RNF213 p.R4810K Variant Carriers with Intracranial Arterial Stenosis Have a Low Atherosclerotic Burden

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Aim: The ring finger protein 213 gene (*RNF213*) p.R4810K variant is a major susceptibility gene for intracranial arterial stenosis in East Asia. We hypothesized that if intracranial arterial stenosis is induced by a non-atherosclerotic mechanism similar to moyamoya disease, the patients with *RNF213* p.R4810K variant may have a lower cumulative atherosclerotic burden than the non-carriers.

Methods: A total of 112 participants with intracranial arterial stenosis were enrolled in this multicenter cross-sectional study. We compared the prevalence of atherosclerotic risk factors and three different cardiovascular risk scores (Essen Stroke Risk Score, Framingham Risk Score, and Suita Risk Score) between the *RNF213* p.R4810K variant carriers and non-carriers. Patients with moyamoya disease were excluded from the study.

Results: The *RNF213* p.R4810K variant carriers were younger than the non-carriers ($P<0.001$). The prevalence of each atherosclerotic risk factor was not significant, but it tended to be lower in the variant carriers. The Essen Stroke Risk Score (carriers: 2.3 ± 1.5 vs. non-carriers: 2.9 ± 1.5, $P=0.047$), Framingham Risk Score (10.7 ± 6.4 vs. 15.3 ± 6.2, $P=0.001$), and Suita Risk Score (35.4 ± 15.8 vs. 48.7 ± 15.2, $P<0.001$) were significantly lower in the variant carriers. Among the three risk scores, the Suita score showed the highest predictive accuracy for the variant carriers.

Conclusions: *RNF213* p.R4810K variant carriers have a lower cumulative atherosclerotic burden than non-carriers among patients with intracranial arterial stenosis. New therapeutic approaches beyond the standard management of atherosclerotic risk factors are required to prevent the development of intracranial arterial stenosis.

Introduction

Intracranial arterial stenosis accounts for 30%–50% of the causes of ischemic stroke in East Asia[1, 2]. Recently, the p.R4810K variant of the ring finger protein 213 gene (*RNF213*), which was identified as a susceptibility gene for moyamoya disease[3, 4], was found to be a strong risk factor for intracranial arterial stenosis[5, 6] and ischemic stroke[7]. Approximately 22%–50% of patients with intracranial arterial stenosis reportedly have this variant[5, 6], which is much higher than the prevalence in the general population

$(1.3\%–2.0\%)$[7, 8].

The mechanisms by which this variant causes intracranial arterial stenosis have not yet been elucidated, but it has been reported that the RNF213 protein is involved in caveolin-1-mediated endothelial cell function and angiogenesis[9] and the variant carriers have smaller diameters of intracranial arteries, suggesting negative remodeling[10, 11]. On the other hand, positive remodeling is known to occur in response to atherosclerotic plaque formation[12]. These findings suggest that intracranial arterial stenosis, generally thought to be caused by atherosclerosis, occurs via a different mechanism in variant carriers. We hypothesized that if intracranial arterial stenosis is induced by a non-atherosclerotic mechanism, *RNF213* p.R4810K variant carriers may have a lower cumulative atherosclerotic burden than non-carriers.

Aim

In this study, we compared the prevalence of atherosclerotic risk factors between *RNF213* p.R4810K variant carriers and non-carriers among patients with intracranial arterial stenosis. In addition, the cumulative atherosclerotic burden was estimated using three different cardiovascular risk scores and compared between the two groups. Because variant carriers have a higher rate of recurrent stroke[13], it is clinically important to identify the variant carriers in patients with intracranial arterial stenosis. Therefore, we examined the optimal cut-off values of each risk score for predicting the *RNF213* variant carriers.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design and Participants

This multicenter cross-sectional observational study was conducted at three medical centers in Kansai district of Japan: Osaka University Hospital, Osaka; National Cerebral and Cardiovascular Center, Osaka; and Kobe City Medical Center General Hospital, Hyogo. Participants were recruited from these three medical centers. Written informed consent was obtained from all participants. This study was approved by the Ethics Committee for Clinical Research of Osaka University (approval number: 845) and each medical center. Patients who met the following criteria were included: (1) patients aged over 20 years; (2) patients with intracranial arterial stenosis defined as a diameter reduction of ≥ 50% or occlusion

in the intracranial internal carotid artery, M1–M2 segment of the middle cerebral artery, A1–A2 segment of the anterior cerebral artery, P1–P2 segment of the posterior cerebral artery, or the basilar artery; and (3) available *RNF213* genetic test. We excluded patients diagnosed with intracranial arterial occlusion due to cardiogenic embolism or moyamoya disease according to the Japanese guidelines for moyamoya disease[14].

At Osaka University Hospital and Kobe City Medical Center General Hospital, 124 candidates with intracranial arterial stenosis were recruited from a prospectively collected database between January 2019 and February 2021. Among them, 13 patients diagnosed with moyamoya disease were excluded. Informed consent was obtained from 68 patients, and 2 were excluded because their blood samples were not available. At the National Cerebral and Cardiovascular Center, 4802 patients were admitted with acute ischemic stroke between January 2011 and December 2018. After providing their informed consent, 68 patients with intracranial arterial stenosis underwent *RNF213* genotyping, and 22 patients diagnosed with cardioembolic stroke or moyamoya disease were excluded. Finally, 112 patients with intracranial arterial stenosis were enrolled in this study. Among the 112 enrolled patients, 11 (9.8%) were included in the previous study[7].

RNF213 Genotyping Examination

Peripheral blood samples were obtained from all enrolled patients. Genotyping of *RNF213* p.R4810K was performed using a fully automated gene analysis system (GTS-7000; Shimadzu Corporation, Kyoto, Japan or LightCycler 96 system; Roche, Basel, Switzerland). These systems allow the direct detection of single-nucleotide polymorphisms from 1 μL of whole blood samples using real-time polymerase chain reaction[15]. The primer sequences used in the current analysis were 5'-TTCCAGAACGTCCAGCAAGT-3' (forward) and 5'-ACAGTCCTGGTCCTGTCAGA-3' (reverse). The probe sets used were 5'-CTCCATCAGAGGCTTCCT-3' and 5'-CTCCATCAAAGGCTTCCT-3'. As a result of *RNF213* p.R4810K variant genotype analysis, the GA and AA variants for p.R4810K were defined as variant carriers and the wild-type homozygous (GG) as non-carriers.

Imaging Analysis

The diagnosis of intracranial arterial stenosis was based on magnetic resonance angiography or computed tomography angiography findings. If a patient underwent more than one examination, the initial examination was used for the analysis. All

images were evaluated by an experienced neurologist (MO) who was blinded to the clinical information.

Estimation of Cumulative Atherosclerotic Burden

Patient characteristics, including age, sex, presence of atherosclerotic risk factors, history of cerebrovascular and cardiovascular diseases, blood pressure values, and laboratory data at the time of initial evaluation were extracted from a prospectively collected database and electronic medical records of each medical center. To estimate the cumulative arteriosclerotic burden, we calculated three different cardiovascular risk scores for each individual: 1) Essen Stroke Risk Score[16], 2) Framingham Risk Score[17], and 3) Suita Risk Score[18], as described in previous papers.

In brief, the Essen Stroke Risk Score is a tool for predicting the recurrence rate of stroke and combined cardiovascular events within 1 year, derived from a data subset from the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events trial[19]. The score is calculated based on the presence or absence of eight risk factors (0–9 points): age, hypertension, diabetes mellitus, previous myocardial infarction, other cardiovascular disease, current smoking, peripheral arterial disease, and additional transient ischemic attack or ischemic stroke[16]. The Framingham Risk Score is one of the most standard tools for assessing the 10-year risk of cardiovascular disease. The score is calculated as a function of age, sex, total cholesterol, high-density lipoprotein (HDL) cholesterol, systolic blood pressure, antihypertensive use, diabetes, and smoking habits (–5 to 31 points)[17]. The Suita Risk Score is a new prediction algorithm for cardiovascular disease within 10 years based on a large urban cohort study in Japan[20], which is calculated based on the variables in the Framingham Risk Score (age, sex, total cholesterol, HDL cholesterol, blood pressure, diabetes, and smoking habit) and the severity of chronic kidney disease according to the estimated glomerular filtration rate (1–104 points)[18]. Because data pertaining to the serum HDL cholesterol level and diastolic blood pressure were not available in one patient for each variable, mean imputation was used to calculate the Framingham Risk Score and Suita Risk Score.

Statistical Analysis

Categorical variables are presented as numbers and percentages and compared using the Chi-squared test. Continuous variables are presented as mean ± standard deviation and compared using the *t*-test. Multivariate logistic regression analyses were used to calculate the adjusted odds ratios (ORs) and 95% confidence intervals (CIs). Associations of atherosclerotic risk scores with the *RNF213* p.R4810K variant were analyzed by *t*-test. For clinical application, cut-off values for predicting the p.R4810K variant were determined from the receiver operating characteristic (ROC) curve using the Youden index. Statistical significance was defined as a *P*-value <0.05. A Bonferroni correction was applied for multiple comparisons in ROC analyses, with significance set at $P < 0.016$ (0.05/3). All statistical analyses were performed using JMP Pro version 16.0.0 (SAS Institute Inc. Cary, NC, USA).

Results

Patient Characteristics and *RNF213* Variant

The mean age of the 112 participants was 62.0 ± 17.6 years, and 48% were female. No patient was homozygous for the AA genotype for p.R4810K, 30 (27%) were heterozygous for the GA genotype, and 82 (73%) were wild-type. **Table 1** shows the characteristics of the carriers and non-carriers. The *RNF213* p.R4810K variant carriers were 11.9 years younger than the non-carriers ($P < 0.001$). The prevalence of atherosclerotic risk factors, such as hypertension, dyslipidemia, diabetes, and smoking habits, was not significantly different between the carriers and non-carriers. After adjusting for age and sex, there were no significant differences in the prevalence of atherosclerotic risk factors between the carriers and non-carriers. Similarly, blood pressure values, serum cholesterol levels, and estimated glomerular filtration rates were not significantly different between the two groups.

As shown in **Table 1**, all p.R4810K carriers and 96% of non-carriers had intracranial arterial stenosis or occlusion in the anterior circulation, whereas 20% of carriers and 24% of non-carriers had stenosis or occlusion in the posterior circulation. In comparison with the non-carriers, the variant carriers had a significantly higher prevalence of multiple intracranial arterial stenosis or occlusion (OR: 2.70, 95% CI: 1.14–6.41, $P = 0.022$). This significance was maintained after adjusting for age and sex.
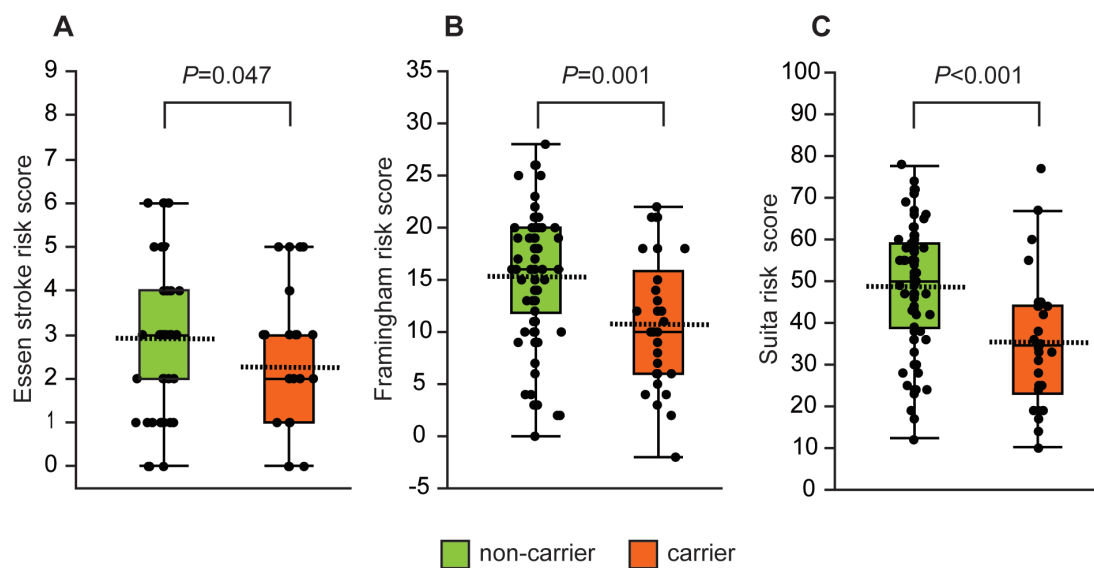
Cumulative Atherosclerotic Burden and RNF213 Variant

Fig. 1 shows the distribution of the three different cardiovascular risk scores for p.R4810K carriers and non-carriers. Essen Stroke Risk Scores were significantly lower in carriers than in non-carriers (2.3 ± 1.5 vs. 2.9 ± 1.5, $P = 0.047$). Similarly, Framingham Risk Scores and Suita Risk Scores were significantly lower in carriers (10.7 ± 6.4 vs. 15.3 ± 6.2, $P = 0.001$; 35.4 ± 15.8 vs. 48.7 ± 15.2, $P < 0.001$,

Table 1. Patient characteristics of the *RNF213* p.R4810K variant carriers and non-carriers

	Non carrier N = 82	Carrier N = 30	*P* value	Odds ratio	Adj. odds ratio
Age (year)	65.2 ± 17.1	53.3 ± 16.0	0.001	0.96 (0.93–0.99)	
Female	37 (45%)	17 (57%)	0.279	1.59 (0.68–3.70)	
Hypertension	64 (78%)	21 (70%)	0.378	0.66 (0.26–1.68)	0.57 (0.17–1.85)
Dyslipidemia	56 (68%)	19 (63%)	0.621	0.80 (0.33–1.93)	0.98 (0.38–2.51)
Diabetes mellitus	21 (26%)	6 (20%)	0.539	0.73 (0.26–2.02)	0.90 (0.29–2.74)
Smoking habit	26 (32%)	9 (30%)	0.863	0.92 (0.37–2.29)	0.59 (0.19–1.82)
Previous TIA or stroke	35 (43%)	17 (57%)	0.189	1.76 (0.75–4.09)	0.75 (0.31–1.86)
Previous myocardial infarction	7 (9%)	2 (7%)	0.747	0.77 (0.15–3.91)	0.66 (0.12–3.80)
Other cardiovascular disease	12 (12%)	3 (10%)	0.735	0.79 (0.21–3.02)	0.62 (0.14–2.65)
Peripheral artery disease	0 (0%)	0 (0%)			
Systolic blood pressure (mmHg)	131.0 ± 14.4	128.2 ± 17.3	0.394	0.99 (0.96–1.02)	0.98 (0.95–1.01)
Diastolic blood pressure (mmHg)	78.5 ± 12.6	80.1 ± 16.8	0.594	1.01 (0.98–1.04)	0.98 (0.95–1.02)
Total-cholesterol (mmol/L)	5.0 ± 1.0	4.9 ± 0.8	0.516	1.00 (0.98–1.01)	0.67 (0.40–1.13)
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	0.935	0.96 (0.34–2.70)	0.50 (0.14–1.74)
LDL-cholesterol (mmol/L)	2.9 ± 0.8	2.8 ± 0.8	0.447	0.81 (0.47–1.39)	0.62 (0.02–1.66)
eGFR (ml/min/1.73m^2)	68.2 ± 22.6	72.3 ± 21.2	0.381	1.01 (0.99–1.03)	0.99 (0.96–1.01)
Anterior circulation stenosis/occlusion	79 (96%)	30 (100%)	0.288		
Posterior circulation stenosis/occlusion	20 (24%)	6 (20%)	0.626	0.78 (0.28–2.16)	1.23 (0.40–3.73)
Multiple stenosis/occlusion	32 (39%)	19 (63%)	0.022	2.70 (1.14–6.41)	2.74 (1.09–6.91)

TIA, transient ischemic attack; HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate; Adj. odds ratio, odds ratio adjusted for age and sex.

Fig. 1. Comparison of three cardiovascular risk scores between the *RNF213* p.R4810K variant carriers and non-carriers

Essen Stroke Risk Score (A), Framingham Risk Score (B), and Suita Risk Score (C) were used to estimate the cumulative atherosclerotic burden. The variant carriers had a significantly lower cumulative atherosclerotic burden than the non-carriers in all three risk scores. Box plots indicate the 25th and 75th percentiles (box), median (black line), and mean value (dotted line), and whiskers extend to the upper and lower limits within 1.5 times the interquartile range from the box.
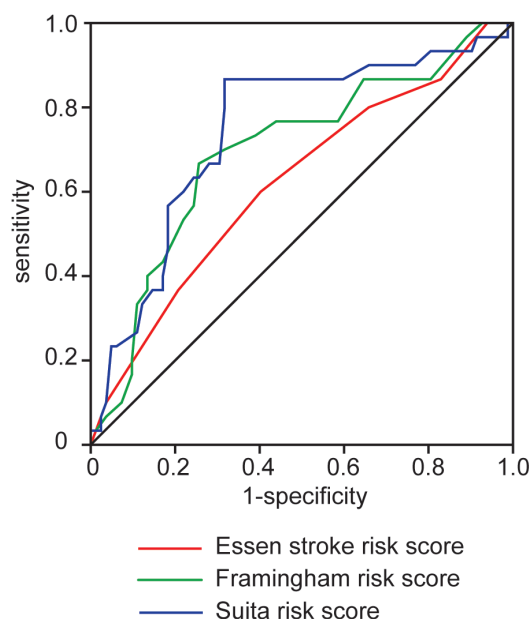
Fig. 2. Receiver operating curves for predicting the *RNF213* p.R4810K variant carrier

The Suita Risk Score had the largest AUC (0.75), followed by the Framingham Risk Score (0.70) and Essen Stroke Risk Score (0.62).

Table 2. The optimal cut-off values of each risk score for predicting the *RNF213* variant carrier

	Range	Cut-off value	Sensitivity	Specificity	Odds ratio	Adj. odds ratio
Essen stroke risk score	[0, 6]	2	0.60	0.60	2.23 (0.95–5.23)	0.74 (0.22–2.53)
Framingham risk score	[-2, 28]	12	0.67	0.74	5.81 (2.35–14.39)	10.35 (1.96–54.55)
Suita risk score	[10, 78]	45	0.87	0.68	14.00 (4.43–44.25)	20.19 (4.25–95.96)

Range indicates the maximum and minimum values in this population. Adj. odds ratio indicates odds ratio adjusted for age, sex, and the presence of multiple intracranial arterial stenosis.

respectively).

To identify the impact of age on risk scores, we first performed sensitivity analyses by setting the age score to 0 for each risk score. The Essen Risk Score showed no significant difference between the carriers and non-carriers (1.9 ± 1.2 vs. 2.0 ± 1.2, $P=0.836$). The Framingham Risk Score was marginally lower in carriers (3.8 ± 3.7 vs. 5.2 ± 3.5, $P=0.076$) than in non-carriers. Meanwhile, the Suita Risk Score was significantly lower in carriers (-5.4 ± 10.0 vs. -1.4 ± 8.7, $P=0.040$) even after disregarding the score of age. Next, because of the uneven distribution of variant carriers in the young population, we excluded patients under 50 years of age ($n=33$) from the analyses. The Framingham Risk Score and Suita Risk Score were still significantly lower in carriers (15.3 ± 5.0 vs. 17.8 ± 4.2, $P=0.046$; 46.6 ± 13.2 vs. 54.9 ± 9.8, $P=0.007$, respectively), but there was no difference in the Essen Stroke Risk Score between the two groups (3.3 ± 1.3 vs. 3.3 ± 1.4, $P=0.990$).

ROC Analysis

The ROC curves and area under the curve (AUC) for each risk score are shown in **Fig. 2**. The largest AUC was obtained for the Suita Risk Score (0.75), followed by the Framingham Risk Score (0.70) and the Essen Stroke Risk Score (0.62). The AUC of the Suita Risk Score was significantly larger than that of the Essen Stroke Risk Score ($P=0.002$) and tended to be larger than that of the Framingham Risk Score ($P=0.066$). The optimal cut-off value for predicting the *RNF213* p.R4810K variant carrier was 2 for the Essen Stroke Risk Score (sensitivity, 0.60; specificity, 0.60), 12 for the Framingham Risk Score (sensitivity, 0.67; specificity, 0.74), and 45 for the Suita Risk Score (sensitivity, 0.87; specificity 0.68) **(Table 2)**.

When a Suita Risk Score of ≤ 45 points was adopted as the cut-off point, the OR for predicting the variant carrier was 14.00 (95% CI: 4.43–44.25; **Table 2**). In a multivariate logistic regression model where age, sex, the presence of multiple stenosis, and

Suita Risk Score of ≤ 45 points were included, only the Suita Risk Score of ≤ 45 points was an independent predictor of the *RNF213* p.R4810K variant (adjusted OR: 20.19, 95% CI: 4.25–95.96, *P* <0.001).

Discussion

The present study showed that all three different cardiovascular risk scores were significantly lower in the *RNF213* p.R4810K variant carriers than in non-carriers, yet the prevalence of each atherosclerotic risk factor was not significantly different between the two groups. Among the three risk scores, the Suita Risk Score showed the highest predictive accuracy for the variant carriers, and a Suita Risk Score of ≤ 45 points was an independent predictor of the variant carriers. These results suggest that the variant carriers had a lower cumulative atherosclerotic burden than the non-carriers, and that the Suita Risk Score can be a useful tool to screen variant carriers in East Asian patients with intracranial arterial stenosis.

In this study, the p.R4810K variant carriers were younger and included more females than non-carriers. The prevalence of multiple intracranial stenosis was higher in the carriers. These results are consistent with those of previous studies[7, 13, 21]. Similar to the present study, it was reported that the prevalence of atherosclerotic risk factors, such as hypertension, dyslipidemia, and diabetes, was slightly lower in the carriers, but the difference was not significant[13, 21]. It is recognized that each single risk factor, such as hypertension and dyslipidemia, has a small effect on the development of atherosclerosis, but the combined effect of multiple risk factors can additively increase the risk[22]. It is important to evaluate the cumulative atherosclerotic burden to assess the individual risk of cardiovascular disease. We used the three risk scores in this study. The Essen Stroke Risk Score, developed in Europe, adopts a simple and easy scoring system, in which all risk factors are scored in a binary manner (1, present; 0, absent), and the severity is not considered. Therefore, the predictive accuracy may be inferior to the other two scores. The Framingham Risk Score, which was developed in the United States, is the most popular tool for assessing the cardiovascular risk. However, it has been reported that the Framingham Risk Score tends to overestimate the risk of cardiovascular disease in the Asian population[23] because the absolute risk of cardiovascular events is much lower in Asia[24]. In Asian population studies, chronic kidney disease has been reported to be an independent risk factor for cardiovascular disease[25]. Based on these characteristics in Asia, the Suita Risk

Score was developed to accurately predict the risk of cardiovascular disease in the Asian population[18]. In this study performed in Japan, the Suita Risk Score showed the highest predictive accuracy among the three risk scores. Regional characteristics are likely to contribute to these differences among the three risk scores.

Because all three risk scores are strongly affected by age, we performed sensitivity analyses using two different approaches to evaluate the influence of age. The Suita Risk Score was significantly lower, and the Framingham Risk Score was marginally lower in the variant carriers, even after disregarding the influence of age. Multivariate logistic analysis also showed that the Suita Risk Score was an independent predictor of variant carriers even after adjusting for age and sex. A large Japanese population study[26] showed that the mean value of the Suita Risk Score in the general population was approximately 43, which is higher than that of 35.4 in the variant carriers with intracranial stenosis. Because the average age was different between the two populations, it is not possible to simply compare these two values; however, these results indicate that the *RNF213* p.R4810K variant carriers develop intracranial arterial stenosis even if they have a lower cumulative atherosclerotic risk than the general population.

Two possible mechanisms were considered for the development of intracranial arterial stenosis in variant carriers. First, the *RNF213* variant enhances susceptibility to vessel wall injury, and the accumulation of minor atherosclerotic risks accelerates intracranial atherosclerosis under the condition of vascular vulnerability. In this case, more aggressive management of atherosclerotic risk factors from the early stage may reduce the development and progression of intracranial stenosis in variant carriers. Another hypothesis is that a non-atherosclerotic mechanism causes intracranial vessel narrowing independent of conventional atherosclerotic risk factors, similar to that of child-onset moyamoya disease. Our results appear to support the second hypothesis; however, further comparisons of long-term prognosis between *RNF213* variant carriers with and without a cumulative atherosclerotic burden are necessary to elucidate the exact mechanisms. Recently, it has been found that RNF213 affects vascular endothelial cell functions and angiogenesis through caveolin-1 and NFAT1[9, 27]. RNF213 also influences fat metabolism[28] and inflammation via the NF-kB pathway[29]. Research on further approaches to these non-atherosclerotic pathways may lead to the development of new treatments for intracranial arterial stenosis.

This study had several limitations. First, because clinical information was obtained from previous medical records, the prevalence of atherosclerosis risks can be underestimated because of insufficient diagnostic surveillance. Second, sensitivity analyses were performed to remove the influence of age on risk scores, but this might be insufficient because some parameters, such as blood pressure and estimated glomerular filtration rate, are also affected by age. Third, because only Japanese patients were enrolled in this study, it is not clear whether these results can be adopted in other countries. Further multinational studies, especially in East Asia, where many asymptomatic carriers of the variant live, are warranted to verify these results.

In conclusion, this study showed that the *RNF213* p.R4810K variant carriers had a lower cumulative atherosclerotic burden than the non-carriers among patients with intracranial arterial stenosis. Our results suggest that new therapeutic approaches beyond the standard management of atherosclerotic risk factors are required to prevent the development of intracranial arterial stenosis.

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This study has no conflicts of interest to disclosure.

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