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Distribution of Moyamoya Disease Susceptibility Polymorphism p.R4810K in *RNF213* in East and Southeast Asian Populations

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Running head: p.R4810K in *RNF213* in East and Southeast Asian Populations

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Abstract

Moyamoya disease is an idiopathic vascular disorder of the intracranial arteries. We previously identified *ring finger 213* (*RNF213*) as the strongest susceptibility gene for moyamoya disease in East Asian people by a genome-wide linkage analysis and exome analysis. The coding variant p.R4810K in *RNF213* was strongly associated with moyamoya disease in Japanese (odds ratio: 338.94, $P= 1.05 \times 10^{-100}$) and Korean (odds ratio: 135.63, $P= 7.59 \times 10^{-27}$) people, and much less strongly associated in Chinese people (odds ratio: 14.70, $P= 2.63 \times 10^{-5}$). In the present study, we investigated the distribution of p.R4810K in *RNF213* in 2,508 participants from East and Southeast Asian countries using a TaqMan probe. p.R4810K was detected at an allele frequency of about 1.00% in 4 of 11 investigated locations in China. In contrast, p.R4810K was detected homogeneously at relatively high frequencies of 1.00–1.72% in all investigated locations in Korea and Japan, including Okinawa. p.R4810K was not detected in Southeast Asian populations. The susceptibility population to moyamoya disease was estimated to be 16.16 million people in East Asian countries, i.e., 11.41 million Chinese, 1.36 million Korean and 3.39 million Japanese people. The number of patients with moyamoya disease, which was estimated at approximately one per 300 carriers of p.R4810K, was considered to be 53,800 in East Asian populations.

Introduction

Moyamoya disease [MIM 607151] is a rare idiopathic progressive disorder characterized by occlusive lesions in the supraclinoid internal carotid artery and its main branches in the circle of Willis. A fine vascular network that resembles "puffs of smoke" ("moyamoya" in Japanese) develops to compensate for the blood flow around the occlusive regions.[12,13] Moyamoya disease has the highest prevalences in East Asian countries such as Japan, Korea and China compared with other countries.[2,7,10]

We had identified a susceptibility locus for familial moyamoya disease on 17q25.3.[11] Significant associations with genes in this region had been reported,[4,8] but rigorous identification awaited further study. The *ring finger 213* (*RNF213*) gene was finally identified as a susceptibility gene for moyamoya disease by the rigorous traditional positional cloning approach, exome analysis and functional analysis using zebrafish.[9] Overall, 74.5% of East Asian patients with moyamoya disease carry the rare founder variant p.R4810K in *RNF213*. However, a gradient of prevalence of patients with moyamoya disease was observed, with 90% in Japanese, 79% in Korean and 23% in Chinese people. On the other hand, no gradient of prevalence was observed in three general populations. In total, 2.4% of East Asian general populations carry p.R4810K. The minor allele frequencies of p.R4810K in general populations were 1.4%, 1.3% and 1.0% for Japanese, Korean and Chinese people, respectively.[9] These similar allele frequencies of p.R4810K in the three East Asian general populations could not explain the lower frequency of Chinese patients with moyamoya disease compared with Japanese and Korean patients with moyamoya disease. In Japan, moyamoya disease occurs in approximately one of 300 carriers.[8] If so, this discrepancy in the allele frequency between cases and controls in Chinese people might be attributable to

selection bias in the general populations in the previous study.[9] The primary aim of the present study was to estimate the susceptibility population to moyamoya disease by conducting a large scale screening of p.R4810K in general populations in East and Southeast Asia. The secondary aim was to estimate the patients with moyamoya disease associated with p.R4810K in *RNF213*.

Materials and Methods

Ethical statement

Ethical approval for this study was given by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G140; approval date: 10/18/2004), by the Seoul National University Hospital Institutional Review Board (approval number: H0507-509-153; approval date: 8/24/2005). The study participants were recruited in these institutes. The subjects, who participated in this study after 2000 and were recruited by School of Medicine, Kyoto University. All subjects gave written informed consent. Those, who donated blood samples before 2000 who were recruited by Tohoku University School of Medicine, or Akita University School of Medicine, or Kyoto University School of Medicine, gave verbal informed consents. The application of blood samples which were donated before 2000 for genetic analysis were also approved by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G140; approval date: 10/18/2004).

Subjects

A total of 2,508 unrelated participants from East Asian and Southeast Asian

countries were recruited, comprising 587 Chinese, 294 Korean, 1474 Japanese, 103 Vietnamese and 50 Filipino people. The Japanese participants were recruited from mainland Japan including Hokkaido, Honshu, Shikoku, Kyushu and Okinawa. The Korean participants were recruited from five locations (Table 1). The Chinese participants were recruited from 11 locations (Table 1). Blood samples were obtained from two sources. One source was the Kyoto University Human Specimen Bank, which collected blood samples from the 1990s and 2000s as previously described.[5,6] These blood samples included Chinese, Korean, Japanese and Philippine samples. The other source involved samples collected in an international collaboration that included Vietnam and Seoul, South Korea.

DNA isolation

Peripheral blood (10 mL) was collected from all participants. Genomic DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Maryland,USA) according to the manufacturer's protocol. The quality and concentration of the extracted DNA were assessed using an infinite M200 PRO (TECAN, Tokyo, Japan). The DNA was stored in a freezer at –20°C until analysis.

Genotyping of p.R4810K

Genotyping of p.R4810K in all participants was conducted using a TaqMan probe (Custom TaqMan SNP Genotyping Assays; Applied Biosystems, Foster City, CA, USA) and a 7300/7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocols. Briefly, the PCR amplifications were performed with 1–20 ng of purified genomic DNA, 0.1 μL of 40× SNP Genotyping Assay, 6.25 μL of 2×

TaqMan Universal PCR Master Mix, No AmpErase UNG and 5.15 μL of DNase-free water. The final reaction volume was 12.5 μL/well in 96-well plates. The standard protocol for the cycling conditions was hold for 10 min at 95°C for AmpliTaq Gold enzyme activation, followed by 40 cycles of 15 sec at 92°C for denaturation and 1 min at 60°C for annealing/extension. After each PCR amplification, an endpoint plate read was performed using the Real-Time PCR System (Applied Biosystems). The associated Sequence Detection System (SDS) software uses the fluorescence measurements taken during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample.

Results

Demographic characteristics of the participants

The demographic features of all participants in this study are shown in Table 1. The sampled cities covered 234.02 million people. A total of 2,508 participants from three East Asian countries and two Southeast Asian countries were recruited from 1987 to 2009. Briefly, the recruited subjects comprised 587 participants from 11 Chinese locations, 294 from five Korean locations, 1474 from five Japanese locations, 103 from one Vietnamese location and 50 from one Philippine location. Most of the participants were females, with the exception of 608 Japanese males and 80 Korean males. Information on age was only available for the participants from certain locations. Angiography was only conducted for 384 Japanese and 46 Korean participants.

Geographic distribution of p.R4810K in East and Southeast Asian populations

The geographic distributions of p.R4810K in the different ethnicities are shown in Table 2 and Figure 1. p.R4810K was detected in East Asian populations, but not in Southeast Asian populations. p.R4810K was detected at allele frequencies of 0.43%, 1.36% and 1.36% in the Chinese, Korean and Japanese populations, respectively. p.R4810K was detected at an allele frequency of about 1.00% in 4 of 11 investigated locations in China, suggesting that the distribution of p.R4810K was heterogeneous and limited to certain specific locations in China. In contrast, p.R4810K was homogenously spread throughout Korea and Japan at relatively high allele frequencies of 1.00–1.72%. p.R4810K was not detected in 49 Taiwanese, 103 Vietnamese and 50 Filipino participants.

Discussion

In the present study, we confirmed the presence of similar allele frequencies of p.R4810K in *RNF213* in Japanese and Korean general populations. However, the expansion of the population examined revealed that the allele frequency of p.R4810K in Chinese population was 0.43%, being one-third of the frequency in Japanese or Korean populations. Therefore, our previous observation was considered to be attributable to selection bias. [9] The lower prevalence of p.R4810K in the Chinese general population might be proportional to the lower carrier rate (i.e., 23%) in Chinese patients. In accordance with this observation, while a single dominant polymorphism is associated with Japanese or Korean patients, various polymorphisms in *RNF213* may be associated with Chinese patients. In fact, we observed five additional mutations in Chinese patients while no additional mutations were observed in Korean or Japanese patients.[9]

In the present study, magnetic resonance angiography was only conducted for

limited numbers of participants. This limitation did not affect our results because of the very low prevalences of moyamoya disease in the general populations. The estimated total numbers of carriers in the three ethnicities were 11.41 million for Chinese, 1.36 million for Korean and 3.39 million for Japanese people. An assumption that moyamoya disease occurs in one of 300 carriers with p.R4810K[8, 9] yields estimated numbers of patients with moyamoya disease attributable to p.R4810K of 38,000 in Chinese, 4,500 in Korean and 11,300 in Japanese people. Although these numbers provide rough and minimum estimates because only p.R4810K variant is counted, a large number of patients suggest that more attention should be paid to moyamoya disease in East Asian countries.

Differences in the clinical profiles of moyamoya disease have been recognized among the three countries.[1,3] However, the differences between the clinical profiles of Korean and Japanese patients are unlikely to be explained by genetic differences. It is also speculated that additional factor(s) to the genetic factor are needed to explain the variation in the clinical phenotypes and low penetrance among carriers.[8, 9] Therefore, unknown modifier factor(s) may also be attributable to the differences in the clinical phenotypes. Further research is needed to identify such factor(s), which may also be crucial for the development of moyamoya disease among carriers of p.R4810K in *RNF213*.

Acknowledgments

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Figure legend

Figure 1. Locations of the sampling sites in East and Southeast Asian countries.

re 1


Dehui
Hokkai
Huludao
Beijing
Seoul
Jinan
Chonan
Haman
Honshu
Baoji
Xian
Pusan
Shikoku
Shanghai
Jeju-do
Kyushu
Changsha
Heping
Okinawa
Nanning
Tainan
Hanoi
Manila

Table 1. Demographic features of the participants in the East and Southeast Asian populations.

Area	Country	Location	Size of population (million)	Male: Female	Age (y): mean ± SD	Angiography[a]
East Asia	China	Dehui	1.00	0: 50	NA	0
		Huludao	2.82	0: 50	NA	0
		Beijing	19.61	0: 50	38.4 ± 10.6	0
		Jinan	6.81	0: 50	NA	0
		Xian	8.47	0 : 94	NA	0
		Baoji	3.72	0 : 48	NA	0
		Shanghai	23.02	0: 50	NA	0
		Changsha	7.04	0: 50	NA	0
		Heping	4.60	0: 46	NA	0
		Nanning	6.66	0: 50	NA	0
		Tainan	1.88	0: 49	36.4 ± 11.5	0
		Total	85.63	0: 587	37.4 ± 11.1	0
	Korea	Seoul	10.46	80: 25	42.2 ± 13.3	46
		Chonan	0.58	0: 29	34.6 ± 8.8	0
		Haman	0.06	0: 46	43.6 ± 6.8	0
		Pusan	3.60	0: 49	39.4 ± 7.8	0
		Jeju-do	0.53	0: 65	NA	0
		Total	15.23	80: 214	40.9 ± 10.9	46
	Japan	Mainland	123.62	608: 766	57.7 ± 14.1	384
		Okinawa	1.38	0: 100	47.5 ± 7.2	0
		Total	125.00	608: 866	57.0 ± 14.0	384
Southeast Asia	Vietnam	Hanoi	6.50	0: 103	22.8 ± 10.2	0
	Philippines	Manila	1.66	0: 50	NA	0

SD: standard deviation; NA: not applicable.

[a]Angiography: conventional digital subtraction angiography, magnetic resonance angiography, computed tomography angiography and others.

Table 2. Geographic distribution of p.R4810K in *RNF213* in East and Southeast Asian populations.

Area	Country	Location	Genotype of p.R4810K			Sample size	Minor allele frequency (%)	Total Population (million)	Estimated population with p.R4810K in a country (million)
			GG	GA	AA				
East Asia	China	Dehui	50	0	0	50	0.00		
		Huludao	50	0	0	50	0.00		
		Beijing	49	1	0	50	1.00		
		Jinan	50	0	0	50	0.00		
		Xian	92	2	0	94	1.06		
		Baoji	48	0	0	48	0.00	1340.00	11.41
		Shanghai	49	1	0	50	1.00		
		Changsha	50	0	0	50	0.00		
		Heping	46	0	0	46	0.00		
		Nanning	49	1	0	50	1.00		
		Tainan	49	0	0	49	0.00		
		Total	582	5	0	587	0.43		
	Korea	Seoul	102	3	0	105	1.43		
		Chonan	28	1	0	29	1.72		
		Haman	45	1	0	46	1.09	50.00	1.36
		Pusan	48	1	0	49	1.02		
		Jeju-do	63	2	0	65	1.54		
		Total	286	8	0	294	1.36		
	Japan	Mainland	1339	32	3	1374	1.38		
		Okinawa	98	2	0	100	1.00	125.00	3.39
		Total	1437	34	3	1474	1.36		
Southeast Asia	Vietnam	Hanoi	103	0	0	103	0.00	90.50	0.00
	Philippines	Manila	50	0	0	50	0.00	94.00	0.00

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Identification of *RNF213* as a Susceptibility Gene for Moyamoya Disease and Its Possible Role in Vascular Development

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Abstract

Background: Moyamoya disease is an idiopathic vascular disorder of intracranial arteries. Its susceptibility locus has been mapped to 17q25.3 in Japanese families, but the susceptibility gene is unknown.

Methodology/Principal Findings: Genome-wide linkage analysis in eight three-generation families with moyamoya disease revealed linkage to 17q25.3 ($P<10^{-4}$). Fine mapping demonstrated a 1.5-Mb disease locus bounded by D17S1806 and rs2280147. We conducted exome analysis of the eight index cases in these families, with results filtered through Ng criteria. There was a variant of p.N321S in *PCMTD1* and p.R4810K in *RNF213* in the 1.5-Mb locus of the eight index cases. The p.N321S variant in *PCMTD1* could not be confirmed by the Sanger method. Sequencing *RNF213* in 42 index cases confirmed p.R4810K and revealed it to be the only unregistered variant. Genotyping 39 SNPs around *RNF213* revealed a founder haplotype transmitted in 42 families. Sequencing the 260-kb region covering the founder haplotype in one index case did not show any coding variants except p.R4810K. A case-control study demonstrated strong association of p.R4810K with moyamoya disease in East Asian populations (251 cases and 707 controls) with an odds ratio of 111.8 ($P=10^{-119}$). Sequencing of *RNF213* in East Asian cases revealed additional novel variants: p.D4863N, p.E4950D, p.A5021V, p.D5160E, and p.E5176G. Among Caucasian cases, variants p.N3962D, p.D4013N, p.R4062Q and p.P4608S were identified. *RNF213* encodes a 591-kDa cytosolic protein that possesses two functional domains: a Walker motif and a RING finger domain. These exhibit ATPase and ubiquitin ligase activities. Although the mutant alleles (p.R4810K or p.D4013N in the RING domain) did not affect transcription levels or ubiquitination activity, knockdown of *RNF213* in zebrafish caused irregular wall formation in trunk arteries and abnormal sprouting vessels.

Conclusions/Significance: We provide evidence suggesting, for the first time, the involvement of *RNF213* in genetic susceptibility to moyamoya disease.

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Competing Interests: Kyoto University has applied to the Patent Office, Japan, for a patent on this gene. The authors confirm that this does not alter their adherence to all the PLos One policies on sharing data and materials.

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Introduction

Moyamoya disease is an idiopathic disorder characterized by occlusive lesions in the supraclinoid internal carotid artery and its main branches in the circle of Willis. To compensate for the blood flow around the occlusive region, a fine vascular network develops that resembles "puffs of smoke" (Figure 1A) [1]. The unique appearance of moyamoya vessels described by Suzuki and Takaku

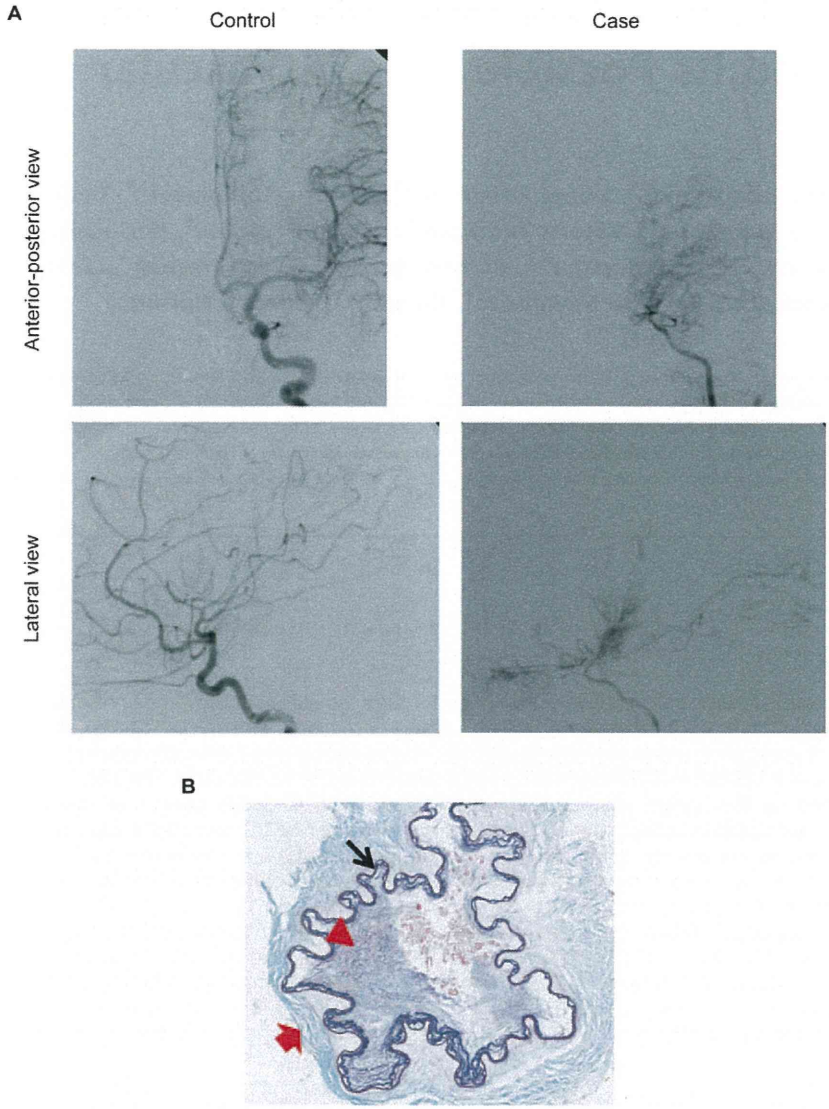

Figure 1. Clinical features of moyamoya disease. (**A**) An anterior-posterior and lateral views of left internal carotid angiography in a 9-year-old child with moyamoya disease (right) and an 8-year-old control child (left). An occlusion with moyamoya vessels can be seen around the terminal portion of the internal carotid artery and proximal portions of the anterior cerebral artery and middle cerebral artery in the affected child. (**B**) Intimal hyperplasia in the middle cerebral artery from an autopsy of a 69-year-old case. Intimal hyperplasia (red arrowhead), atrophic media (red arrow) and winding internal elastic lamina (black arrow) can be seen with Elastica Masson staining, ×200.
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in 1969 [2] spurred international recognition of moyamoya disease [MIM 607151] (Online Mendelian Inheritance in Man in Appendix S1). Moyamoya disease occurs worldwide [3], but its prevalence is highest in East Asian countries, including Japan (1 in 10,000), Korea and China [4], [5]. It is known to cause stroke in neonates and children, and therefore pathological clues for early diagnosis and novel therapeutic approaches are needed [6].

The occlusive lesions are caused by excessive proliferation of smooth muscle cells (SMCs), which specifically occurs in arteries in the skull with compensatory bypass vessels (Figure 1B) [7]. These occlusive lesions are similar to those of atherosclerosis in that both types of lesion exhibit endothelial injury, SMC proliferation and intimal hyperplasia. However, there are major differences between the two types: in moyamoya vessels, infiltrating cellular components such as macrophages and lipid deposits are not observed [8]. A recent study revealed that accelerated vascular remodeling might underlie the pathological consequences of moyamoya disease [9]. Clinical investigations have revealed elevated levels of certain growth factors or cytokines in the cerebrospinal fluid, serum or blood vessels of patients with moyamoya disease [10]–[15]. These factors are assumed to be associated with vascular remodeling.

Epidemiological studies have revealed several risk factors associated with moyamoya disease, including Asian ethnicity, female gender and a family history of the disease [4]. Given that 15% of moyamoya patients have a family history of the disease, genetic factors are suspected to underlie its pathogenesis.

Several studies have explored genetic factors and revealed four loci associated with moyamoya disease: 3p24–p26 [16], 6q25 [17], 8q23 [18] and 17q25 [19]. The locus on 17q25, first mapped by

Yamauchi *et al.* [19], was replicated in 2008 [20], [21]. In our previous study, a strong association of a variant (ss161110142) was demonstrated in East Asian patients, suggesting a possible East Asian founder haplotype for moyamoya disease [21]. The major aim of the present study was to identify the causative gene for moyamoya disease. In the present study, we conducted exome analysis and identified ring finger protein 213 (*RNF213*; DDBJ/EMBL/GenBank accession number AB537889) [National Center for Biotechnology Information (NCBI) in Appendix S1] as a susceptibility gene. In this study, we cloned *RNF213* and determined its open reading frame (ORF). Compared with the previously registered *RNF213* sequence [NM_020914.4] (NCBI in Appendix S1), our *RNF213* clone possesses a 2,500-bp longer 3′ untranslated region (UTR) and lacks exon 4. Since we found that AB537889 seems to be the major transcript of *RNF213*, the descriptions of *RNF213* in this paper are primarily based on the newly determined ORF unless otherwise stated.

Methods

Ethical statement

Ethical approval for this study was given by the Institutional Review Board and Ethics Committee of Kyoto University School of Medicine, Kyoto University, Japan (approval number: G140; approval date: 10/18/2004); by the Ethics Committee of the medical faculty of the University of Tübingen (Ethik-Kommission der Medizinischen Fakultät des Universitätsklinikums Tübingen; permit number: 273/2009BO1; approval date: 1/1/2009); by Seoul National University Hospital Institutional Review Board (approval number: H0507-509-153; approval date 8/24/2005); by the Medical Ethics Committee of Beijing Children's Hospital Institutional Review Board, Capital Medical University, China (approval number: 2005-31; approval date: 3/15/2005); and by the Ethics Committee of University Hospital and the Faculty of Medicine of Palacky University in Olomouc (reference number: 62/10; approval date: 8/18/2008). Participants were recruited in these institutes. All subjects gave written informed consent, or for those considered too young to consent, informed consent was given by their parent or guardian.

Subjects

Diagnostic criteria were based on the Japanese Research Committee on moyamoya disease of the Ministry of Health, Welfare and Labour, Japan (RCMJ) criteria [22]. Information on family histories, gender, age of onset, onset of symptoms and unilateral or bilateral moyamoya disease was obtained either by interview or by clinical chart review, as previously reported [21], [23].

We recruited East Asian cases (Japanese, Korean and Chinese), as described previously [21]. The participants comprised 41 Japanese families and one Korean family (Figure S1 and Table S1), 209 unrelated cases (120 Japanese, 37 Korean and 52 Chinese). We recruited 757 unrelated controls: 384 Japanese, 223 Korean and 100 Chinese (Table S2) and an additional 50 Chinese adult females [Mean age at participation (years ± SD): 38.4±10.5].

We also recruited a Caucasian family of Czech ethnicity (Figure S2A) and 7 unrelated Czech and 42 German cases with moyamoya disease at the University of Tubingen (Germany), and Palacky University and University Hospital (Olomouc, Czech Republic). The mean age (years ± SD) at diagnosis was 30.4±14.7, and the sex ratio of males:females was 16:33. The 284 Caucasian controls were recruited from Palacky University and University Hospital ($n = 120$), the University of Tubingen ($n = 164$) and another 100 Caucasian control samples (the Coriell Caucasian Panel) were bought from the Coriell Institute. The mean age (years ± SD) was 43.9±17.9 and the sex ratio of males:females was 176:208. No magnetic resonance imaging screening of the controls was conducted.

Linkage, haplotype and segregation analyses

We conducted a parametric genome-wide linkage analysis using GENEHUNTER [24] (GENEHUNTER Ver2.1_r6: Appendix S1) in the eight largest three-generation families (peds. 2, 10, 14, 15, 17–20) (Figure 2) with an ABI Prism Linkage Mapping Set (Version 2; Applied Biosystems) with 382 markers, 10 cM apart, for 22 autosomes, and fine-mapping markers designed according to information from the uniSTS reference physical map (http://www.ncbi.nlm.nih.gov/genome/sts/). Unaffected members were treated as "phenotype unknown" because of the incomplete and age-dependent penetrance of moyamoya disease [20]. We considered obligate carriers and subjects with unilateral occlusion or stenosis around the circle of Willis as affected, as previously reported [20]. The disease allele frequency was set at 0.00002 and a phenocopy frequency of 0.000001 was assumed, as previously reported [20]. Population allele frequencies were assigned equal portions for individual alleles. We performed multipoint analyses for autosomes and obtained logarithm of the odds (LOD) scores. Haplotypes were constructed with GENEHUNTER and segregation was investigated using the constructed haplotypes.

To calculate the statistical significance level of the linkage to 17q25.3 in the eight families, we applied a bootstrap method using simulation, as previously reported [25]. We simulated recombination events for 22 chromosomes in these eight families, calculated multipoint LOD scores by GENEHUNTER and obtained the maximum total LOD scores in each trial. The simulation was run 10,000 times.

Exome analysis

We conducted exome analysis on one index case from each of the eight families (peds. 2, 10, 14, 15, 17–20) (Figure 2), who were diagnosed with moyamoya disease based on RCMJ criteria. We targeted all protein-coding regions as defined by RefSeq37 (RefSeq in Appendix S1). Approximately 210,121 coding exons in the 20,794 target genes from 10μg of genomic DNA from each case were captured using an Agilent SureSelect Human All Exon kit, following the manufacturer's protocols. Briefly, DNA was sheared by acoustic fragmentation (Covaris) and purified using a QIAquick PCR Purification kit (Qiagen). The quality of the fragmentation and purification was assessed with an Agilent 2100 Bioanalyzer. The fragment ends were repaired and adaptors were ligated to them (NEBNext DNA sample prep; New England Biolabs). The resulting DNA library was purified using a QIAquick PCR Purification kit, amplified by PCR and captured by hybridization to the biotinylated RNA library "baits" (Agilent). The whole-exome DNA library was sequenced on an Illumina Genome Analyzer IIx in 76-bp paired-end reads using two channels. Sequence reads were mapped to the reference human genome (Ghr37/hg19) (NCBI and UCSC Genome Browser in Appendix S1) using ELANDv2 software (Illumina). Variant detection was performed with CASAVA software (version 1.7; Illumina). Sequence calls were filtered to include only those with >8 coverage, >30 consensus quality and >20 mapping quality. Candidate variants were filtered by SAMtools (Appendix S1). For comparison, we used an exome database of five Japanese controls, who do not have histories of stroke, for which the analysis was conducted by the same protocol and experimental procedures.

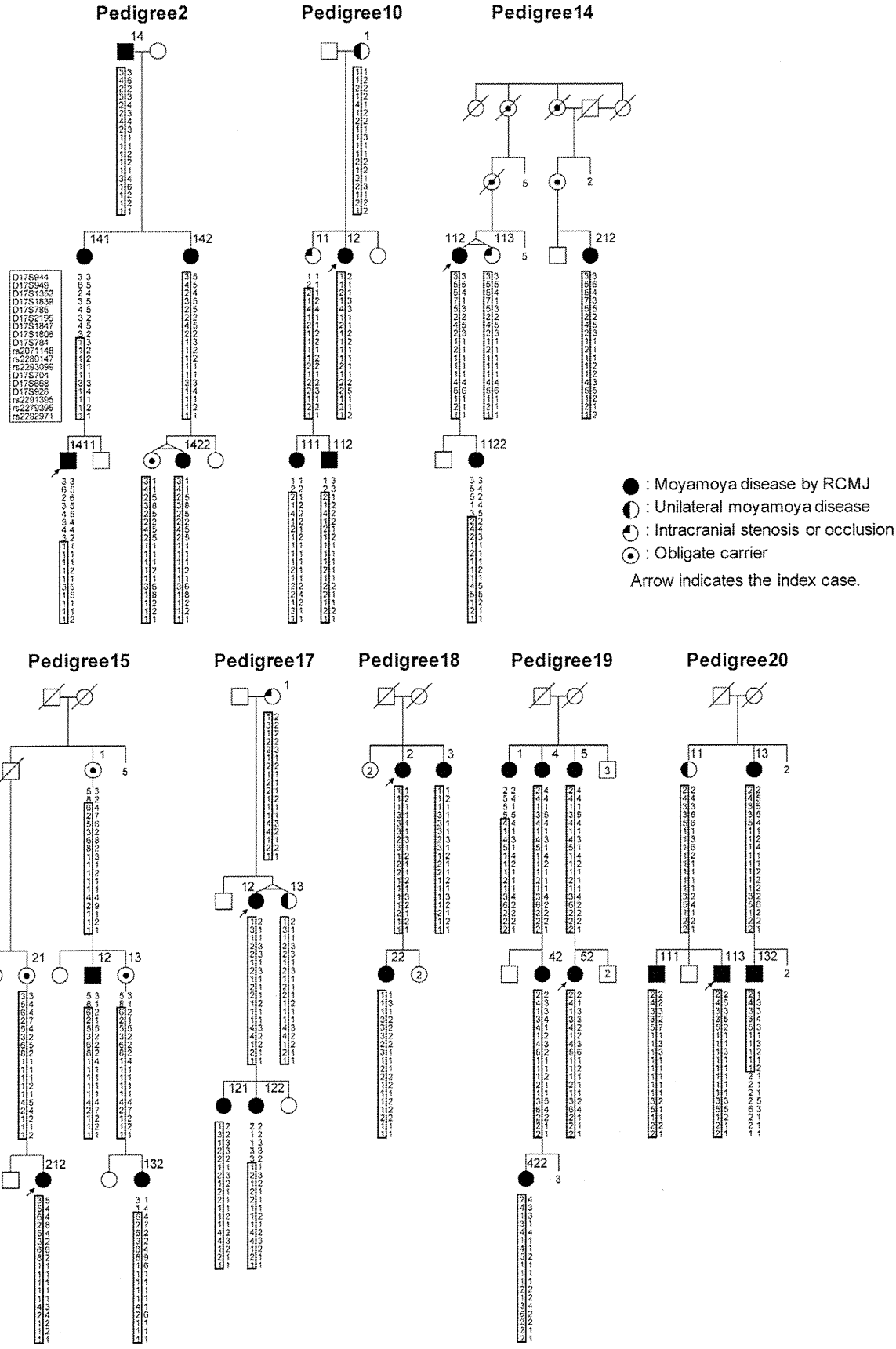
Pedigree2
Pedigree10
Pedigree14
Pedigree15
Pedigree17
Pedigree18
Pedigree19
Pedigree20
D17S944
D17S949
D17S1353
D17S1839
D17S785
D17S2199
D17S1847
D17S1806
D17S784
rs2071148
rs2280147
rs2293099
D17S704
D17S668
D17S926
rs2291395
rs2279395
rs2282971
: Moyamoya disease by RCMJ
: Unilateral moyamoya disease
: Intracranial stenosis or occlusion
: Obligate carrier
Arrow indicates the index case.

Figure 2. The eight three-generation families and haplotype construction with fine markers. For familial index cases in the eight pedigrees, the haplotypes are indicated by the boxes constructed in GENEHUNTER [24]. The smallest disease haplotype spanning from D17S1806 to rs2280147 was estimated by the region common to both 1411 in the pedigree 2 and 132 in pedigree 20.
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Direct sequencing of *RNF213* and *PCMTD1* by the Sanger method

Genomic DNA was extracted from blood samples with a QIAamp DNA Blood Mini kit (Qiagen). The whole *RNF213* gene was sequenced, including the 5′ promoter region at least 2 kb upstream of the first exon, 50–300 bp of each intron upstream and downstream of each exon, all the exons and throughout the 3′ UTR. Target sequences, including the skipped exon 4 were based on the open reading frame of NM_020914.4. Sequencing exon 8 of *PCTDM1* was conducted to confirm the exome result. Sequencing primers were commercially synthesized (PROLIGO Primers & Probes) (Table S3) (Appendix S1). The PCR products were separated on an ABI Prism 3130 Avant DNA sequencer (Applied Biosystems, Tokyo, Japan).

Deep sequencing of introns and intergenic regions of *RNF213*

To search for a hidden causative variant, we sequenced the entire 260-kb genomic region from the 5′ end of *SLC26A11* (bp 78194200) to the 5′ end of *NPTX1* (bp 78450404) (NCBI in Appendix S1) in the index case of pedigree 11, who was homozygous at 17q25.3 because of parental consanguineous marriage, using two bacterial artificial chromosome (BAC) clones (bp 78194200–78238718 and bp 78345170–78450404) (Table S4) or by direct sequencing. The entire RNF213 gene including promoters, 5′UTR, introns, exons and 3′UTR was sequenced in a control sample in the same way as for the index case of pedigree 11. Since we failed to sequence intron 15 of *RNF213*, we used Southern blotting to detect a possible expansion. Details of the BAC cloning and Southern blotting are described in the Text S1. The primer sequences are listed in Table S3.

Genotyping of five rare variants (ss179362671, ss179362673 (p.R4810K), ss179362674, ss179362675 ss161110142) and 34 other single nucleotide polymorphisms (SNPs)

Typing of the five rare variants and 34 SNPs was conducted using Taqman probes (TaqMan SNP Genotyping Assays; Applied Biosystems) using a 7300/7500 Real-Time PCR System (Applied Biosystems) (dbSNP 131 and Hapmap database in Appendix S1). The transmitted haplotypes in 42 families were constructed using GENEHUNTER. Details of the 39 SNPs are described in the Text S1. The allele frequencies were determined in Japanese controls.

Copy number variations (CNVs)

We performed genotyping with Affymetrix 500K arrays (GeneChip® Mapping 250/500 K; Affymetrix, Inc.) according to the manufacturer's protocol. Genomic DNA samples donated by the index cases of pedigrees 5, 11 and 18 and the control spouse of 2 of pedigree 18 were analyzed. The genome-wide call rates were >95% for all the genomic DNAs. CNVs were identified from the intensities using the Affymetrix GeneChip® Chromosome Copy Number Analysis Tool software V.4.01. CNVs were observed using CNAG viewer (Version 2.0; Takara).

Association study with p.R4810K (ss179362673)

Association studies were conducted between 161 Japanese cases (41 index cases from Japanese families and unrelated 120 cases) and 384 Japanese controls, between 38 Korean cases (the index case from a Korean family and 37 cases) and 223 Korean controls and between 52 Chinese cases and 100 Chinese controls (Tables S1 and S2). Cases were exclusively diagnosed as moyamoya by RCMJ criteria.

The association was investigated using SNP & Variation Suite V7 (Golden Helix, Inc. in Appendix S1). Allelic, additive, dominant and recessive models were tested. Population-attributable risk (PAR) was defined as PAR = 100*(K–y)/K, where K is the population prevalence and y is the phenocopy proportion that was estimated from the number of cases with the risk allele [21].

Screening of *RNF213* polymorphisms in controls

Five *RNF213* variants, p.D4863N, p.E4950D, p.A5021V, p.D5160E and p.E5176G were screened in 757 East Asian controls: 384 Japanese, 223 Korean, and 150 Chinese. Four variants, p.N3962D, p.D4013N, p.R4062Q and p.P4608S, were screened in 384 Caucasian controls: 120 Czech, 164 German, and 100 Caucasian. The screening methods are described in the Text S1 and in Table S5.

Immortalized cell lines, cell culture and transient transfection

Lymphoblastoid cell lines (LCLs) were isolated and immortalized with Epstein-Barr virus for five unrelated controls (three individuals, the spouse of individual 2 in pedigree 18 and the spouse of individual 12 in pedigree 17) and six cases (individuals 2 and 22 in pedigree 18, individual 11 in pedigree 11 and individuals 12, 121 and 122 in pedigree 17). HeLa cells and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) and coronary artery smooth muscle cells (CASMCs) were grown in EBM-2 and SmBM-2 (Lonza), respectively. Expression plasmids were transfected into cells using Lipofectamine LTX (Invitrogen) in accordance with the manufacturer's instructions.

Cloning of *RNF213* and construction of an expression vector

We cloned *RNF213* and constructed wild-type and various mutants of the *RNF213* expression vector as described in the Text S1. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB537889 (NCBI in Appendix S1).

Detection of splicing products of *RNF213* cDNA or *FLJ35220* cDNA and real-time quantitative PCR

A Human Total RNA Master Panel II (Clontech Inc) and total RNA from the Artery (BioChain) were reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). We tested whether *RNF213* cDNA skipped exon 4 of *RNF213* by molecular sizing and direct sequencing of cDNA isolated from the RNA of the above human tissues, HUVECs, and LCLs isolated from the five controls and six cases. The PCR primers were designed to cover exons 3–5 as described in the Text S1. We also tested whether or not *FLJ35220* cDNA skipped exon 11 of *FLJ35220* due to G>A substitution in the