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 predominantly 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 UTHouston 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.\(^4\) 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).\(^5\)

**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,\(^6\) 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
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 \textit{RNF213} Variants in Patients With MMD

The \textit{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 \textit{RNF213}).\textsuperscript{7} Therefore, exons 43, 44, 45, and 60, along with the flanking introns, were initially sequenced in 86 MMD probands to determine whether the \textit{RNF213} p.R4810K Asian founder mutation and rare variants in the RING finger domain were present in this US-based cohort. \textit{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. \textit{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 \textit{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 \textit{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 \textit{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 \textit{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.

\begin{table}
\centering
\caption{Demographic Characteristics}
\begin{tabular}{|l|c|}
\hline
Variable & n=110 (Range or Percentage) \\
\hline
Median age, y (IQR) & 28 (9–40) \\
Gender & \\
Men & 27 (24.5%) \\
Women & 83 (75.5%) \\
Ethnicity & \\
European American & 82 (74.5%) \\
Hispanic & 6 (5.5%) \\
Black & 6 (5.5%) \\
Korean & 6 (5.5%) \\
Japanese & 2 (1.8%) \\
Filipino & 2 (1.8%) \\
Indian & 1 (0.9%) \\
Bangladeshi & 1 (0.9%) \\
Chinese & 1 (0.9%) \\
Pakistani & 1 (0.9%) \\
Vietnamese & 1 (0.9%) \\
Japanese/Filipino & 1 (0.9%) \\
Laterality & \\
Bilateral & 78 (70.9%) \\
Unilateral & 28 (25.5%) \\
Unknown & 4 (3.6%) \\
\hline
\end{tabular}
\end{table}

IQR indicates interquartile range.
Rare variants in the Exome Variant Server database were spread evenly throughout \textit{RNF213} gene, whereas rare variants identified in our cohort were all located in exons 42 through 68, which encodes the C terminus of \textit{RNF213}, with a single exception, the p.A529del variant (Figure 1). We have also performed exome sequencing analysis on 86 unrelated probands...
Table 2. *RNF213* Rare Variants Identified and Predicted Effect of Amino Acid Substitutions

<table>
<thead>
<tr>
<th>As Alt</th>
<th>A529del*</th>
<th>R3922Q+</th>
<th>C3997Y</th>
<th>D4013N</th>
<th>R4019C</th>
<th>I4076V</th>
<th>K4115del+</th>
<th>D4237E+</th>
<th>K4732T+</th>
<th>R4810K</th>
<th>E4950_F4951ins?–</th>
<th>V5163+</th>
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<td>0</td>
<td>0.843</td>
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<td>0.228</td>
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</tr>
<tr>
<td>GERP†</td>
<td>...</td>
<td>3.22</td>
<td>4.54</td>
<td>4.67</td>
<td>–6.62</td>
<td>4.32</td>
<td>...</td>
<td>–3.21</td>
<td>–1.11</td>
<td>2.04</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>
| MutationTaster | | P‡ | P‡ | D§ | P‡ | P‡ | D§ | P‡ | P‡ | D§ | P‡ | ... | ...
| Polyphen2_HVAR | | Benign | Pro | D‡ | Benign | Pos D‡ | Benign | ... | Benign | Benign | Benign | ... | Benign |
| SIFT | | Damaging | Tolerated | Damaging | Tolerated | Damaging | ... | Damaging | Damaging | Damaging | ... | Damaging |
| MutationAssessor | | Low | High | Low | Medium | Medium | ... | Low | Neutral | Medium | ... | Medium |
| Cscore_PHRED | | 10.59 | 14.8 | 16.19 | 19.28 | 9.963 | ... | 5.689 | 8.108 | 6.746 | ... | 1.25 |
| MAF_EVS-EA# | | 0 | 0 | 0 | 0 | 0.00093 | 0 | 0 | 0.000581 | 0 | 0 | 0.000116 |
| MAF_EVS-AA# | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1kG_ASN_AF** | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1kG_ASN_AF** | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

‡ indicates families in which *RNF213* variants were identified through exome sequencing.
*Conservation.
†Conservation.
‡Polymorphism.
§Disease causing.
¶Possibly damaging.
#Minor allele frequencies in European American and black cohorts in the NHLBI Exome Sequencing Project.
**Minor allele frequencies in 1000 Genome project.
¶¶Japanese cohort in phase 1 of 1000 Genomes project.

*RNF213* rare 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),7 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 *RNF213* variant different than the p.R4810K founder mutation. A novel 3 bp deletion (p.K4115del) was confirmed to be de novo in a child with severe and early onset MMD (MM131; Figure 2B). Segregation of the *RNF213* rare variant with MMD was confirmed in 3 of 20 families and decreased penetrance for MMD was noted in 5 families (Figure 2B). Monozygotic twins with the p.D4237E alteration were both diagnosed with MMD at 40 years (MM139; Figure 2B). One twin presented to medical attention with transient ischemic attacks and imaging revealed several ischemic infarcts and bilateral MMD. This diagnosis prompted cerebrovascular screening in the other twin, which revealed bilateral MMD; both underwent revascularization procedures. Only 1 variant, p.R3922Q variant, was found to be discordant with the disease status in a family (MM006; Figure 2B).

*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.7 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 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.

Discussion

The results of this study indicate that the Asian founder mutation in 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 RNF213 p.R4810K variant was not identified in patients with MMD of European, Hispanic, or black descent. However, other rare variants in 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 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 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
identified \*\textit{RNF213} variant, p.D4013N, with disease was con-

firmed in family MM096.

Although limited segregation could be done in the families

with novel \*\textit{RNF213} variants, there is evidence suggesting that

many of these variants are disease causing. All but one

of these variants are located in the C terminus of the \*\textit{RNF213}

protein, which is where the \*\textit{RNF213} p.R4810K founder vari-

ant is located, and also where other variants have been

identified in patients with MMD.\textsuperscript{6,7} The variants are either not present or present at extremely low frequencies in the Exome

Variant Server database. The only variant with evidence that it

may not be disease causing is p.R3922Q, which does not

segregate with disease in the family. It is interesting to note that all of the \*\textit{RNF213} variants identified in patients with

MMD to date are predicted to produce a mutant protein (ie,

no frame-shift or nonsense mutations predicted to lead to
degradation of the message have been identified).

In comparison with our data (Table 2), the Exome Variant

Server database reports 397 variants in \*\textit{RNF213}. A total of

284 of these (71.5\%) have a MAF<0.03\%, including 11 stop-
gain, 4 frame-shift, 3 splice site, and 266 missense variants.

PolyPhen-2 analysis suggests that 125 of these missense vari-

ants are benign, 55 are possibly damaging, 81 are probably
damaging, and 5 have unknown effects. A total of 185 of the

397 rare variants (46.6\%) are located in exons 2 to 41 and 99

(24.9\%) are located in exons 42 to 68. In addition, 1 frame-shift

variant, c.2735del1, has a MAF of 0.59\% in ESP database but

is not have evidence of other features of atherosclerotic lesions,
such as cholesterol deposition and inflammatory cells.\textsuperscript{26} \*\textit{ACTA2}

mutations in human smooth muscle cells and \*\textit{Acta2} (\*\*\textit{Acta2}^+\ )
deficiency in mouse smooth muscle cells have both been

shown to increase rates of proliferation of these cells.\textsuperscript{20,27} The

\*\textit{Acta2}^+ 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 \*\textit{RNF213} have been made,

these models have not provided further information on the con-

nection between \*\textit{RNF213} alterations and MMD.

MMD is a progressive disease in the majority of patients,

including in individuals with the \*\textit{RNF213} p.R4810K vari-

ant.\textsuperscript{1,25–30} Prevention of strokes and the resulting comorbid-

ties depends on the early identification of at risk individuals

predisposed to MMD. Early diagnosis allows for timely sur-
gical 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 \*\textit{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 \*\textit{RNF213} p.R4810K

variant. Three-dimensional time-of-flight MR angiography

imaging for individuals with the \*\textit{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 \*\textit{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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None.
References

Rare Variants in an Ethnically Diverse Population With Moyamoya Disease

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