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RNF213 p.R4810K variant in moyamoya disease in adults and children from a Malaysian tertiary center

[1,2]Jun Hui Ho *BSc*, [1]Kay Sin Tan *FRCP*, [3]Wei Kang Lim *MPaed*, [3]Choong Yi Fong *FRCPCH*, [1]Tsun Haw Toh *MRCP*, [1]Kar Foo Lau *MRCP*, [1]Zhen Shun Chan *MBBS*, [1]Mei-Ling Sharon Tai *MRCP*, [4]Kartini Rahmat *FRCR*, [4]Farah Diana Tarmizi Thayaparan *MBBS*, [5]Sumitha Murugesu *MRCPCH*, [2]Ching Ching Ng *PhD*

[1]Division of Neurology, Department of Medicine, Faculty of Medicine, Universiti Malaya, Kuala Lumpur; [2]Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur; [3]Division of Paediatric Neurology, Department of Paediatrics, Faculty of Medicine, Universiti Malaya, Kuala Lumpur; [4]Department of Biomedical Imaging, Faculty of Medicine, Universiti Malaya, Kuala Lumpur; [5]Paediatric Neurology Unit, Tunku Azizah Hospital, Kuala Lumpur, Malaysia

Abstract

Background: *Ring finger protein 213* (*RNF213*) is a major susceptibility gene for moyamoya disease (MMD). A single nucleotide variant known as c.14429G>A (p.R4810K, rs112735431) is strongly associated with MMD, weakly associated with moyamoya syndrome (MMS) and intracranial atherosclerosis (ICAS) in East Asians. The percentage of patients carrying p.R4810K and the effect sizes vary between different racial backgrounds. We aimed to investigate the prevalence of p.R4810K in MMD, MMS, and ICAS patients in Malaysia. *Methods*: We genotyped p.R4810K in DNA extracted from 9 MMD, 13 MMS, 15 ICAS cases, and 45 controls using TaqMan SNP genotyping assay. Clinical and neuroradiological data was collected for each patient and the distribution of genotype frequencies compared between cases and controls and tested for association. *Results*: Two of seven (28.6%) Chinese MMD cases had heterozygous p.R4810K (GA) genotype. The remaining MMD cases (5 Chinese and 2 Malays), all 13 MMS, 15 ICAS cases and 45 controls had homozygous wild-type (GG) genotype. Higher frequency of GA genotype was observed in Chinese MMD patients compared with controls, indicating an association between p.R4810K and Chinese MMD subgroup under a dominant model (P=0.0398).
Conclusion: This is the first study reporting p.R4810K in a multiracial Asian population. The p.R4810K missense mutation was detected in MMD cases of Chinese descent in Malaysia. Further studies are needed to identify other susceptibility variants and genes in Malaysian patients with moyamoya and ICAS.

Keywords: *RNF213*, p.R4810K, rs112735431, moyamoya, intracranial atherosclerosis, Malaysian

INTRODUCTION

Moyamoya disease (MMD) is a rare cerebrovascular disease characterized by slowly progressive stenosis of the internal carotid arteries and the formation of an abnormal vascular network (moyamoya vessels) at the base of the brain. In the past decade, a human gene known as *ring finger protein 213* (*RNF213*) was first identified as a major susceptibility gene for MMD.[1,2] *RNF213* encodes a ubiquitously expressed 591 kDa (5,207 amino acid) protein that exerts both AAA+ ATPase and ubiquitin ligase activities.[1-3] The RNF213 protein is implicated in various processes such as angiogenesis, vasculogenesis, lipid metabolism and cell-autonomous immunity.[3] A single nucleotide variant of *RNF213*, c.14429 G>A (NM_001256071.3) (p.R4810K, rs112735431) is strongly and significantly associated with MMD in East Asians, including Japanese, Korean and Chinese. Among these 3 races, the odds ratio

Address correspondence to: Prof. Dr. Kay Sin Tan, Division of Neurology, Department of Medicine, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia. Tel: +603-79493191, Email: tanks_neuro@um.edu.my; Associate Prof. Dr. Ching Ching Ng, Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia. Tel: +603-79675872, Email: ccng@um.edu.my

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(OR) for Japanese was found to be the highest (OR: 338.94, 95% CI: 147.82-777.84), followed by Korean (OR: 135.63, 95% CI: 43.03-427.52), and Chinese (OR: 14.70, 95% CI: 3.05-70.81).[1] While *RNF213* p.R4810K is the most important genetic risk factor for MMD in East Asians, this variant was not found in both Caucasian MMD cases and controls.[1] In addition, some studies have been conducted to understand the pathological role of p.R4810K variant. It was found that the overexpression of p.R4810K could reduce the angiogenicity in endothelial cells (ECs)[4,5] and increase susceptibility to hypoxia.[4] Moreover, p.R4810K overexpression in 293T and HeLa cells also decreased ubiquitylation globally compared to the controls and wild-type RNF213 overexpressing cells[6] and increased risk of genomic instability by mitotic abnormality.[7] However, the pathological effects of the p.R4810K variant in MMD has not been completely elucidated.

Moyamoya syndrome (MMS) or quasi-moyamoya disease (QMMD) refers to moyamoya vasculopathy associated with underlying disease entities.[8] These underlying medical conditions include atherosclerosis, autoimmune disease, Down's syndrome, irradiation, hyperthyroidism, and sickle cell disease. A patient with known MMD and associated underlying diseases, will be classified as of MMS or QMMD, whereas a patient with MMD without any associated underlying diseases or with unrelated underlying disease will be excluded from MMS or QMMD diagnosis.[8,9] Association between p.R4810K and MMS remains uncertain, given the relative paucity of information. Recent studies have found that p.R4810K was significantly associated with MMS, with OR ranged from 50.6 to 89.0 and $P<0.01$.[10-12] On the other hand, it has also been reported that none of the non-atherosclerotic MMS patient carried the p.R4810K variant, suggesting no association between the variant and non-atherosclerotic MMS.[13]

Intracranial atherosclerosis (ICAS) is a common cause of stroke in Asia. The underlying progressive pathological processes lead to thromboembolism and subsequent transient or permanent cerebral ischemic events with a high risk of recurrent stroke or infarct extension.[14] It has been known that the rate of ICAS is disproportionately higher in Asian and African American patients than in Caucasian patients.[14-17] The p.R4810K has also been reported for ICAS in East Asian populations.[18,19] Several studies have found significant associations between p.R4810K and ICAS, with the highest OR observed in Japanese (OR: 16.8, 95% CI: 3.81-74.5, $P<0.0001$)[20], followed by Korean (OR: 14.3, 95% CI: 2.80-73.2, $P=0.001$)[21], and Chinese (OR: 4.38, 95% CI: 1.61-11.89, $P=0.004$)[22], but the strength of association was weaker compared to MMD.[20-22] *RNF213* p.R4810K is associated with early-onset stroke with intracranial arterial stenosis and was found more frequently in women.[23]

Malaysia has substantial genetic diversity from South and East Asia from its multiracial population originating in Austronesia (Malay)[24], Southern China (Han Chinese)[25] and India (predominantly Southern Indian).[26] As there are associations between *RNF213* p.R4810K, moyamoya angiopathy and ICAS in East Asian populations, and the lack of similar studies in other populations, we aimed to investigate the prevalence of *RNF213* p.R4810K in patients with MMD, MMS, and ICAS in Malaysia.

METHODS

Study subjects

The study was approved by the Universiti Malaya Medical Center-Medical Research Ethics Committee (Ref: MREC 201847-6207). Written informed consent was obtained from all adult participants and from parents of participants if the participants were below 18 years old. Clinical and neuroradiological data was collected for each patient with diagnostic evaluation (cerebral angiography and/or magnetic resonance angiography (MRA). A total of 9 MMD, 12 MMS, and 15 ICAS cases were recruited from Universiti Malaya Medical Center (UMMC) and 1 MMS case was recruited from Kuala Lumpur Hospital, from February 2019 to May 2021 based on the following selection inclusion criteria:

1. Moyamoya disease (MMD)
   Cerebral angiography and/or MRA: Stenosis or occlusion of the terminal portion of the intracranial internal carotid artery or proximal portions of the anterior and/or middle cerebral artery; abnormal vascular networks in vicinity of occlusive or stenotic lesions in the arterial phase.
   MRA: Abnormal vascular networks in the basal ganglia (with 2 or more visible flow voids in basal ganglia).
2. Moyamoya syndrome (MMS)
   Moyamoya pathology arising secondary to cranio-therapeutic irradiation, trisomy 21, sickle cell disease, thalassemia, neurofibromatosis

(NF), autoimmune diseases, renal artery stenosis, hyperthyroidism, and PHACE syndrome (cervicofacial haemangiomas).[9]

3. Intracranial atherosclerosis (ICAS)
   Patients with large artery intracranial stenosis without features of MMD were identified from an ongoing young stroke registry, with the following criteria: patients admitted due to ischaemic stroke, between the age of 18 and 49 years, with completed cerebrovascular diagnostics (conventional angiography and/or MRA) demonstrating at least 50% stenosis or occlusion at terminal portions of the ICA and/or proximal MCA. Based on TOAST criteria[27], patients with potential sources of cardioaortic embolism, extracranial atherosclerosis with significant (>50%) stenosis on the relevant extracranial arteries, other stroke mechanisms (coagulopathy, vasculitis, arterial dissection), or incomplete evaluations were excluded.

The selection criteria of MMD was based on the criteria of Research Committee on the Pathology and Treatment of Spontaneous Occlusion of the Circle of Willis; Health Labour Sciences Research Grant for Research on Measures for Intractable Diseases, Japan.[9] A total of 45 control participants (20 males and 25 females) were recruited with a mean age of 32.29 ± 14.17 years. These control participants had no typical symptoms for MMD, MMS and ICAS and were not screened by cerebral angiography, MRA, TCD, or any other tests. All controls were recruited from paediatric and adult neurology clinics of UMMC. All the cases and controls were unrelated and demographic data obtained from hospital records.

Genomic DNA extraction and p.R4810K genotyping

Venous blood samples were obtained from each participant. Genomic DNA (gDNA) was extracted from the blood samples using Exgene™ Blood SV Midi Kit (GeneAll, Seoul, South Korea) following manufacturer's protocol. Concentration of DNA was measured by NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and diluted to working concentration of 20 ng/μL for genotyping. Genotyping of p.R4810K in all participants was performed using TaqMan SNP Genotyping Assays (Assay ID: C_153120198; Applied Biosystems, Foster City, USA) performed on QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, USA), according to manufacturer's protocol (TaqMan® GTXpress™ Master Mix). The genotype of each sample was determined based on the fluorescence signals generated by PCR amplification. To evaluate concordance of the genotyping results, about 20% of the samples were randomly selected for re-genotyping.

Verification of RNF213 p.R4810K positive cases

Participants carrying *RNF213* p.R4810K variant identified using TaqMan SNP genotyping assay were further verified by direct Sanger sequencing. PCR primers were adopted from Wang *et al*.[28] and produced a 176 bp amplicon harboring p.R4810K variant. Forward primer: 5'-TTT TGG CCT TGC AAA GGG ATC T-3' and Reverse primer: 5'-CCA CCC TGT TCC CCT ATG CAG T-3'. Conventional PCR was performed in a total volume of 25 μL: 5.25 μL of Q5 reaction mixture (5 μL of 5× Q5 reaction buffer and 0.25 μL of Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA)), 0.5 μL of 10 mM dNTPs (Thermo Fisher Scientific, Waltham, USA), 1.25 μL of 10 μM primers, 5 μL of input gDNA (20 ng/μL), and PCR grade water (milliQ) to final volume. The PCR thermal cycling conditions were as follows: initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 66°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 2 min. Purified PCR products were sent to the service provider (Bio Basic Inc., Markham, Canada) in Singapore for DNA sequencing.

Statistical analysis

The analysis of demographic characteristics and the distribution of p.R4810K genotypes among the participants was performed using SPSS Statistics version 23.0 (SPSS Inc., Chicago, USA). Continuous variables were presented as mean (± standard deviation) and median (range: min – max), and categorical variables were presented as frequency (n) and proportions (%). Fisher's exact test to test for association was performed using online VassarStats (http://vassarstats.net/odds2x2.html?). A two-tailed $P<0.05$ was considered statistically significant.

RESULTS

Demographic characteristics of study subjects

This study comprised 37 patients with 9 MMD (24.32%), 13 MMS (35.14%) and 15 ICAS (40.54%) patients and 45 controls. The highest number of patients studied in each group was

Malaysian Chinese, followed by Malay and Indian. The median age at diagnosis for ICAS (40.00 years, range 22-48 years) was higher compared to that of MMD (23.00 years, range 6-50 years) and MMS (26.00 years, range 0.5-43 years). The median age at diagnosis below 18 years for MMD (8.00 years, range 6-8.7 years) was higher than that of MMS (4.20 years, 0.5-15 years). The median age of control participants was 37.00 years (range 3-54 years). Demographic characteristics of the study subjects are summarized in Table 1.

Distribution of p.R4810K genotypes between cases and controls

Genotype distribution of *RNF213* p.R4810K in cases and controls are shown in Table 2. *RNF213* p.R4810K missense mutation (allele A) was detected in two Chinese MMD cases. Both cases were heterozygotes (GA genotype). Their p.R4810K genotypes were verified by direct Sanger sequencing (Figure 1) and clinical information is shown in Table 3. However, the variant was not found in Malaysian Chinese MMS (n = 8), Malaysian Malay MMD (n = 2) and MMS (n = 4), and Malaysian Indian MMS (n = 1) cases, nor in any ICAS cases (n = 15) and control participants (n = 45). Carrier rates of p.R4810K variant was higher in Malaysian Chinese MMD (28.6%, 2/7) compared to the Chinese controls (0%, 0/26). We observed an association between p.R4810K and Chinese MMD subgroup under a dominant model (*P*=0.0398).

DISCUSSION

To the best of our knowledge, this was the first ever study in the Malaysian population.

Table 1: Demographic characteristics of study participants

Demographic characteristics	Case (N = 37)			Control (N = 45)
	MMD (n = 9)	MMS (n = 13)	ICAS (n = 15)	
Sex, n (%)				
Male	4 (44.4)	2 (15.4)	12 (80.0)	20 (44.4)
Female	5 (55.6)	11 (84.6)	3 (20.0)	25 (55.6)
Race, n (%)				
Chinese	7 (77.8)	8 (61.5)	7 (46.7)	26 (57.8)
Malay	2 (22.2)	4 (30.8)	5 (33.3)	14 (31.1)
Indian	0 (0)	1 (7.7)	3 (20.0)	5 (11.1)
Age at diagnosis, y				
Mean (SD)	24.97 (15.97)	22.99 (17.38)	39.40 (6.63)	NA
Median (range)	23.00 (6 – 50)	26.00 (0.5 – 43)	40.00 (22 – 48)	NA
Age at diagnosis (<18y), n (%)	3 (33.3)	6 (46.2)	0 (0)	NA
Mean (SD)	7.57 (1.40)	6.13 (6.02)	0	NA
Median (range)	8.00 (6 – 8.7)	4.20 (0.5 – 15)	0	NA
Age at diagnosis (≥18y), n (%)	6 (66.7)	7 (53.8)	15 (100)	NA
Mean (SD)	33.67 (11.60)	37.43 (6.85)	39.40 (6.63)	NA
Median (range)	32.50 (22 – 50)	41.00 (26 – 43)	40.00 (22 – 48)	NA
Age at recruitment, y				
Mean (SD)	NA	NA	NA	32.29 (14.17)
Median (range)	NA	NA	NA	37.00 (3 – 54)
Age at recruitment (<18y), n (%)	NA	NA	NA	9 (20.0)
Mean (SD)	NA	NA	NA	9.44 (4.00)
Median (range)	NA	NA	NA	8.00 (3-14)
Age at recruitment (≥18y), n (%)	NA	NA	NA	36 (80.0)
Mean (SD)	NA	NA	NA	38.00 (9.00)
Median (range)	NA	NA	NA	39.00 (18-54)

Abbreviations: N/n: number of participants; SD: standard deviation; y: year; NA: not applicable.

Table 2: Distribution of p.R4810K genotypes among MMD, MMS, and ICAS cases, and controls

Race	Group		p.R4810K Genotype, n (%)		Total, n
			GG	GA	
Chinese	Cases	MMD	5 (71.4)	2 (28.6)	7
		MMS	8 (100)	0 (0)	8
		ICAS	7 (100)	0 (0)	7
	Controls		26 (100)	0 (0)	26
Malay	Cases	MMD	2 (100)	0 (0)	2
		MMS	4 (100)	0 (0)	4
		ICAS	5 (100)	0 (0)	5
	Controls		14 (100)	0 (0)	14
Indian	Cases	MMD	0 (0)	0 (0)	0
		MMS	1 (100)	0 (0)	1
		ICAS	3 (100)	0 (0)	3
	Controls		5 (100)	0 (0)	5

Abbreviation: n: number of participants.

We found an association of p.R4810K variant with MMD in the Malaysian Chinese subgroup. The mutation was identified in 28% of Chinese MMD patients. Our finding was similar with previous studies conducted in the Han Chinese population, in China and Taiwan. In these studies, the proportions of MMD patients carrying at least one allele of p.R4810K (GA and AA genotypes) ranged from 12.9% to 23.8%[1,30-33], while the proportion of controls with this variant ranged from 0.4% to 1.3%.[1,31,33] For Japanese and Koreans, the proportions of MMD patients carrying p.R4810K variant ranged from 72.7% to 90.1% and from 67.4% to 88.0%, respectively, higher than Chinese MMD patients.[1,20,34-37] In contrast, the variant was detected in 1.8% to 3.7% of Japanese controls[1,20,34,35] and in 2.0% to 2.7% of Korean controls.[1,21] Notably, the allele frequency of p.R4810K variant in Chinese general population was lower than in Japan and Korea. Lower prevalence of p.R4810K within Chinese general population might be the reason for the lower carrier rate of p.R4810K variant among Chinese MMD patients.[38]

Furthermore, a recent study from India found that the variant was detected in 5 out of 65 Indian MMD patients (7.7%), but not in the 104 controls. All the 5 MMD patients were homozygous for p.R4810K variant (AA) while their parents were heterozygous (GA) with a clearly documented history of disease in one affected parent suggesting an autosomal dominant inheritance pattern of the disease. These 5 patients were from West Bengal and Tamil Nadu of India and the mutation was absent in individuals from Malayalam speaking population of Kerala.[39] *RNF213* p.R4810K mutation was not detected in the two Malay MMD cases in our study. In addition, studies

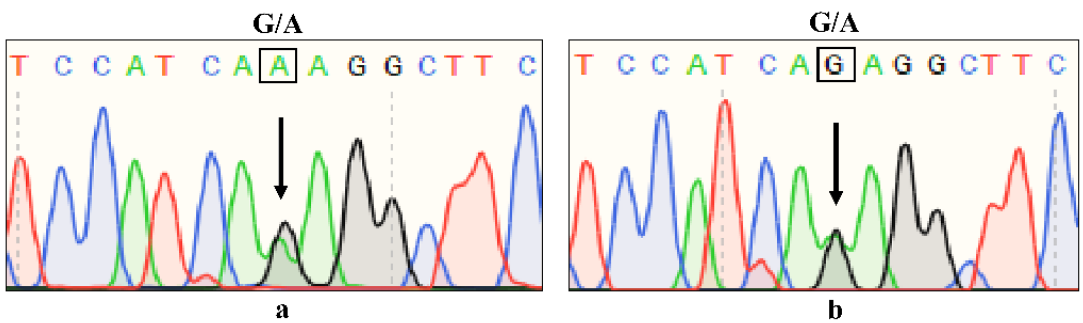

Figure 1. The chromatograms showing the p.R4810K genotypes of 2 Chinese MMD p.R4810K carriers. The chromatograms show that both MMD cases (a: Patient 1 and b: Patient 2) carried the p.R4810K variant (double peaks) at position c.14429 G>A (arrow), and both are heterozygous (GA). The black boxes show the positions of nucleotides corresponding to the peaks.

Table 3: Clinical information of 2 Malaysian Chinese MMD cases carrying heterozygous p.R4810K variant (GA genotype)

Item		Patient 1[29]	Patient 2
Sex		Female	Male
Race		Chinese	Chinese
Age at diagnosis (years)		25	8
Initial infarct distribution		R ACA	Bilateral frontal
Vessel imaging	CCA	Normal	Normal
	ICA	Bilateral proximal ICA stenosis, R ICA cavernous occlusion	Bilateral distal ICA occlusion
	MCA	Bilateral MCA beaded	Normal
	ACA	Bilateral ACA beaded	Normal
	Other vessels	NA	Bilateral PCA and SCA stenosis
Stroke recurrence		Yes	No
Surgical intervention		No	Right-sided multiple burr holes for indirect revascularization, Left burr hole
Risk factors		Dyslipidemia	None
Family history of stroke		Unknown	Unknown
Medications		Antiplatelet	Antiplatelet, anti-convulsant

Abbreviations: NA: not applicable; R: right; CCA: common carotid artery; ICA: internal carotid artery; MCA: middle cerebral artery; ACA: anterior cerebral artery; PCA: posterior cerebral artery; SCA: superior cerebellar artery.

in Caucasian patients did not demonstrate this mutation.[1,2,40] Instead, several rare missense *RNF213* variants were reported in Caucasians, including p.N3962D, p.R3922Q, p.D4013N, p.R4019C, and p.V4146A[1,41,42] suggesting genetic heterogeneity and further highlighting the need for racial-specific MMD variants identification.

Studies have shown that the age of onset for MMD patients with GA and AA genotype was significantly lower than those with GG genotype.[33] Patients with AA genotype displayed the lowest median age of onset as compared to GG and GA[33,34,39,43,44] and had rapid disease progression leading to significant neurological deficits with severe and wide distribution of vasculopathy.[39,43] Moreover, patients with family history of MMD are more likely to have p.R4810K variant.[1,2,33,45]

None of the Malaysian patients with MMS and ICAS carried p.R4810K variant. This could be due to the small sample size in the current study and greater difficulty in obtaining an association signal because of the much lower carrier rate of p.R4810K in MMS and ICAS patients. The proportion of MMS patients carrying the p.R4810K variant varied widely across different populations. A Chinese study found that 11.9% of patients with MMS carried p.R4810K variant.[11] In the Japanese studies, the proportion ranged from 0.0% to 80.0%. Interestingly, 2[8,13] out of 3 cited studies found no significant association between p.R4810K and MMS. Such a wide range of proportions may be due to their small sample sizes. It should be noted that the associated underlying diseases recruited into these studies were different, the only common underlying disease found in these 3 studies was hyperthyroidism.[8,10,13] Furthermore, a Korean study discovered that 18.7% patients with MMS had the same variant, but the MMS-associated underlying disease included into their study was neurofibromatosis type 1 (NF1).[12] The MMS-associated underlying diseases included in our present study were NF1, hyperthyroidism, α-thalassemia, and PHACE syndrome.

For ICAS, previous studies have found that the proportions of ICAS patients carrying p.R4810K was much lower than those in MMD. The proportion ranged from 0.8% to 7.0% in Chinese ICAS patients.[22,46,47] As for Japanese and Koreans, the proportions of patients were higher as compared to that in Chinese ICAS patients, ranging from 9.6% to 24.3%[20,23,48,49] and from 6.5% to 33.2%[18,19,21,50], respectively. In terms of age of onset, Miyawaki *et al*. found that heterozygous

and homozygous p.R4810K were significantly associated with earlier age of onset of ICAS as compared to those with wild-type genotype.[20]

Our study has a few limitations. First, the sample size for each disease category in each racial group was small; therefore, any statistical analysis attempt would be prone to biases. Second, most of the participants were only recruited from one tertiary hospital with only one patient recruited from another government hospital, thus there may be a selection bias as these cases were referred to major tertiary hospitals. Third, we only investigated the prevalence of p.R4810K but did not evaluate less common variants in the *RNF213* gene. Therefore, the p.R4810K variant may not be sufficient to elucidate the genetics of moyamoya angiopathy and ICAS in the Malaysian population.

In conclusion, the *RNF213* p.R4810K variant was present in 2 out of 7 (28.6%) Malaysian Chinese MMD cases but was not present in cases clinically classified as MMS and ICAS, in control participants, and in Malays and Indians. It significantly increases the risk of MMD in Malaysian Chinese. Further research with a larger sample size is required to identify susceptibility genes and variants for Malaysian patients with MMD, MMS and ICAS.

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DISCLOSURE

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Conflict of interest: None

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