Differentially expressed circular RNA profile in hemorrhagic and ischemic moyamoya disease

MENG ZHAO[1,2*], WENJIE LI[1,2*], XUN YE[1,2], QIAN ZHANG[1,2], YAN ZHANG[1,2] and JIZONG ZHAO[1,2]

[1]Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing 100070, P.R. China;
[2]China National Clinical Research Center for Neurological Diseases, Beijing 100070, P.R. China

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Abstract. Hemorrhage is a frequent manifestation in patients with moyamoya disease (MMD). Compared with MMD patients with other subtypes, patients with hemorrhagic MMD (hMMD) are at higher risk of poor prognostic outcomes, Circular RNAs (circRNAs) frequently display dysregulated expression in several human diseases. In the present study, the role of circRNAs in the pathogenesis of hemorrhage in MMD was investigated. Microarray profiling on 12 moyamoya disease samples, consisting of six hMMD and six matching ischemic MMD (iMMD) samples, was performed. Reverse transcription-quantitative PCR was then used to confirm the microarray analysis findings. Bioinformatics tools, including Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis, were used for further assessment. A network map of circRNA-microRNA-gene interactions was also constructed. In total, 3,607 differentially expressed circRNAs, in which 1,940 circRNAs were upregulated and 1,967 circRNAs were downregulated, were identified in hMMD (fold change ≥2.0 and P<0.05) samples. Gene Ontology revealed that the differentially expressed circRNAs were mainly involved in 'cell cycle phase transition' and 'mitotic cell cycle phase transition'. In addition, the ubiquitin mediated proteolysis pathway was found to be the most significantly enriched pathway in hMMD samples. The results of the present study suggested that clusters of circRNAs were differently expressed in hMMD compared with those in iMMD samples, which provides novel insights into hemorrhage in moyamoya disease pathophysiology and potential targets for future therapy.

Correspondence to: Dr Jizong Zhao, Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, 119 South 4th Ring West Road, Fengtai, Beijing 100070, P.R. China
E-mail: zhaojizong@bjtth.org

*Contributed equally

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Introduction

Moyamoya disease (MMD) is characterized by the progressive occlusion of bilateral carotid forks, which are associated with moyamoya vessels formation at the base of the brain (1,2). MMD tends to be more prevalent in Asian countries. Since the etiology of MMD remains poorly understood, the criteria for the diagnosis of MMD are mainly based on characteristic angiographic findings. Ischemic attack and hemorrhage are two of the most common presentations of MMD. In particular, the hemorrhagic subtype is associated with a poor clinical course, which is found in 20% patients with MMD (3), but only 50% patients experience adequate recovery after the first hemorrhagic event (4). There is no clinical scale for stratifying the occurrence of ischemic MMD (iMMD) and hemorrhagic MMD (hMMD), nor is there a tool for the prediction of rebleeding or hemorrhagic transformation. Previous studies have suggested that different molecular mechanisms underlie the two subtypes.

RNA expression studies offer unique opportunities for understanding the pathogenesis of a wide variety of neurological diseases (5,6). Several studies suggest that non-coding RNA, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are associated with hemorrhagic neurological diseases (7-10). However, it remains unclear whether altered expression of such non-coding RNAs directly results in hemorrhage in cerebrovascular diseases. Circular RNAs (circRNAs) form another class of stable, single-stranded, non-coding RNAs that are formed by back-splicing events through exon or intron circularization (11-13). Previous studies have reported that circRNAs can regulate gene expression on transcriptional or post-transcriptional levels by functioning as miRNA sponges (11,14). In addition, stroke has been reported to alter the expression of circRNAs with possible functional implications in poststroke pathophysiology (15). Since circRNA-based research is an emerging area of investigation, to the best of our knowledge, circRNA expression is abnormally expressed in MMD (16). No study to date has investigated the circRNA expression profile in hMMD.

Therefore, the present study focused on the potential differences in the circRNA expression profile between hMMD and iMMD. circRNA dysregulation may serve a role in the hemorrhage of MMD. CircRNAs may become potential future biological targets and prognostic indicators for hemorrhage in MMD.

Materials and methods

Patient selection and sample collection. In total, adult patients diagnosed with MMD presenting with hemorrhage or transient ischemic attack were recruited from Beijing Tiantan Hospital (Beijing, China) between March and July 2016. The diagnosis of MMD adhered to the guidelines established by the Research Committee on Moyamoya Disease of the Ministry of Health, Labor, and Welfare of Japan (17). Inclusion criteria were: Age ≥18 years; diagnosis of definite MMD per Research Committee guidelines; presenting with either hemorrhage or transient ischemic attack; no prior surgical revascularization; no other cerebrovascular conditions; no systemic diseases that could affect RNA expression; informed consent provided. Exclusion criteria encompassed pediatric patients, those with quasi-MMD (moyamoya syndrome) and individuals with history of other cerebrovascular conditions, hypertension or diabetes to mitigate potential confounding factors. Patients with conditions known to influence RNA expression were excluded, including autoimmune diseases, chronic infections, malignancies, chronic inflammatory conditions, recent major surgeries, and any use of immunosuppressive or anti-inflammatory medications. Finally, 12 patients (5 males and 7 females, age 22-50 years old) were included in microarray analysis.

Whole venous blood samples (12 patients with MMD for microarray and 22 patients for validation, 3 ml each) were obtained from the patients with MMD 2 weeks post-symptom onset, prior to any revascularization procedures. Blood samples were collected between March and July 2016 with patients' written informed consent for biobanking and future research use. The specific circRNA analysis protocol was reviewed and approved by the Ethics Committee Review Board of Beijing Tiantan Hospital (approval no. KYSQ2020-161-01) prior to conducting the molecular studies.

RNA extraction and microarray analysis. Total RNA was isolated from blood samples using TRIzol® reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RNA quantity and integrity were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and agarose gel electrophoresis, respectively. Sample labeling and circRNA array hybridization were performed according to the manufacturer's protocol (Arraystar, Inc.). CircRNA enrichment, amplification and fluorophore-labeled cRNA synthesis were performed using the Super RNA Labeling kit (Arraystar, Inc.), followed by purification with the RNeasy Mini kit (Qiagen GmbH). The labeled cRNAs were hybridized onto the Arraystar Human circRNA Array (6x7K; Arraystar, Inc.) for 17 h at 65˚C in an Agilent Hybridization Oven (Agilent Technologies, Inc.). Post-hybridization, arrays were washed, fixed and scanned using a G2505C scanner (Agilent Technologies, Inc.). Raw data extraction was conducted using Feature Extraction v.11.0.1.1 software (Agilent Technologies, Inc.). Subsequent data processing, including quantile normalization, was performed using R v.3.3 software (R Foundation for Statistical Computing; https://www.r-project.org). Differentially expressed circRNAs were defined as those with fold changes ≥2.00 and P<0.05. Hierarchical clustering was employed to visualize distinct circRNA expression patterns among samples.

Reverse transcription-quantitative (RT-q) PCR. To validate the microarray data, RT-qPCR was conducted. Cells were harvested at approximately 80% confluence (approximately $1x10^6$ cells/ml) for RNA extraction. Total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. CircRNA expression levels were quantified using a ViiA 7 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Divergent primers were designed to specifically amplify circRNAs and differentiate them from their linear isoforms (Table I). The PCR cycling conditions were as follows: initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. β-actin served as the internal control. The expression level of each circRNA was calculated as a fold change using the $2^{-\Delta\Delta Cq}$ method (18).

Bioinformatics analysis and circRNA/miRNA gene network construction. The microarray data are in the supplementary tables. All raw relevant datasets are in the supplementary materials. The parent linear mRNAs of differentially expressed circRNAs were subjected to Gene Ontology (GO) analysis (http://www.geneontology.org) to elucidate the functional enrichment of these coding genes. Pathway analyses were conducted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/).

A circRNA/miRNA gene network was constructed for the differentially expressed circRNAs identified from microarray and RT-qPCR validation experiments. CircRNA/miRNA interactions were predicted using the Arraystar miRNA target prediction software (version 1.0, Arraystar, Inc.; https://www.arraystar.com), which integrates TargetScan and miRanda algorithms (19). miRNA target gene analysis was performed using miRTarBase (20). All miRNA gene targets were experimentally validated with strong evidence (western blotting or Reporter assay). The circRNA/miRNA gene network was visualized using Cytoscape 2.8.2 (https://cytoscape.org).

Statistical analysis. All data are presented as the mean ± standard error. Statistical comparisons were performed using paired t-tests or independent t-tests as appropriate. All statistical analyses were conducted using R v.3.3 software (R Foundation for Statistical Computing; https://www.r-project.org). P<0.05 was considered to indicate a statistically significant difference.

Results

Overview of circRNA profiles. A total of 12 patients with MMD, namely 6 hMMD and 6 age and sex matched patients with iMMD, were enrolled into the present study (Table SI). The expression profiles of human circRNAs were obtained by microarray analysis (Table SII). Differentially expressed circRNAs with statistical significance (fold changes ≥2.0 and P<0.05) between hMMD and iMMD groups were identified using a volcano plot (Fig. 1A) and scatter plot (Fig. 1B). A total of 3,607 circRNAs with expression change >2x were identified (Table SIII). Compared with iMMD, 1,940 circRNAs

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Primer
β-actin	Forward: 5' GTGGCCGAGGACTTTGATTG3'
	Reverse: 5' CCTGTAACAACGCATCTCATATT3'
hsa_circRNA_103572	Forward: 5' ATGTGGAAAATTTCCTAGAAGC 3'
	Reverse: 5' AGGTCTGTCATCACTCTGAGGT 3'
hsa_circRNA_103574	Forward: 5' GTACCTAAATTAACAATGGCGA 3'
	Reverse: 5' AAGGGGTGAAGCATGACCT 3'
hsa_circRNA_029937	Forward: 5' GGCCATAGGAAAAGGATACAG 3'
	Reverse: 5' CTCTAGGTCCCAAGAATTTACC 3'
hsa_circRNA_104293	Forward: 5' GCACAGATCTGATTCTGAACGT 3'
	Reverse: 5' TCATTGGATATGTCCTGATAGTCC 3'
hsa_circRNA_025016	Forward: 5' TATTCCCTTTCCAGAAGATGAT 3'
	Reverse: 5' CATAGTTGGAACCAGGTTGG 3'
hsa_circRNA_091419	Forward: 5' CGTGTTTTCCTCTCTGAATCTG 3'
	Reverse: 5' TCGCTTAATCCTGAAAGTCTTG 3'
hsa_circRNA_060184	Forward: 5' ACCCGCCATGGGAGTGTG 3'
	Reverse: 5' GGGGCTTCCAGCAGTGCT 3'

circRNA, circular RNA.

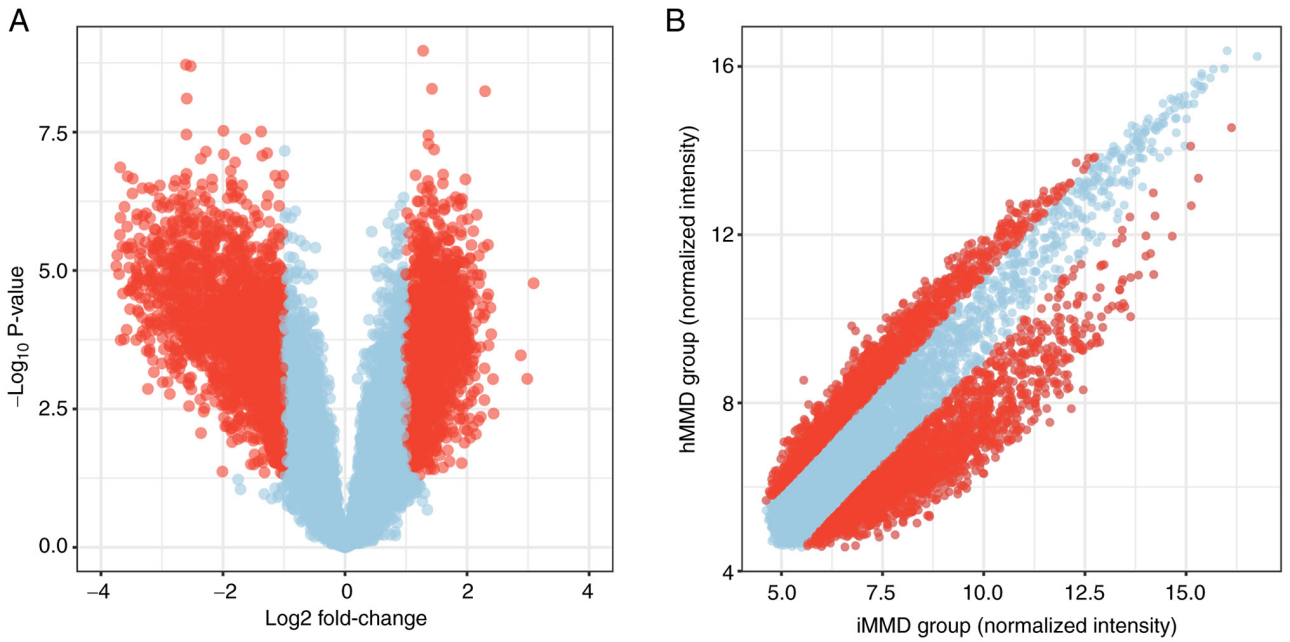

Figure 1. Differential expression and correlation analysis of circRNA in MMD. (A) Volcano plots and (B) scatter plots of circRNA expression profiles in MMD and control groups. Red dots represent differentially expressed circRNAs (P<0.05 and fold change ≥2.0). circRNAs, circular RNAs; MMD, moyamoya disease.

were upregulated and 1,967 circRNAs were downregulated in hMMD samples. Hierarchical clustering revealed the circRNA expression patterns between hMMD and iMMD were significantly different (Fig. 2). The complete dataset from the present study is available in the supplementary materials, including all raw microarray data files and associated documentation.

RT-qPCR validation of the microarray data. RT-qPCR was conducted in a group of 11 hMMD and 11 iMMD samples to verify the differential expression of the candidate circRNAs. A total of seven circRNAs were selected for RT-qPCR validation based on: i) Magnitude of differential expression (fold change >2), ii) statistical significance (P<0.05), and iii) predicted interactions with MMD-relevant pathways. A total of seven circRNAs, including four upregulated circRNAs (circRNA-025016, circRNA-104293, circRNA-060184 and circRNA-091419) and three downregulated circRNAs (circRNA-029937, circRNA-103574 and circRNA-103572), were selected for further assessment. The results obtained from RT-qPCR were consistent with the RNA sequencing data

Table II. Top five GO biological processes of upregulated and downregulated target genes with most significance in hMMD compared with iMMD.

GO term	Fold enrichment	P-value
A, Upregulated target genes		
GO:0006402 mRNA catabolic process	2.979113618	4.34E-11
GO:0000956 nuclear-transcribed mRNA catabolic process	3.061329416	4.82E-11
GO:0000184 nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	3.571550985	5.75E-10
GO:0006614 SRP-dependent cotranslational protein targeting to membrane	3.840377404	3.09E-09
GO:0006613 cotranslational protein targeting to membrane	3.714413025	3.26E-09
B, Downregulated target genes		
GO:0044770 cell cycle phase transition	2.764948269	4.06E-13
GO:0044772 mitotic cell cycle phase transition	2.818120352	4.06E-13
GO:0018205 peptidyl-lysine modification	2.811021812	2.10E-11
GO:0016570 histone modification	2.719584675	2.12E-11
GO:0007059 chromosome segregation	2.764948269	1.57E-08

GO, Gene Ontology; MMD, moyamoya disease; h, hemorrhagic; i, ischemic.

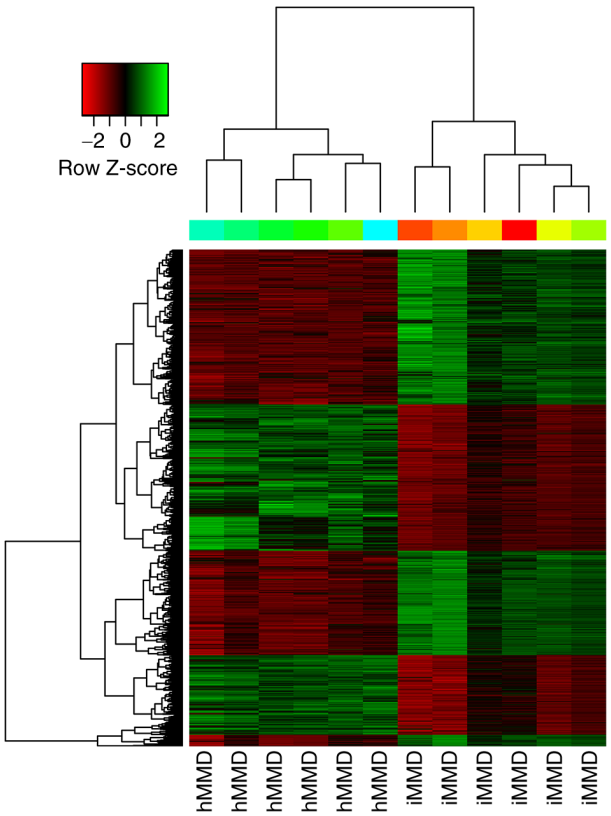

Figure 2. Heat map of differentially expressed circRNAs between MMD and control groups. circRNAs, circular RNAs; MMD, moyamoya disease; h, hemorrhagic; i, ischemic.

(Fig. 3), RT-qPCR validation confirmed significant upregulation of circRNA-025016, circRNA-104293, circRNA-060184 and circRNA-091419 in hMMD samples compared to iMMD. Conversely, circRNA-029937, circRNA-103574 and circRNA-103572 showed significant downregulation in hMMD samples.

Bioinformatics analysis. GO and KEGG analysis of differentially expressed circRNAs was next performed (Fig. 4). The GO and KEGG pathway analyses revealed distinct patterns between hMMD and iMMD (Fig. 4). Fig. 4A shows upregulated circRNAs in hMMD were enriched in mRNA catabolic processes and protein targeting pathways. Fig. 4B indicates downregulated circRNAs were mainly involved in cell cycle regulation. In KEGG analysis, Fig. 4C demonstrates upregulated pathways including AGE-RAGE signaling and focal adhesion, while Fig. 4D shows downregulated pathways with ubiquitin-mediated proteolysis being the most significant. In GO analysis of biological processes, 'mRNA catabolic process' and 'nuclear-transcribed mRNA catabolic process' were two of the biological processes with the most significance in upregulated circRNAs in hMMD samples. By contrast, 'cell cycle phase transition' and 'mitotic cell cycle phase transition' were the biological processes with the most significance amongst the downregulated circRNAs in the hMMD samples (Tables II and SIV).

KEGG pathway analysis demonstrated 10 enrichment pathways in the upregulated circRNAs and 36 enriched pathways in the downregulated circRNAs (Tables III and SV). Among them, the 'Ubiquitin mediated proteolysis' (Fig. 5) and 'Cell cycle' pathways were the top enriched pathways with the most significance. Fig. 5 shows the ubiquitin-mediated proteolysis pathway from the KEGG database. The red boxes highlight differentially expressed genes between hMMD and iMMD, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). This pathway's dysregulation in hMMD suggests altered

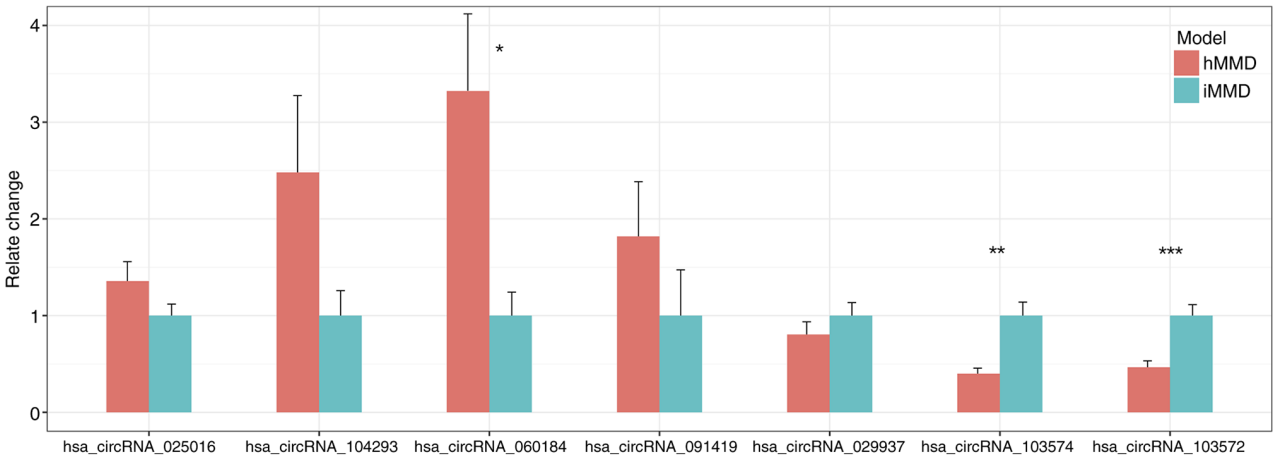

Figure 3. Reverse transcription-quantitative PCR validation of expression levels of circRNAs identified by microarray analysis. Samples were prepared in triplicate. The error bar stands for standard deviations. *P<0.05, **P<0.01; ***P<0.001. circRNAs, circular RNAs; MMD, moyamoya disease; h, hemorrhagic; i, ischemic.

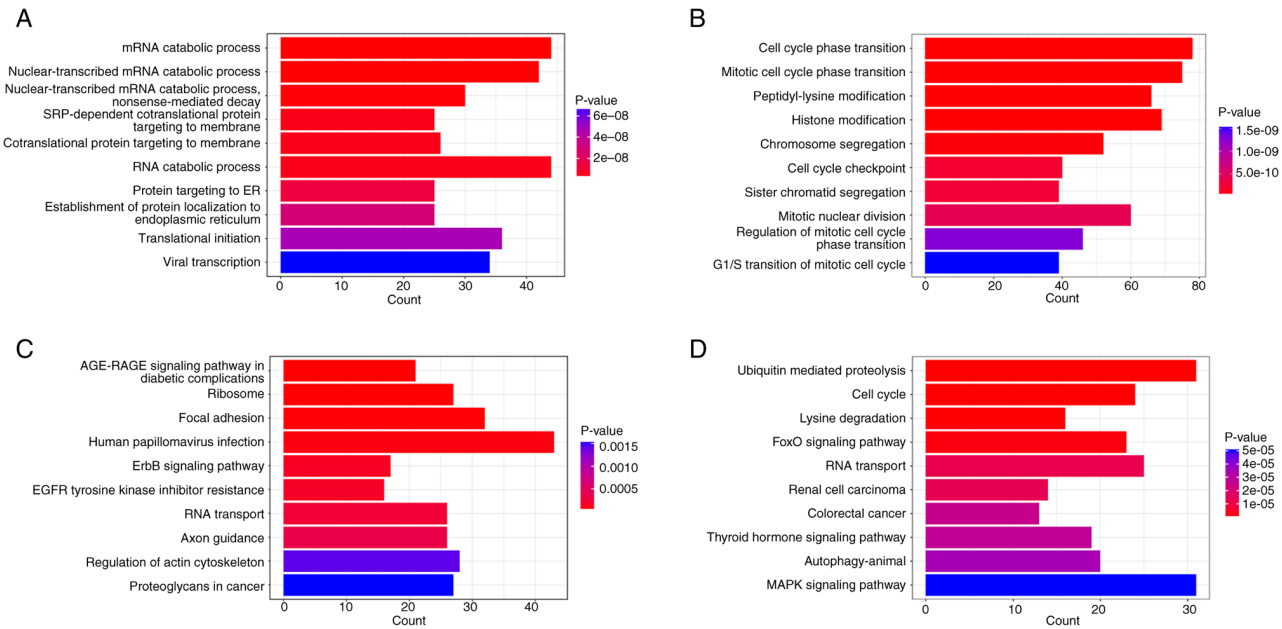

Figure 4. GO and Kyoto Encyclopedia of Genes and Genomes pathway analysis of the differentially expressed circRNAs. Top 10 GO biological processes for (A) upregulated and (B) downregulated circRNAs. Top 10 enriched pathways for (C) upregulated and (D) downregulated circRNAs. circRNAs, circular RNAs; GO, Gene Ontology.

protein degradation may contribute to vessel wall instability. The validated circRNAs, 34 predicted miRNAs and 308 target genes were next used to construct the circRNA/miRNA network (Fig. 6).

Discussion

In the present study, the circRNA expression of hMMD and iMMD were comprehensively profiled by microarray analysis. Compared with iMMD samples, a total of 3,607 significantly differentiated circRNAs in hMMD were detected. Subsequently, the differentially expressed circRNAs were identified to be involved in several biological processes and signaling pathways, such as 'mRNA catabolic process' and 'ubiquitin mediated proteolysis', according to GO analysis and KEGG pathway analysis. In addition, a network map of circRNA/miRNA gene interactions was also constructed for the validated significantly differentiated circRNAs. These results suggested that there was a significant difference in the circRNA expression profile between the iMMD and hMMD samples. Several key circRNAs may show promise as candidate biomarkers for hemorrhage in MMD.

CircRNAs have been recently found to be pervasively transcribed in the genome (11,21). It was previously reported that circRNAs can reverse the inhibitory effects of miRNAs on their target mRNAs by directly binding to miRNAs through miRNA response elements (22). In addition, previous studies suggested that circRNAs are enriched in the brain and may participate in regulating synaptic function and neural plasticity (23,24). Dysregulated circRNAs have been reported to be associated with several human diseases, including neurological disease, cardiovascular system diseases and cancers (25-27).

Table III. KEGG pathways of upregulated and downregulated target genes with most significance in hMMD compared with iMMD.

KEGG term	Fold enrichment	P-value
A, Upregulated target genes		
hsa04933 AGE-RAGE signaling pathway in diabetic complications	2.914065180	6.42E-06
hsa03010 Ribosome	2.408564077	1.44E-05
hsa04510 Focal adhesion	2.209083152	1.51E-05
hsa05165 Human papillomavirus infection	1.863478364	4.39E-05
hsa04012 ErbB signaling pathway	2.715598947	1.00E-4
B, Downregulated target genes		
hsa04120 Ubiquitin mediated proteolysis	3.950900593	2.02E-11
hsa04110 Cell cycle	3.379438385	1.02E-07
hsa00310 Lysine degradation	4.735032313	1.13E-07
hsa04068 FoxO signaling pathway	3.042347940	1.33E-06
hsa03013 RNA transport	2.552694686	1.23E-05

KEGG, Kyoto Encyclopedia of Genes and Genomes; MMD, moyamoya disease; h, hemorrhagic; i, ischemic.

Circular antisense non-coding RNA in the INK4 locus (cANRIL) was documented to influence INK4/ADP ribosylation factor expression and increase the risk of atherosclerotic vascular disease (28). This finding suggests that circRNAs are involved in the development of atherosclerotic cerebrovascular disease (23). The mechanism of MMD remains poorly understood. However, genetic and environmental factors were considered to be vital in the development of the vascular stenosis and MMD vessel formations (29).

A previous genome-wide association study has shown Ring Finger 213 (RNF213) to be an important MMD susceptibility gene (30). Vasculogenesis and angiogenesis, which require endothelial cell proliferation and migration, form the two central processes involved in the development of biological revascularization (31). The arteriogenesis process, which refers to the formation of collateral circulation, is typically activated by the increased fluid shear stress generated by the pressure difference between perfusion territories (32). The proliferation of endothelial and smooth muscle cells may lead to aberrant angiogenesis (29). The associated changes in circulating endothelial/smooth muscle progenitor cells (33), angiogenesis (33-35) and caveolin (36), may also be involved. The pathogenesis of MMD had also been associated with non-coding RNAs in previous studies. miRNAs, which are small non-coding RNAs ~23 nucleotides in length, can negatively regulate the expression of proteins by altering their gene expression through post-transcriptional repression or mRNA degradation (37). miRNAs have been reported to serve an important role in the regulation of proliferation and aging of various tissues. A previous microarray study on miRNAs profiles in serum from patients with MMD suggested that elevated serum levels of miRNAs are associated with RNF213 (38). Another study previously revealed that the increased expression of miRNA Let-7c in patients with MMD may also contribute to MMD pathogenesis by targeting RNF213 expression (39). In addition, annexin 1, which is expressed in endothelial and smooth muscle cells (SMC), is a gene target of miRNA-196a to mediate apoptosis and inhibition of cell proliferation (29).

Although there is notable heterogeneity in clinical symptoms depending on the age of onset and ethnicity (40-42), patients with MMD typically present with ischemic and/or hemorrhagic stroke. These two subtypes may have pathogenic differences. The proposed pathophysiologic mechanism for hemorrhage in MMA is long-term hemodynamic stress to collateral vessels (43). Theoretically, impaired perfusion results in hemodynamic stress on the vessel wall and facilitates dilation or micro-aneurysm formation in collateral vessels. Dilatation and abnormal branching of the anterior choroidal artery and/or posterior communicating artery are viable predictors of hemorrhage in adult patients with MMD (44). In addition, one previous study suggested that by using 7T time-of-flight magnetic resonance angiography, ventricular micro-aneurysms in MMD angiopathy collateral vessels can be detected (45). The non-coding RNAs have been suggested to be involved in other hemorrhagic cerebral vascular diseases. A previous study suggests that lncRNAs may contribute to the pathogenesis of cerebral aneurysms by regulating loss of the contractile SMC phenotype (46). The distinction between hemorrhagic and ischemic presentations of MMD has important clinical implications. Zhao *et al* (47) previously demonstrated that patients with hMMD show distinct imaging characteristics and collateral patterns compared to iMMD, suggesting different pathophysiological mechanisms. The circRNA findings of the present study provided molecular support for this, revealing divergent expression patterns between subtypes. The identified circRNA networks may help explain the different propensities for hemorrhage compared with ischemia (16). The different circRNA expression may

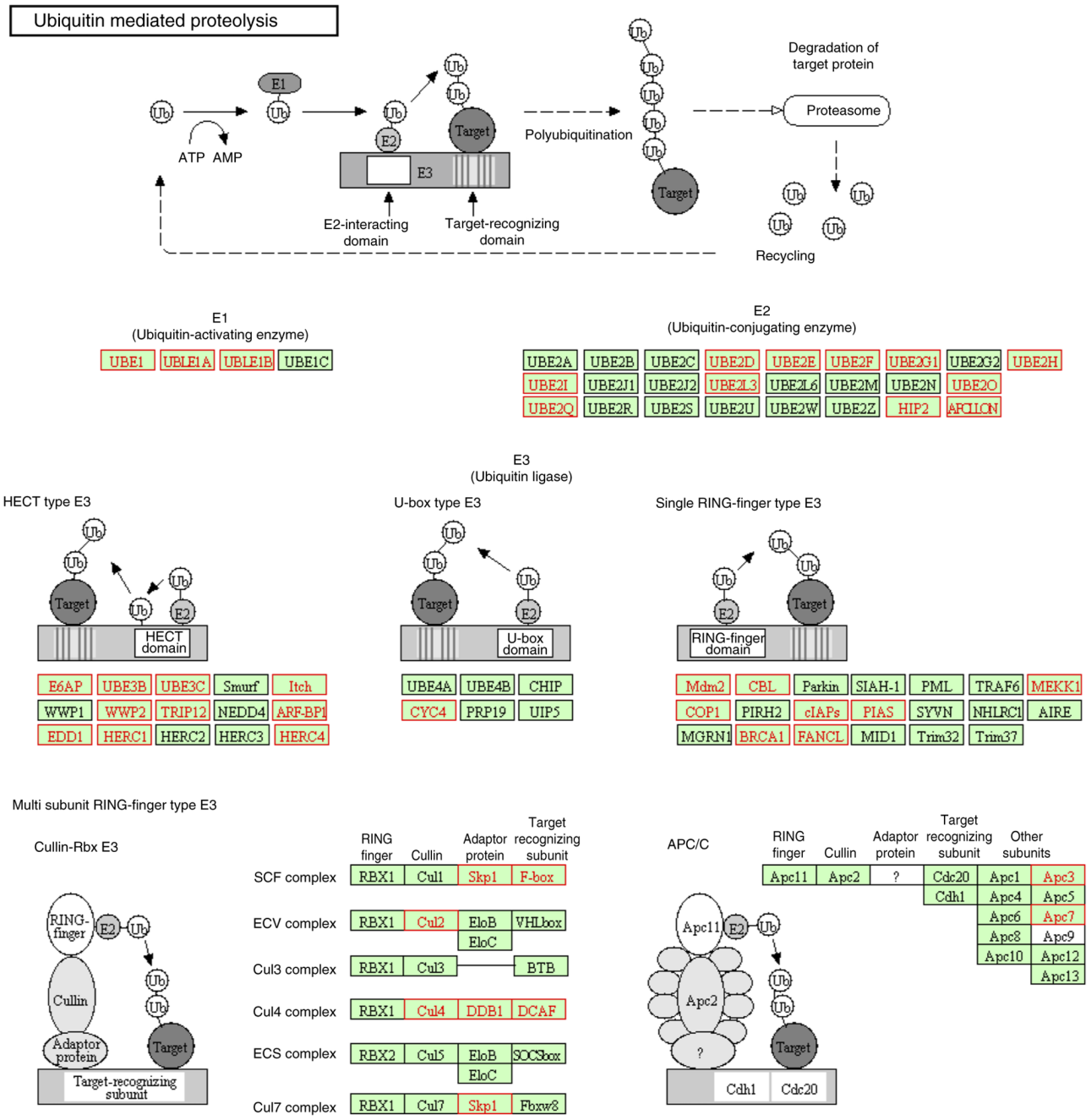

Figure 5. Target genes in the ubiquitin mediated proteolysis pathway. Target genes are shown in red. The image was generated based on Kanehisa Laboratories and Kyoto Encyclopedia of Genes and Genomes pathways.

serve a role in different vessel formation in different MMD subgroups.

In addition, circRNAs may serve roles in the pathogenesis of hMMD by regulating SMC proliferation and TGF-β signaling. As shown in the network (Fig. 6), circRNA-0005873 may serve a role in the expression of TGF-β by regulating miRNA-141-3p. Alterations in normal TGF-β signaling have been implicated in the pathophysiology of several vascular disorders, including atherosclerosis and primary pulmonary hypertension (48). Another study suggested that TGF-β is one of the underlying factors contributing to the development of thoracic aortic aneurysm (49). As shown in the network, pappalysin-1 (PAPPA) was the direct target gene of miRNA-141. A previous study has also suggested that miRNA-141 can inhibit vascular SMC proliferation through targeting PAPPA (50).

The ubiquitin mediated proteolysis pathway was detected as the top significant pathway according to KEGG analysis. These results suggested that circRNAs may serve important roles in proteolysis processes in hemorrhage. Proteases, including thrombin and MMPs, were previously reported to have complex functions in the brain under both normal and pathological conditions (49). MMPs are endopeptidases that can degrade components of the extracellular matrix. The MMPs serve an important role in normal and atherosclerotic blood vessels by being involved in plaque disruption (51). Increased vascular MMP2 or MMP9 expression is involved in the pathogenesis of spontaneous intracranial hemorrhage

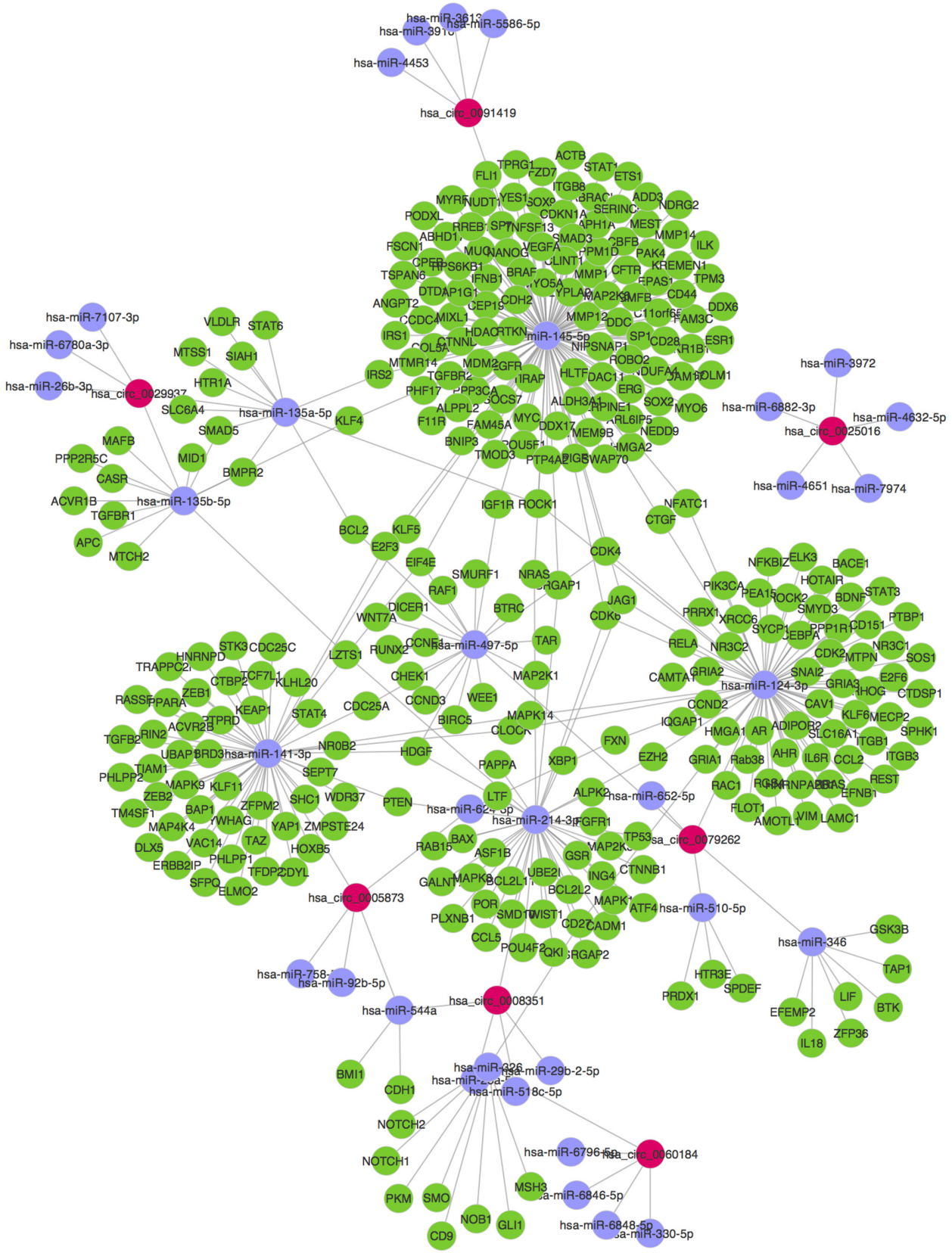

Figure 6. Co-expression network of the validated circRNAs and predicted miRNAs and targeted genes. The red dots represent circRNAs, purple dots represent miRNAs and the green dots represent genes. circRNAs, circular RNAs; miRNAs, microRNAs.

in patients with cerebral amyloid angiopathy (52). Abdominal aortic aneurysm expansion is likely to result from increasing proteolysis related to increasing MMP-9 expression (53).

Proteolysis has been associated with the rupture of abdominal aortic aneurysms. A previous study has shown that Smad7 can interact with the heteromeric TGF-β receptor complex and

recruits the E3 ubiquitin-ligases Smurf1 and Smurf2, targeting the receptors for degradation to terminate the signaling response (54). These factors may work in concert to modify and direct the response to signals through this complex pathway. Additionally, MMPs have been reported to serve a significant role in regulating angiogenesis, the process of new blood vessel formation (55). An enhanced understanding of the molecular mechanisms involved in hemorrhage in MMD may potentially lead to novel therapeutic strategies against this potentially lethal condition. While acute vascular events such as stroke can alter circRNA expression patterns, emerging evidence suggests circRNAs may also play causative roles in vascular pathology. The findings in the present study of distinct circRNA profiles between hMMD and iMMD suggested these molecules could be both markers and mediators of disease progression. Further mechanistic studies are needed to fully elucidate the complex interplay between circRNAs and vascular remodeling in MMD.

The present study had several limitations that warrant consideration. All samples were acquired from a single ethnic group in mainland China. Consequently, different circRNA signatures may exist across diverse ethnic groups. A limitation of the present study is the relatively small sample size. While it detected significant differences between groups, larger cohorts will be needed to validate these findings and establish clinical utility of circRNA biomarkers. In addition, the functions of circRNAs in MMD were analyzed based on bioinformatics predictions. While this approach is widely used in non-coding RNA research, future studies on the specific interactions of circRNAs with miRNAs and the downstream effects on hMMD and iMMD signaling pathways are necessary. A key limitation is the descriptive nature of the current findings and a lack of mechanistic studies.

To conclude, to the best of the authors' knowledge, the present study represented the first comparison of circRNA expression profiles in hMMD and iMMD samples. These findings expanded on the understanding of the mechanisms underlying hemorrhage in MMD, which may provide novel insights for developing therapeutic interventions for hemorrhagic complications of MMD.

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Availability of data and materials

The datasets generated in the current study are not publicly available to ncbi.nlm.nih.gov due to institutional policy and regional data sharing restrictions but may be requested from the corresponding author. Raw data are provided in the supplementary materials of this article.

Authors' contributions

MZ and JZ participated in study conceptualization and methodology development. WL supervised the project, acquired funding, and provided resources. XY performed data curation, formal analysis and statistical analysis. QZ and YZ were responsible for investigation, experimental work and data validation. MZ drafted the original manuscript, while YZ and JZ reviewed and edited the manuscript. QZ and YZ were responsible for confirming the authenticity of all raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The patients provided written informed consent for biobanking and future research use. The specific circRNA analysis protocol was reviewed and approved by the Ethics Committee Review Board of Beijing Tiantan Hospital (Beijing, China; approval no. KYSQ2020-161-01) prior to conducting the molecular studies.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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