RNF213 Rare Variants in an Ethnically Diverse Population With Moyamoya Disease

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Background and Purpose—Moyamoya disease (MMD) is a rare, genetically heterogeneous cerebrovascular disease resulting from occlusion of the distal internal carotid arteries. A variant in the Ring Finger 213 gene (RNF213), altering arginine at position 4810 (p.R4810K), is associated with MMD in Asian populations. However, there are a lack of data on the role of RNF213 in patients with MMD of additional ethnicities and diasporic Asian populations. We investigate the contribution of RNF213 alterations to MMD in an ethnically diverse population based in the United States.

Methods—We initially sequenced RNF213 exons 43, 44, and 45 (encoding the eponymous RING finger domain) and exon 60 (encoding p.R4810K) in 86 ethnically diverse patients with MMD. Comprehensive exome sequencing data from 24 additional patients with MMD was then analyzed to identify RNF213 variants globally. Segregation of variants with MMD and other vascular diseases was assessed in families.

Results—RNF213 p.R4810K was identified in 56% (9/16) of patients with MMD of Asian descent and not in 94 patients of non-Asian descent. 3.6% (4/110) of patients had variants in the exons encoding the RING finger domain. Seven additional variants were identified in 29% (7/24) of patients with MMD who underwent exome sequencing. Segregation analysis supported an association with MMD for 2 variants and a lack of association with disease for 1 variant.

Conclusions—These results confirm that alterations in RNF213 predispose patients of diverse ethnicities to MMD, and that the p.R4810K variant predisposes individuals of Asian descent in the United States to MMD. (Stroke. 2014;45:3200-3207.)

Key Words: moyamoya disease ■ RNF213 protein, human ■ stroke

Moyamoya disease (MMD) is a vasculopathy characterized by progressive stenosis of the internal carotid arteries and its proximal branches accompanied by the development of a compensatory collateral vessel network. Affected individuals often present with strokes, transient ischemic attacks, and intracerebral hemorrhage. There are 2 incidence peaks for MMD, one in children ≈5 years of age and another in adults in their mid-40s. Patients with MMD present with a variety of comorbidities, including headaches, migraines, seizures, and cognitive impairment. Medical treatments to reverse or inhibit the progression of the arterial occlusion are not currently available. Therefore, neurosurgical intervention in the form of direct and indirect revascularization procedures to reduce the risk for strokes has remained the mainstay treatment for these patients. Epidemiological data have shown a higher incidence and prevalence of MMD in Japan, MMD occurs in ≥2 members of a family in ≈9% to 15% of cases, which supports a genetic basis to disease predisposition. Pedigree analysis of these families with MMD suggests an autosomal dominant inheritance pattern with reduced penetrance. A strong association between a RNF213 variant (p.R4810K) and MMD has been demonstrated in Japanese, Korean, and Chinese patients but not in individuals of European descent. The RNF213 p.R4810K variant has been shown to segregate with disease in Asian families and predominately demonstrates an autosomal dominant inheritance pattern with reduced penetrance. In addition to the p.R4810K variant found in East Asian patients, other RNF213 variants...
have been identified in both East Asians and Europeans (patients with MMD from Germany and Czechoslovakia). Interestingly, the frequency of RNF213 variants in Europeans was lower than in Asian patients, and RNF213 p.R4810K was not identified in Europeans.

Because MMD occurs in diverse ethnic populations, we sought to examine the prevalence of RNF213 genetic variants in a multiethnic cohort of patients with MMD from the United States. In this study, we identified the RNF213 p.R4810K variant in patients with MMD of Asian descent and confirm that RNF213 rare variants are associated with MMD in patients of non-Asian descent. Interestingly, RNF213 rare variants in non-Asian families also segregate with early onset occlusive diseases, such as coronary artery disease (CAD), but additional studies are needed to confirm that RNF213 variants also predispose to the additional vascular diseases.

Materials and Methods
Study Population and Sample Collection
This study was approved by the University of Texas Health Science Center at Houston Institutional Review Board, and informed consent was obtained from study participants. A total of 110 families who had ≥1 family member diagnosed with MMD were recruited or referred to an ongoing research study at UTHealth between 2007 and 2013. Demographic data, vascular disease presentation, radiological findings, and surgical and clinical histories were abstracted from the patient medical records when available or obtained by patient self-report. Three generation family histories were obtained via interviews conducted by a genetic counselor or medical student. Consenting relatives of the affected probands with MMD were recruited into the study if available. In addition to MMD, diagnoses of other vascular diseases, such as stroke, myocardial infarction, stenosis of other arteries, arterial aneurysms and dissections, and congenital defects affecting the heart or other vascular structures (eg, aortic coarctation), were recorded for probands and their family members. Medical records documenting disease and risk factor status were obtained when available.

Diagnosis of MMD was based on MR, computed tomography, or diagnostic 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. Patients diagnosed with both unilateral and bilateral MMD were included in this study. Exclusion of other causes of arterial occlusion, such as atherosclerosis, was completed via medical record and imaging review. Premature CAD and stroke were defined as onset of disease at the age of ≤55 years in men and ≤60 years in women. Individuals of all ethnicities diagnosed with MMD at any age were included in this study. Syndromic cases of MMD (Moyamoya syndrome) and those with another established genetic cause for their MMD (eg, ACTA2 mutations) were excluded.

Blood or buccal cells were collected for DNA extraction. Mutation status of individuals was determined by DNA sequencing or inferred based on their location in the pedigree.

DNA Sequencing
Based on Ensembl, RNF213 has 5 splice variants and the longest isoform is NM_001256071, which includes 67 coding exons encoding 5207 amino acids.8 Bidirectional Sanger sequencing of exons 43 to 45 along with exon 60 was performed to identify rare RNF213 functional variants and to confirm the rare variants identified by exome sequencing. Polymerase chain reaction and sequencing primers were designed 60 to 120 bp from the intron–exon boundaries. Polymerase chain reaction was performed using HotStar Taq DNA polymerase (Qiagen Inc, Valencia, CA). Polymerase chain reaction products were treated with EXOSAP-IT (Affymetrix, Inc, Cleveland, OH) to digest the primers and subsequently sequenced using BigDye chemistry (Applied Biosystems, Foster City, CA). The sequencing product was purified using BigDye XTerminator (Applied Biosystems) and then loaded on an ABI3730xl sequencing instrument using the Rapid36 run module. Sequencing results were analyzed using Mutation Surveyor software (SoftGenetics, State College, PA).

Exome Sequencing
One microgram of barcoded shotgun library was hybridized for capture of probes targeting 64 Mb of coding exons (Roche/NimbleGen SeqCap EZ Cap v2) according to the manufacturer’s protocol, and custom blockers complimentary to the full length of the flanking adaptor and barcodes were added. Enriched libraries were amplified via polymerase chain reaction before sequencing (BioRad iProof). Pooled, barcoded libraries were sequenced via paired-end 50 bp reads with an 8 bp barcode read on Illumina HiSeq sequencers. Read data from a flow-cell lane were treated independently for alignment and quality-control purposes in instances where the merging of data from multiple lanes was required. Variant detection and genotyping were performed with the UnifiedGenotyper tool from GATK (version 1.5.29). Variant data for each sample were formatted as raw calls that contained individual genotype data for 1 or multiple samples and were flagged with the filtration walker (GATK) for marking sites that were of lower quality and potential false-positives.

Exome analysis was performed using the Variant Association Tools platform,18 with prioritization based on segregation of rare, damaging variants with disease in families. Additional analyses were also performed using single nucleotide polymorphism and Variation Suite version 8.0.1 (Golden Helix, Bozeman, MT). Heterozygous variants that potentially altered amino acids and were observed at a minor allele frequency (MAF) <0.03% in the NHLBI Exome Sequencing Project were considered candidate mutations. The MAF for each variant was also checked in 1000 Genomes (http://1000genomes.org). For each variant, conservation scores at nucleotide residues were derived from the UCSC Genome Bioinformatics Web site (http://genome.ucsc.edu), and additional bioinformatics analyses were performed using CADD, MutationAssessor, MutationTaster, SIFT, PolyPhen2, and PROVEAN.11–16

Statistics
The segregation of the RNF213 p.D4013N variant in family MMD096 was assessed by 2-point linkage analysis and was performed using the MLINK program of the FASTLINK package.17,18 All individuals with MMD were designated as affected. Additional linkage analyses were performed designating individuals with MMD, premature onset CAD, stroke, or subarachnoid hemorrhage as affected. All linkage analyses were done using affected individuals only, an autosomal dominant mode of inheritance, and a disease allele frequency of 0.0001. For each model, simulation analyses were performed using the MSIM of the SLINK package to obtain the expected maximum LOD score given the pedigree structure, affection status, and availability of genotype data.19

Results
Demographic and Clinical Description of Patients With MMD
In this study, DNA from 110 probands with MMD underwent targeted sequencing of RNF213 or whole exome sequencing (Table 1). All affected individuals who were sequenced had diagnoses of MMD of unknown genetic cause. Of the 110 total probands, the 24 affected individuals who underwent exome sequencing were more likely to be early onset cases with unaffected parents, familial MMD probands, or probands with MMD and other comorbid vascular diseases. The median age of diagnosis of the MMD probands was 28 years, with a range of 9 months to 59 years and the average age of diagnosis...
was 26.7 years. Approximately 75.5% of the MMD cohort was women (83/110). Of the patients whose medical records documented the laterality of their MMD, 73.6% had bilateral involvement (78/106). The majority of the MMD families were of European descent (74.5%; 82/110), but Hispanic, black, and Korean patients each made up 5.5% of the cohort. The remainder of the cohort was composed of individuals from a variety of other Asian ethnicities.

Identification of RNF213 Variants in Patients With MMD

The RNF213 variant previously associated with MMD in Asian patients, p.R4810K, is located in exon 60, and the 4 possibly disease-causing variants (p.N3962, p.D4013N, p.R4062, and p.P4608S) identified in European MMD patients are located in exons 43 to 45, which encode the RING finger domain (1 of 3 functional domains identified in RNF213). Therefore, exons 43, 44, 45, and 60, along with the flanking introns, were initially sequenced in 86 MMD probands to determine whether the RNF213 p.R4810K Asian founder mutation and rare variants in the RING finger domain were present in this US-based cohort. RNF213 p.R4810K was identified in 56% (9/16) of the unrelated MMD families of Asian descent but was not identified in any European American or Hispanic families. RNF213 p.R4810K was confirmed to segregate with MMD in 2 of the 9 families with this variant (MM121, MM056; Figures 1 and 2A; Table 2) but was not fully penetrant, as previously reported. This variant was also identified in novel groups not previously reported. These included patients of Bangladeshi, Indian, and Filipino origin (Table 3). Four rare RNF213 variants located in and around the RING finger domain were identified via Sanger sequencing in this cohort (p.C3997Y, p.I4076V, p.D4013N, and p.R4019C; Table 2; Figures 1 and 2). These rare variants were novel based on an Ensembl search or had a MAF<0.03% in the Exome Variant Server database (http://evs.gs.washington.edu/EVS/).

To determine whether other RNF213 rare variants were present in patients with MMD apart from exons that were sequenced, exome sequencing was performed on 36 individuals from 24 unrelated families, including 24 individuals with MMD and 12 unaffected parents of patients with childhood onset MMD. Gene variants identified via exome sequencing were filtered, and only rare variants that changed the amino acid sequence with a MAF<0.03% in the Exome Variant Server database (http://evs.gs.washington.edu/EVS/) were pursued for further investigation. The MAF threshold was set at 0.03% because we sought to identify RNF213 rare variants that conferred a significant risk for disease and expected to identify variants that are uncommon in the general population, since MMD is a rare condition. Seven RNF213 rare variants were identified via exome analysis in 7 unrelated families, including a 21 bp in-frame insertion starting at amino acid 4951, p.R3922Q, p.D4237E, p.K4732T, p.V5163I, and two 3 bp deletions, p.A529del and p.K4115del (Table 2 and Table 3). All variants were confirmed by Sanger sequencing.

Table 1. Demographic Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>n=110 (Range or Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y (IQR)</td>
<td>28 (9–40)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>27 (24.5%)</td>
</tr>
<tr>
<td>Women</td>
<td>83 (75.5%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
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<tr>
<td>European American</td>
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<tr>
<td>Hispanic</td>
<td>6 (5.5%)</td>
</tr>
<tr>
<td>Black</td>
<td>6 (5.5%)</td>
</tr>
<tr>
<td>Korean</td>
<td>6 (5.5%)</td>
</tr>
<tr>
<td>Japanese</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Filipino</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Indian</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Bangladeshi</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Chinese</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Pakistani</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Vietnamese</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Japanese/Filipino</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Laterality</td>
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<tr>
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<td>78 (70.9%)</td>
</tr>
<tr>
<td>Unilateral</td>
<td>28 (25.5%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (3.6%)</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range.
Rare variants in the Exome Variant Server database were spread evenly throughout RNF213 gene, whereas rare variants identified in our cohort were all located in exons 42 through 68, which encodes the C terminus of RNF213, with a single exception, the p.A529del variant (Figure 1). We have also performed exome sequencing analysis on 86 unrelated probands.
with thoracic aortic aneurysms and dissections and only identified 3 rare RNF213 variants (Figure 1; D. M. Milewicz, MD, PhD, unpublished data, 2014). All 3 rare variants found in the patients with thoracic aortic aneurysms and dissections were located in the exons encoding the N terminus of RNF213 protein. Furthermore, rare RNF213 variants were found at a significantly higher frequency in the MMD cohort when compared with our thoracic aortic aneurysms and dissections cohort (P < 0.05).

RNF213 variants were identified in 8 of 82 European Americans and in 2 of 6 Hispanic families. Only one of these rare variants had been previously identified in a patient with MMD (p.D4013N), and 8 of these variants are novel and not present in exome databases (Table 2). Six of the 8 missense variants are predicted to be possibly damaging or damaging by 2 of 4 functional prediction programs. A family of Asian descent (Japanese/Filipino) was found to have a rare variant with MMD was confirmed in 3 of the 4 functional prediction programs. A family of Asian descent (Japanese/Filipino) was found to have a rare variant with MMD was confirmed in 3 of 4 Asian families (44.5%), who had a history of multiple intracranial aneurysms. In MM044, the proband also had unilateral renal artery stenosis.

RNF213 Variants and Other Vascular Diseases
Two of the MMD probands with RNF213 rare variants presented with comorbid vascular diseases (Figure 2B). The proband in MM060 was diagnosed with coarctation of the aorta at 6 years old, thoracic aortic disease at 35 years old, and had a history of multiple intracranial aneurysms. In MM044, the proband also had unilateral renal artery stenosis.

Although MMD was the primary disease identified in the RNF213 alteration carriers, other vascular diseases occurred in members of half the families, including premature CAD and stroke, subarachnoid hemorrhage, aortic coarctation, thoracic aortic aneurysm, and stenosis of other arteries. Premature CAD and stroke were seen in 4 of the 9 Asian families (44.5%), who carry the p.R4810K mutation (MM144, MM067, MM073, and MM056); segregation analysis was not possible in these families because of the unavailability of familial samples (Figure 2A).

Additional and diverse vascular diseases were also present in families with other RNF213 variants. The most informative family for assessing vascular diseases in family members with a RNF213 variant was MM096, who carried a previously reported RNF213 variant for MMD, p.D4013N. This family had 3 family members who presented with MMD, but individuals with the variant also presented with early onset CAD and stroke and subarachnoid hemorrhage (Figure 2B). In addition, a 14-year-old girl who was at risk for inheriting the p.D4013N variant died suddenly of a stroke without MMD being previously diagnosed. For 2-point linkage analysis using only family members affected with MMD, the observed LOD is equal to expected maximum LOD at 1.20. For analysis using all family members affected with all vascular diseases, the observed LOD score was 1.81, which is close to the expected...
maximum LOD of 2.08. Therefore, the observed LOD score obtained using only affected individuals in family MM096 only is equal to or close to expected maximum LOD, indicating that the \textit{RNF213} variant is likely to be disease causing.

Approximately 45% (5/11) of the families with the non-Asian founder variants had other vascular diseases (MM089, MM096, MM011, MM060, and MM044; Figure 2B). The mother of the proband in MM089 also has the novel p.I4076V variant and was diagnosed with CAD in her 40s. Although a sample for segregation analysis in MM011 was not available, the proband’s father was diagnosed with unilateral carotid artery stenosis and a paternal uncle died from complications secondary to pulmonary artery stenosis at 35 years of age.

\section*{Discussion}

The results of this study indicate that the Asian founder mutation in \textit{RNF213}, p.R4810K, is also present in Asian American patients with MMD and segregates with disease in these families in an autosomal dominant manner with reduced penetrance. It is notable that the \textit{RNF213} p.R4810K variant was not identified in patients with MMD of European, Hispanic, or black descent. However, other rare variants in \textit{RNF213} were identified in European and Hispanic American populations. Of the 24 MMD probands who underwent exome sequencing, 22 were of European American descent and 2 were Hispanic, and \textit{RNF213} variants were identified in 5 of the 22 European American families (23%) and in both of the Hispanic American probands. Thus, the data presented here confirm that the Asian founder mutation, p.R4810K, is a major predisposing allele in Asian Americans, encompassing individuals from Japan, China, Korea, Philippines, India, and Bangladesh. In addition, these data provide novel evidence that novel \textit{RNF213} variants are present in a substantial proportion of MMD in patients of European and Hispanic descent, and strong genetic data indicated that 2 of these variants are disease causing. A novel de novo variant p.K4115del was identified in an affected individual with severe, early onset MMD in family MM131, and segregation of a previously
Occlusive lesions in the large arteries show some pathological features that are similar to atherosclerotic lesions, specifically increased proliferation of smooth muscle–like cells in the lumen or intimal layer. However, MMD occlusive lesions do not have evidence of other features of atherosclerotic lesions, such as cholesterol deposition and inflammatory cells. ACTA2 mutations in human smooth muscle cells and Acta2 (Acta2\textsuperscript{−/−}) deficiency in mouse smooth muscle cells have both been shown to increase rates of proliferation of these cells.\textsuperscript{20,21} The Acta2\textsuperscript{−/−} proliferation could be blocked both in vitro and in vivo using imatinib, which blocks signaling through tyrosine kinase receptors, such as the platelet-derived growth factor receptors. Although mouse models deficient in RNF213 have been made, these models have not provided further information on the connection between RNF213 alterations and MMD.

MMD is a progressive disease in the majority of patients, including in individuals with the RNF213 p.R4810K variant.\textsuperscript{1,20–28} Prevention of strokes and the resulting comorbidities depends on the early identification of at risk individuals predisposed to MMD. Early diagnosis allows for timely surgical intervention to reduce the risk of stroke and possibly decrease cognitive deficits. For example, screening for MMD in patients with unilateral MMD and in high-risk populations, such as those with neurofibromatosis 1 or Down syndrome, has been shown to decrease the prevalence of strokes in these patients.\textsuperscript{31–33} These data and the results presented here suggest that diagnostic screening for the RNF213 p.R4810K variant should be pursued in patients with MMD of Asian descent, and family members screened for the variant if the MMD index case is positive. Baseline cerebrovascular screening for MMD is indicated in family members with the RNF213 p.R4810K variant. Three-dimensional time-of-flight MR angiography imaging for individuals with the RNF213 p.R4810K would be reasonable given the sensitivity for detecting disease and lack of radiation exposure. The frequency of cerebrovascular imaging in asymptomatic p.R4810K carriers should be tailored individually until more data are made available on outcomes. The data presented here also suggest that screening for rare variants in the C-terminal domain of RNF213 (exons 42–68) should be considered for all patients with MMD. Variants previously determined to cause MMD or those that segregate with disease in families could be used to identify other family members at risk for MMD. Ultimately, prevention of future strokes and the resulting comorbidities depends on the early identification of at risk individuals predisposed to MMD.

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Disclosures

None.
References

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