0.002**
	(%)	92.2%	7.8%	
	Stroke (n)	215	33	
	(%)	86.7%	13.3%	
rs574603859 (A/T) chr2: 188301544	control (n)	12375	645	**0.003**
	(%)	95.0%	5.0%	
	Stroke (n)	245	3	
	(%)	98.8%	1.2%	
rs147565266 (T/A) chr2: 188311515	control (n)	12967	53	**0.022**
	(%)	99.6%	0.4%	
	Stroke (n)	244	4	
	(%)	98.4%	1.6%	

Homo, homozygote; Hetero, heterozygote.

studied SNPs in *CLR* is shown. Pair-wise SNP R-squared D' linkage values (multiplied by 100) are also shown. We then selected five SNPs from among the *CLR* variants (rs77035639, rs3815524, rs75380157, rs574603859, and rs147565266) to avoid similar haplotypes. Similarly, *RAMP2* SNP (rs753152), which is associated with stroke, was selected for analysis. The distributions of genotypes and alleles of the evaluated SNPs are summarized in **Supplementary Table 2**. **Supplementary Fig. 2** shows exons (shown as boxes) 1−4 for *RAMP2*, and exons 1−15 for *CLR*. For analysis, we compared the associations between genotypes and stroke, after combining the heterozygous and minor homozygous alleles because of the small number of minor homozygotes alleles.

Table 1 shows the distribution of stroke for each SNP. The major homozygotes had significantly higher incidences of stroke compared with the heterozygotes and minor homozygotes, except for rs574603859. SNP rs574603859 showed an inverse ratio between major homozygotes and the other genotypes. **Table 2** summarizes the baseline characteristics of the participants divided into two groups, classified by major homozygous alleles versus heterozygous and minor homozygous alleles. None of the SNPs showed a constant tendency for these characteristics. **Table 3** shows the distribution of obesity, ischemic heart disease, hypertension, dyslipidemia, and diabetes for each SNP. SNP rs574603859 was associated with a higher incidence of obesity in the heterozygotes and minor homozy-

Table 2. Characteristics of study participants for each single nucleotide polymorphism (SNP).

Genotype	rs753152				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	5890	44.8%	447	47.1%	0.188
Age (year)	13137	54.8 ± 9.4	950	55.0 ± 9.5	0.462
BMI (kg/m^2)	10578	23.2 ± 3.4	752	23.1 ± 3.5	0.809
Systolic blood pressure (mmHg)	10514	128 ± 20.2	747	128 ± 19.1	0.528
Diastolic blood pressure (mmHg)	10513	78.2 ± 12.3	747	77.9 ± 11.7	0.441
Triglyceride (mg/dl)	10861	128 ± 96.5	792	130 ± 94.0	0.585
Total cholesterol (mg/dl)	9947	211 ± 34.7	749	211 ± 36.0	0.821
nonHDL-C (mg/dl)	9946	148 ± 35.1	749	149 ± 36.1	0.781
HDL-C (mg/dl)	10863	62.7 ± 16.3	792	62.3 ± 15.8	0.458
Hemoglobin A1C (%)	8057	5.55 ± 0.73	581	5.61 ± 0.74	0.055
eGFR (mL/min/1.73 m^2)	10509	78.8 ± 15.1	774	78.3 ± 14.9	0.374
Alcohol drinking					
0 g/d	5912	45.8%	433	46.6%	
0.1−22.9 g/d	4224	32.7%	305	32.8%	
23−45.9 g/d	1412	10.9%	101	10.9%	0.870
46.0 + g/d	1359	10.5%	90	9.7%	
Smoking	2471	18.8%	171	18.0%	0.574
Regular physical activity	3830	29.2%	289	30.5%	0.417

Genotype	rs77035639				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	6114	44.9%	223	46.2%	0.609
Age (year)	13604	54.8 ± 9.4	483	54.5 ± 9.3	0.587
BMI (kg/m^2)	10937	23.2 ± 3.4	393	23.3 ± 3.2	0.562
Systolic blood pressure (mmHg)	10874	128 ± 20.1	387	128 ± 20.4	0.605
Diastolic blood pressure (mmHg)	10873	78.2 ± 12.2	387	77.6 ± 12.3	0.318
Triglyceride (mg/dl)	11246	128 ± 96.3	407	129 ± 96.0	0.868
Total cholesterol (mg/dl)	10326	211 ± 34.7	370	211 ± 36.1	0.966
nonHDL-C (mg/dl)	10325	148 ± 35.1	370	149 ± 36.5	0.879
HDL-C (mg/dl)	11248	62.7 ± 16.3	407	62.5 ± 15.6	0.795
Hemoglobin A1C (%)	8347	5.55 ± 0.73	291	5.63 ± 0.91	0.171
eGFR (mL/min/1.73 m^2)	10899	78.7 ± 15.1	384	78.2 ± 13.8	0.527
Alcohol drinking					
0 g/d	6136	45.9%	209	44.5%	
0.1−22.9 g/d	4376	32.7%	153	32.6%	
23−45.9 g/d	1458	10.9%	55	11.7%	0.856
46.0 + g/d	1396	10.4%	53	11.3%	
Smoking	2548	18.7%	94	19.5%	0.682
Regular physical activity	3985	29.3%	134	27.7%	0.475

(Cont Table 2)

Genotype	rs3815524				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	5613	45.0%	724	44.6%	0.750
Age (year)	12464	54.8 ± 9.4	1623	54.7 ± 9.4	0.816
BMI (kg/m^2)	10010	23.2 ± 3.4	1320	23.2 ± 3.3	0.950
Systolic blood pressure (mmHg)	9949	128 ± 20.1	1312	128 ± 20.1	0.868
Diastolic blood pressure (mmHg)	9948	78.3 ± 12.2	1312	77.9 ± 12.1	0.374
Triglyceride (mg/dl)	10288	128 ± 96.3	1365	130 ± 96.3	0.447
Total cholesterol (mg/dl)	9426	211 ± 34.8	1270	212 ± 34.2	0.703
nonHDL-C (mg/dl)	9425	148 ± 35.2	1270	149 ± 35.0	0.382
HDL-C (mg/dl)	10289	62.7 ± 16.3	1366	62.3 ± 15.8	0.372
Hemoglobin A1C (%)	7659	5.56 ± 0.74	979	5.55 ± 0.66	0.684
eGFR (mL/min/1.73 m^2)	9966	78.8 ± 15.2	1317	78.3 ± 14.1	0.237
Alcohol drinking					
0 g/d	5615	45.8%	730	46.1%	
0.1−22.9 g/d	4007	32.7%	522	32.9%	
23−45.9 g/d	1336	10.9%	177	11.2%	0.848
46.0 + g/d	1293	10.6%	156	9.8%	
Smoking	2345	18.8%	297	18.3%	0.635
Regular physical activity	3655	29.4%	464	28.6%	0.542

Genotype	rs75380157				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	5843	45.5%	494	44.5%	0.777
Age (year)	12978	54.8 ± 9.4	1109	54.6 ± 9.4	0.629
BMI (kg/m^2)	10439	23.2 ± 3.4	891	23.0 ± 3.2	0.280
Systolic blood pressure (mmHg)	10377	128 ± 20.1	884	128 ± 20.3	0.845
Diastolic blood pressure (mmHg)	10376	78.3 ± 12.2	884	77.9 ± 12.3	0.367
Triglyceride (mg/dl)	10734	128 ± 96.1	919	129 ± 98.9	0.663
Total cholesterol (mg/dl)	9829	211 ± 34.8	867	211 ± 34.4	0.888
nonHDL-C (mg/dl)	9829	148 ± 35.2	867	149 ± 34.7	0.930
HDL-C (mg/dl)	10735	62.7 ± 16.3	920	62.5 ± 15.6	0.723
Hemoglobin A1C (%)	7954	5.56 ± 0.74	684	5.56 ± 0.71	0.794
eGFR (mL/min/1.73 m^2)	10393	78.8 ± 15.2	890	78.1 ± 14.0	0.190
Alcohol drinking					
0 g/d	5841	45.8%	504	46.5%	
0.1−22.9 g/d	4169	32.7%	360	33.2%	
23−45.9 g/d	1393	10.9%	120	11.1%	0.637
46.0 + g/d	1348	10.6%	101	9.3%	
Smoking	2424	18.7%	218	19.7%	0.424
Regular physical activity	3801	29.3%	318	28.7%	0.679

(Cont Table 2)

Genotype	rs574603859				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	6044	45.1%	293	43.0%	0.287
Age (year)	13405	54.8 ± 9.4	682	54.8 ± 9.2	0.889
BMI (kg/m^2)	10778	23.1 ± 3.4	552	23.6 ± 3.5	**0.001**
Systolic blood pressure (mmHg)	10711	128 ± 20.1	550	130 ± 20.6	**0.020**
Diastolic blood pressure (mmHg)	10710	78.2 ± 12.2	550	79.0 ± 12.1	0.137
Triglyceride (mg/dl)	11082	128 ± 95.0	571	133 ± 118	0.192
Total cholesterol (mg/dl)	10183	211 ± 34.8	513	212 ± 34.4	0.871
nonHDL-C (mg/dl)	10182	148 ± 35.2	513	150 ± 34.8	0.393
HDL-C (mg/dl)	11084	62.7 ± 16.3	571	61.9 ± 15.9	0.213
Hemoglobin A1C (%)	8235	5.55 ± 0.72	403	5.59 ± 0.94	0.277
eGFR (mL/min/1.73 m^2)	10732	78.7 ± 15.1	551	79.4 ± 14.7	0.302
Alcohol drinking					
0 g/d	6033	45.8%	312	46.7%	
0.1−22.9 g/d	4325	32.8%	204	30.5%	
23−45.9 g/d	1432	10.9%	81	12.1%	0.558
46.0 + g/d	1378	10.5%	71	10.6%	
Smoking	2523	18.8%	119	17.5%	0.392
Regular physical activity	3905	29.2%	214	31.4%	0.210

Genotype	rs147565266				*p* value
	Major Homo		Hetero + Minor Homo		
	n	mean ± SD (%)	n	mean ± SD (%)	
Sex (male)	6305	45.0%	32	50.8%	0.376
Age (year)	14024	54.8 ± 9.4	63	56.0 ± 8.3	0.312
BMI (kg/m^2)	11277	23.2 ± 3.4	53	23.3 ± 3.2	0.739
Systolic blood pressure (mmHg)	11209	128 ± 20.1	52	133 ± 26.2	0.189
Diastolic blood pressure (mmHg)	11208	78.2 ± 12.2	52	79.6 ± 14.6	0.501
Triglyceride (mg/dl)	11599	128 ± 95.9	54	151 ± 161	0.293
Total cholesterol (mg/dl)	10644	211 ± 34.7	52	215 ± 36.6	0.446
nonHDL-C (mg/dl)	10643	148 ± 35.1	52	152 ± 39.1	0.488
HDL-C (mg/dl)	11601	62.7 ± 16.2	54	62.9 ± 21.2	0.950
Hemoglobin A1C (%)	8592	5.56 ± 0.73	46	5.63 ± 0.84	0.520
eGFR (mL/min/1.73 m^2)	11230	78.7 ± 15.1	53	80.2 ± 14.2	0.482
Alcohol drinking					
0 g/d	6318	45.9%	27	43.5%	
0.1−22.9 g/d	4513	32.8%	16	25.8%	
23−45.9 g/d	1505	10.9%	8	12.9%	0.226
46.0 + g/d	1438	10.4%	11	17.7%	
Smoking	2632	18.8%	10	15.9%	0.631
Regular physical activity	4105	29.3%	14	22.2%	0.268

Homo, homozygote; Hetero, heterozygote.

Table 3. Genotype and allele distributions in obesity, ischemic heart disease, hypertension, dyslipidemia, and diabetes.

SNPs	Genotype	Obesity			Ischemic heart disease			Hypertension			Dyslipidemia			Diabetes		
		≥25	<25	*p* value	(−)	(+)	*p* value	(−)	(+)	*p* value	(−)	(+)	*p* value	(−)	(+)	*p* value
rs753152	Major Homo	7823 74.0%	2755 26.0%	0.729	12006 97.0%	374 3.0%	0.774	6624 63.0%	3889 37.0%	0.432	6396 63.5%	3676 36.5%	0.846	7496 93.0%	561 7.0%	**0.030**
	Hetero+	561	191		883	29		482	265		476	278		526	55	
	Minor Homo	74.6%	25.4%		96.8%	3.2%		64.5%	35.5%		63.1%	36.9%		90.5%	9.5%	
rs77035639	Major Homo	8097 74.0%	2840 26.0%	0.643	12447 97.0%	388 3.0%	0.678	6864 63.1%	4009 36.9%	0.832	6642 63.5%	3810 36.5%	0.414	7758 92.9%	589 7.1%	0.163
	Hetero+	287	106		442	15		242	145		230	144		264	27	
	Minor Homo	73.0%	27.0%		96.7%	3.3%		62.5%	37.5%		61.5%	38.5%		90.7%	9.3%	
rs3815524	Major Homo	7423 74.2%	2587 25.8%	0.301	11399 97.0%	351 3.0%	0.388	6275 63.1%	3673 36.9%	0.878	6067 63.6%	3473 36.4%	0.498	7105 92.8%	554 7.2%	0.323
	Hetero+	961	359		1490	52		831	481		805	481		917	62	
	Minor Homo	72.8%	27.2%		96.6%	3.4%		63.3%	36.7%		62.6%	37.4%		93.7%	6.3%	
rs75380157	Major Homo	7718 73.9%	2721 26.1%	0.632	11878 97.0%	366 3.0%	0.349	6541 63.0%	3835 37.0%	0.611	6316 63.5%	3634 36.5%	1.000	7381 92.8%	573 7.2%	0.395
	Hetero+	666	225		1011	37		565	319		556	320		641	43	
	Minor Homo	74.7%	25.3%		96.5%	3.5%		63.9%	36.1%		63.5%	36.5%		93.7%	6.3%	
rs574603859	Major Homo	8001 74.2%	2777 25.8%	**0.012**	12255 97.0%	385 3.0%	0.803	6778 63.3%	3932 36.7%	0.085	6550 63.5%	3759 36.5%	0.575	7648 92.9%	587 7.1%	0.921
	Hetero+	383	169		634	18		328	222		322	195		374	29	
	Minor Homo	69.4%	30.6%		97.2%	2.8%		59.6%	40.4%		62.3%	37.7%		92.8%	7.2%	
rs147565266	Major Homo	8345 74.0%	2932 26.0%	1.000	12833 97.0%	402 3.0%	1.000	7079 63.2%	4129 36.8%	0.111	6841 63.5%	3933 36.5%	0.570	7981 92.9%	611 7.1%	0.378
	Hetero+	39	14		56	1		27	25		31	21		41	5	
	Minor Homo	73.6%	26.4%		98.2%	1.8%		51.9%	48.1%		59.6%	40.4%		89.1%	10.9%	

Homo, homozygote; Hetero, heterozygote.

gotes. SNP rs753152 was associated with a higher incidence of diabetes in the heterozygotes and minor homozygotes. The other four SNPs showed no significant association with these diseases.

To determine the relationship of the SNPs with stroke in consideration of environmental factors, a logistic regression analysis adjusted for age, sex, research area, alcohol intake, current smoking, regular physical activity, obesity, hypertension, diabetes, dyslipidemia, and ischemic heart disease was performed. For the logistic regression analysis, the major homozygous genotypes were used as the reference group and the heterozygous and minor homozygous genotypes were used as the exposed group in the dominant model. **Table 4** shows model I adjusted for basic characteristics (age, sex, research area), model II adjusted for lifestyle, and model III adjusted for anamnesis. *RAMP2* SNP rs753152 was associated with a significantly higher OR in model I (OR, 1.773; 95% CI, 1.194−2.634). The *CLR* SNPs were associated with a significantly higher OR in model I (OR, 1.448−3.735) in participants with stroke, excluding rs574603859. SNP rs574603859 had a lower OR in model I (OR, 0.238; 95% CI, 0.076−0.745) between major homozygotes and the others. The model II results were similar to those for model I. In model III, rs574603859 showed no significant OR. The lack of statistical significance when adjusting for anamnesis indicated that rs574603859 has no strong effect on the risk of stroke.

Table 4. Associations between the *RAMP2* and *CLR* gene variants and stroke.

SNPs	Model Ⅰ			Model Ⅱ			Model Ⅲ		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value	OR	95% CI	*p*-value
rs753152	1.773	1.194-2.634	**0.005**	1.784	1.200-2.652	**0.004**	1.767	1.059-2.947	**0.029**
rs77035639	2.015	1.199-3.385	**0.008**	2.084	1.239-3.506	**0.006**	2.250	1.177-4.302	**0.014**
rs3815524	1.448	1.023-2.051	**0.037**	1.482	1.046-2.099	**0.027**	1.908	1.259-2.891	**0.002**
rs75380157	1.845	1.270-2.683	**0.001**	1.877	1.290-2.731	**0.001**	2.115	1.338-3.342	**0.001**
rs574603859	0.238	0.076-0.745	**0.014**	0.245	0.078-0.768	**0.016**	0.276	0.068-1.126	0.073
rs147565266	3.735	1.325-10.52	**0.013**	3.997	1.412-11.31	**0.009**	5.316	1.775-15.92	**0.002**

Model Ⅰ: adjusted for age, sex, research area
Model Ⅱ: adjusted for age, sex, research area, alcohol intake, current smoking, regular physical activity, obesity
Model Ⅲ: adjusted for age, sex, research area, alcohol intake, current smoking, regular physical activity, obesity, hypertension, diabetes, dyslipidemia, ischemic heart disease

Discussion

There is considerable evidence to suggest that the pathogenesis of stroke is affected by not only genetic factors, but also environment interactions[9]. Previous studies showed that a history of hypertension, dyslipidemia, diabetes, physical inactivity, diet, waist-to-hip ratio, current smoking, cardiac causes, and alcohol consumption were associated with risk of stroke[32, 33]. There was a J-shaped association between high amounts of alcohol and increased risk of both ischemic and hemorrhagic stroke[34]. Therefore, we defined age, sex, research area, alcohol consumption status, current smoking, regular physical activity, obesity, hypertension, diabetes, dyslipidemia, and ischemic heart disease as independent variables in the logistic regression analyses. To the best of our knowledge, this is the first study to investigate the relationships between *RAMP2* and *CLR* and stroke in the light of gene-environment interactions in humans.

The pathogenesis of stroke is very complex and is associated closely with vascular dysfunction and disruption. Indeed, similar to chronic obstructive pulmonary disease, systemic inflammation and oxidative stress might play important roles in increasing the risk of stroke by promoting vascular dysfunction and platelet hyperactivity[35]. A review study showed that ADM has strong anti-oxidation and anti-inflammation activities[14]. Moreover, ADM acts via CLR/RAMP2 to prevent brain injury in both acute and chronic cerebral ischemia[36], and exerts crucial vasoprotective effects following vascular injury[37]. The vascular ADM-CLR/RAMP2 system is critical in the regulation of vascular integrity, including the maintenance of vascular structure, and the regulation of angiogenesis and vasoprotection against vascular injury[14, 19]. Studying the ADM-CLR/RAMP2 system should reveal the mechanisms underlying the functional integrity of the vascular system, and could serve as the basis for novel approaches to therapy and preventive medicine.

RAMP expression is modulated by various agents in cell culture and in animal models of human disease[38]. For example, marked changes induced in the cardiovascular and renal systems provided evidence of an important role for dynamic RAMP regulation in those systems. Studies suggest that regulation of RAMPs might modulate the pathophysiology of conditions linked to RAMP-interacting G protein-coupled receptors. For example, human SNP studies described the relationship between *CLR* and essential hypertension and primary angle closure glaucoma[39, 40]. Polymorphisms in the *ADM* gene have also been reported to have a possible association with essential hypertension, dysglycemia, and adrenomedullin levels[41-45]. Genetic variants of the ADM-CLR/RAMP2 system might affect vascular homeostasis and cerebrovascular/cardiovascular disease. Several studies have revealed interactions of SNPs with stroke[46-49]. In these studies, the functional genetic polymorphisms were located in the promoters, which could cause differences in the plasma levels of the encoded target protein; were located in coding exons, leading to amino acid changes; or were located in an intron. Although we demonstrated that an intron-located SNP is not functional, it could have other effects, such as influencing splicing or regulatory processes; for example, the binding of transcription factors to the gene. Furthermore, as a tag SNP, the polymorphism might be representative of many other variants, which could regulate the function of the receptor.

The limitations of our study include its cross-sectional design. However, case-control studies can be used to assess previously identified candidate regions and to determine target selections more precisely. In general, strokes can be divided into three subtypes: ischemic, lacunar, and hemorrhagic. In this study, we

did not investigate the subtypes of stroke because we used a self-administered questionnaire to judge anamnesis. By contrast, a previous study reported that stroke and myocardial infarct seem sensitive enough to use self-administered questionnaire for judgment at baseline in Japanese cohort studies[50]. In a future (follow-up) survey, we plan to assess the participants by looking up the actual medical records; therefore, we expect these additional data will lead to further detailed analysis of genetic variants of *ADM* receptor genes in accordance with the stroke subtypes and cardiovascular disease. In addition, we only assessed Japanese participants in the present study, and further studies in other ethnic groups are needed to validate our findings.

Conclusions

In conclusion, the association of the *RAMP2* and *CLR* genes with stroke in a Japanese cohort implicates these genes in the pathogenesis of stroke, although further investigation is required to confirm their associations. It will be interesting to determine whether the polymorphisms of *RAMP2* and *CLR* are responsible for functional changes, and to reveal the underlying mechanism, given the potentially important role that *ADM* receptor genes play in stroke and/or vascular fragility.

Conflict of Interest

There are no conflicts of interest.

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Reference

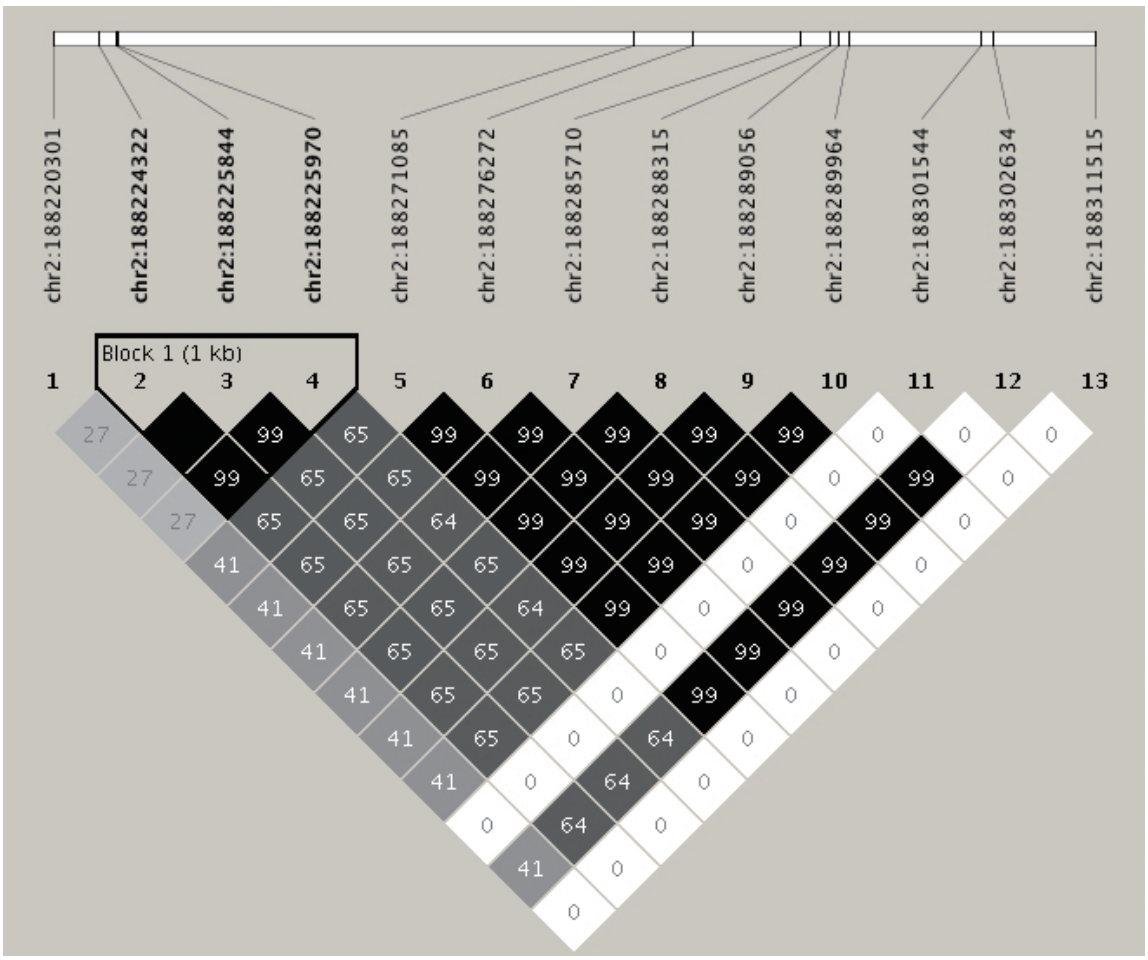
1) Cleaver O and Melton DA: Endothelial signaling during development. Nat Med, 2003; 9: 661-668
2) Carmeliet P: Angiogenesis in health and disease. Nat Med, 2003; 9: 653-660
3) Miller AA, Budzyn K and Sobey CG: Vascular dysfunction in cerebrovascular disease: mechanisms and therapeutic intervention. Clin Sci (Lond), 2010; 119: 1-17
4) Higashi Y, Noma K, Yoshizumi M and Kihara Y: Endothelial function and oxidative stress in cardiovascular diseases. Circ J, 2009; 73: 411-418
5) Mochizuki N: Vascular integrity mediated by vascular endothelial cadherin and regulated by sphingosine 1-phosphate and angiopoietin-1. Circ J, 2009; 73: 2183-2191
6) Dejana E: Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol, 2004; 5: 261-270
7) Lanktree MB, Dichgans M and Hegele RA: Advances in genomic analysis of stroke: what have we learned and where are we headed? Stroke, 2010; 41: 825-832
8) Bevan S and Markus HS: Genetics of common polygenic ischaemic stroke: current understanding and future challenges. Stroke Res Treat, 2011; 2011: 179061
9) Markus HS: Stroke genetics. Hum Mol Genet, 2011; 20: R124-131
10) Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H and Eto T: Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun, 1993; 192: 553-560
11) Abe M, Sata M, Nishimatsu H, Nagata D, Suzuki E, Terauchi Y, Kadowaki T, Minamino N, Kangawa K, Matsuo H, Hirata Y and Nagai R: Adrenomedullin augments collateral development in response to acute ischemia. Biochem Biophys Res Commun, 2003; 306: 10-15
12) Brain SD and Grant AD: Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev, 2004; 84: 903-934
13) Kato J, Tsuruda T, Kita T, Kitamura K and Eto T: Adrenomedullin: a protective factor for blood vessels. Arterioscler Thromb Vasc Biol, 2005; 25: 2480-2487
14) Koyama T, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Kawate H and Shindo T: Adrenomedullin-RAMP2 System in Vascular Endothelial Cells. J Atheroscler Thromb, 2015; 22: 647-653
15) McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG and Foord SM: RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature, 1998; 393: 333-339
16) Parameswaran N and Spielman WS: RAMPs: The past, present and future. Trends Biochem Sci, 2006; 31: 631-638
17) Shindo T, Kurihara Y, Nishimatsu H, Moriyama N, Kakoki M, Wang Y, Imai Y, Ebihara A, Kuwaki T, Ju KH, Minamino N, Kangawa K, Ishikawa T, Fukuda M, Akimoto Y, Kawakami H, Imai T, Morita H, Yazaki Y, Nagai R, Hirata Y and Kurihara H: Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. Circulation, 2001; 104: 1964-1971

18) Ichikawa-Shindo Y, Sakurai T, Kamiyoshi A, Kawate H, Iinuma N, Yoshizawa T, Koyama T, Fukuchi J, Iimuro S, Moriyama N, Kawakami H, Murata T, Kangawa K, Nagai R and Shindo T: The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. J Clin Invest, 2008; 118: 29-39
19) Koyama T, Ochoa-Callejero L, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Iinuma N, Arai T, Yoshizawa T, Iesato Y, Lei Y, Uetake R, Okimura A, Yamauchi A, Tanaka M, Igarashi K, Toriyama Y, Kawate H, Adams RH, Kawakami H, Mochizuki N, Martinez A and Shindo T: Vascular endothelial adrenomedullin-RAMP2 system is essential for vascular integrity and organ homeostasis. Circulation, 2013; 127: 842-853
20) Fritz-Six KL, Dunworth WP, Li M and Caron KM: Adrenomedullin signaling is necessary for murine lymphatic vascular development. J Clin Invest, 2008; 118: 40-50
21) Hamajima N: The Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) to detect gene-environment interactions for cancer. Asian Pacific journal of cancer prevention: Asian Pac J Cancer Prev, 2007; 8: 317-323
22) Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H and Hishida A: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis, 2009; 53: 982-992
23) Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ and Sham PC: PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet, 2007; 81: 559-575
24) Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM and Lee JJ: Second-generation PLINK: rising to the challenge of larger and richer datasets. GigaScience, 2015; 4: 7
25) Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA and Reich D: Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet, 2006; 38: 904-909
26) Patterson N, Price AL and Reich D: Population structure and eigenanalysis. PLoS Genet, 2006; 2: e190
27) Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA and Abecasis GR: A global reference for human genetic variation. Nature, 2015; 526: 68-74
28) Yamaguchi-Kabata Y, Nakazono K, Takahashi A, Saito S, Hosono N, Kubo M, Nakamura Y and Kamatani N: Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am J Hum Genet, 2008; 83: 445-456
29) Delaneau O, Marchini J and Zagury JF: A linear complexity phasing method for thousands of genomes. Nat Methods, 2011; 9: 179-181
30) Das S, Forer L, Schonherr S, Sidore C, Locke AE, Kwong A, Vrieze SI, Chew EY, Levy S, McGue M, Schlessinger D, Stambolian D, Loh PR, Iacono WG, Swaroop A, Scott LJ, Cucca F, Kronenberg F, Boehnke M, Abecasis GR and Fuchsberger C: Next-generation genotype imputation service and methods. Nat Genet, 2016; 48: 1284-1287
31) Barrett JC, Fry B, Maller J and Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics, 2005; 21: 263-265
32) O'Donnell MJ, Chin SL, Rangarajan S, Xavier D, Liu L, Zhang H, Rao-Melacini P, Zhang X, Pais P, Agapay S, Lopez-Jaramillo P, Damasceno A, Langhorne P, McQueen MJ, Rosengren A, Dehghan M, Hankey GJ, Dans AL, Elsayed A, Avezum A, Mondo C, Diener HC, Ryglewicz D, Czlonkowska A, Pogosova N, Weimar C, Iqbal R, Diaz R, Yusoff K, Yusufali A, Oguz A, Wang X, Penaherrera E, Lanas F, Ogah OS, Ogunniyi A, Iversen HK, Malaga G, Rumboldt Z, Oveisgharan S, Al Hussain F, Magazi D, Nilanont Y, Ferguson J, Pare G and Yusuf S: Global and regional effects of potentially modifiable risk factors associated with acute stroke in 32 countries (INTERSTROKE): a case-control study. Lancet, 2016; 388: 761-775
33) Isabel C, Calvet D and Mas JL: Stroke prevention. Press Med, 2016; 45: e457-e471
34) Ikehara S, Iso H, Yamagishi K, Kokubo Y, Saito I, Yatsuya H, Inoue M and Tsugane S: Alcohol consumption and risk of stroke and coronary heart disease among Japanese women: the Japan Public Health Center-based prospective study. Prev Med, 2013; 57: 505-510
35) Austin V, Crack PJ, Bozinovski S, Miller AA and Vlahos R: COPD and stroke: are systemic inflammation and oxidative stress the missing links? Clin Sci (Lond), 2016; 130: 1039-1050
36) Igarashi K, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Kawate H, Yamauchi A, Toriyama Y, Tanaka M, Liu T, Xian X, Imai A, Zhai L, Owa S, Koyama T, Uetake R, Ihara M and Shindo T: Pathophysiological roles of adrenomedullin-RAMP2 system in acute and chronic cerebral ischemia. Peptides, 2014; 62: 21-31
37) Xian X, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Tanaka M, Koyama T, Kawate H, Yang L, Liu T, Imai A, Zhai L, Hirabayashi K, Dai K, Tanimura K, Liu T, Cui N, Igarashi K, Yamauchi A and Shindo T: Vasoprotective activities of the adrenomedullin-RAMP2 system in endothelial cells. Endocrinology, 2017; 158: 1359-1372
38) Udawela M, Hay DL and Sexton PM: The receptor activity modifying protein family of G protein coupled receptor accessory proteins. Semin Cell Dev Biol, 2004; 15: 299-308
39) Sano M, Kuroi N, Nakayama T, Sato N, Izumi Y, Soma M and Kokubun S: Association study of calcitonin-receptor-like receptor gene in essential hypertension. Am J Hypertens, 2005; 18: 403-408
40) Awadalla MS, Burdon KP, Thapa SS, Hewitt AW and Craig JE: A cross-ethnicity investigation of genes previously implicated in primary angle closure glaucoma. Mol Vis, 2012; 18: 2247-2254
41) Verweij N, Mahmud H, Mateo Leach I, de Boer RA, Brouwers FP, Yu H, Asselbergs FW, Struck J, Bakker SJ, Gansevoort RT, Munroe PB, Hillege HL, van Veldhuisen DJ, van Gilst WH, Sillje HH and van der Harst P: Genome-wide association study on plasma levels of midregional-proadrenomedullin and C-terminal-pro-endothelin-1. Hypertension, 2013; 61: 602-608
42) Ong KL, Tso AW, Leung RY, Cherny SS, Sham PC, Lam TH, Cheung BM and Lam KS: A genetic variant in the gene encoding adrenomedullin predicts the development

of dysglycemia over 6.4 years in Chinese. Clin Chim Acta, 2011; 412: 353-357
43) Glorioso N, Herrera VL, Didishvili T, Ortu MF, Zaninello R, Fresu G, Argiolas G, Troffa C and Ruiz-Opazo N: Sex-specific effects of NLRP6/AVR and ADM loci on susceptibility to essential hypertension in a Sardinian population. PLoS one, 2013; 8: e77562
44) Cheung BM, Ong KL, Tso AW, Leung RY, Cherny SS, Sham PC, Lam TH and Lam KS: Plasma adrenomedullin level is related to a single nucleotide polymorphism in the adrenomedullin gene. Eur J Endocrinol, 2011; 165: 571-577
45) Chen S, Lu X, Zhao Q, Wang L, Li H and Huang J: Association of adrenomedullin gene polymorphisms and blood pressure in a Chinese population. Hypertens Res, 2013; 36: 74-78
46) Yi X, Wu L, Liao D, Wang C and Zhang B: Interactions Among CYP2C8, EPHX2, and CYP4A11 Variants and CYP Plasma Metabolite Levels in Ischemic Stroke. J Atheroscler Thromb, 2016; 23: 1286-1293
47) Zhong H, Cai Y, Cheng J, Cai D, Chen L, Su C, Li K, Chen P, Xu J and Cui L: Apolipoprotein E Epsilon 4 Enhances the Association between the rs2910164 Polymorphism of miR-146a and Risk of Atherosclerotic Cerebral Infarction. J Atheroscler Thromb, 2016; 23: 819-829
48) Zhang L, Yang J, Xue Q, Yang D, Lu Y, Guang X, Zhang W, Ba R, Zhu H and Ma X: An rs13293512 polymorphism in the promoter of let-7 is associated with a reduced risk of ischemic stroke. J Thromb Thrombolysis, 2016; 42: 610-615
49) Yi X, Lin J, Wang Y, Zhou Q, Wang C, Cheng W and Chi L: Association of Cytochrome P450 Genetic Variants with Clopidogrel Resistance and Outcomes in Acute Ischemic Stroke. J Atheroscler Thromb, 2016; 23: 1188-1200
50) Yamagishi K, Ikeda A, Iso H, Inoue M and Tsugane S: Self-reported stroke and myocardial infarction had adequate sensitivity in a population-based prospective study JPHC (Japan Public Health Center)-based Prospective Study. J Clin Epidemiol, 2009; 62: 667-673

Supplementary Table 1. Genetic variants of the *RAMP2* and *CLR* loci.

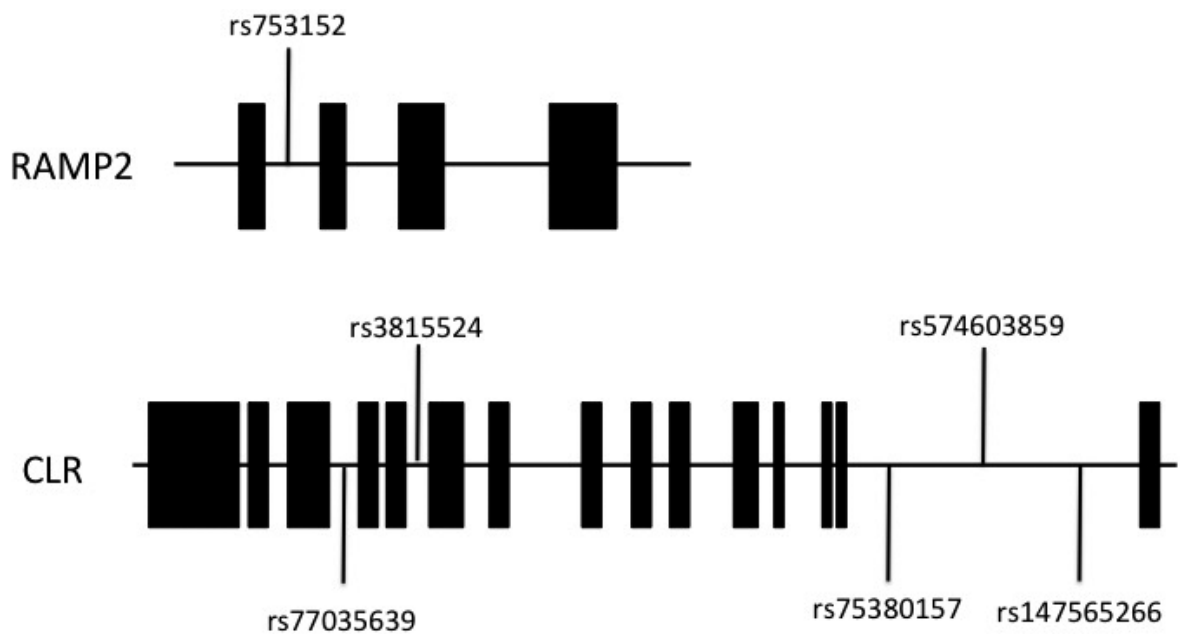
Gene	chromosome	Position							
RAMP2	17	40913366	188216783	188225844	188236012	188252411	188269685	188287434	188298742
		40913505	188216807	188225970	188236025	188252561	188269709	188287456	188298787
CLR	2	188206953	188217247	188226414	188236053	188252608	188270337	188288165	188300228
		188207245	188217379	188226520	188236279	188253796	188270560	188288259	188300873
		188207585	188217500	188226900	188236354	188253902	188270687	188288266	188301149
		188207611	188217501	188227008	188236378	188254046	188270864	188288315	188301544
		188208012	188217706	188227300	188236458	188254173	188270914	188289056	188302053
		188208120	188217738	188227302	188236819	188254581	188270925	188289079	188302587
		188208130	188218342	188227302	188237045	188255091	188271085	188289172	188302634
		188208290	188218458	188227613	188237868	188255237	188271819	188289174	188302770
		188208736	188218683	188227754	188238101	188255432	188272092	188289448	188303700
		188209158	188218937	188227921	188238103	188255549	188272460	188289795	188303845
		188209159	188218946	188228516	188238334	188255912	188272951	188289849	188303979
		188209179	188219052	188228911	188239574	188256158	188273829	188289964	188304095
		188209709	188219186	188229007	188239647	188256237	188275146	188289971	188304213
		188210214	188219325	188229335	188239809	188256685	188275930	188290419	188304446
		188210256	188219447	188229622	188240559	188256910	188275982	188290490	188304891
		188210257	188219468	188229739	188241519	188257985	188276272	188290717	188305046
		188210415	188219975	188229820	188241522	188258904	188276515	188290969	188305110
		188210586	188220301	188230146	188241953	188259620	188276584	188290969	188305797
		188210673	188220317	188230333	188242861	188260401	188276906	188291105	188306215
		188210960	188220384	188230588	188243363	188260747	188276987	188291382	188306229
		188211005	188220446	188230692	188243503	188260912	188277123	188291723	188306543
		188211112	188220865	188230976	188243620	188260913	188277455	188291869	188306768
		188211296	188221302	188231072	188243633	188260921	188277682	188292242	188306985
		188211443	188221350	188231216	188243658	188261266	188278035	188292458	188307038
		188211568	188221547	188231433	188243684	188261890	188278203	188293391	188307334
		188211568	188221648	188231645	188243940	188261922	188278226	188293438	188307429
		188211610	188221793	188231887	188244430	188262362	188278525	188293545	188307608
		188211789	188221911	188232223	188245890	188262468	188278822	188293614	188307747
		188212371	188221943	188232805	188246023	188263003	188279122	188293635	188307962
		188212423	188222351	188233502	188247648	188263325	188279606	188293921	188308056
		188213235	188222428	188233524	188247843	188264246	188280382	188294598	188308240
		188213336	188222469	188233714	188248440	188264602	188280870	188294980	188308604
		188213538	188222560	188234194	188248594	188264833	188280896	188295534	188308640
		188213819	188222561	188234520	188248663	188265242	188282330	188295611	188308853
		188214239	188222581	188234678	188248727	188265543	188282703	188296132	188309875
		188214694	188222928	188234844	188249420	188266531	188283002	188296488	188310118
		188214823	188222946	188234928	188250234	188266951	188283061	188297133	188310305
		188214924	188223256	188235033	188250476	188267147	188283123	188297160	188311515
		188215045	188223889	188235100	188250718	188267147	188284000	188297214	188311900
		188215156	188224057	188235162	188250860	188267193	188284127	188297348	188311992
		188215209	188224322	188235258	188251427	188267470	188284824	188297349	
		188215241	188224457	188235340	188251432	188268001	188285455	188297388	
		188215292	188224928	188235611	188251535	188268541	188285710	188298159	
		188215299	188225030	188235956	188251702	188268931	188286217	188298197	
		188216078	188225240	188236000	188251972	188269235	188286516	188298341	



Supplementary Fig. 1. The linkage disequilibrium analyses of 13 *CLR* (calcitonin-receptor-like receptor) SNPs associated with stroke.
The haplotype structure and the position of the studied single nucleotide polymorphisms in the *CLR* gene exhibited a statistically significant association with stroke.

Supplementary Table 2. Allele and genotype frequencies of the *RAMP2* and *CLR* genes in the participants

SNP	Allele frequency	Genotype frequency	n	*P* for Hardy-Weinberg equilibrium
rs753152 (T/G)				
TT	T=0.965	T/T=0.933	13137	
TG		T/G=0.065	921	0.003
GG	G=0.035	G/G=0.002	29	
rs77035639 (A/G)				
AA	A=0.983	A/A=0.965	13604	
AG		A/G=0.034	476	0.177
GG	G=0.017	G/G=0.001	7	
rs3815524 (G/C)				
GG	G=0.940	G/G=0.885	12464	
GC		G/C=0.110	1552	0.003
CC	C=0.060	C/C=0.005	71	
rs75380157 (A/T)				
AA	A=0.959	A/A=0.921	12978	
AT		A/T=0.076	1073	0.006
TT	T=0.041	T/T=0.003	36	
rs574603859 (A/T)				
AA	A=0.975	A/A=0.952	13405	
AT		A/T=0.047	664	0.001
TT	T=0.025	T/T=0.001	18	
rs147565266 (T/A)				
TT	T=0.998	T/T=0.996	14024	
TA		T/A=0.004	63	0.790
AA	A=0.002	A/A=0.000	0	



Supplementary Fig. 2. Organization of the *RAMP2* (receptor activity-modifying protein 2) and *CLR* (calcitonin-receptor-like receptor) genes and locations of the SNPs used in the present study. Closed boxes indicate exons and lines represent introns.