Frequency and significance of rare RNF213 variants in patients with adult moyamoya disease

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Abstract

Purpose
Moyamoya disease (MMD) is a rare cerebrovascular disorder characterized by stenosis of the internal carotid arteries with compensatory development of collateral vessels. Although a founder variant of RNF213, p.Arg4810Lys (c.14429G>A, rs112735431), is a major genetic risk factor for MMD in East Asians, the frequency and disease susceptibility of other variants in this gene remain largely unknown. In the present study, we investigated the association of RNF213 variants with MMD in Korean patients and population controls.

Methods
For all RNF213 variants listed in the Human Gene Mutation Database (HGMD) as disease-causing or likely disease-causing mutations for MMD, genotyping was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Genetic data from 264 adult patients with MMD were analyzed and compared with two control populations comprised of 622 and 1,100 Korean individuals, respectively.

Results
Among the 30 RNF213 variants that were listed in the HGMD, p.Arg4810Lys was identified in 67.4% (178/264) of patients with MMD and showed a significantly higher allele frequency than in the controls, giving an odds ratio of 63.29 (95% confidence interval, 33.11–120.98) for the 622 controls and 48.55 (95% confidence interval, 31.00–76.03) for the 1100 controls. One additional variant, p.Ala5021Val (c.15062C>T, rs138130613), was identified in 0.8% (2/264) of patients; however, the allele frequencies were not significantly different from those in the controls.
Conclusions
These results suggest that, in our cohort of Korean patients, the p.Arg4810Lys is the only variant that is strongly associated with MMD among the 30 RNF213 variants listed in the HGMD.

Introduction
Moyamoya disease (MMD; OMIM 607151) is a rare cerebrovascular condition characterized by a progressive steno-occlusive vasculopathy of the large intracranial arteries with compensatory development of collateral vessels [1]. Affected individuals often present with strokes, transient ischemic attacks, and intracerebral hemorrhage [1]. The majority of moyamoya patients are of Asian descent. The annual incidence of MMD is estimated to be 0.35–0.94 per 100,000 people in Japan [1,2], and approximately one-tenth that amount in Europe [3]. Epidemiological data have shown a high incidence and prevalence of MMD in Korea. The annual incidence has steadily increased from 1.7 to 2.3 per 100,000 people from 2007 to 2011 [4].

A strong association between RNF213 p.Arg4810Lys (c.14429G>A, rs112735431) and increased susceptibility for MMD have been reported in previous studies [5,6]. RNF213 p.Arg4810Lys is a major genetic risk factor in East Asian MMD patients, demonstrating a predominantly autosomal dominant inheritance pattern with reduced penetrance [6]. The frequency of the RNF213 p.Arg4810Lys variant in East Asian patients was 73–79% [5–7]; therefore, other genetic factors contribute to the onset and progression of MMD. In addition to the RNF213 p.Arg4810Lys variant, other RNF213 variants have been identified in both East Asian and European patients with MMD [8–10]. However, because of the limited numbers of MMD patients used in these studies [5,6], further study in a large Korean population is necessary. Therefore, we investigated the frequency of MMD-related RNF213 variants, including RNF213 p.Arg4810Lys, in a cohort of Korean patients with MMD compared with control populations.

Materials and methods
Study subjects and RNF213 variant list
We included patients with a diagnosis of MMD at Samsung Medical Center (a tertiary referral hospital in Seoul, Korea) between February 2013 and December 2015. A diagnosis of MMD was based on transfemoral cerebral angiogram findings demonstrating stenosis or occlusion of the terminal portion of the internal carotid artery with the formation of collateral vessels compensating for the arterial occlusion. Based on the Stop Stroke Study Trial of Org 10172 in Acute Stroke Treatment (SSS-TOAST), patients with potential sources of cardioaortic embolism, extracranial atherosclerosis with significant stenosis (≥50%) on the relevant extracranial arteries, other stroke mechanisms (coagulopathy, vasculitis, arterial dissection, etc.), or incomplete evaluations were excluded.

We selected all variants listed in the Human Gene Mutation Database (HGMD professional version of 2015.3) as disease-causing or likely disease-causing RNF213 variant for MMD. This database provides known gene lesions responsible for human inherited diseases, which are selected on the basis of published studies in the literature [11]. A total of 30 RNF213 variants are summarized in Table 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) genotyping was performed for all selected RNF213 variants.
Genomic DNA extracted from peripheral blood leukocytes were subjected to MALDI-TOF MS genotyping. Control data were obtained from the Korean reference genome database for healthy Korean subjects (http://152.99.75.168/KRGDB/). The control data consisted of that from 622 and 1,100 Korean individuals. Demographic information including sex and age for the control group were not available due to the Korean Personal Information Protection. In the final step, we compared genotype data between patients and healthy controls. This study was approved by the Institutional Review Board of Samsung Medical Center (IRB approval 2008-02-046-057).

**Table 1. Thirty RNF213 variants associated with moyamoya disease.**

| Location | Nucleotide | Amino acid | HGMD accession | dbSNP |
|----------|------------|------------|----------------|-------|
| Exon 9   | c.1587_1589delCGC | p.Ala531del | CD1410268 | NA |
| Exon 26  | c.4865C>T      | p.Ala1622Val | CM154727 | NA |
| Exon 41  | c.11671A>G     | p.Met3891Val | CM110690 | NA |
| Exon 42  | c.11797G>A     | p.Val3933Met | CM154728 | NA |
| Exon 43  | c.11884A>G     | p.Asn3962Asp | CM116652 | rs138615753 |
| Exon 44  | c.11990G>A     | p.Cys3997Tyr | CM1410266 | NA |
| Exon 44  | c.12020C>G     | p.Pro4007Arg | CM129860 | NA |
| Exon 44  | c.12037G>A     | p.Asp4013Asn | CM1111820 | rs397514563 |
| Exon 44  | c.12055C>T     | p.Arg4019Cys | CM1410267 | rs139265462 |
| Exon 45  | c.12185G>A     | p.Arg4062Gln | CM116653 | NA |
| Exon 45  | c.12226A>G     | p.Ile4076Val | CM1410269 | NA |
| Exon 46  | c.12391C>T     | p.Arg4131Cys | CM154726 | NA |
| Exon 48  | c.12554A>C     | p.Lys4185Thr | CM1414304 | NA |
| Exon 51  | c.13100A>T     | p.Gln4367Leu | CM129861 | NA |
| Exon 56  | c.13699G>A     | p.Val4567Met | CM110691 | rs145282452 |
| Exon 56  | c.13756A>C     | p.Thr4586Pro | CM129858 | NA |
| Exon 57  | c.13822C>T     | p.Pro4608Ser | CM116654 | NA |
| Exon 57  | c.13891C>G     | p.Leu4631Val | CM129862 | NA |
| Exon 59  | c.14195A>C     | p.Lys4732Thr | CM1410270 | rs148776624 |
| Exon 59  | c.14248G>A     | p.Glu4750Lys | CM155454 | NA |
| Exon 59  | c.14293G>A     | p.Val4765Met | CM110692 | NA |
| Exon 60  | c.14429G>A     | p.Arg4810Lys | CM110689 | rs112735431 |
| Exon 62  | c.14587G>A     | p.Asp4863Asn | CM116647 | NA |
| Exon 63  | c.14780G>A     | p.Asp4927Gln | CM155455 | NA |
| Exon 63  | c.14850G>C     | p.Glu4950Asp | CM116648 | rs371441113 |
| Exon 65  | c.15062C>T     | p.Ala5021Val | CM116649 | rs138130613 |
| Exon 67  | c.15408G>A     | p.Met5136I  | CM129859 | rs376505157 |
| Exon 68  | c.15480C>G     | p.Asp5160Glu | CM116650 | NA |
| Exon 68  | c.15487G>A     | p.Val5163Ile | CM1410271 | rs201733659 |
| Exon 68  | c.15527A>G     | p.Glu5176Gly | CM116651 | NA |

Abbreviations: dbSNP, single nucleotide polymorphism database; HGMD, Human Gene Mutation Database; NA, not available.

*RNF213* reference accession number: NM_001256071.1

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RNF213 genotyping using MALDI-TOF MS analysis

Detection of RNF213 variants was carried out by high-throughput multiplex analysis on a Sequenom MassARRAY MALDI-TOF MS (Sequenom, San Diego, CA, USA). We designed
specific primers to flank the mutation sites as well as extension primers to bind adjacent to the mutation sites using MassARRAY assay design software (S1 Table). The method involves a multiplex primary polymerase chain reaction (PCR) followed by an iPLEX reaction with a single primer that is specific for each genotype.

A primary PCR was performed by combining 1 μl of genomic DNA (10 ng μl⁻¹) with 4 μl of PCR cocktail in a 384-well plate. The PCR cocktail was comprised of 875.5 μl of water, 230.4 μl of PCR buffer, 184.3 μl of 25 mM MgCl₂, 23.1 μl of dNTP mix (25 mM each dNTP), 460.8 μl of primer mix (500 nM each), and 46.1 μl of PCR enzyme. The plate was then subjected to cycling on a thermal cycler (Model T1 plus; Biometra, Goettingen, Germany), and the cycling conditions were as follows: 1 cycle at 94˚C for 4 min, 45 cycles at 94˚C for 20 s, 1 cycle at 56˚C for 30 s, 1 cycle at 72˚C for 1 min, and a final extension step at 72˚C for 3 min. Allele discrimination reactions were carried out by adding 2 μl of iPLEX reaction mix to the dephosphorylated primary PCR reaction mix. Reactions were cycled at 94˚C for 30 s, followed by 40 cycles at 94˚C for 5 s, 1 cycle at 52˚C for 5 s, 1 cycle at 80˚C for 5 s, and 1 cycle at 72˚C for 3 min. The products were spotted onto a 384-spot SpectroCHIP with a MassARRAY Nanodispenser (Sequenom) and analyzed on a MassARRAY Analyzer Compact (Sequenom). If a variant or “no call” was detected in MALDI-TOF MS genotyping, the corresponding exon and intron regions of the genomic DNA were sequenced to confirm the status.

DNA sequencing for RNF213 variants

Targeted coding exons and flanking introns of RNF213 were amplified using primer sets designed by the authors. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. PCR was performed on a Thermal Cycler 9700 (Applied Biosystems, Foster City, CA, USA). The PCR products were sequenced on an ABI Prism 3730xl genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were compared with the reference sequence for RNF213 (NM_001256071.1). All mutations and their putative effects at the protein level were renamed according to the Human Genome Variation Society guidelines.

Statistics

To assess the data quality of RNF213 variants, P-values were calculated by an exact test for Hardy-Weinberg equilibrium. To analyze genotype and allele frequency data, differences between the control and disease groups were compared by a χ² or Fisher’s exact test as appropriate. The calculations were performed with VassarStats (http://vassarstats.net/), and 95% confidence intervals were calculated for each value. All P-values were based on two-sided comparisons, and P-values < 0.05 were considered statistically significant.

Results

Characteristics of the study population

Two hundred sixty-four patients with MMD were included in this study: 185 (70.1%) were female, and the average age was 44.4 ± 14.2 years (range: 18–81 years) (Table 2). One hundred sixty-two patients (61.4%) showed bilateral involvement of the internal carotid arteries on transfemoral cerebral angiogram, and 102 (38.6%) patients showed unilateral involvement. Eighty-four (31.8%) patients had ischemic stroke, 55 (20.8%) patients had TIA, 17 (6.4%) patients had hemorrhagic stroke, and 108 (40.9%) patients were asymptomatic (Table 2).
RNF213 genotype and allele frequencies in control and patient groups

Genotype distributions of RNF213 variants were in Hardy-Weinberg equilibrium. To investigate the associations between RNF213 variants and MMD, genotype and allele frequencies were analyzed using samples from 264 Korean patients with MMD and two sets of controls comprised of 622 and 1100 individuals, respectively.

The genotype and allele frequencies at the 30 RNF213 variant loci are listed in Table 3. RNF213 p.Arg4810Lys was identified in 67.4% (178/264) of patients with MMD. The allele frequency of RNF213 p.Arg4810Lys was significantly higher in MMD patients than in the 622 controls (33.90% in the MMD group versus 0.80% in the control group; \( P < 0.0001 \)) and in the 1100 controls (33.90% in the MMD group versus 1.05% in the control group; \( P < 0.0001 \)). The odds ratio for an association between the RNF213 p.Arg4810Lys variant allele and MMD was 63.29 (95% confidence interval, 33.11–120.98) in the 622 controls and 48.55 (95% confidence interval, 31.00–76.03) in the 1100 controls.

RNF213 p.Ala5021Val (c.15062C>T, rs138130613) RNF213 missense variant was identified in 2 patients with MMD (0.8%, 2/264); however, the allele frequency was not significantly different compared to that of the control group (0.38% in the MMD group versus 0.24% in control group; \( P = 1.00 \)). No other RNF213 variant was identified in patients with MMD.

Discussion

The major finding of this study is that the RNF213 p.Arg4810Lys variant is the main predisposing allele for MMD in our cohort of 30 previously known mutations. Another rare variant, RNF213 p.Ala5021Val, was identified, but did not significantly associate with MMD. Our findings are in line with previous research demonstrating that RNF213 p.Arg4810Lys is the strongest founder variant common in East Asian MMD patients [5,6].

RNF213 on chromosome 17q25 has been recognized as the major susceptibility gene for MMD in East Asians [5,6], and the p.Arg4810Lys variant has been identified in 95% of patients with familial MMD, as well as in 80% of patients with sporadic MMD [5]. A recent study conducted in a mouse model deficient in RNF213 addressed the potential role of RNF213 alteration in the development of aberrant vascular networks in chronic ischemia [12]. However, other functional missense variants of RNF213 have been identified in both East Asians and European patients with MMD [9,10,13]. Several RNF213 non-p.Arg4810Lys variants were recently found in Caucasian and East/South Asian cases of MMD [6,10,14,15]. Additionally,
clinical manifestations and angiographic findings differ between Caucasian and East Asians [16]. The RNF213 p.Arg4810Lys variant is reportedly associated with the ischemic-type MMD, whereas RNF213 non-p.Arg4810Lys variants are associated with hemorrhagic-type MMD [14]. In the present study, 52.6% (139/264) of MMD patients had cerebral ischemia (TIA or cerebral infarction). Only 6.4% (17/264) of the patients had hemorrhage at diagnosis. Although the variant allele was observed at higher frequency in the ischemia group than in the hemorrhage group, this difference was not significant (35.6% and 20.6%, respectively; \( P = 0.08 \)). Further studies of larger cohorts of Asians are required to clarify the association between the RNF213 p.Arg4810Lys variant and clinical characteristics.

### Table 3. Allele frequencies of 30 RNF213 variants in Korean patients with moyamoya disease and two population controls.

| RNF213 genotype | Sample size | MMD patients \( (N = 264) \) | Controls * \( (N = 622) \) | Controls * \( (N = 1100) \) | \( P \)-value † |
|----------------|-------------|-----------------|-----------------|-----------------|-----------|
| | | Carrier frequency % (95% CI) | MAF % (95% CI) | MAF % (95% CI) | MAF % (95% CI) |
| p.Ala531del | 264 | 0 | 0 | 0 | 0 | NA |
| p.Ala1622Val | 264 | 0 | 0 | 0 | 0 | NA |
| p.Met3891Val | 264 | 0 | 0 | 0 | 0 | NA |
| p.Val3933Met | 264 | 0 | 0 | 0 | 0 | NA |
| p.Asn3962Asp | 264 | 0 | 0 | 0 | 0 | NA |
| p.Cys3997Tyr | 264 | 0 | 0 | 0 | 0 | NA |
| p.Pro4007Arg | 264 | 0 | 0 | 0 | 0 | NA |
| p.Asp4013Asn | 264 | 0 | 0 | 0 | 0 | NA |
| p.Arg4019Cys | 264 | 0 | 0 | 0 | 0 | NA |
| p.Arg4062Gln | 264 | 0 | 0 | 0 | 0 | NA |
| p.Ile4076Val | 264 | 0 | 0 | 0 | 0 | NA |
| p.Arg4131Cys | 264 | 0 | 0 | 0 | 0 | NA |
| p.Lys4185Thr | 264 | 0 | 0 | 0 | 0 | NA |
| p.Gln4367Leu | 264 | 0 | 0 | 0 | 0 | NA |
| p.Val4567Met | 264 | 0 | 0 | 0 | 0.08 (0.01–0.45) | NA |
| p.Thr4586Pro | 264 | 0 | 0 | 0 | 0 | NA |
| p.Pro4608Ser | 264 | 0 | 0 | 0 | 0 | NA |
| p.Leu4631Val | 264 | 0 | 0 | 0 | 0 | NA |
| p.Lys4732Thr | 264 | 0 | 0 | 0 | 0 | NA |
| p.Glu4750Lys | 264 | 0 | 0 | 0 | 0 | NA |
| p.Val4765Met | 264 | 0 | 0 | 0 | 0 | NA |
| p.Arg4810Lys | 86 | 177 | 1 | 264 | 67.42 (61.55–72.79) | 33.90 (29.99–38.04) | 0.80 (0.43–1.47) | 1.05 (0.7–1.57) | <0.0001 |
| p.Asp4863Asn | 264 | 0 | 0 | 0 | 0 | NA |
| p.Arg4927Gln | 264 | 0 | 0 | 0 | 0 | NA |
| p.Glu4950Asp | 264 | 0 | 0 | 0 | 0 | NA |
| p.Ala5021Val | 262 | 2 | 0 | 0 | 0.76 (0.21–2.72) | 0.38 (0.10–1.37) | 0.24 (0.08–0.7) | 0.14 (0.05–0.41) | 1.00 |
| p.Met5136I | 264 | 0 | 0 | 0 | 0 | NA |
| p.Asp5160Glu | 264 | 0 | 0 | 0 | 0 | NA |
| p.Val5163Ile | 264 | 0 | 0 | 0 | 0 | NA |
| p.Glu5176Gly | 264 | 0 | 0 | 0 | 0 | NA |

Abbreviations: CI, confidence interval; Het, heterozygous; Hom, homozygous; MAF, minor allele frequency; NA, not available; WT, wild type. Significant results are shown in bold.

*Data from the Korean Reference Genome Database (http://152.99.75.168/KRGDB/)

†MMD patients vs. controls (\( N = 622 \) or \( N = 1100 \))

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Our study revealed that ~33% of Korean MMD patients in our cohort did not harbor susceptibility variants of RNF213, indicating that further studies are required to discover other genetic risk factors for MMD. Several susceptibility genes have been identified for MMD: MMD-2 (gene: RNF213) [5,6], MMD-5 (ACTA2) [17], and MMD-6 with achalasia (GUCY1A3) [18]. Loci for the disorder have been mapped to chromosome 3p (MMD-1) [19], 8q23 (MMD-3) [20], and Xq28 (MMD-4) [21]. Further studies on other modifying factors, such as microRNAs and their polymorphisms and biomarkers (e.g., endothelial progenitor cells) are needed [22,23].

Our study was limited in that we could not determinate whether other RNF213 rare variants apart from the 30 previously discovered RNF213 variants were present in patients with MMD. Exome and genome sequencing could be useful tools to identify novel susceptibility genes or variants for MMD. Additionally, our study had a limited sample size. Further studies with larger cohorts including pediatric patients with MMD are needed. Lastly, intracranial stenosis can be caused by atherosclerosis or other causes (e.g., dissection) in adult patients. All patients underwent conventional angiography and high-resolution magnetic resonance imaging to preclude non-MMD pathologies in selected cases, especially when vascular studies showed controversial results in the diagnosis of MMD [24].

Conclusions
We confirmed that, in our cohort, RNF213 p.Arg4810Lys was strongly associated with MMD among the 30 RNF213 variants listed in the HGMD. Our analysis also indicates that other susceptibility genes exist, providing further insight into the pathogenesis of MMD.

Supporting information
S1 Table. PCR primers used in this study for the MALDI-TOF MS genotyping. (DOCX)

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