Genetic Variants of RAMP2 and CLR are Associated with Stroke

Teruhide Koyama¹, Nagato Kuriyama¹, Etsuko Ozaki¹, Daisuke Matsui¹, Isao Watanabe¹, Wakiko Takeshita¹, Komei Iwai¹, Yoshiyuki Watanabe¹, Masahiro Nakatochi², Chisato Shimano³, Keitaro Tanaka³, Isao Oze⁴, Hidemi Ito⁶, Hirokazu Uemura⁵, Sakurako Katsuura-Kamano⁵, Rie Ibusuki⁶, Ippei Shimoshikiryo⁶, Naoyuki Takashima⁷, Aya Kadota⁷, Sayo Kawai⁹, Tae Sasaki⁹, Rieko Okada⁹, Asahi Hishida⁹, Mariko Naito⁹, Kiyonori Kuriki¹⁰, Kaori Endoh¹⁰, Norihiro Furusyo¹¹, Hiroaki Ikezaki¹¹, Sadao Suzuki¹², Akihiro Hosono¹², Haruo Mikami¹³, Yohko Nakamura¹³, Michiaki Kubo¹⁴ and Kenji Wakai⁹

¹Department of Epidemiology for Community Health and Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
²Center for Advanced Medicine and Clinical Research, Nagoya University Hospital, Nagoya, Japan
³Department of Preventive Medicine, Faculty of Medicine, Saga University, Saga, Japan
⁴Division of Molecular and Clinical Epidemiology, Aichi Cancer Center Research Institute, Nagoya, Japan
⁵Department of Preventive Medicine, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan
⁶Department of International Islands and Community Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
⁷Department of Public Health, Shiga University of Medical Science, Shiga, Japan
⁸Center for Epidemiologic Research in Asia, Shiga University of Medical Science, Shiga, Japan
⁹Department of Preventive Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan
¹⁰Laboratory of Public Health, School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan
¹¹Department of Environmental Medicine and Infectious Disease, Kyushu University, Fukuoka, Japan
¹²Department of Public Health, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
¹³Cancer Prevention Center, Chiba Cancer Center Research Institute, Chiba, Japan
¹⁴Laboratory for Genotyping Development, Center for Integrative Medical Sciences, RIKEN, Yokohama, Japan

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

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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. Current and novel therapeutic approaches aimed at improving vascular function provide real benefits with respect to reducing cerebrovascular disease. 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. 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. Genetic contributions to strokes may result from common variants with small effect sizes, rare variants with large effect sizes, or their combination. However, environmental risk factors are associated with the pathogenesis of stroke, and considerable evidence suggests that gene-environment interactions are important.

Adrenomedullin (ADM) is a vasoactive peptide first identified in human pheochromocytoma. Although ADM is secreted by various organs and tissues, it is produced mainly by vascular endothelial cells and serves a number of physiological functions. The ADM receptor system in humans.

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-associate diseases. 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 (m²). Obesity was defined as a BMI ≥ 25.0 kg/m². 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, ‘gō’ (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. Anamnysis 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 m²) = 194 × creatinine⁻¹.094 × age⁻⁰.287 (for men) and eGFR (mL/min/1.73 m²) = 194 × creatinine⁻¹.094 × age⁻⁰.287 × 0.739 (for women). Each blood sample was centrifuged and the plasma was separated and stored at −80°C 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°C 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 Human-OmniExpressExome-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. Principal component analysis (PCA) and genotyping and quality control filtering resulted in 14,091 participants and 14,087 participants, respectively.

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
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 homozygotes alleles.

Table 1. Genotype and allele distributions in stroke.

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

Homo, homozygote; Hetero, heterozygote.
### Table 2. Characteristics of study participants for each single nucleotide polymorphism (SNP).

| Genotype                        | rs753152 | rs77035639 |
|---------------------------------|----------|------------|
|                                 | Major Homo | Hetero + Minor Homo | $p$ value |
|                                 | n | mean ± SD (%) | n | mean ± SD (%) | n | mean ± SD (%) | 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/m²)**                 | 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 m²)**       | 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 |
|---------------------------------|------------|
|                                 | Major Homo | Hetero + Minor Homo | $p$ value |
|                                 | n | mean ± SD (%) | n | mean ± SD (%) | 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/m²)**                 | 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 m²)**       | 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 |
| Genotype                          | rs3815524 |                  | rs3815524 |                  | p value |
|----------------------------------|-----------|----------------|-----------|----------------|--------|
|                                   | Major Homo | Hetero + Minor Homo |                                   |                  |        |
| 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/m²)                      | 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 m²)            | 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 |                  | rs75380157 |                  | p value |
|-----------------------------------|------------|----------------|------------|----------------|--------|
|                                   | Major Homo | Hetero + Minor Homo |                                   |                  |        |
| 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/m²)                      | 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 m²)            | 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 | rs147565266 |
|-----------------------------------|-------------|-------------|
|                                   | Major Homo  | Hetero + Minor Homo | p value |
|                                   | 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/m²)                       | 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 m²)             | 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      |

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 | 2755 | 0.729 | 12006 | 374 | 0.777 | 6624 | 3889 | 0.432 | 6396 | 3676 | 0.846 | 7496 | 561 |
|        | Hetero | 561 | 191 | 0.643 | 883 | 29 | 0.678 | 482 | 265 | 0.832 | 476 | 278 | 0.414 | 526 | 55 |
|         | Minor Homo | 74.6% | 25.4% | 0.301 | 96.8% | 3.2% | 0.878 | 62.5% | 37.5% | 0.498 | 61.5% | 38.5% | 0.907 | 90.7% | 9.3% |
| rs77055659 | Major Homo | 8097 | 2840 | 0.632 | 11399 | 351 | 0.349 | 6275 | 3673 | 1.000 | 6067 | 3473 | 0.432 | 7105 | 554 |
|         | Hetero | 287 | 106 | 0.301 | 442 | 15 | 0.388 | 242 | 145 | 1.000 | 230 | 144 | 0.498 | 264 | 27 |
|         | Minor Homo | 73.0% | 27.0% | 0.301 | 96.7% | 3.3% | 0.388 | 62.5% | 37.5% | 0.498 | 61.5% | 38.5% | 0.907 | 90.7% | 9.3% |
| rs3815524 | Major Homo | 7423 | 2587 | 0.301 | 97.0% | 3.0% | 0.878 | 63.1% | 36.9% | 0.498 | 63.6% | 36.4% | 0.907 | 92.8% | 7.2% |
|         | Hetero | 961 | 359 | 0.301 | 1490 | 52 | 0.388 | 831 | 481 | 0.498 | 805 | 481 | 0.917 | 62 |
|         | Minor Homo | 72.8% | 27.2% | 0.301 | 96.6% | 3.4% | 0.388 | 63.3% | 36.7% | 0.498 | 62.6% | 37.4% | 0.907 | 93.7% | 6.3% |
| rs75380157 | Major Homo | 7718 | 2721 | 0.632 | 11878 | 366 | 0.349 | 6541 | 3835 | 0.611 | 6316 | 3634 | 1.000 | 7381 | 573 |
|         | Hetero | 666 | 225 | 0.632 | 1011 | 37 | 0.349 | 565 | 319 | 0.611 | 556 | 320 | 0.907 | 641 | 43 |
|         | Minor Homo | 73.9% | 26.1% | 0.632 | 97.0% | 3.0% | 0.611 | 63.0% | 37.0% | 0.611 | 63.5% | 36.5% | 0.907 | 92.8% | 7.2% |
| rs574603859 | Major Homo | 8001 | 2777 | 0.012 | 12255 | 385 | 0.803 | 6778 | 3932 | 0.085 | 6550 | 3759 | 0.575 | 7648 | 587 |
|         | Hetero | 383 | 109 | 0.012 | 634 | 18 | 0.803 | 328 | 222 | 0.085 | 322 | 195 | 0.575 | 374 | 29 |
|         | Minor Homo | 69.4% | 30.6% | 0.012 | 97.2% | 2.8% | 0.803 | 59.6% | 40.4% | 0.575 | 62.3% | 37.7% | 0.929 | 92.9% | 7.1% |
| rs147565266 | Major Homo | 8345 | 2932 | 1.000 | 12833 | 402 | 1.000 | 7079 | 4129 | 1.000 | 6841 | 3933 | 1.000 | 7981 | 611 |
|         | Hetero | 39 | 14 | 1.000 | 56 | 1 | 1.000 | 27 | 25 | 1.000 | 31 | 21 | 1.000 | 41 | 5 |
|         | Minor Homo | 73.6% | 26.4% | 1.000 | 98.2% | 1.8% | 1.000 | 51.9% | 48.1% | 1.000 | 59.6% | 40.4% | 1.000 | 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.
There is considerable evidence to suggest that the pathogenesis of stroke is affected by not only genetic factors, but also environment interactions. 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. There was a J-shaped association between high amounts of alcohol and increased risk of both ischemic and hemorrhagic stroke. 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. A review study showed that ADM has strong anti-oxidation and anti-inflammation activities. Moreover, ADM acts via CLR/RAMP2 to prevent brain injury in both acute and chronic cerebral ischemia, and exerts crucial vasoprotective effects following vascular injury. 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. 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. 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. Polymorphisms in the ADM gene have also been reported to have a possible association with essential hypertension, dysglycemia, and adrenomedullin levels. 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. 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. 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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| Gene | chromosome | Position |
|------|------------|----------|
| **RAMP2** | 17 | 40913366 |
| | | 40913505 |
| CLR | 2 | 188206953 |
| | | 188207245 |
| | | 188207585 |
| | | 188207611 |
| | | 188208012 |
| | | 188208120 |
| | | 188208130 |
| | | 188208290 |
| | | 188208736 |
| | | 188209158 |
| | | 188209159 |
| | | 188209179 |
| | | 188209709 |
| | | 188210214 |
| | | 188210256 |
| | | 188210257 |
| | | 188210415 |
| | | 188210586 |
| | | 188210673 |
| | | 188210960 |
| | | 188211005 |
| | | 188211112 |
| | | 188211296 |
| | | 188211443 |
| | | 188211568 |
| | | 188211568 |
| | | 188211610 |
| | | 188211789 |
| | | 188212371 |
| | | 188212423 |
| | | 188213235 |
| | | 188213336 |
| | | 188213538 |
| | | 188213819 |
| | | 188214239 |
| | | 188214694 |
| | | 188214823 |
| | | 188214924 |
| | | 188215045 |
| | | 188215156 |
| | | 188215209 |
| | | 188215241 |
| | | 188215292 |
| | | 188215299 |
| | | 188216078 |
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          | 28   |                                 |
| rs77035639 (A/G)| A=0.983          | A/A=0.965          | 13604|                                |
| AA              | A/G=0.034        |                    | 476  | 0.177                           |
| AG              |                   |                    |      |                                 |
| GG              | G=0.017          | G/G=0.001          | 7    |                                 |
| rs3815524 (G/C)| G=0.940          | G/G=0.885          | 12464|                                |
| GG              |                   |                    |      |                                 |
| GC              | G/C=0.110        |                    | 1552 | 0.003                           |
| CC              | C=0.060          | C/C=0.005          | 71   |                                 |
| rs75380157 (A/T)| A=0.959          | A/A=0.921          | 12978|                                |
| AA              | A/T=0.076        |                    | 1073 | 0.006                           |
| AT              |                   |                    |      |                                 |
| TT              | T=0.041          | T/T=0.003          | 36   |                                 |
| rs574603859 (A/T)| A=0.975         | A/A=0.952          | 13405|                                |
| AA              | A/T=0.047        |                    | 664  | 0.001                           |
| AT              |                   |                    |      |                                 |
| TT              | T=0.025          | T/T=0.001          | 18   |                                 |
| rs147565266 (T/A)| T=0.998         | T/T=0.996          | 14024|                                |
| TT              |                   |                    |      | 0.790                           |
| TA              | T/A=0.004        |                    | 63   |                                 |
| 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.