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RAPID COMMUNICATION

Whole exome sequencing and functional validation identify *CAPN1* variants as a cause of Chinese moyamoya disease

Moyamoya disease (MMD, MIM 607151) is a rare vascular condition that has high recurrence, mortality, and disability rates, and an effective treatment for this disease is currently lacking. The main symptoms of affected children and adults include ischemic and hemorrhagic strokes, with an age of onset that follows a bimodal distribution trend at approximately 5 and 40 years of age. Affected individuals are at risk of intracranial hemorrhagic or ischemic stroke, seizures, cognitive impairment, and developmental delays. A strong ethnicity-related effect, combined with family aggregation, suggests a genetic basis for predisposition to MMD. Ring finger protein (RNF213) p.R4810K is only found in 20% of Chinese patients with MMD.[1] Other pathogenic variants underlying MMD are yet to be identified.

Whole exome sequencing (WES) was applied to analyze the candidate variants of all six families without *RNF213* p.R4810K (Fig. 1A; Table S1). A detailed screening flowchart of the variants is illustrated in Figure S1. Thirty pathogenic or damaging variants potentially associated with MMD were derived using pathogenicity prediction algorithms and knowledge-based prioritization (Table S2). A total of 173 MMD cases without *RNF213* p.R4810K and 114 controls were used for multiplex PCR and sequencing of the selected candidate variants (Table S3). The control population included our controls and the general population in the public database. After analysis, 12 variants were identified in this cohort. Frequencies of four candidate variants (*NLRP14* rs11041150, *AHI1* rs1186817196, *CAPN1* rs139570056, and *BLM* rs55880859) were significantly higher in the cases than in at least one control population database (Table S4). Except for c.1246C > T (p.R416C) in *BLM* (rs55880859) for participant 14 of family D, the other variants were validated by Sanger sequencing in the corresponding individuals. Previous gene studies on MMD and its rare pathogenic genes based on WES were conducted using Japanese, Chinese, and European cohorts. However, duplication and cross-verification are lacking. Interestingly, the identified variant did not pass the screening process of this study but finally appeared in the candidate gene list. The different races, large genetic heterogeneity, different screening criteria, and limited sample size of our study may lead to failure to reproduce the results.

According to the published susceptibility genes or proteins, biological processes, pathways, and disease-related databases most related to the pathogenesis of MMD, comprehensive and reasonable evidence exists for the selection of *CAPN1* as the only gene for in-depth study in the next stage. Surprisingly, CAPN1 was found to interact with filamin A (FLNA) (Fig. 1B), which was identified as one of the two known substrates [*NFAT1* (nuclear factor of activated T cells 1) and *FLNA*] of *RNF213*. Accumulated *FLNA* may lead to negative (contractile) remodeling of blood vessels by damaging vascular integrity, which is an important process leading to smoky angiogenesis.[2] *CAPN1* p.R616C was predicted to exist at the end of the first EF-hand domain (589—617, 714 aa) (Fig. 1C). Furthermore, two additional *CAPN1* variants (p.D128N and p.S646L) were identified in two cases through comprehensive exome sequencing of *CAPN1* (Fig. 1C). The newly identified variants, *CAPN1* p.R616C and p.S646L, were located at the end of the first and second EF-hands. Many calcium-binding proteins belong to the same evolutionary family and share a calcium-binding domain known as the EF-hand. Ca^{2+} binding induces a conformational change in the EF-hand motif, leading to activation or inactivation of target proteins. Therefore, *CAPN1* p.R616C may cause the EF-hand domain to weaken or even lose its ability to bind to Ca^{2+}, thereby inhibiting the cleavage ability of FLNA and leading to its accumulation.[3] Conservation alignment revealed that the mutated amino acids of *CAPN1* are conserved among

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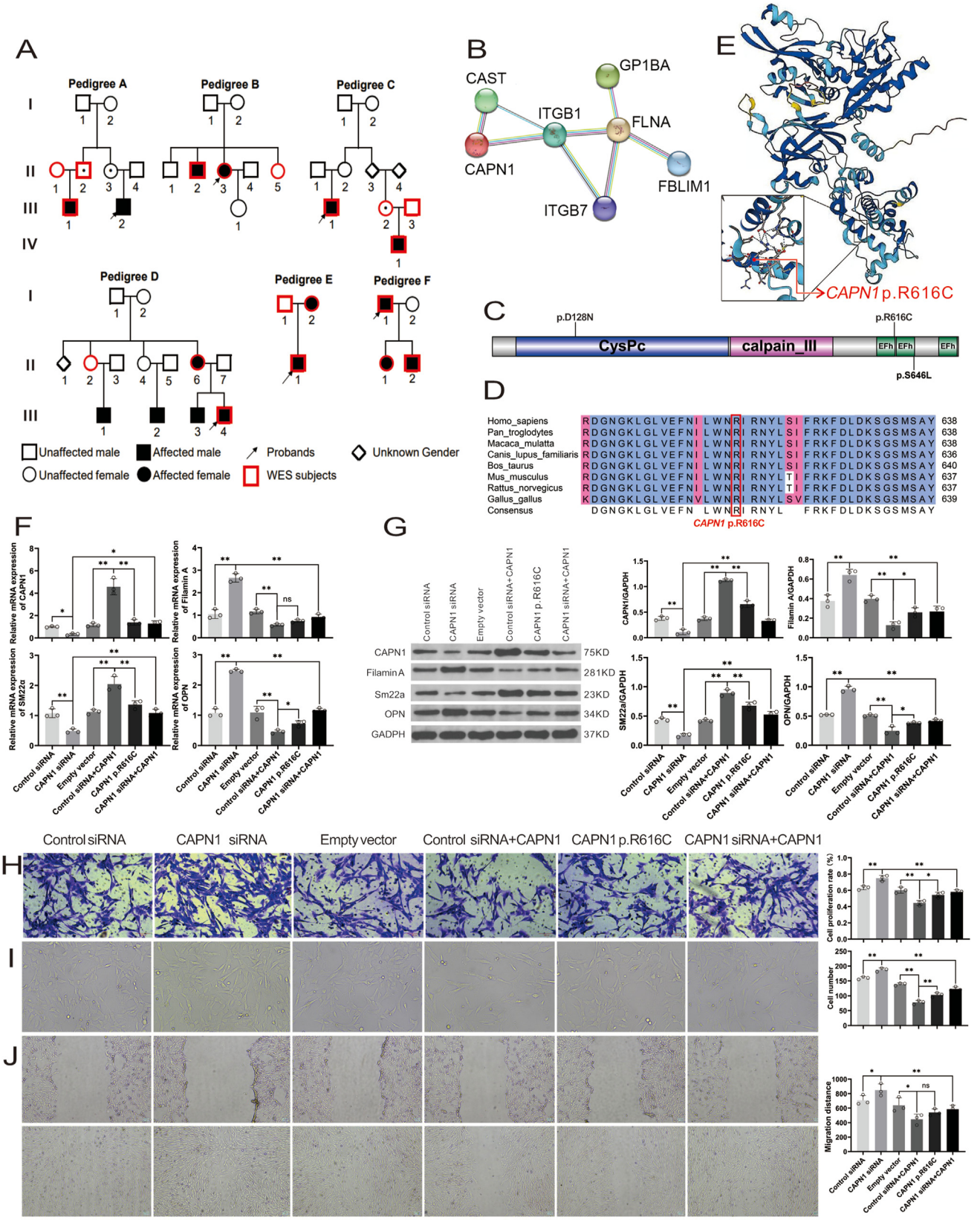

Figure 1 Identification, filtering, bioinformatics analysis, and *in vitro* verification of biomolecules' functional verification of *CAPN1* variants. **(A)** Pedigrees of six Chinese moyamoya disease (MMD) families. WES, whole exome sequencing. **(B)** Protein—protein interaction network analyzed using the STRING database. The network codes represent the proteins produced by corresponding protein-coding genes that are mainly associated with ion channels. **(C)** Locations of the *CAPN1* variants, p.D128N, p.R616C, and

different species (Fig. 1D) and a three-dimensional protein model of CAPN1 is conducted (Fig. 1E).

Our replicate association study results indicated that *CAPN1* p.R616C was significantly associated with the risk of MMD (Table S6). The baseline characteristics of all participants included in this analysis and carrying *CAPN1* variants screened at each stage of the study were summarized in Table S5 and Table S7, respectively.

We then constructed the *CAPN1* silencing, overexpression, and p.R616C mutated plasmids. *In vitro* transfected SV40T immortalized primary human artery smooth muscle cells (IPHASMC) indicated that silencing of *CAPN1* leads to an increase in the expression of *FLNA* and osteopontin (*OPN*), and a decrease in the expression of smooth muscle 22α (*SM22*α), promoting the phenotypic transformation of VSMCs (Fig. 1F and G). The immunofluorescence test results of *CAPN1* verified that the silencing, overexpression, and mutant plasmids were successfully constructed, which also indicated that most of *CAPN1* was probably located in the cytoplasm (Fig. S2). Next, we used CCK-8, transwell, and scratch wound assays to determine the VSMC proliferation, invasion, and migration capacities. The proliferation rate (Fig. 1H), cell migration number (Figs. 1I), and 48-h migration distance (Fig. 1J) of VSMCs were increased by *CAPN1* silencing.

CAPNs are involved in the maintenance of vascular endothelial cell integrity and angiogenesis. Previous studies suggested that *CAPN1* mediates matrix metalloproteinase (MMP)-2 expression and consequently affects the invasion of fibroblasts and leukemic cells.[4] Therefore, the regulation of *CAPN1* on MMPs may be crucial in characteristic angiogenesis and vascular remodeling of MMD; however, the specific mechanism must be clarified *in vitro* and *in vivo*. Notably, a certain interaction was found between *FLNA* and *CAPN1* based on the protein interaction network model. Coincidentally, previous studies found that *FLNA* is one of the two potential substrates of RNF213 E3 ubiquitin ligase.[5] *FLNA* plays a vital role in the differentiation and migration of VSMCs and signal transduction in these cells. Therefore, *FLNA* is known to contribute to the change in VSMC phenotype from a "contractile" to a "synthetic" phenotype, which leads to the over-proliferation of VSMCs, secretion of elevated amounts of extracellular matrix, and acceleration of vascular remodeling. Therefore, this remodeling might be associated with the negative (constrictive) remodeling of vessels, another characteristic feature of MMD.

A functional link between *RNF213* variants and MMD has not been established. However, studies on two substrates (*NFAT*1 and *FLNA*) in RNF213 and related biological pathways, especially the role of WNT/Ca^{2+}/NFAT signaling during angiogenesis, are emerging. Based on previous evidence, owing to insufficient proteasomal degradation by mutated *RNF213*, WNT/Ca^{2+}/NFAT signaling was enhanced. Further, *FLNA* and *NFAT1* levels were revealed to be increased upon silencing of *RNF213*.[2] Therefore, the following assumptions can be made: first, *CAPN1* and *RNF213* have similarities in their action on *FLNA*; second, the Wnt/Ca^{2+} signaling pathway plays an important role in the angiogenesis and vascular remodeling of MMD, and its upstream and downstream regulatory factors must be further studied; and finally, *CAPN1* and *RNF213* may play a synergistic role in the pathogenesis of MMD, and many unknown substrates and some functional variants must be identified and verified.

Herein, we report the pedigree-based WES to identify potential rare, large-effect pathogenic variants and verify the variant in the largest sample size in Chinese patients with MMD. We reported this systematic verification study that combined bioinformatics analysis and experimental verification of biomolecules based on the identification of a new variation. *CAPN1* p.R616C significantly increased the risk of MMD and had a loss-of-function effect on *CAPN1* expression. Furthermore, our mechanistic investigation into the role of *CAPN1* in VSMC revealed that *CAPN1* knockout contributes to the failure of *FLNA* cleavage, promotes phenotypic transformation of smooth muscle cells, significantly enhances their proliferation and migration, and promotes vascular remodeling and angiogenesis.

p.S646L, are shown below in the schematic diagram of the domain structure of the CAPN1 (714 amino acids in human) protein. CysPc, calpain-like thiol protease family (peptidase family C2), calcium-activated neutral protease (large subunit). Calpain_III, a group of cysteine peptidases belonging to the MEROPS peptidase family C2 (calpain family, clan CA). A type example is calpain, which is an intracellular protease involved in many important cellular functions that are regulated by calcium (EFh domain: EF-hand, calcium-binding motif). EF-hands are calcium-binding motifs that occur at least in pairs. Links between disease states and genes encoding EF-hands, particularly the S100 subclass, are emerging. Each motif consists of a 12-residue loop flanked on either side by a 12-residue alpha-helix. The location marked with red dots is the location of the *CAPN1* p.R616C variant. **(D)** Conservation alignment shows that the mutated amino acids of CAPN1 are conserved among different species. **(E)** Construction of the three-dimensional model of the calpain-1 catalytic subunit using the AlphaFold Protein Structure Database. **(F)** *CAPN1* silencing promotes human aortic vascular smooth muscle cell (HAVSMC) phenotype transformation. mRNA levels of *CAPN1*, filamin A (*FLNA*), contractile and synthetic indicator proteins, smooth muscle 22α (*SM22*α), and osteopontin (*OPN*) in HAVSMCs. **(G)** Representative images of Western blot analysis for CAPN1, FLNA, SM22α, OPN, and GAPDH. Relative grey values of the protein bands are shown, and the statistical results of protein expression were calculated ($n = 6$). At least three independent experiments were performed for each group. **(H)** Representative images (left) and quantification of the cell viability results (right) from Cell Counting Kit-8 assay. **(I)** Representative images (left) and quantification of the results (right) obtained from the transwell assay. **(J)** Representative images (left) and quantification (right) of the scratch migration assay results ($n = 6$). At least three independent experiments were performed for each group. *$p < 0.05$, **$p < 0.001$.

Ethics declaration

The ethical issues involved in this study were examined and approved by the Ethics Committee of China Medical University and the Chinese PLA General Hospital. All patients and immediate family members signed written informed consent forms.

Author contributions

Yue Wang, Zhengxing Zou, Wanyang Liu, Lian Duan: Conceptualization, methodology, software, writing - original draft preparation, reviewing, editing, and funding acquisition; Zhibin Yang, Zhengshan Zhang: Data curation, software, validation, reviewing, and editing; Jun Xu, Fangbin Hao: Visualization, investigation, reviewing, and editing; Juan Shen, Cong Han: Investigation, software, validation, reviewing, and supervision. All authors participated in the interpretation of data and the manuscript preparation and review. All authors read and approved the final draft.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.101090.

References

1. Zhang Q, Liu Y, Zhang D, et al. RNF213 as the major susceptibility gene for Chinese patients with moyamoya disease and its clinical relevance. *J Neurosurg*. 2017;126(4):1106—1113.
2. Scholz B, Korn C, Wojtarowicz J, et al. Endothelial RSPO3 controls vascular stability and pruning through non-canonical WNT/Ca^{2+}/NFAT signaling. *Dev Cell*. 2016;36(1):79—93.
3. Blanchard H, Grochulski P, Li Y, et al. Structure of a calpain Ca^{2+}-binding domain reveals a novel EF-hand and Ca^{2+}-induced conformational changes. *Nat Struct Biol*. 1997;4(7):532—538.
4. Popp O, Heidinger M, Ruiz-Heinrich L, Ries C, Jochum M, Gil-Parrado S. The calpastatin-derived calpain inhibitor CP1B reduces mRNA expression of matrix metalloproteinase-2 and-9 and invasion by leukemic THP-1 cells. *Biol Chem*. 2003;384(6):951—958.
5. Zhu G, Chen H, Zhang W. Phenotype switch of vascular smooth muscle cells after siRNA silencing of filamin. *Cell Biochem Biophys*. 2011;61(1):47—52.

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