MicroRNA Expression in Circulating Leukocytes and Bioinformatic Analysis of Patients with Moyamoya Disease

Kaijiang Kang

Beijing Tiantan Hospital, Capital Medical University

Yuan Shen

Beijing Tiantan Hospital, Capital Medical University

Qian Zhang

Beijing Tiantan Hospital, Capital Medical University

Jingjing Lu

Beijing Tiantan Hospital, Capital Medical University

Yi Ju

Beijing Tiantan Hospital, Capital Medical University

Ruijun Ji

Beijing Tiantan Hospital, Capital Medical University

Na Li

Beijing Tiantan Hospital, Capital Medical University

Jianwei Wu

Beijing Tiantan Hospital, Capital Medical University

Bo Yang

Beijing Tiantan Hospital, Capital Medical University

Jinxi Lin

China National Clinical Research Center for Neurological Diseases

Xianhong Liang

China National Clinical Research Center for Neurological Diseases

Dong Zhang

Beijing Tiantan Hospital, Capital Medical University

Xingquan Zhao (✉ zxq@vip.163.com)

Beijing Tiantan Hospital, Capital Medical University

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Abstract

Background and purpose- MicroRNAs (miRNAs) in exosomes had been implicated differentially expressed in MMD patients, but the miRNAs expression in circulating leukocytes remains unclear. This study was investigated on the differential expression of miRNAs in peripheral leukocytes between MMD patients and healthy adults, and among patients with subtypes of MMD.

Methods- A total of 30 patients with MMD and 10 healthy adults were enrolled in a stroke center from October 2017 to December 2018. The gene microarray was used to detect the differential expression profiles of miRNA in leukocytes between MMD patients and controls, and the differentially expressed miRNAs were verified by the method of real-time PCR. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to explore the key signaling pathways and possible pathogenesis of MMD.

Results- The microarray results showed 12 differentially expressed miRNAs in leukocytes of MMD patients compared with controls (fold change > 2.0 and $P < 0.05$), of which 7 miRNAs were up-regulated (miRNA-142-5p, miRNA-29b-3p, miRNA-424-5p, miRNA-582-5p, miRNA-6807-5p, miRNA-142-3p, miRNA-340-5p), and 5 miRNAs were down-regulated (miRNA-144-3p, miRNA-144-5p, miRNA-451a, miRNA-486-5p, miRNA-363-3p). The real-time PCR confirmed 7 differentially expressed miRNAs ($P < 0.05$), of which 4 miRNAs (miRNA-29b-3p, miRNA-142-3p, miRNA-340-5p, miRNA-582-5p) were up-regulated, and 3 miRNAs (miRNA-363-3p, miRNA-451a and miRNA-486-5p) were down-regulated. Both GO and KEGG analysis suggested that the Wnt signaling pathway may be involved in the pathogenesis of MMD. In addition, miRNAs were also differentially expressed among patients with subtypes of MMD.

Conclusion- This study indicated that miRNAs are differentially expressed in peripheral leukocytes between MMD patients and healthy adults, and among patients with subtypes of MMD. The Wnt signaling pathway is probably involved in the pathogenesis of MMD.

Introduction

Moyamoya disease (MMD) is a relatively rare cerebrovascular disease associated with recurrent stroke, and the prevalence of MMD have increased around the world, especially in Japan, Korea, and China[1–6]. Currently, the etiology and pathogenesis of MMD are still unclear, so there is a lack of reliable molecular biological markers and effective drugs[7–9]. The incidence of MMD has been demonstrated as racial propensity and familial clustering, and some genes have been indicated to be abnormally expressed, which suggests that the MMD may be closely associated with gene expressions[8–12]. MMD can be manifested as ischemic, hemorrhagic, or asymptomatic, and hemorrhagic stroke is one of the main factors leading to acute death and severe disability in patients with MMD[13, 14]. However, the mechanisms and predictors of hemorrhagic stroke associated with MMD are still limited to the vascular or hemodynamic characteristics, while the underlying pathophysiological mechanisms and biological processes are still unclear[15–18].

MicroRNA (miRNA) is a kind of endogenous small RNA with a length of about 18–24 nucleotides, which plays an important regulatory role in the normal metabolism of cells and the development of diseases by inhibiting the translation of messenger RNA (mRNA) into proteins or promoting the degradation of mRNA[19, 20]. Although miRNA has been investigated for several years, its relationship with MMD remains to be elucidated. It has been indicated that miRNAs were abnormally expressed in peripheral blood and cerebrospinal fluid of patients with MMD[21–24]. However, previous studies had mostly focused on the differences of miRNAs in exosomes, which cannot fully reflect the actual expression of intracellular miRNAs. Also, studies have shown that some mononuclear cells in peripheral blood can differentiate into vascular endothelial progenitor cells and vascular smooth muscle progenitor cells, and the pathophysiological process of MMD is often accompanied by inflammatory changes involving a variety of cytokines, suggesting that MMD may be closely associated with peripheral leukocytes[25, 26].

In this study, we investigated the differential expression of miRNA in peripheral leukocytes between MMD patients and healthy adults, and among patients with subtypes (hemorrhagic, ischemic, and asymptomatic) of MMD, to explore the pathogenesis of MMD, and provide the basis for clinical diagnosis, outcome prediction and therapeutic strategy of MMD.

Materials And Methods

Study population

The study was performed according to the guidelines from the Helsinki Declaration, and was approved by the Research Ethics Committee of the hospital. Written informed consent was obtained from all the subjects or their legally authorized representatives. A total of 30 patients, who were diagnosed with MMD in our stroke center from October 2017 to December 2018, were consecutively included in this study. The inclusion criteria included: 1) the age was 18-65 years; 2) diagnosed as MMD according to the 2012 Japanese Moyamoya disease diagnosis and treatment guidelines[27]; 3) the patient's informed consent to the study protocol. The exclusion criteria included: 1) moyamoya syndrome indicated by clinical manifestations, high-resolution MRI, or laboratory examinations; 2) with history of stroke in the past three months; 3) patients who had received revascularization surgery; 4) patients with immunological diseases, tumors, or pregnancy. In addition, 10 healthy adults with matching gender and age were included as normal controls.

Sample collection and RNA extraction

Peripheral venous blood samples (about 6 ml) were taken from the antecubital vein into an anticoagulant drying tube under fasting conditions. The whole blood was centrifuged at 2600 rpm for 10 min at room temperature within an hour. The white blood cells were separated from the middle layer, and the mixed red blood cells were removed with red blood cell lysis buffer. A kind of RNA stabilizer (RNAlater, Thermo Fisher Scientific, Baltics UAB, Vilnius, Lithuania) was added to the cryotube containing white blood cells

and was stored at 4 °C for 24 hours, then stored in -80 °C refrigerator for standby. Trizol Reagent (Invitrogen, Carlsbad, CA, USA) was used for leukocyte denaturation. The miRNeasy Mini Kit (Qiagen p/n 217004) was used for RNA collection and purification according to the manufacturer's protocol. The quality evaluation of the total RNA was performed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

miRNA microarray

The Agilent Human (8*60K) V21.0 miRNA microarrays (Agilent, Santa Clara, CA, USA) were performed using a Gene Expression Hybridization Kit (Agilent's miRNA Complete Labeling and Hyb Kit-p/n5190-0456) according to the manufacturer's instructions. Slides were washed in staining dishes with a Gene Expression Wash Buffer Kit (Agilent, Santa Clara, CA, USA) and scanned by an Agilent Microarray Scanner with default settings according to the manufacturer's instructions. The data was extracted using an Agilent Feature Extraction (AFE) software (version 10.7.1.1), and the raw data were normalized by Quantile algorithm using Gene Spring Software 12.6 (Agilent Technologies). Differentially expressed miRNAs were identified through fold-change (> 2) and P value (< 0.05) filtering using R software (version 3.2.3) with the samr package.

Real-time PCR

Several differentially expressed miRNAs were quantified by using real-time polymerase chain reaction (RT-PCR) based on the Taqman method. Total RNAs were reversely transcribed using the Taqman MicroRNA Reverse Transcription Kit (ABI, 4366597) according to the manufacturer's instructions. RT-PCR was performed using QuantStudio 5 Real-Time PCR System (ABI, USA) and QuantStudio™ Design & Analysis Software with the Taqman Universal PCR Master Mix (ABI, 4440049). The program was as follows: 95°C, 10 min; (95°C, 15 s; 60°C, 1 min) 40 cycles. The expression level of each miRNA was calculated as fold change adopting the $2^{-\Delta\Delta C_t}$ method. We conducted three replicate experiments to test the reproducibility. The homogenous miRNA-16 was used as an internal reference for the analysis.

Bioinformatics analysis

In this study, we used TargetScan (<http://www.targetscan.org/>, TargetScanHuman 7.2) to identify the targets of differentially expressed miRNAs. We defined the predicted target genes by no less than 10 differentially expressed miRNAs as potential functional targets in MMD. To determine the biological relationship between the potential miRNA target genes, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed using DAVID Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>, DAVID Bioinformatics Resources 6.8). The significance threshold was set to 0.05 in our enrichment analysis.

Statistical analysis

The statistical analysis was performed using SPSS (version 22.0, IBM-SPSS, Chicago, IL, USA) and R software (version 3.2.3). Genes with a two-sided *P* value of <0.05 and fold change >2.0 were regarded as statistically significant genes.

Results

Demographic information and clinical characteristics

The study recruited 30 patients with MMD (including 10 hemorrhagic MMD patients, 12 ischemic MMD patients, and 8 asymptomatic MMD patients), and 10 healthy adults with matched demographic characteristics. The detailed characteristics of the included subjects were shown in Table 1.

miRNA microarray

In analysis of 30 MMD patients compared with 10 controls, 9 miRNAs were found to be differentially expressed, with 5 up-regulated (miRNA-142-5p, miRNA-29b-3p, miRNA-424-5p, miRNA-582-5p, miRNA-6807-5p) and 4 down-regulated (miRNA-144-3p, miRNA-144-5p, miRNA-451a, miRNA-486-5p) (Table 2, Figure 1). In addition, miRNA expression was significantly different in 6 samples (4 in the MMD group and 2 in the control group). After the elimination of these 6 samples, miRNA-142-3p, miRNA-340-5p, miRNA-363-3p were also differentially expressed in MMD patients (miRNA-142-3p and miRNA-340-5p up-regulated, while miRNA-363-3p down-regulated).

In analysis of 10 hemorrhagic MMD patients compared with 10 controls, 17 miRNAs were found to be differentially expressed, with 13 up-regulated (miRNA-101-3p, miRNA-142-3p, miRNA-142-5p, miRNA-148a-3p, miRNA-29b-3p, miRNA-340-5p, miRNA-374a-5p, miRNA-424-5p, miRNA-450a-5p, miRNA-542-3p, miRNA-582-5p, miRNA-590-5p, miRNA-6807-5p) and 4 down-regulated (miRNA-144-3p, miRNA-144-5p, miRNA-451a, miRNA-486-5p). In analysis of 8 asymptomatic MMD patients compared with 10 controls, 7 miRNAs were found to be differentially expressed, with 3 up-regulated (miRNA-142-3p, miRNA-29b-3p, miRNA-6515-5p) and 4 down-regulated (miRNA-144-3p, miRNA-144-5p, miRNA-451a, miRNA-486-5p). In analysis of 12 ischemic MMD patients compared with 10 controls, 3 miRNAs were found to be differentially expressed (miRNA-29b-3p and miRNA-424-5p up-regulated, while miRNA-486-5p down-regulated) (Figure 2).

In addition, miRNA-486-5p was found to be differentially down-regulated in hemorrhagic MMD patients, compared with ischemic MMD patients. Also, miRNA-6515-5p was found to be differentially up-regulated in asymptomatic MMD patients, compared with symptomatic (hemorrhagic and ischemic) MMD patients.

Real-time PCR

The RT-PCR verification of 10 differentially expressed miRNAs was performed between 30 MMD patients and 10 controls, including miRNA-142-5p, miRNA-29b-3p, miRNA-424-5p, miRNA-582-5p, miRNA-144-3p, miRNA-451a, miRNA-486-5p, miRNA-142-3p, miRNA-340-5p, miRNA-363-3p. The probe was synthesized by Thermo Fisher Scientific, and the target sequence of the probe is shown in Table 3. Three independent experiments had strong reproducibility, and all of the 10 genes were identified successfully. However, two normal control samples had higher overall CT values than other samples, but miRNA-16 (internal reference) was relatively lower, which led to higher overall expression and affected the overall analysis results, so the two samples were removed during the final analysis.

Compared with 10 healthy adults, 7 miRNAs had differential expression in 30 MMD patients ($P < 0.05$), of which miRNA-29b-3p ($P < 0.001$), miRNA-142-3p ($P = 0.013$), miRNA-340-5p ($P = 0.002$), miRNA-582-5p ($P = 0.005$) was up-regulated, while miRNA-363-3p ($P = 0.007$), miRNA-451a ($P < 0.001$), miRNA-486-5p ($P = 0.001$) was down-regulated (Figure 3).

Compared with 10 healthy adults, 9 miRNAs had differential expression in 10 hemorrhagic MMD patients ($P < 0.05$), of which miRNA-29b-3p ($P = 0.002$), miRNA-142-3p ($P = 0.004$), miRNA-142-5p ($P = 0.047$), miRNA-340-5p ($P < 0.001$), miRNA-582-5p ($P = 0.003$) were up-regulated, while miRNA-451a ($P = 0.001$), miRNA-486-5p ($P = 0.035$), miRNA-144-3p ($P = 0.009$), miRNA-363-3p ($P = 0.002$) were down-regulated. Compared with 10 healthy adults, 6 miRNAs had differential expression in 8 asymptomatic MMD patients ($P < 0.05$), of which miRNA-29b-3p ($P = 0.004$), miRNA-142-3p ($P = 0.001$), miRNA-340-5p ($P = 0.009$), miRNA-582-5p ($P = 0.005$) were up-regulated, while miRNA-363-3p ($P = 0.015$), miRNA-451a ($P = 0.009$) were down-regulated. Compared with 10 healthy adults, 4 miRNAs had differential expression in 12 ischemic MMD patients ($P < 0.05$), of which miRNA-29b-3p ($P = 0.006$), miRNA-142-5p ($P = 0.035$), miRNA-340-5p ($P = 0.030$) were up-regulated, while miRNA-451a ($P = 0.030$) was down-regulated.

In addition, the miRNA-142-3p was up-regulated in hemorrhagic MMD patients and miRNA-340-5p was up-regulated in hemorrhagic and the asymptomatic MMD patients compared with ischemic MMD patients ($P < 0.05$). The miRNA-486-5p was down-regulated in hemorrhagic and asymptomatic MMD patients, but there was no statistically significant difference ($P > 0.05$). There was no significant differential expression between the hemorrhagic and the asymptomatic MMD patients.

Target gene prediction and bioinformatics analysis

We used the TargetScan target gene prediction program to predict the potential target genes of differentially expressed miRNAs by microarray and RT-PCR. According to the 7 differentially expressed miRNAs in peripheral leukocytes of MMD patients, a total of 3728 target genes were predicted. Of the 3728 target genes, the biological processes and signal transduction pathways that may be involved in the enrichment analysis are carried out through GO and KEGG. The associated biological processes and signal pathways were screened (according to the criteria: P value ≤ 0.05 , number of target genes ≥ 10)

and sequenced according to the enrichment degree, and the top 30 biological processes and signal pathways are shown in Figure 4. Both the GO and KEGG analysis suggested that the Wnt signaling pathway may be involved in the pathogenesis of MMD (Figure 4, Figure 5).

Table 1

Demographic and clinical characteristics of the subjects

ID	Subtypes	Gender	Age (years)	Suzuki stage	
				Left	Right
HM_01	Hemorrhagic MMD	Female	32	4	4
HM_02	Hemorrhagic MMD	Female	43	2	3
HM_03	Hemorrhagic MMD	Female	24	3	3
HM_04	Hemorrhagic MMD	Female	49	6	6
HM_05	Hemorrhagic MMD	Male	49	6	3
HM_06	Hemorrhagic MMD	Male	42	5	5
HM_07	Hemorrhagic MMD	Female	50	3	3
HM_08	Hemorrhagic MMD	Female	34	3	3
HM_09	Hemorrhagic MMD	Male	50	0	3
HM_10	Hemorrhagic MMD	Female	42	4	4
IM_01	Ischemic MMD	Male	34	3	3
IM_02	Ischemic MMD	Female	36	3	1
IM_03	Ischemic MMD	Female	46	3	3
IM_04	Ischemic MMD	Male	35	3	3
IM_05	Ischemic MMD	Female	30	4	3
IM_06	Ischemic MMD	female	46	3	3
IM_07	Ischemic MMD	Male	47	4	3
IM_08	Ischemic MMD	Female	21	2	3
IM_09	Ischemic MMD	Female	29	2	2
IM_10	Ischemic MMD	Male	39	5	4
IM_11	Ischemic MMD	Male	37	2	2
IM_12	Ischemic MMD	Male	39	2	2
AM_01	Asymptomatic MMD	Male	42	2	2
AM_02	Asymptomatic MMD	Male	34	5	3
AM_03	Asymptomatic MMD	Female	29	3	3
AM_04	Asymptomatic MMD	Female	38	3	3
AM_05	Asymptomatic MMD	Female	34	4	4

AM_06	Asymptomatic MMD	Female	48	2	2
AM_07	Asymptomatic MMD	Male	50	3	3
AM_08	Asymptomatic MMD	Female	33	6	5
C_01	Control	Male	42	-	-
C_02	Control	Male	35	-	-
C_03	Control	Male	35	-	-
C_04	Control	Male	34	-	-
C_05	Control	Female	34	-	-
C_06	Control	Female	32	-	-
C_07	Control	Female	33	-	-
C_08	Control	Female	35	-	-
C_09	Control	Female	31	-	-
C_10	Control	Female	33	-	-

HM: hemorrhagic moyamoya disease; IM: ischemic moyamoya disease; AM: asymptomatic moyamoya disease; C: controls

Table 2

Differentially expressed miRNAs in microarray analysis between 30 MMD patients and 10 controls

Systematic Name	P values	Fold change (abs)	Regulation
miRNA-142-5p	0.000020	2.020448	up
miRNA-144-3p	0.000958	2.043367	down
miRNA-144-5p	0.002665	2.413351	down
miRNA-29b-3p	0.000006	2.420699	up
miRNA-424-5p	0.000043	2.309035	up
miRNA-451a	0.000068	2.478956	down
miRNA-486-5p	0.000165	5.521476	down
miRNA-582-5p	0.001293	2.228220	up
miRNA-6807-5p	0.000010	2.227829	up

Table 3

Target sequence of the probe in real-time PCR

miRNA	Target sequence of the probe
miRNA-16	UAGCAGCACGUAAAUAUUGGCG
miRNA-29b	UAGCACCAUUUGAAAUCAGUGUU
miRNA-142-3p	UGUAGUGUUUCCUACUUAUGGA
miRNA-142-5p	CAUAAAGUAGAAAGCACUACU
miRNA-144	UACAGUAUAGAUGAUGUACU
miRNA-340	UUAUAAAGCAAUGAGACUGAUU
miRNA-363	AAUUGCACGGUAUCCAUCUGUA
miRNA-424	CAGCAGCAAUUCAUGUUUUGAA
miRNA-451	AAACCGUUACCAUUACUGAGUU
miRNA-486	UCCUGUACUGAGCUGCCCCGAG
miRNA-582-5p	UUACAGUUGUUCAACCAGUUACU

Discussions

The results of this study suggested that miRNA-29b-3p, miRNA-142-3p, miRNA-340-5p, and miRNA-582-5p were significantly up-regulated, while miRNA-363-3p, miRNA-451a, miRNA-486-5p were significantly down-regulated in peripheral leukocytes of patients with MMD compared with healthy controls. In addition, miRNAs were also differentially expressed among patients with subtypes of MMD.

Previous studies had suggested that miRNA-106b, miRNA-130a, miRNA-126, miRNA-125-3p, miRNA-196a, let-7c were abnormally expressed in the peripheral serum of MMD patients[21, 23, 24]. Also, the miRNA-3679-5p, miRNA-6165, miRNA-6760-5p, and miRNA-574-5p had been indicated to be differentially expressed in the cerebrospinal fluid of MMD patients[22]. The miRNA-196a was suggested to regulate cell proliferation and apoptosis by regulating the expression of ANXA1 gene in endothelial cells and vascular smooth muscle cells, which may be related to the onset of MMD[23]. It was indicated that the serum let-7c in MMD patients was significantly increased, which can bind to the 3'non-coding region of RNF213 transcribed mRNA, thereby affecting the biological activity of RNF213[21]. The RNF213 mutation is currently considered to be a susceptible gene for MMD in Asian populations, which may be involved in the pathogenesis of MMD[28]. In the present study, however, these miRNAs did not show abnormal expression in peripheral leukocytes of MMD patients compared with controls, which suggested that there may be significant differences in the expression of miRNA between peripheral leukocytes and serum of patients with MMD. The circulating serum miRNAs reflect only the level of extracellular miRNAs secreted

by a variety of cells through exosomes, but cannot fully reflect the true expression of specific intracellular miRNAs, which impeded further investigations on the miRNA-related target genes and cell functions.

The pathological characteristics of MMD are mainly manifested as a gradual degeneration of the smooth muscle in the arterial media, and the abnormal proliferation of the smooth muscle in the arterial intima, leading to progressive stenosis and occlusion of the involved vessels, accompanied by the development of collaterals[7–9]. The present study indicated that several biological processes or signal transduction pathways may be involved in the pathophysiological process of MMD, including vascular smooth muscle cell proliferation and metastasis, apoptosis, neovascularization, immune and inflammatory reactions, and thrombosis.

Both the GO analysis and KEGG analysis suggested that the Wnt signaling pathway may be involved in the pathogenesis of MMD (Fig. 4, Fig. 5). The Wnt signaling pathway is a complex regulatory network consisting of the secreted Wnt protein family, the transmembrane receptor Frizzled family, CK1, Dishevelled, GSK3, APC, Axin, β -catenin, and TCF/Lef family[29]. When the extracellular ligand binds to the cell surface receptor, the intracellular segment of the surface receptor was activated, which transmits extracellular signals into the cells. Previous studies have shown that the Wnt signaling pathway is activated in some cardiovascular and cerebrovascular diseases, and plays an important role in the proliferation, migration, apoptosis, and differentiation of vascular smooth muscle cells, which is one of the mechanisms of MMD[30–33]. Therefore, the role of the Wnt signaling pathway in MMD is worthy of further investigation.

In addition, miRNAs were also differentially expressed among patients with subtypes of MMD. Patients with MMD need to undergo a chronic conversion of cerebral hemodynamics and feeding arteries, from the original internal carotid artery system to the external carotid artery system or vertebrobasilar artery system. Depending on the degree or weight of vascular occlusion and collateral vasodilation, MMD can be manifested as ischemic, asymptomatic, or hemorrhagic[8, 9]. In this study, the microarray results demonstrated an increasing number of differentially expressed miRNAs from ischemic MMD to asymptomatic MMD to hemorrhagic MMD compared with controls. Also, the real-time PCR results suggested that the miRNA-142-3p was up-regulated in hemorrhagic MMD patients, and the miRNA-340-5p was up-regulated in hemorrhagic and asymptomatic MMD patients compared with ischemic MMD patients. This indicated that miRNA may be involved in the process of arterial occlusion and collateral development.

In this study, we found that the miRNAs are abnormally expressed in peripheral leukocytes of patients with MMD. After further verification of real-time PCR with larger sample size, and verification of target genes regulation and cell function regulation, these differentially expressed miRNAs are likely to become new molecular biological markers of MMD and associated stroke, and hold a promise to become new potential targets of therapeutic strategy.

Potential limitations of our studies should be mentioned. First, all of the patients in this study were enrolled from a single center, so potential selection bias may be inevitable. We, however, tried our best to

reduce in-house selection bias by collecting patients consecutively. Second, the case-control nature of the study necessitates further prospective cohorts to confirm our conclusions. Third, the sample size of this study is relatively small, and further PCR verification is needed in a large sample of people. Fourth, we used target gene prediction and functional enrichment analysis to indirectly obtain the biological processes that may be involved in the pathogenesis, which lacked verification of miRNA's regulation of target genes and cell functions, and further vitro cytology experiments are necessitated.

Conclusions

This study indicated that miRNAs are differentially expressed in peripheral leukocytes between MMD patients and healthy adults, and among patients with subtypes of MMD. The Wnt signaling pathway is probably involved in the pathogenesis of MMD.

Declarations

Ethics approval and consent to participate

The study was performed according to the guidelines from the Helsinki Declaration, and it was approved by the Institutional Review Board (IRB) of the hospital. Written informed consent was obtained from all patients or their legally authorized guardian.

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

None

Funding

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Authors' Contributions

KK and YS contributed to the conception/design of the study, the acquisition, analysis and interpretation of data, drafting of the manuscript, final approval of the version to be published. QZ, JL, YJ, RJ, NL, JW, BY, JL and XL were responsible for the acquisition of data, final approval of the version to be published. XZ and DZ were responsible for the conception/design of the study, revision of the work, critical revision of the manuscript, final approval of the version to be published, and agreement to be accountable for all aspects of the work.

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Figures

Figure 1

Heat map of differentially expressed miRNAs between MMD patients and healthy controls

Figure 2

Heat map of differentially expressed miRNAs between hemorrhagic MMD patients (A), ischemic (B), asymptomatic MMD patients (C) and healthy controls

Figure 3

Validation of microarray results using quantitative real-time PCR

Figure 4