PHACTR1 is associated with disease progression in Chinese Moyamoya disease

Yongbo Yang [1], **Jian Wang** [2], **Qun Liang** [3], **Yi Wang** [1], **Xinhua Chen** [1], **Qingrong Zhang** [1], **Shijie Na** [1], **Yi Liu** [4], **Ting Yan** [5], **Chunhua Hang** [Corresp., 1], **Yichao Zhu** [Corresp. 6, 7]

[1] Department of Neurosurgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu, China

[2] Department of Neurosurgery, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, Changzhou, Jiangsu, China

[3] Drum Tower Clinical Medical College, Nanjing Medical University, Nanjing, Jiangsu, China

[4] Department of Neurosurgery, West China Hospital, Sichuan University, Chengdu, Sichuan, China

[5] Safety Assessment and Research Center for Drug, Pesticide and Veterinary Drug of Jiangsu Province, Nanjing Medical University, Nanjing, Jiangsu, China

[6] Department of Physiology, Nanjing Medical University, Nanjing, Jiangsu, China

[7] State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, Jiangsu, China

Corresponding Authors: Chunhua Hang, Yichao Zhu
Email address: hang_neurosurgery@163.com, zhuyichao@njmu.edu.cn

Moyamoya disease (MMD) is a progressive stenosis at the terminal portion of internal carotid artery and frequently occurs in East Asian countries. The etiology of MMD is still largely unknown. We performed a case-control design with whole-exome sequencing analysis on 31 sporadic MMD patients and 10 normal controls with matched age and gender. Patients clinically diagnosed with MMD was determined by digital subtraction angiography (DSA). Twelve predisposing mutations on 7 genes associated with the sporadic MMD patients of Chinese ancestry (*CCER2, HLA-DRB1, NSD-1, PDGFRB, PHACTR1, POGLUT1*, and *RNF213)* were identified, of which 8 single nucleotide variants (SNVs) were deleterious with CADD PHRED scaled score >15. Sanger sequencing of 9 cases with disease progression and 22 stable MMD cases validated that SNV (c.13185159G>T, p.V265L) on *PHACTR1* was highly associated with the disease progression of MMD. Finally, we knocked down the expression of PHACTR1 by transfection with siRNA and measured the cell survival of human coronary artery endothelial cell (HCAEC) cells. PHACTR1 silence reduced the cell survival of HCAEC cells under serum starvation cultural condition. Together, these data identify novel predisposing mutations associated with MMD and reveal a requirement for PHACTR1 in mediating cell survival of endothelial cells.

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[1] Department of Neurosurgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Rd., Nanjing 210008, China.

[2] Department of Neurosurgery, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, 29 Xinglong Rd., Changzhou 213003, China.

[3] Drum Tower Clinical Medical College, Nanjing Medical University, 101 Longmian Av., Nanjing 211166, China.

[4] Department of Neurosurgery, West China Hospital, Sichuan University, 37 Guoxue Rd., Chengdu 610041, China.

[5] Safety Assessment and Research Center for Drug, Pesticide and Veterinary Drug of Jiangsu Province, Nanjing Medical University, 101 Longmian Av., Nanjing 211166, China.

[6] Department of Physiology, Nanjing Medical University, 101 Longmian Av., Nanjing 211166, China.

[7] State Key Laboratory of Reproductive Medicine, Nanjing Medical University, 101 Longmian Av., Nanjing 211166, China.

# Equal contribution to this work

* Correspondence to: Chunhua Hang M.D., hang_neurosurgery@163.com and Yichao Zhu Ph.D., zhuyichao@njmu.edu.cn

Short title: PHACTR1 mediates MMD

Authors' e-mail:

Yongbo Yang: yangyongbo3000@163.com
Jian Wang: wj.624@163.com
Qun Liang: liangyiming1991@163.com
Yi Wang: glnxgb@163.com
Xinhua Chen: cxhxiaonei@126.com
Qingrong Zhang: njzhangqingrong@aliyun.com
Shijie Na: nashijie@163.com
Yi Liu: Neurosurgeon@live.cn
Ting Yan: yanting@njmu.edu.cn
Chunhua Hang: hang_neurosurgery@163.com
Yichao Zhu: zhuyichao@njmu.edu.cn

Abstract

Moyamoya disease (MMD) is a progressive stenosis at the terminal portion of internal carotid artery and frequently occurs in East Asian countries. The etiology of MMD is still largely unknown. We performed a case-control design with whole-exome sequencing analysis on 31 sporadic MMD patients and 10 normal controls with matched age and gender. Patients clinically diagnosed with MMD was determined by digital subtraction angiography (DSA). Twelve predisposing mutations on 7 genes associated with the sporadic MMD patients of Chinese ancestry (*CCER2, HLA-DRB1, NSD-1, PDGFRB, PHACTR1, POGLUT1*, and *RNF213)* were identified, of which 8 single nucleotide variants (SNVs) were deleterious with CADD PHRED scaled score >15. Sanger sequencing of 9 cases with disease progression and 22 stable MMD cases validated that SNV (c.13185159G>T, p.V265L) on *PHACTR1* was highly associated with the disease progression of MMD. Finally, we knocked down the expression of PHACTR1 by transfection with siRNA and measured the cell survival of human coronary artery endothelial cell (HCAEC) cells. PHACTR1 silence reduced the cell survival of HCAEC cells under serum starvation cultural condition. Together, these data identify novel predisposing mutations associated with MMD and reveal a requirement for PHACTR1 in mediating cell survival of endothelial cells.

Keywords: Moyamoya disease; Whole-exome sequencing; Mutation; PHACTR1

Introduction

Moyamoya disease (MMD) is a progressive stenosis at the terminal portion of internal carotid artery (ICA) with compensatory development of a hazy network of basal collaterals called Moyamoya vessels (Scott & Smith 2009; Suzuki & Takaku 1969). The prevalence of MMD is the highest in East Asian countries, including Japan, Korea and China (Kuroda & Houkin 2008; Miao et al. 2010). The annual incidences of MMD in China and Japan are 0.43 and 0.54 per 100,000, which are significantly higher than that in the USA (0.086 per 100,000) and Europe (0.3 per 100,000) (Kraemer et al. 2008; Kuriyama et al. 2008; Miao et al. 2010; Uchino et al. 2005). Epidemiology studies had revealed several risk factors associated with MMD, such as Asian ethnicity, female gender and family history (Ganesan & Smith 2015). Although the heritability of MMD is unknown, the genetic components may play an important role in the etiology of MMD (Inayama et al. 2018).

Previous studies have explored and revealed several genetic loci associated with MMD, such as 3q24-p26, 6q25, 8q23, 17q25 (Ikeda et al. 1999; Inoue et al. 2000; Sakurai et al. 2004; Yamauchi et al. 2000). A polymorphism of c.14576G>A in the *RNF213* gene (*RNF213*) on the 17q25-ter region was identified as a novel susceptibility gene for MMD in Japanese and Chinese populations with a founder effect (Liu et al. 2011). *RNF213* is correlated with early onset and severe forms of MMD. Recently, rare variants on the C-terminal of *RNF213* were found correlated with MMD arteriopathy in patients of European ancestry (Guey et al. 2017). While a genome-wide association study involving a large case-control study among Chinese ancestry revealed 10 novel loci could be responsible for MMD, which extended our knowledge of MMD (Duan et al. 2018). However, the etiology of MMD is far more complicated than we expected, and further study on genome wide association is necessary.

Disease progression is among the most frequent reason for clinical symptomatic events. The clinical characteristics has been increasingly recognized as a prevalent cause of disease progression in those patients with natural course, such as early age onset, autoimmune factors and family heritability, but the evaluation is mostly focused on clinical characteristics (Dlamini et al.

2019; Grangeon et al. 2019; Jiang et al. 2018; Kim & Jeon 2014). The relationship between genetic risk factor and disease progression in MMD patients remains unknown.

In this study, we performed a case-control design with whole-exome sequencing analysis on 31 sporadic MMD patients (9 cases with disease progression and 22 stable MMD cases) and 10 normal controls with matched age and gender. Predisposing mutations were discovered and then validated by Sanger sequencing. We identified 12 predisposing mutations on 7 genes associated with the sporadic MMD patients of Chinese ancestry and further validated that SNV (c.13185159G>T, p.V265L) on *PHACTR1* was highly associated with the disease progression of MMD. Finally, we evaluated the effect of PHACTR1 on cell survival of endothelial cells.

Material and methods

Subjects

We enrolled 31 MMD subjects and 10 non-related healthy controls with matched age and gender during 2018 to 2019 at Department of Neurosurgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China. Subjects recruited into this study were all Chinese ancestry. The study was reviewed and approved by the research ethics committee, Nanjing Drum Tower Hospital of Nanjing University Medical School (2018-173-01). Written informed consent was taken from each participant.

Patients with MMD determined by digital subtraction angiography (DSA) were based on the guidelines established by the Japanese Research Committee on Moyamoya disease of the Ministry of Health, Welfare and Labor, Japan (RCMJ) (Fukui 1997). Information on family histories, gender, age, onset symptoms were obtained by interview. Cases with additional evidence of arthrosclerosis, meningitis, autoimmune diseases, brain neoplasm, Down syndrome, Recklinghausen disease, irradiation or other obvious specific etiologies were excluded. All included MMD subjects all first time came to the hospital and diagnosed with the MMD. All these MMD subjects did not have any drug treatment for MMD or other conditions.

Treatment and clinical follow-up

Through the use of single-photon emission computed tomography (SPECT), cerebral blood flow (CBF) was semi-quantitatively measured for all MMD patients 1 month after the initial onset. After preoperative imaging evaluation, surgical revascularization, including superficial temporal artery to middle cerebral artery anastomosis combined with encephalo-duroarterio-synangiosis (EDAS), was conducted in the hemisphere with intracranial hemorrhage or with more severe ischemia. If a patient elected not to undergo surgery, conservative management was used instead. After baseline investigation and surgical intervention, all patients underwent clinical follow-up. Brain MRI and MRA were performed every 6 to 12 months. During the follow-up period, if major intracranial artery stenosis progression was suspected on MRA or cerebrovascular events occurred, cerebrovascular DSA was performed within 1 week. The presence or absence of disease progression was evaluated at the final follow-up visit which defined as the progression of any major intracranial artery stenosis >50% in the ICA and/or the posterior cerebral artery (PCA). Representative cases with disease progression are shown in Figure 1.

Blood sample extraction and storage

Three tubes of 15 mL peripheral blood samples were collected and subjected to DNA extraction (Qiagen). DNA extraction method is seen in the exome sequencing analysis section. DNA samples were used immediately to next experiments, or store at -80°C for later usage.

Whole-exome sequencing and Sanger sequencing

We conducted exome sequencing analysis on 31 sporadic MMD subjects and 10 non-related healthy control subjects. Peripheral blood samples were collected from the subjects, and DNA was extracted using the QIAamp™ DNA and Blood Mini kit (Qiagen™, Munich, Germany), and sheared using acoustic fragmentation (Covaris) and purified using a QIAquick PCR Purification Kit (Qiagen). The whole-exome DNA library was sequenced on an Illumina HiSeq X Ten platform and performed as previously described (Stachler et al. 2015).

The Sanger sequencing was performed in Genscript Ltd. (Nanjing, China).

Bioinformatics analysis

Raw sequencing reads and all qualified reads were processed with an in-house bioinformatics pipeline, which followed as previously described (Stachler et al. 2015). Duplicated fragments were marked by Picard v1.141. After converting the data into bam format, GATK BaseRecalibration module was used to improve the base quality and then HaplotypeCaller module was used to discover genetic variants (SNV/INDEL). SnpEff 4.3i and Gemini v0.18.0 were used for functional annotation with Online Mendelian Inheritance in Man (OMIM), the Exome Aggregation Consortium (ExAC) Browser, MutationTaster2 and the Combined Annotation Dependent Depletion (CADD).

Variants filtering and selection

Variants were excluded if they had a call rate <98%, major allele frequency <1%, abnormal heterozygosity > (mean±3SD), or significant deviation from Hardy–Weinberg equilibrium among controls ($P_{hwe}<1\times10^{-4}$). The non-coding, synonymous, impact_severity=low, MAF (minor allele frequency) >0.01 variants were excluded from the raw data. SO_IMPACT=HIGH was referenced using MAF database including ESP, 1KG, ExAC database. The definition of "SO_IMPACT=HIGH" is based on the Gemini (https://gemini.readthedocs.io/en/latest/content/database_schema.html#details-of-the-impact-and-impact-severity-columns). The filtered variants was compared with known MMD related genes, such as *RNF213, CCER2, HLA-DRB1*. SNVs with CADD PHRED scaled score >15 were regarded as deleterious.

siRNA transfection and Western blotting

Human coronary artery endothelial cell (HCAEC) was kindly gifted from Dr. Shengnan Li (Nanjing Medical University) and cultured in DMEM plus 10% fetal bovine serum (FBS, Hyclone,

Waltham, MA). SiRNA targeting *PHACTR1* (sc-95456, Santa Cruz Biotechnology, Santa Cruz, CA) and scrambled siRNA were transiently transfected into HCAECs using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). HCAEC cells 48 h after transfection were lysed with ice-cold RIPA lysis buffer. The SDS-PAGE was performed as the standard procedure. Anti-PHACTR1 (sc-514800, Santa Cruz Biotechnology) and anti-β-actin (Sigma, St. Louis, MO) were used. Protein bands were visualized with ECL reagent (Thermo Scientific, Rockford, IL) and recorded by Tanon 5200 Multi Imaging Workstation (Tanon, Shanghai, China).

Cell survival assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used for the assessment of HCAEC cell survival and performed as previously described (Zhu et al. 2012).

Statistical analysis

Continuous variables were described as mean ± SD, and categorical variables were presented as number and percentage. An independent *t*-test, and a chi-square or Fisher exact test were used to compare patients with and without disease progression. A P value < 0.05 was considered significant. All statistical analyses were performed using SPSS 23.0 (IBM, Chicago, Illinois).

Results

Characteristic of subjects and variants quantity

The mean age (years±SD) of all enrolled cases was 33±8.46, and sex ratio of male: female was 0.34. The mean age and sex ratio of these healthy controls were 35.1±6.58 and 0.3, which matched the disease group (Table 1). Of the 31 patients, 14 (45.2%) presented as intracranial hemorrhage and 17 (54.8%) presented as ischemic stroke at baseline. Among these 31 subjects, 9 of them suffered from disease progression in 10 hemispheres during the follow-up periods including 8 in the anterior circulation and 2 in the posterior circulation, and the other 22 cases were diagnosed as stable MMD disease.

By comparing with whole-exome sequencing results of the 31 sporadic MMD subjects with the human genome GRCh37.75, total 15,206 genes were successfully picked out. The main sequencing quality and depth were 85.42 M±11.32 M reads, 99.69±0.16 map (%), 111.87±12.42 depth, suggesting a high quality of whole-exome sequencing. Total 196,365 variants were found including 176,582 site nucleotide polymorphisms (SNP), 8,906 insertions (INS) and 10,877 deletions (DEL) (Table 2). Total 117,748 (51.359%) variants were mis-senses, 1,525 (0.665%) variants were non-senses, and 109,992 (47.976%) variants were silent types. By comparing with the healthy subjects, 10,241 variants in exons were discovered.

Significant predisposing mutations associated with disease progression

By comparing with the known MMD-related genes, 12 predisposing mutations on 7 gene exons had from medium to high impact on gene modification (Table 3). These genes includes *RNF213, CCER2, HLA-DRB1, NSD-1, PDGFRB, PHACTR1* and *POGLUT1,* 8 of these SNVs were deleterious with CADD PHRED scaled score >15. Next, we performed Sanger sequencing to validate the association between predisposing mutations and MMD disease progression. SNV (c.13185159G>T, p.V265L) on *PHACTR1* was found in 3 cases with disease progression and shown significantly associated with its progression.

The effect of PHACTR1 on cell survival

We hypothesized that PHACTR1, one of 7 genes mentioned above, may mediate cell survival of human endothelial cells. We knocked down the expression of PHACTR1 by transfection with siRNA targeting PHACTR1 (Figure 2A), and then measured the cell survival of HCAEC cells (Figure 2B). Western blotting revealed that siRNA targeting PHACTR1 largely downregulated the expression of PHACTR1 in HCAEC cells, insulting in a ~80% decrease (Figure 2A). After the successive 96 h measurement of cell survival rate, we found that PHACTR1 silence reduced the cell survival of HCAEC cells under serum starvation cultural condition for 96 h, but not for 24~72 h (Figure 2B).

222

223 **Discussion**

224 All of the recruited sporadic MMD patients are all first-time being diagnosed with this
225 disease, and did not receive any clinical treatment or drugs administration for any other medical
226 conditions. *RNF213* was identified as a novel susceptible gene for MMD in East Asian population.
227 This study identified 4 novel susceptibility loci and confirmed the previous reported susceptibility
228 gene *RNF213*. Furthermore, we identified *HLA-DRB1* variants are found in the sporadic MMD
229 patients. *HLA-DRB1* play a central role in the immune system by presenting peptides derived from
230 extracellular proteins (Ji et al. 2018; Lauterbach et al. 2014). Although MMD patients with auto-
231 immune diseases were excluded from this study, immune system dysfunction is still associated
232 with the MMD pathophysiological process. *CCER2* was reported as a biomarker for MMD by
233 Japanese colleagues (Mukawa et al. 2017). *NSD-1* is a transcriptional factor (Jo et al. 2016). In
234 this study, we found a variant on *CCER2* locus and two mutations on *NSD-1*.

235 Our results suggest that cytoskeleton system and cardiovascular development are involved
236 with the MMD pathological process. *PDGFRB* and *PHACTR1* work on the actin cytoskeleton
237 system, and *PDGFRB* regulates cardiovascular development (Onel et al. 2018; Perez-Hernandez
238 et al. 2016). *POGLUT1* works on the NOTCH signaling pathway to regulate developments (Wu
239 et al. 2017). According to Sanger sequencing, we found that SNV (c.13185159G>T, p.V265L) on
240 *PHACTR1* was significantly associated with MMD disease progression. Owing to the lack of
241 appropriate animal model for MMD, we provisionally checked the effect of genes associated with
242 MMD susceptibility on immortal endothelial cells. We found PHACTR1 mediates the cell survival
243 of endothelial cells under serum starvation cultural condition. Nevertheless, the precise
244 mechanisms of MMD needs to be further studied on molecular and cellular levels.

245 The main limitation of the study is the small size of samples. The subjects are difficult to
246 recruit, we have attempted to perform a meta-analysis to strengthen or our conclusions. We have
247 gone through three databases and obtained 76 articles about MMD (Pubmed, n=23; Embase, n=33;
248 Web of science, n=20). The duplicated articles (n= 42) were removed. Then, 29 articles were

249 excluded through sceening title or/and abstract, full text, due to review, animal studies, case report
250 and/or unrelated outcomes, moyamoya syndrome or other diseases. After reading the full text of
251 13 sceened articles, we have not found these 8 SNVs mentioned in Table 3. So, we are
252 unfortunately unable to perform a meta-analysis in the present research status.
253
254 **Conclusions**
255 We perform whole-exome sequencing on 31 sporadic MMD subjects and 10 healthy volunteers,
256 and identify 12 predisposing mutations of 7 genes (*RNF213, CCER2, HLA-DRB1, NSD-1,*
257 *PDGFRB, PHACTR1* and *POGLUT1*) associated with MMD and SNV (c.13185159G>T,
258 p.V265L) on *PHACTR1* associated with its progression. We preliminarily provide the rational
259 evidence of the effect of PHACTR1 on endothelial cell survival and indicate its involvement in
260 the pathophysiological process of MMD.
261
262 **Abbreviations**
263 MMD, Moyamoya disease; HCAEC, human coronary artery endothelial cell; DSA, digital
264 subtraction angiography; MCA, middle cerebral artery; SNV, single nucleotide variant; MAF,
265 minor allele frequency.
266
267 **ADDITIONAL INFORMATION AND DECLARATIONS**
268 **Funding**
269 This work was supported by grants from Key Project supported by Medical Science & Technology
270 Development Foundation of Nanjing Department of Health (ZKX15014) to Yongbo Yang, Jiangsu
271 Postdoctoral Foundation of Jiangsu Provincial Department of Human Resources & Social Security
272 (1501122B) to Yongbo Yang, and the Science and Technology Foundation of Nanjing Medical
273 University (2017NJMU001) to Ting Yan.
274
275 **Competing Interests**

The authors declare there are no competing interests.

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366 **Figure Legends**

367

368 **Figure 1. Representative cases of disease progression in MMD.**

369 (A) right internal carotid angiograms of a 48 y/r man, showing artery stenosis progression in the
370 proximal portion of right MCA (arrows). (B) right posterior cerebral angiograms of a 51 y/r
371 woman, showing artery stenosis progression in the proximal portion of right PCA (arrows).

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373 **Figure 2. PHACTR1 silence reduces the cell survival of HCAEC cells.**

374 (A) HCAEC cells were transiently transfected with siRNA targeting PHACTR1 or scrambled
375 siRNA. Western blotting verified the knockdown efficiency of siRNA targeting PHACTR1. β-
376 actin as the loading control. (B) HCAEC cells transfected with siRNA targeting PHACTR1 or
377 scrambled siRNA were cultured under serum starvation cultural condition for successive 96 h. Cell
378 survival rate was assessed by cell survival assays. PHACTR1 silence reduced the cell survival of
379 HCAEC cells under serum starvation cultural condition for 96 h.

Table 1(on next page)

Table 1. Demographic and clinical data of MMD cases and healthy controls

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Characteristics	MMD Group	Healthy Group
Age (Mean±SD)	33±8.46	35.10±6.58
Gender (Male/Female)	0.34	0.30
Ages<18y (%)	7 (22.58%)	2 (20%)
Ages≥18y (%)	24 (77.42%)	8 (80%)
Family history	No	No
Disease progression (%)	9 (29.03%)	---
Stable disease (%)	22 (70.97%)	---
Other combined severe conditions	No	No

Table 2(on next page)

Table 2. Total amount of variants found on every chromosome

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Chromosome	Length	Variants
1	249,250,621	19,250
2	243,199,373	13,079
3	198,022,430	10,188
4	191,154,276	7,014
5	180,915,260	8,074
6	171,115,067	12,066
7	159,138,663	10,358
8	146,364,022	6,694
9	141,213,431	8,301
10	135,534,747	8,454
11	135,006,516	11,547
12	133,851,895	9,960
13	115,169,878	3,211
14	107,349,540	6,748
15	102,531,392	7,578
16	90,354,753	9,221
17	81,195,210	10,799
18	78,077,248	3,263
19	59,128,983	13,925
20	63,025,520	4,873
21	48,129,895	2,394
22	51,304,566	5,289
X	155,270,560	3,838
Y	59,373,566	241
Total	**3,095,677,412**	**196,365**

3

Table 3(on next page)

Table 3. Associations between predisposing mutations and disease progression in MMD.

1 Missence variant.

2 According to Sanger sequencing.

3 *P* value for χ^2 test.

4 CADD PHRED-like scaled C-scores = -10*log_10 (rank/total), the recommended deleterious threshold was >15 for scaled C-scores.

1 **Table 3. Associations between predisposing mutations and disease progression in MMD.**

Locus	Gene	Position	Ref	Alt[1]	Codon change	Amino acid change	Qual	Depth	Impact	CADD raw score	CADD PHRED scaled score[4]	Progression /Stable	wildtype[2]	Mutant[2]	*P* value[3]
19q13.2	***CCER2***	39401715	C	T	c. 39401715C>T	p.G67R	1763.4	3451	Medium	1.187	14.37	Progression	9	0	0.71
												Stable	21	1	
6p21.32	***HLA-DRB1***	32549352	G	C	c. 32549352G>C	p.P212A	372.77	6450	Medium	1.940	18.63	Progression	8	1	0.71
												Stable	22	0	
		32551942	T	C	c. 32551942T>C	p.D105G	2688.4	16207	Medium	2.289	21.90	Progression	8	1	0.71
												Stable	22	0	
5q35.3	***NSD1***	176562643	T	C	c. 176562643T>C	p.I180T	1044.4	2736	Medium	2.862	23.30	Progression	9	0	0.71
												Stable	21	1	
		176638711	A	G	c. 176638711A>G	p.H1104R	1108.4	2199	Medium	0.003	2.68	Progression	9	0	0.71
												Stable	21	1	
5q32	***PDGFRB***	149513304	G	A	c. 149513304G>A	p.P260L	2883.4	4330	Medium	2.866	23.30	Progression	9	0	0.71
												Stable	21	1	
6p24.1	***PHACTR1***	13185159	G	T	c. 13185159G>T	p.V265L	4277.88	4183	Medium	2.622	22.80	Progression	6	3	*0.019*
												Stable	22	0	
3q13.33	***POGLUT1***	119211265	A	T	c. 119211265A>T	p.M387L	1367.4	2013	Medium	0.846	12.20	Progression	9	0	0.71

												Stable	21	1	
17q25.3	***RNF213***	78291014	A	T	c. 78291014A>T	p.Q995H	736.4	3008	Medium	0.677	10.91	Progression	9	0	0.71
												Stable	21	1	
		78319385	T	G	c. 78319385T>G	p.I2466S	3066.4	3554	Medium	3.499	25.10	Progression	9	0	0.71
												Stable	21	1	
		78320960	T	C	c. 78320960T>C	p.V2991A	2086.4	3562	Medium	1.924	18.48	Progression	9	0	0.71
												Stable	21	1	
		78321631	A	G	c. 78321631A>G	p.I3215V	1814.4	3802	Medium	2.739	23.10	Progression	9	0	0.71
												Stable	21	1	

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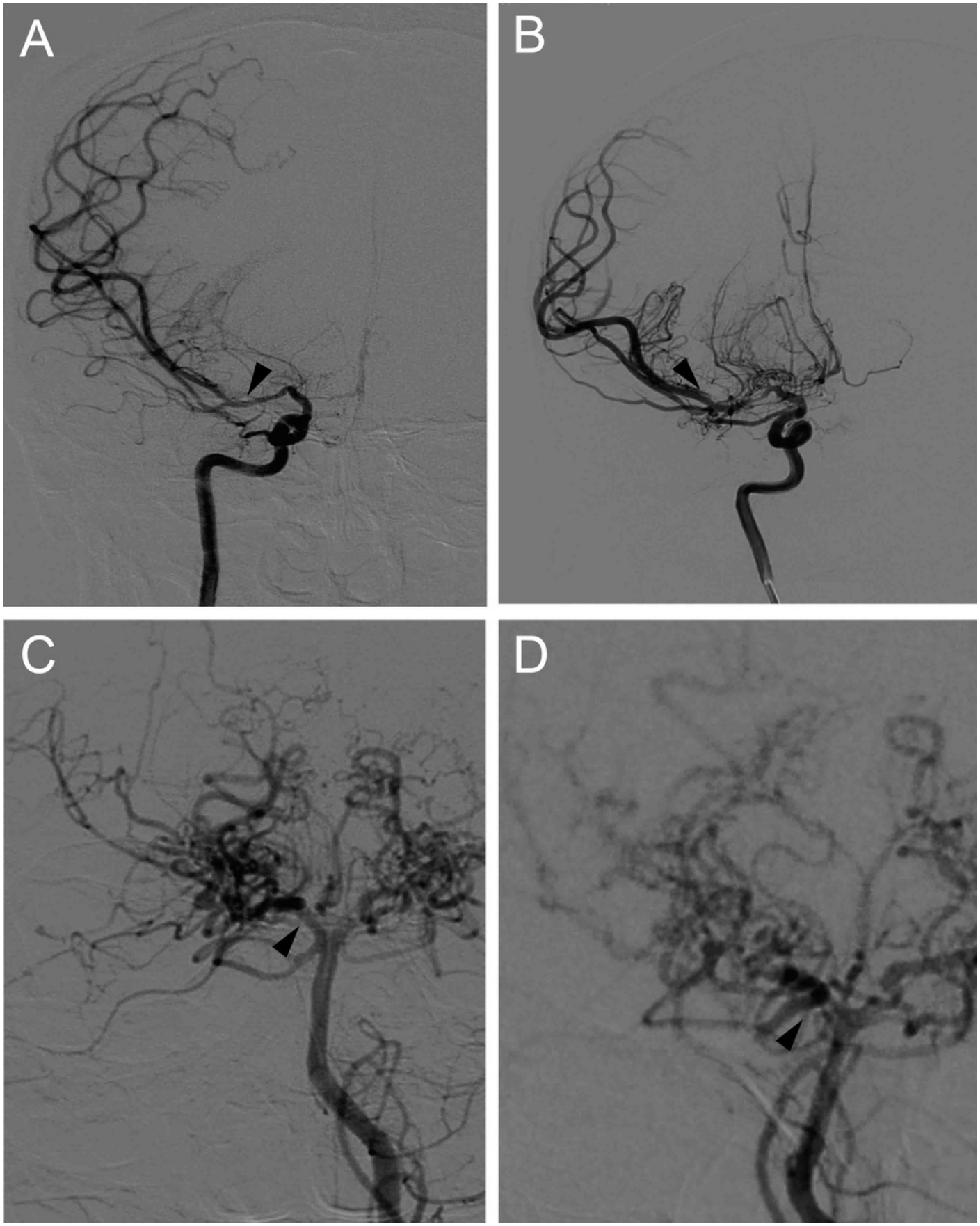
A
B
C
D

Figure 2

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