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ORIGINAL ARTICLE

Genome-Wide Association Study of Intracranial Artery Stenosis Followed by Phenome-Wide Association Study

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Abstract

The genetic background of intracranial artery stenosis (ICAS), a major cause of ischemic stroke, remains elusive. We performed the world's first genome-wide association study (GWAS) of ICAS using DNA samples from Japanese subjects, to identify the genetic factors associated with ICAS and their correlation with clinical features. We also conducted a phenome-wide association study (PheWAS) of the top variant identified via GWAS to determine its association with systemic disease. The GWAS involved 408 patients with ICAS and 349 healthy controls and utilized an Asian Screening Array of venous blood samples. The PheWAS was performed using genotypic and phenotypic data of the Biobank Japan Project, which contained information on 46 diseases and 60 quantitative trait data from > 150,000 Japanese individuals. The GWAS revealed that the East Asian-specific functional variant of *RNF213*, rs112735431 (c.14429G > A, p.Arg4810Lys), was associated with ICAS (odds ratio, 12.3; 95% CI 5.5 to 27.5; $P = 7.8 \times 10^{-10}$). Stratified analysis within ICAS cases demonstrated that clinical features of those with and without the risk allele were different. PheWAS indicated that high blood pressure and angina were significantly associated with *RNF213* rs112735431. The first GWAS of ICAS, which stratifies subpopulations within the ICAS cases with distinct clinical features, revealed that *RNF213* rs112735431 was the most significant variant associated with ICAS. Thus, *RNF213* rs112735431 shows potential as an important clinical biomarker that characterizes pleiotropic risk in various vascular diseases, such as blood pressure and angina, thereby facilitating personalized medicine for systemic vascular diseases in East Asian populations.

Keywords Intracranial artery stenosis · Genome-wide association study · *RNF213* · Phenome-wide association study · Hypertension · Angina

Introduction

Intracranial artery stenosis (ICAS) is a common cause of ischemic stroke worldwide [1, 2]. ICAS is caused by atherosclerotic changes in the background of lifestyle-related diseases such as hypertension, diabetes mellitus, and dyslipidemia [3–5]. Aggressive medical treatments, such as antiplatelet therapy, which are commonly used to prevent cerebral infarction, especially severe stenosis, pose a high stroke risk [6]. Therefore, pathophysiological mechanisms underlying ICAS need elucidation. ICAS is more common in Hispanic, Black, and East Asian populations, including Japanese, than in European populations [7, 8]. This implies the involvement of genetic factors in the prevalence of ICAS [9].

Genome-wide association studies (GWAS), which were performed in an attempt to identify genetic factors that could be linked to stroke, have revealed numerous stroke-related genetic factors [10, 11]. Ischemic stroke is divisible into several subtypes, and previous GWAS which investigated ischemic stroke conformed to the TOAST classification (classical Trial of ORG10172 in Acute Stroke Treatment classification). These subtypes include large-artery stroke

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(LAS) caused by atherosclerosis of the external and major intracranial arteries [12]. Cerebral infarction caused by ICAS was included in LAS, and, to the best of our knowledge, no GWAS specific to ICAS has been reported to date [10, 11].

Herein, we conducted the first GWAS of ICAS using DNA samples from Japanese subjects to identify genetic factors associated with ICAS. We also analyzed the associations between genetic factors related to ICAS and its clinical features. Furthermore, we performed a phenome-wide association study (PheWAS) of the top variant of ICAS revealed by GWAS to investigate the association between ICAS-associated variants and other systemic diseases.

Materials and Methods

Study Population

We recruited ICAS patients and healthy volunteers from the Japanese population at the University of Tokyo or from related institutions (Kanto Neurosurgical Hospital, Teraoka Memorial Hospital, and Osaka University Graduate School of Medicine) between November 2011 and March 2019. ICAS diagnosis was based mainly on magnetic resonance angiography (MRA) (1.5 Tesla or 3 Tesla) or digital subtraction angiography (DSA). MRA and DSA images were reviewed by two or more physicians, including at least one neurosurgeon and one radiologist. The criteria for ICAS were as follows: (i) stenosis or occlusion in major intracranial arteries on MRA or DSA; (ii) age > 40 years; (iii) one or more risk factors for atherosclerosis, such as hypertension, diabetes mellitus, dyslipidemia, coronary artery disease, arteriosclerosis obliterans, or a history of smoking; and (iv) no signs of cardiac embolism, dissection, vasculitis, or moyamoya disease (MMD) or quasi MMD. Patients with ICAS included those with both symptomatic and asymptomatic ICAS. Symptomatic ICAS cases were defined as those presenting with cerebral infarction or transient ischemic attack in the area of the stenotic artery, while asymptomatic ICAS cases included those exhibiting unrelated symptoms, such as headache and dizziness or a medical checkup. The following information was extracted from medical records: age; sex; family history of stroke; hypertension; diabetes mellitus; dyslipidemia; coronary artery disease; arteriosclerosis obliterans; and smoking history. Hypertension was defined as a systolic blood pressure ≥ 140 mmHg and a diastolic blood pressure ≥ 90 mmHg at the first visit or receipt of antihypertensive agents. Diabetes mellitus was defined as an HbA1c level ≥ 6.5% or being on anti-diabetic medications. Dyslipidemia was defined as high-density lipoprotein cholesterol level < 40 mg/dL, low-density lipoprotein cholesterol level ≥ 140 mg/dL, triglyceride level ≥ 150 mg/dL, or receiving lipid-lowering treatment. We collected information on the degree and sites of stenosis via MRA or DSA images. The degree of stenosis in each patient shown on MRA or DSA images was calculated using WASID Trail criteria [13]. Controls were recruited from the University of Tokyo or related institutions. No control participants had a history of cerebrovascular disease or suspected stroke symptoms, such as hemiplegia and dysarthria. Most control patients underwent MRA to confirm the absence of cerebrovascular lesions.

Genotyping and SNP Imputation

Peripheral blood samples were obtained from all the enrolled patients. Genomic DNA was extracted from the peripheral blood leukocytes at SRL Inc. (Tachikawa, Tokyo, Japan) using a DNA extraction kit (Talent Srl, Trieste, Italy). The samples were genotyped using an Infinium Asian Screening Array Chip (Illumina, CA, USA). The genotyping array was built using an East Asian reference panel, including whole-genome sequences, which enabled effective genotyping of East Asian populations. Individuals with a low genotyping call rate were excluded as part of a quality control procedure (see Methods; Supplementary Appendix). Genotype data were imputed against reference haplotypes of the 1000 Genomes Project Phase 3 version 5 genotype ($n = 2{,}504$) and Japanese whole-genome sequencing data ($n = 1037$) [14, 15].

Genome-Wide Association Study

The associations between single nucleotide polymorphisms (SNPs) and the risk for ICAS were evaluated using a logistic regression model, assuming that the effects of the imputed allele dosages were addictive. The top five principal components were included as covariates. We set a genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (Supplementary Methods).

Association Between Stroke Risk Loci and ICAS

To assess whether stroke risk loci were associated with ICAS risk, we focused on the 32 stroke risk variants described in the MEGASTROKE study [11] and examined the association statistics of these variants in our ICAS GWAS. The Kolmogorov–Smirnov test was performed to evaluate the inflation of *P*-values in stroke risk variants.

Analysis of the Clinical Feature of ICAS

For ICAS, we compared the clinical features of the two groups with or without the top variant revealed by GWAS of ICAS in the univariate and multivariate analyses using logistic regression analysis. Statistical significance was set

at $P < 0.05$. Multivariate analysis was performed using factors with a P-value ≤ 0.05 in univariate analysis. Analyses were performed using JMP® 14 (SAS Institute Inc., Cary, NC, USA).

Phenome-Wide Association Study

PheWAS of the top variant identified by the GWAS of ICAS was performed using clinical information from the BioBank Japan Project data (46 diseases and 60 quantitative traits, $n > 150{,}000$) [16, 17]. We evaluated the association between *RNF213* rs112735431 and risk for 46 diseases, using a logistic regression model, and normalized values of the 60 quantitative traits via a linear regression model [18, 19]. A phenome-wide significance threshold of $P < 4.7 \times 10^{-4}$ was set by applying the Bonferroni correction to the number of phenotypes (Supplementary Methods).

Results

Study Population

Overall, 408 ICAS patients and 349 healthy controls from the Japanese population were included. The sample quality control process resulted in 388 cases and 327 controls (Supplementary Methods). Case and control characteristics are listed (Table 1). The most common site of stenosis was the middle cerebral artery, which accounted for 67.5% of all cases of ICAS. Representative MRA images of ICAS are shown in Fig. 1.

Table 1 Clinical characteristics of the patients at baseline

	ICAS group	Control group
Number	388	327
Age (mean ± SD) (range)	63.8 ± 11.7 (40–94)	46.2 ± 21.4 (16–91)
Female	167 (43.0%)	157 (48.0%)
Stenosis site		
Middle cerebral artery	262 (67.5%)	N.A
Internal carotid artery	96 (24.7%)	N.A
Vertebral artery	20 (5.2%)	N.A
Basilar artery	5 (1.3%)	N.A
Others	5 (1.3%)	N.A

GWAS of ICAS

A signal corresponding to the genome-wide significance level was observed on chromosome 17, with *RNF213* rs112735431 (c.14429G > A, p.Arg4810Lys) showing the strongest association (odds ratio (OR), 12.3; 95% CI, 5.5 to 27.5; $P = 7.8 \times 10^{-10}$) (Fig. 2). The frequency of allele A in ICAS cases (0.12) was higher than that in controls (0.01). Although we performed a conditional analysis by adding the imputed dosage of *RNF213* rs112735431 to the covariate in order to detect variants showing associations that were independent of *RNF213* rs112735431, no signal indicating an association satisfying the genome-wide significance level was detected (Supplemental Fig. 1). These results suggest that rs112735431 accounts for a majority of ICAS-related genetic risk in the *RNF213* region. Details of associations shown by *RNF213* rs112735431 are shown (Supplemental Table 1).

Association Between Stroke Risk Loci and ICAS

Of the 32 risk variants described in the MEGASTROKE study [11], 25 were available for our ICAS GWAS. These 25 variants exhibited an inflation of test statistics (Supplemental Fig. 2; $P = 0.023$, Kolmogorov–Smirnov test). In particular, three risk variants (rs2107595 at *HDAC9/TWIST1* 7p21, rs17612742 at *EDNRA* 4q31, and rs6825454 at *FGA* 4q31) were nominally associated with ICAS ($P < 0.05$) with the risk increasing allele shared with stroke (Supplemental Table 2). Notably, in the MEGASTROKE study, rs2107595 and rs17612742 showed the strongest associations with LAS compared with other stroke phenotypes.

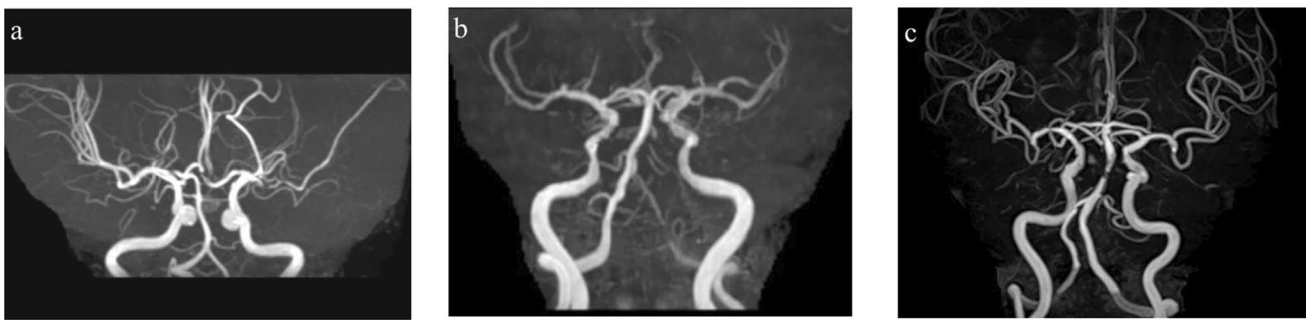

Fig. 1 Representative magnetic resonance angiography images of intracranial artery stenosis. **a** A 68-year-old female with left middle cerebral artery stenosis. **b** A 71-year-old male with vertebral artery stenosis. **c** A 73-year-old male with basilar artery stenosis

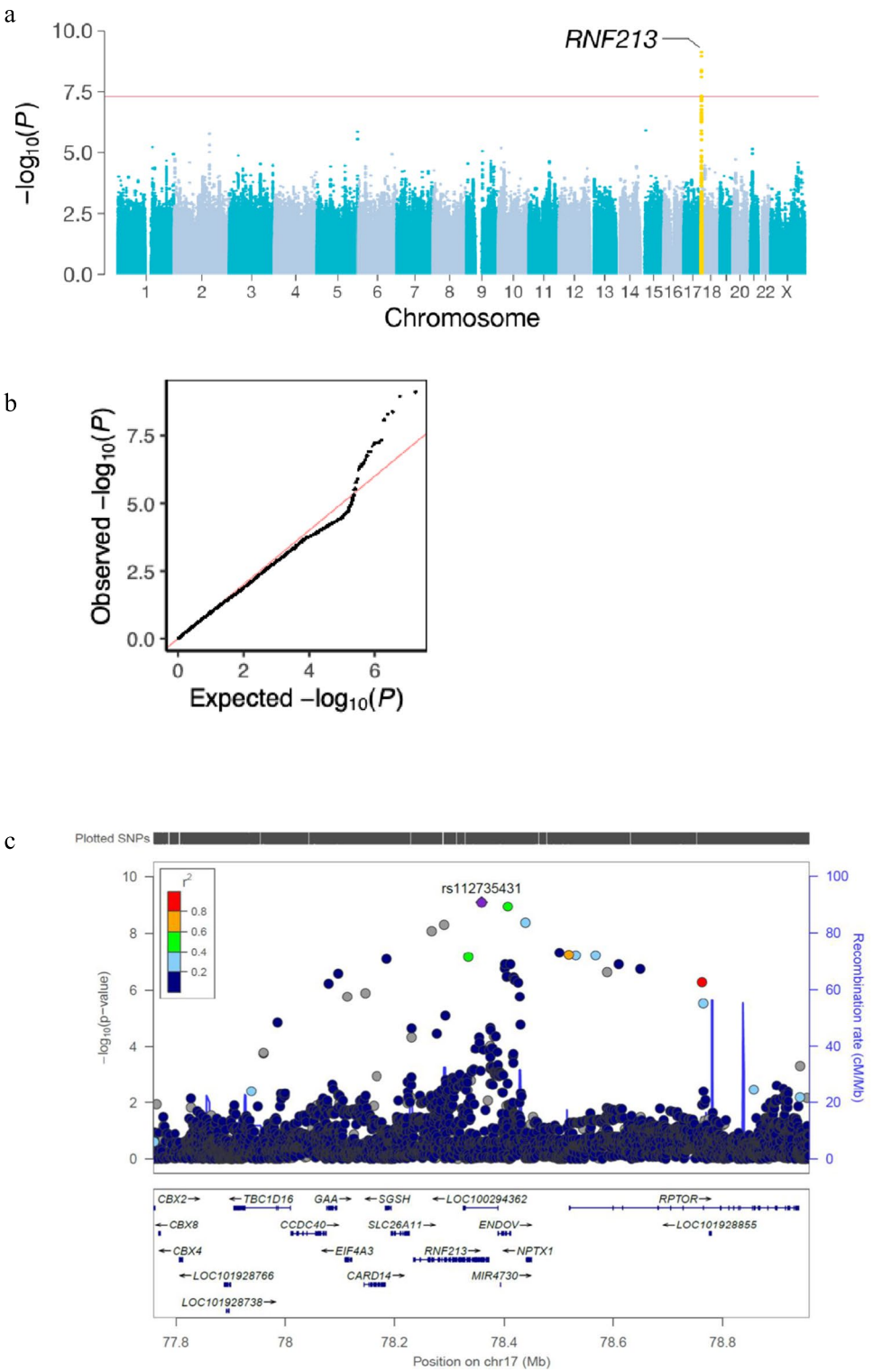

Fig. 2 Genome-wide association study of intracranial artery stenosis. A Manhattan plot of the results of a genome-wide association study (GWAS) of intracranial artery stenosis (ICAS) (**a**). A Manhattan plot showing $-\log_{10}(P)$ of the genome-wide single-marker association test of ICAS in the Japanese population. The red horizontal line represents the genome-wide significance threshold of $P=5.0\times10.^{-8}$. The locus that satisfied the genome-wide significance threshold is colored in yellow. Q-Q plot of ICAS GWAS *P*-values (**b**). The *x*-axis indicates expected $-\log_{10}(P)$ under the null hypothesis, and the *y*-axis indicates the observed $-\log_{10}(P)$. The straight red line indicates the distribution of markers under the null hypothesis. The regional association plot for the lead variant, rs112735431 (**c**). The association of rs112735431 with other single markers in the region was plotted using $-\log_{10}(P)$ (left *y*-axis). The lead variant is represented in purple, and linkage disequilibrium (*r*2) of other markers to the lead variant is shown by a color gradient (based on 1000 Genomes Nov. 2014 ASN). Blue peaks indicate recombination rates (right *y*-axis)

Analysis of the Clinical Feature in ICAS with *RNF213* rs112735431

GWAS of ICAS showed that *RNF213* rs112735431 was the only ICAS-related variant that met GWAS criteria. The *RNF213* rs112735431 variant was detected in 22.9% (89/388; 88 heterozygotes and 1 homozygote) of ICAS patients and stratified analysis within ICAS cases demonstrated distinct clinical features between those with and without this risk allele. In the univariate model, the patients carrying the risk allele were significantly younger than those who were not ($P<0.001$). Cases carrying the risk allele had

a significantly higher family history of stroke ($P < 0.001$) than those who were not. The rate of symptomatic cases was significantly lower in cases carrying the risk allele than in those who were not ($P = 0.03$). For stroke risk factors, cases that were carrying the risk allele showed significantly lower diabetes mellitus ($P = 0.01$) and dyslipidemia ($P = 0.008$) rates, compared to those who were not. For anatomical factors, the cases carrying the risk allele were associated with higher rates of severe stenosis ($P = 0.007$), anterior circulation ($P = 0.04$), proximal stenosis ($P = 0.003$), and posterior cerebral artery involvement cases ($P = 0.01$) compared to those who were not (Table 2). In the multivariate model, family history of stroke ($P = 0.007$, odds ratio (OR) 2.26, 95% confidence interval (CI) 1.25–4.08), diabetes mellitus ($P = 0.02$, OR 0.43, 95% CI 0.20–0.90), and posterior cerebral artery involvement ($P = 0.001$, OR 2.86, 95% CI 1.50–5.45) were associated with the risk allele (Table 2).

PheWAS

To explore the pleiotropic effect of *RNF213* rs112735431 on a variety of human complex traits, we performed a PheWAS of *RNF213* rs112735431, assuming that it exerts clinical effects on the Japanese population. The PheWAS indicated that pulse pressure ($P = 9.9 \times 10^{-25}$), systolic blood pressure ($P = 2.2 \times 10^{-19}$), mean blood pressure ($P = 1.2 \times 10^{-8}$), stable angina (OR, 1.6; 95% CI, 1.4 to 1.8; $P = 9.1 \times 10^{-12}$), and unstable angina (OR, 1.6; 95% CI, 1.3 to 2.0; $P = 1.3 \times 10^{-5}$) were significantly associated with *RNF213* rs112735431 (Table 3; Fig. 3; Supplemental Table 3).

Discussion

We performed the first GWAS of ICAS, a major cause of ischemic stroke. *RNF213* rs112735431 was identified as the most significant variant associated with ICAS. This variant has not been previously identified in a large consortium stroke GWAS [11]. Additionally, we performed the first PheWAS of *RNF213* rs112735431, which indicated that it was significantly associated with systemic vascular diseases, such as high blood pressure and angina.

RNF213 increases the susceptibility of East Asian populations to moyamoya disease (MMD), an idiopathic cerebrovascular malady and a rare cause of stroke, which is characterized by progressive stenosis of intracranial arteries around the terminal portion of the internal carotid artery, with compensatory development of a fine collateral network at the base of the brain (moyamoya vessels) [20, 21]. Several single-variant association studies have indicated that *RNF213* rs112735431 is associated with ICAS [22, 23]. Moreover, *RNF213* rs112735431 is a proven susceptibility gene for non-cardioembolic cerebral infarction in the Japanese population [24]. Our study revealed that *RNF213* rs112735431 was the only ICAS-related variant that met GWAS criteria. According to previous studies, *RNF213* rs112735431 is relatively common in East Asian

Table 2 Comparisons of the clinical characteristics of ICAS patients with and without the *RNF213* rs112735431 risk allele

	All patients	Cases with the risk allele	Cases without the risk allele	Univariate			Multivariate		
				OR	*P* Value	95% CI	OR	*P* Value	95% CI
Age < 64	149 (38.4%)	48 (53.9%)	101 (33.8%)	2.30	0.0007*	1.42–3.71	1.74	0.06	0.99–3.04
Female	167 (43.0%)	46 (51.6%)	121 (40.4%)	1.57	0.06	0.98–2.53			
Family history of stroke	90 (23.2%)	33 (37.5%)	57 (19.1%)	2.55	0.0004*	1.51–4.28	2.26	0.007*	1.25–4.08
Symptomatic cases	228 (58.8%)	43 (48.3%)	185 (61.9%)	0.58	0.03*	0.36–0.94	0.58	0.06	0.33–1.01
Hypertension	291 (75.1%)	60 (68.1%)	231 (77.2%)	0.63	0.08	0.37–1.07			
Diabetes mellitus	99 (25.5%)	13 (14.7%)	86 (28.8%)	0.43	0.01*	0.23–0.81	0.43	0.02*	0.20–0.90
Dyslipidemia	176 (45.5%)	29 (32.9%)	147 (49.1%)	0.51	0.008*	0.31–0.84	0.59	0.06	0.33–1.03
Coronary artery disease	37 (9.5%)	12 (13.6%)	25 (8.3%)	1.73	0.14	0.83–3.60			
Arteriosclerosis obliterans	7 (1.8%)	3 (3.4%)	4 (1.3%)	2.60	0.21	0.57–11.9			
Smoking history	102 (26.3%)	18 (20.4%)	84 (28.1%)	0.66	0.15	0.37–1.17			
Severe stenosis	218 (58.9%)	59 (71.9%)	159 (55.2%)	2.08	0.007*	1.22–3.55	1.66	0.09	0.92–2.99
Anterior circulation	357 (92.7%)	86 (97.7%)	271 (91.2%)	4.13	0.04*	0.96–17.7	2.05	0.34	0.42–10.0
Proximal stenosis	184 (50.0%)	53 (64.6%)	131 (45.8%)	2.16	0.003*	1.30–3.60	1.66	0.05	0.92–3.00
Bilateral stenosis	164 (44.4%)	41 (49.4%)	123 (43.0%)	1.29	0.30	0.79–2.11			
Multiple lesions	218 (59.1%)	55 (66.2%)	163 (56.9%)	1.48	0.13	0.89–2.47			
Posterior cerebral artery involvement	70 (18.9%)	24 (28.9%)	46 (16.1%)	2.12	0.01*	1.20–3.75	2.86	0.001*	1.50–5.45

*Statistical significance: $P < 0.05$, Fisher's exact test, Wilcoxon rank sum test; *OR*, odds ratio; *95% CI*, 95% confidence interval

Table 3 PheWAS of *RNF213* rs112735431

	Category	No. All	No. Case	No. Control	Freq. All	Freq. Case	Freq. Control	Odds ratio (95%CI)	Beta	SE	*P* value
Disease											
Stable angina	Metabolic and cardiovascular	76,295	14,404	61,891	0.011	0.014	0.010	1.56 (1.38–1.78)	-	-	9.1×10^{-12}
Unstable angina	Metabolic and cardiovascular	66,079	4188	61,891	0.010	0.014	0.010	1.61 (1.30–2.00)	-	-	1.3×10^{-5}
Quantitative trait											
Pulse pressure	Blood pressure	131,061	-	-	0.010	-	-	-	0.228	0.022	9.9×10^{-25}
Systolic blood pressure	Blood pressure	131,402	-	-	0.010	-	-	-	0.201	0.022	2.2×10^{-19}
Mean arterial pressure	Blood pressure	131,287	-	-	0.010	-	-	-	0.128	0.022	1.2×10^{-8}

Freq. All: frequency of the effect allele (A allele) in all samples, Freq. Case: Frequency of the effect allele in the case group, Freq. control: frequency of the effect allele in the control group, Beta: effect size of the effect allele, SE: standard error of the effect size

populations but very rare in non-East Asian populations [24]. A study based on the Genome Aggregation Database reported a variant carrier frequency (per 1000) of 7.75 in East Asian and 0.85 in South Asian populations, compared with 0.07 in the Latino/Admixed American population and none in African/African-American, Ashkenazi Jewish, Finnish European, or non-Finnish European populations [25]. Thus, *RNF213* rs112735431 may be the singular factor that explains the higher prevalence of ICAS in the East Asian population. This variant was not previously identified in the MEGASTROKE study [11]. The Asian screening array used for genotyping in this study included rs112735431 as a target SNP, while the arrays used for genotyping in previous GWAS conducted mainly in European populations did not. Therefore, genotyping of rs112735431 in this study was completed without imputation. We believe this contributed directly to the illustration of the association between ICAS and rs112735431.

As for variants other than rs112735431, we assessed whether previously reported stroke risk loci described in the MEGASTROKE study [11] were associated with ICAS risk. Three stroke risk variants (rs2107595 at *HDAC9/TWIST1* 7p21, rs17612742 at *EDNRA* 4q31, and rs6825454 at *FGA* 4q31) were nominally associated with ICAS, although they did not meet the GWAS criteria. Notably, in the MEGASTROKE study, rs2107595 and rs17612742 showed the strongest associations with LAS compared with other stroke phenotypes. These results suggest that ICAS shares genetic etiology with stroke, especially LAS. Furthermore, rs2107595 at 7p21(*HDAC9/TWIST1*) showed the strongest association with ICAS among the stroke variants, where *HDAC9* reportedly regulates arterial inflammation and atherosclerotic plaque vulnerability [26]. Thus, in addition to *RNF213*, *HDAC9* may also be another important genetic factor in ICAS.

Differences between the clinical features of ICAS patients with or without the risk allele, *RNF213* rs112735431, were suggestive. Patients carrying the risk allele had significantly lower rates of diabetes mellitus. This indicates that the risk factors for developing stenosis may differ between those cases with and without the risk allele. This was also substantiated by a previous report [27]. Individuals carrying the risk allele, *RNF213* rs112735431, are more likely to develop ICAS, even in the absence of diabetes mellitus. It is possible that vascular endothelial function is less associated with glucose metabolism in cases with *RNF213* rs112735431. However, further functional analysis of *RNF213* may be essential to confirm this hypothesis.

Anatomically, the proportion of posterior cerebral artery involvement in cases who were carrying the risk allele was significantly higher than that in cases who were not. Clinical differences between ICAS cases with or without the risk allele, *RNF213* rs112735431, indicated that the mechanism

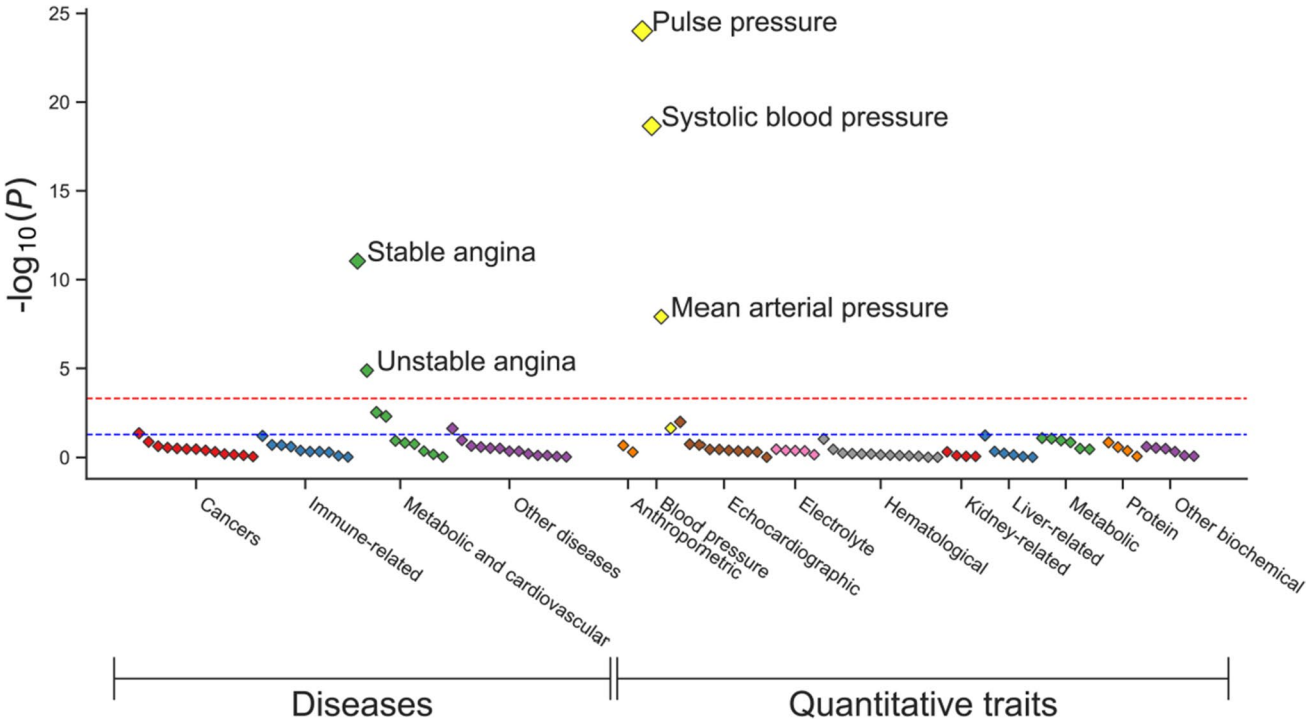

Fig. 3 PheWAS of *RNF213* rs112735431. A Manhattan plot of results of the PheWAS of rs112735431 in the Japanese population. The dotted red horizontal line represents the phenome-wide significance threshold of $P = 4.7 \times 10^{-4}$ via the application of Bonferroni correction on the number of phenotypes. The dotted blue horizontal line represents a P-value of $5.0 \times 10.^{-2}$. Phenotypes meeting the phenome-wide significance thresholds were annotated

underlying vascular stenosis might differ in each group. ICAS patients with or without *RNF213* rs112735431 may therefore require different treatment and risk management strategies. In this study, the ICAS group carrying the risk allele of *RNF213* rs112735431 was younger and had a family history of stroke and proximal stenosis, suggesting that this group carried some factors in common with MMD.

MMD manifests as an idiopathic abnormal progressive stenosis of intracranial arteries, which often develops in younger patients with a family history of stroke and proximal stenosis. The strict diagnostic criteria for MMD [28, 29] that enable a definitive diagnosis of MMD are as follows: (i) a progressive stenotic lesion at the terminal of the internal carotid artery and compensatory development of a fine collateral network at the base of the brain and (ii) the absence of underlying diseases that cause arterial stenosis. However, in actual clinical settings, it may be difficult to clearly diagnose MMD, especially in the elderly, in whom some arteriosclerotic changes may occur spontaneously in the intracranial arteries. Therefore, it is difficult to determine whether intracranial artery stenotic lesions in the elderly are actually due to MMD or arteriosclerosis. The present study suggests that even though ICAS is clinically arteriosclerotic, there are several diseases that are not only arteriosclerotic but also have a gene variant common to MMD and share a common spectrum with MMD [30]. Thus, diagnostic imaging and genetic testing assume greater importance in ICAS and MMD.

Predictors of the impact of the missense variants on protein function (in silico analysis) indicate that *RNF213* rs112735431 (c.14429G > A, p.Arg4810Lys) is benign in PolyPhen-2 (https://genetics.bwh.harvard.edu/pph2/) (version 2.2.2) and tolerated in SIFT (https://sift.jcvi.org/) (version 1.1.3). The combined annotation-dependent depletion (CADD) score was 12.8 (https://cadd.gs.washington.edu/) (version 1.4). Advances in molecular functional analysis of *RNF213* have allowed various pathways related to *RNF213* expression to be identified [31–35]. *RNF213* encodes a large 591 kDa protein with an E3 module and an AAA + ATPase and RING finger domains [20, 21]. *RNF213* rs112735431, which lies in the E3 module, may lead to E3 ligase dysfunction and thereby contribute to the development of MMD [36]. NFAT1 and filamin A have been reported to be substrates for *RNF213* E3 ubiquitin ligase [31]. *RNF213* induces the degradation of NFAT1 and filamin A, attenuates non-canonical Wnt/calcium signaling, and regulates vascular stability and pruning. Loss of RNF213 function suppresses vascular regression and causes overgrowth of blood vessels [31]. An in vitro study indicated that angiogenesis is reduced in vascular endothelial cells derived from iPSCs of MMD

patients carrying *RNF213* rs112735431 [37]. In vitro assays using *RNF213* knocked-out brain endothelial cells displayed clear morphological changes and increased blood–brain barrier permeability [38]. These findings suggest that *RNF213* dysfunction may cause abnormalities in vascular endothelial function. Recent reports have indicated that *RNF213* may be associated with immune function. *RNF213* reportedly plays an antibacterial role by ubiquitinating lipopolysaccharides on the cell surface of bacteria and inducing autophagy [39]. *RNF213* plays a critical role in antigen uptake, processing, and presentation [40]. In an in vivo study, knockdown of *RNF213* in zebrafish caused irregular wall formation in the trunk arteries and abnormal development of craniocervical vessels [20]. However, both knock-out and knock-in of *RNF213* in mice did not result in intracranial artery stenosis [41, 42]. By contrast, induced cerebral hypoperfusion in *RNF213* vascular endothelial cell-specific *RNF213* mutant transgenic mice caused impaired angiogenesis [43]. Thus, multiple secondary factors, such as ischemia, inflammation, and infection, in addition to *RNF213* rs112735431, may contribute to the onset of stenosis [41]. In summary, the detailed mechanism via which *RNF213* rs112735431 causes intracranial artery stenosis remains unclear. Therefore, elucidation of the molecular biological functions of *RNF213* is felt to be indispensable for establishing appropriate therapeutic techniques against ICAS with *RNF213* rs112735431.

RNF213 rs112735431 is reportedly associated with systemic vascular diseases, such as coronary artery stenosis [44], pulmonary artery stenosis [45], pulmonary arterial hypertension [46, 47], and renal artery stenosis [30]. It is also strongly suggested that *RNF213* rs112735431 is associated with vascular stenosis. The above findings suggest that systemic vascular diseases, including ICAS with *RNF213* rs112735431, could be considered distinct entities. Because the molecular function of *RNF213* is currently unknown, we attempted to describe the relationship between *RNF213* rs112735431 and systemic disease as well as other traits, via a PheWAS that analyzed phenotypes affected by this genotype.

PheWAS showed that *RNF213* rs112735431 was associated with pulse pressure, systolic blood pressure, and mean blood pressure, as well as with stable and unstable angina. Our analysis revealed that the association between rs112735431 and systemic vascular disease exceeded PheWAS criteria, confirming that *RNF213* rs112735431 was a systemic vascular disease-related gene variant. A GWAS of blood pressure and coronary artery disease that was performed using the Biobank Japan genome cohort (which was used for PheWAS) illustrated that genomic loci, including rs112735431, were associated with diseases [19, 49]. However, rs112735431 was not directly identified as a disease-associated SNP. We surmised that this might be due to differences in the reference panel used for imputation. The inclusion of reference data derived from 1037 Japanese people in the reference panel used in this study facilitated the imputation of rs112735431 with sufficient reliability. Therefore, we were able to use a more Japanese population-specific reference panel, which had higher imputation quality and more accurate disease recognition than previously reported ones. This process may have allowed fine mapping of the related loci to be completed and the association between rs112735431 and these traits/diseases to be directly elucidated. Thus, utilization of ancestry-specific genotyping SNP arrays and imputation reference panels may help identify ancestry-specific disease-related SNPs in GWAS studies, even for other diseases.

This study may help advocate a model for precision medicine in the field of stroke management. ICAS-associated *RNF213* rs112735431 was significantly associated with hypertension, which is a well-known stroke risk factor. Hypertension and ICAS have a common genetic predisposition. Individuals carrying *RNF213* rs112735431 are more likely to have hypertension and, as a result, more likely to develop ICAS. Since hypertension is a risk factor for cerebrovascular events, controlling blood pressure may be more important for individuals carrying *RNF213* rs112735431. Thus, ischemic stroke may be prevented in persons carrying *RNF213* rs112735431 via timely therapeutic intervention using antihypertensive drugs and regular cerebrovascular MRI exams.

This study included several limitations. The population studied was relatively small for a GWAS. We did not conduct an independent cohort validation study of the results of GWAS of ICAS or the results of analysis of risk factors and clinical features of ICAS with *RNF213* rs112735431. Therefore, this study needs to be replicated in a larger population. Another limitation was that not all control patients were subjected to MRA to confirm the absence of cerebrovascular lesions, resulting in some individuals with asymptomatic ICAS in the control group going undetected. Another limitation was that an evaluation of the association between *RNF213* rs112735431 and external carotid artery lesions, aortic plaque lesions, and renal artery disease was not performed. Evaluation of systemic arterial stenosis may provide important insights and will be a future task of ours.

To the best of our knowledge, this is the first GWAS of ICAS, and it identified *RNF213* rs112735431 as the only variant linked to ICAS. Stratified analysis of ICAS cases illustrated distinct clinical features between those with and without this risk allele, suggesting that the mechanism of stenosis may vary based on the presence or absence of the risk allele. The PheWAS of *RNF213* rs112735431 revealed that this variant affected not only ICAS but also a wide range of phenotypes of systemic vascular diseases, such as blood pressure and angina. This variant may be useful in personalized medicine, with particular reference to the management

of vascular diseases, such as ICAS and angina, via regular monitoring of blood pressure in East Asian populations.

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Author Contribution Satoru Miyawaki, Yukinori Okada, and Nobuhito Saito supervised the study. Shogo Dofuku, Kyuto Sonehara, Satoru Miyawaki, and Yukinori Okada wrote the manuscript. Shogo Dofuku, Kyuto Sonehara, Saori Sakaue, K. Suzuki, Jun Hirata, Meiko Takahashi, and Yukinori Okada conducted data analysis. Shogo Dofuku, Satoru Miyawaki, Hideaki Imai, Masahiro Shimizu, Hiroki Hongo, Yuki Shinya, Kenta Ohara, Yu Teranishi, Atsushi Okano, Hideaki Ono, Hirofumi Nakatomi, Akira Teraoka, Kenichi Yamamoto, Yuichi Maeda, Takuro Nii, Toshihiro Kishikawa, Ken Suzuki, Koichi Matsuda, and Atsushi Kumanogoh collected the samples. Ken Suzuki, Jun Hirata, Meiko Takahashi, and Fumihiko Matsuda constructed the data. All authors read and approved the final manuscript.

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Data Availability The data supporting the findings of this study are available from the corresponding author upon reasonable request from any investigator.

Code Availability Not applicable.

Declarations

Ethics Approval This study was approved by the ethical committee of the University of Tokyo (approval number G10026; approval date, September 12, 2011) and adhered to the principles of the Declaration of Helsinki.

Informed Consent All the participants provided written informed consent with documents approved by the institutional review board of each participating hospital or institution.

Conflict of Interest The authors declare no competing interests.

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