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Integrated Analysis of LncRNA-mRNA Co-Expression Profiles in Patients with Moyamoya Disease

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Moyamoya disease (MMD) is an idiopathic disease associated with recurrent stroke. However, the pathogenesis of MMD remains unknown. Therefore, we performed long noncoding RNA (lncRNA) and messenger RNA (mRNA) expression profiles in blood samples from MMD patients (N = 15) and healthy controls (N = 10). A total of 880 differentially expressed lncRNAs (3649 probes) and 2624 differentially expressed mRNAs (2880 probes) were obtained from the microarrays of MMD patients and healthy controls (P < 0.05; Fold Change >2.0). Gene ontology (GO) and pathway analyses showed that upregulated mRNAs were enriched for inflammatory response, Toll-like receptor signaling pathway, chemokine signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway among others, while the downregulated mRNAs were enriched for neurological system process, digestion, drug metabolism, retinol metabolism and others. Our results showed that the integrated analysis of lncRNA-mRNA co-expression networks were linked to inflammatory response, Toll-like signaling pathway, cytokine-cytokine receptor interaction and MAPK signaling pathway. These findings may elucidate the pathogenesis of MMD, and the differentially expressed genes could provide clues to find key components in the MMD pathway.

Moyamoya disease (MMD) is a congenital disease that is characterized by stenosis of terminal internal carotid arteries and a hazy network of basal collaterals[1]. Recurrent stroke is common among MMD patients, and the standard treatment for MMD is revascularization surgery[2,3]. Although genome-wide and locus-specific association studies identified RNF213 as an important susceptibility gene of MMD[4], few studies focus on the dysregulated genes and the pathogenesis of MMD still remains unknown.

Long noncoding RNAs (LncRNAs) are RNA molecules longer than 200 nucleotides without protein-coding ability[5]. Many studies have revealed a wide range of functional activities of lncRNAs[6,7], including chromatin remodeling, transcriptional control and post-transcriptional processing. The dysregulation of lncRNAs might contribute to inflammatory response[8], and several studies have reported that lncRNAs are associated with various inflammatory conditions[9–12]. TUG1 could decrease inflammation *in vivo*[13], and ANRIL could regulate inflammatory responses through the NF-κB pathway[14]. JMJD1A and MALAT1 can reduce the activity of mitogen-activated protein kinase (MAPK) signaling in glioma cells and gastric cancer[15,16], respectively. Genetic and environmental factors may play important roles in MMD development[17]. In a previous study, lncRNA expression profiles produced completely different clusters, and the MAPK signaling pathway was found to play a core role in this pathway network[18]. Co-expression analysis is widely used to elucidate the relationship between lncRNAs and messenger RNAs (mRNAs)[19,20]. It can elucidate the key lncRNAs and help to find a new regulation mechanism.

Understanding dysregulated lncRNAs and mRNAs is important for the diagnosis and treatment of patients with MMD. Therefore, we performed a microarray to examine lncRNA and mRNA expression profiles in blood samples from MMD patients and healthy controls. A total of 880 differentially expressed lncRNAs (3649 probes)

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Items		MMD	Control
Number		15	10
Sex	Male	8	5
	Female	7	5
Age (Mean ± SD)	Male	32 ± 11.44	31.80 ± 10.30
	Female	32.86 ± 10.57	32.52 ± 10.42
Initial Clinical	IVH	6	ND
	SAH	1	
	TIA	3	
	Infarction	5	
Subgroups	HG	7	ND
	IG	6	
	HIG	2	
mRS Score (Mean ± SD)		1.8 ± 0.68	ND

Table 1. Clinical characteristics of included patients. MMD, Moyamoya disease; IVH, intraventricular hemorrhage; SAH, subarachnoid hemorrhage; TIA, transient ischemic attack; HG, hemorrhagic group; IG, ischemic group; IHG, ischemic and hemorrhagic group; mRs, modified Rankin scale; ND, no data.
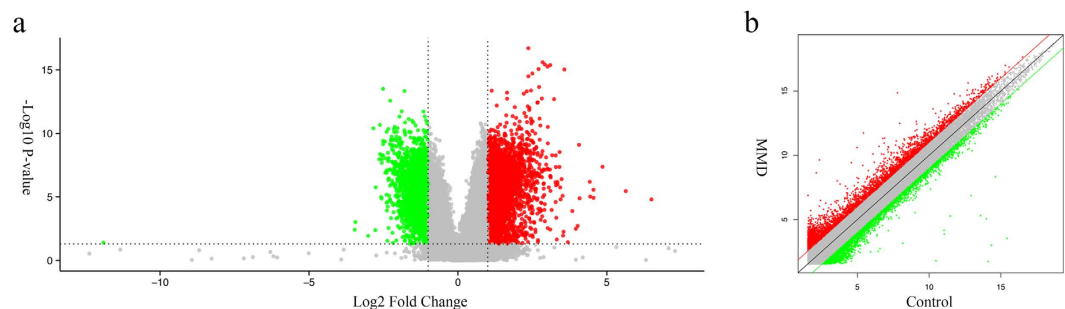

Figure 1. mRNA expression profile in MMD patients and healthy controls. (**a**) Volcano plots of mRNAs expression levels between MMD and control group. (**b**) Scatter plots of mRNAs expression levels between the MMD and control groups. The red dots represented upregulated mRNAs, and the green dots represented downregulated mRNAs (P < 0.05; Fold Change >2.0).

and 2624 mRNAs (2880 probes) were identified. We further performed four co-expression networks in inflammatory response, the Toll-like signaling pathway, cytokine-cytokine receptor interaction and the MAPK signaling pathway. The integrated analysis of the differentially expressed lncRNAs and mRNAs may provide clues to find genes with active roles in pathogenesis of MMD.

Results

The clinical characteristics of included MMD patients.
We included 15 MMD patients and 10 healthy controls in our study. The clinical characteristics of the included patients are shown in Table 1. There were 6 patients with intraventricular hemorrhage (IVH), 1 with subarachnoid hemorrhage (SAH), 3 with transient ischemic attack (TIA) and 5 with infarction as their initial clinical findings. Based on that, we grouped the patients into the hemorrhagic group (HG), the ischemic group (IG) and the ischemic and hemorrhagic group (IHG). The MMD and Control groups had similar age and sex distributions (Table 1).

Identification of differentially expressed lncRNAs and mRNAs.
The list of lncRNAs and their expression profiles were extracted in our previous study[18]. In brief, we identified 880 differentially expressed lncRNAs (3649 probes) and 2624 mRNAs (2880 probes) from the microarrays of MMD patients and healthy controls (P < 0.05; Fold Change >2.0) (Supplementary Table S1). Of those, 1746 upregulated mRNAs and 878 downregulated mRNAs were identified. A volcano plot was created and scatter analyses were conducted to identify differences among mRNAs (Fig. 1a,b). We further created a heat map of differentially expressed mRNAs (Fig. 2).

We also selected several differentially expressed genes randomly and further performed quantitative real-time polymerase chain reaction (qRT-PCR) to examine their expression levels. The qRT-PCR results suggest that the fold changes observed in the microarray analysis were robust (Fig. 3).

Examination of the function of differentially expressed mRNAs.
Gene ontology (GO) and KEGG pathway analyses were conducted to explore the function of the 2624 differentially expressed mRNAs using DAVID (The Database for Annotation, Visualization and Integrated Discovery). The results showed that upregulated genes were enriched for inflammatory response, response to wounding and defense response, etc. (Fig. 4a), while the downregulated genes were enriched for neurological system process, digestion and positive regulation
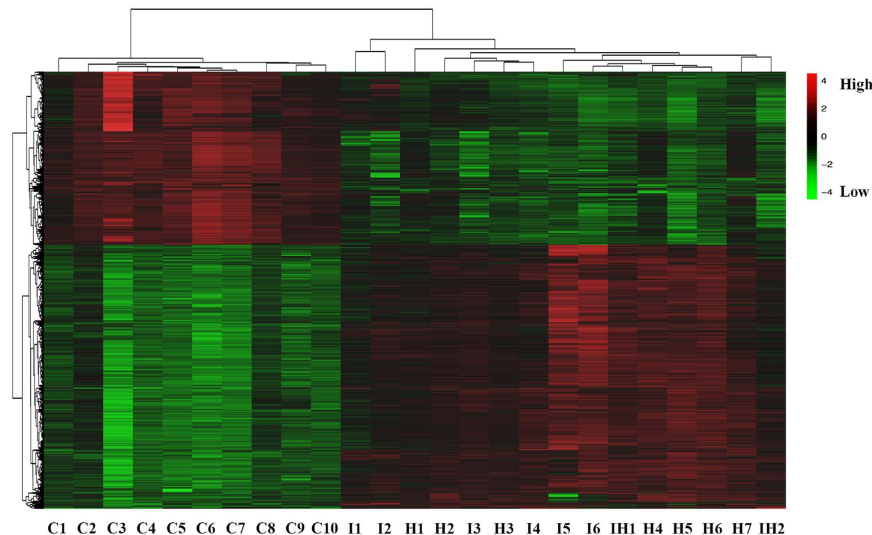
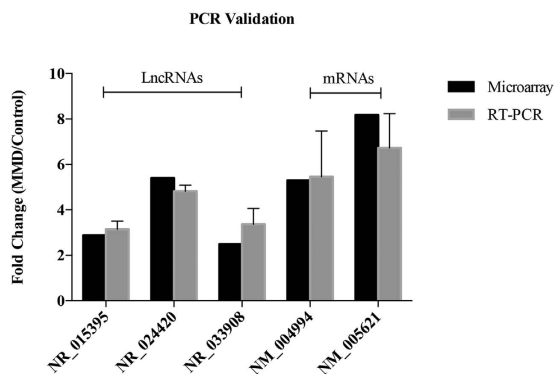**Figure 2. Heat map of differentially expressed mRNAs of MMD patients and healthy controls.**

Figure 3. Validation of microarray results using quantitative real-time polymerase chain reaction (qRT-PCR).

of transcription from RNA polymerase II promoter, etc. (Fig. 4b). Moreover, the KEGG pathway analysis showed that the upregulated genes were enriched for Toll-like receptor signaling pathway, chemokine signaling pathway and MAPK signaling pathway, etc. (Fig. 4c), while the downregulated genes were enriched for drug metabolism, retinol metabolism and olfactory transduction, etc. (Fig. 4d).

LncRNA-mRNA co-expression networks. We performed lncRNA-mRNA co-expression network analysis including 3649 lncRNAs probes and 2880 mRNAs probes. Our results showed that the co-expression networks were linked to inflammatory response, Toll-like signaling pathway, cytokine-cytokine receptor interaction and MAPK signaling pathway (Fig. 5). Thirty-two lncRNAs interacted with 11 mRNAs in the GO term of inflammatory response (Fig. 5a), 26 lncRNAs interacted with 2 mRNAs in the Toll-like signaling pathway (Fig. 5b), 41 lncRNAs interacted with 6 mRNAs in the cytokine-cytokine receptor interaction (Fig. 5c), and 15 lncRNAs interacted with 6 mRNAs in the MAPK signaling pathway (Fig. 5d).

Discussion

MMD is a chronic occlusive cerebrovascular disease of unknown etiology[21], and it is usually diagnosed by radiological findings, such as computed tomography (CT) perfusion and magnetic resonance imaging (MRI)[22]. It has been shown that MMD has a high prevalence in Asian countries, such as China, Japan and South Korea[23–27]. However, there are fewer studies focused on lncRNA-mRNA co-expression in MMD, and the molecular mechanisms behind MMDs remain poorly understood. It has been reported that lncRNAs play important roles in a wide range of functional activities[6,7]. The dysregulation of lncRNAs might contribute towards MMD. Therefore, the integrated analysis of the differentially expressed lncRNAs and mRNAs could help to reveal the pathogenesis of MMD.

Many studies have associated single nucleotide polymorphisms (SNPs) of genes with MMD[28–34]. RNF213 and MMP3 were proposed to be susceptibility genes for MMD[28,30]. It was reported that RNF213 is associated with immune response and that it might act cooperatively with other molecules under inflammatory signals based on bioinformatics data. IFNG and TNFA synergistically activated transcription of RNF213 both *in vitro* and *in vivo*[35].
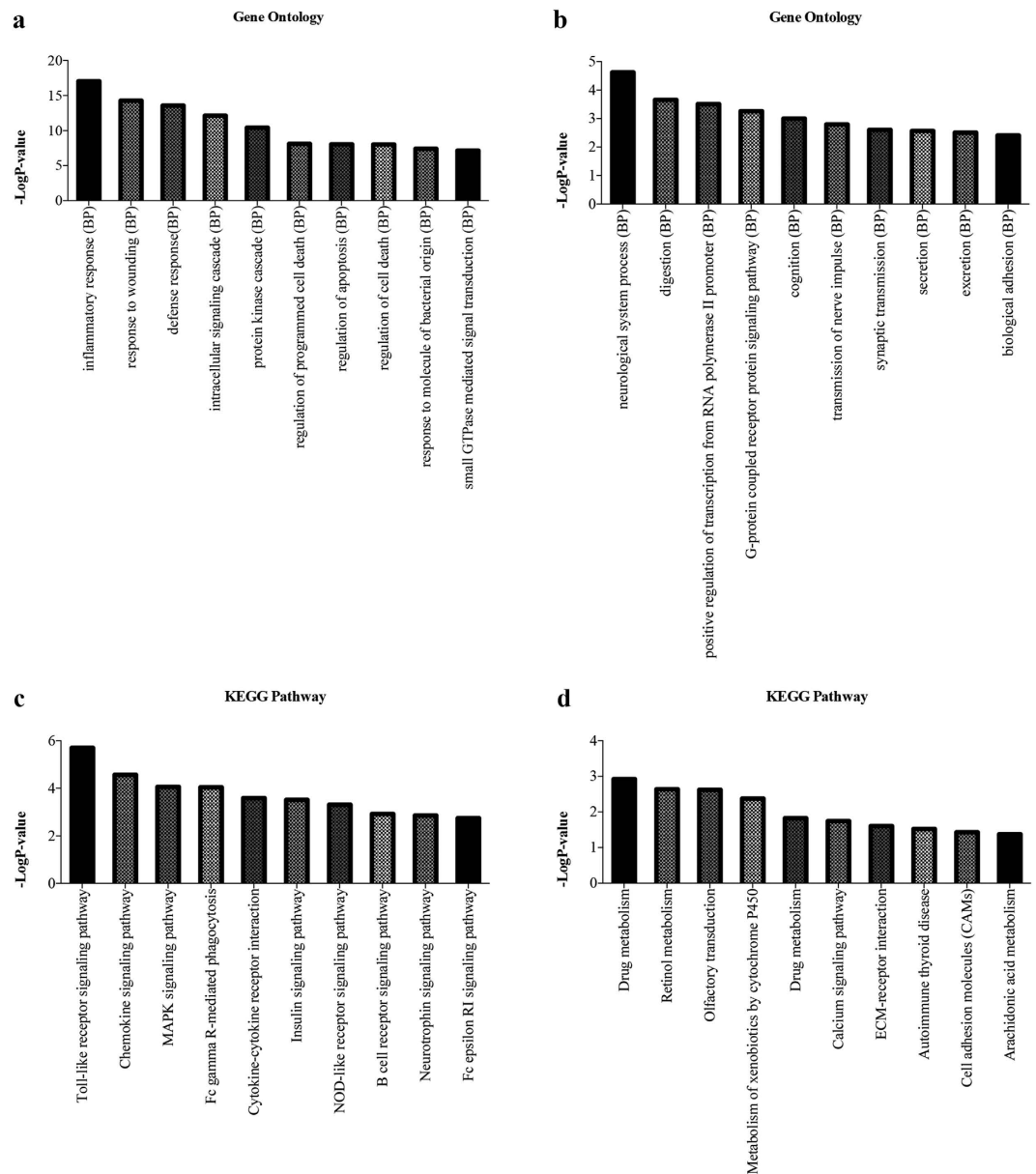
Figure 4. Gene ontology and KEGG Pathway analysis of 2624 differentially expressed mRNAs. (a) The top 10 GO terms upregulated in MMD patients compared with healthy controls. (**b**) The top 10 GO terms downregulated in MMD patients compared with healthy controls. (**c**) The top 10 pathways upregulated in MMD patients compared with healthy controls. (**d**) The top 10 pathways downregulated in MMD patients compared with healthy controls.

However, IFNG and RNF213 were not co-expressed based on the mRNA microarray. The presence of a heterozygous genotype in TIMP2 promoter could be a genetic factor for familial MMD. Moreover, it was reported that TGFB1 could be involved in vascular growth and transformation processes and may play an important role in the development of MMD[34,36]. SNPs may affect the expression of genes for MMD. Based on the microarray database, RNF213 was not differentially expressed between MMDs and controls. However, TGFB1 and TIMP2 were found to be differentially expressed. These findings need to be validated in further studies including more samples.

The co-expression results were based on the expression of lncRNAs and mRNAs for MMD. It was reported that HIF1A could directly bind to the promoter of HOTAIR, which has been identified in a variety of carcinomas[37]. CXCR2 is involved in migration and activation of leukocytes and plays a key role in several inflammatory diseases[38,39]. MALAT1 could downregulate the expression of CXCR2 via miR-22–3p[40]. These were consistent with the results that HOTAIR and MALAT1 were highly associated with HIF1A and CXCR2, respectively (correlation coefficient -0.76, 0.87; $P < 0.01$), based on our microarray. To our knowledge, this is the first study of lncRNA-mRNA co-expression network analysis for patients with MMD. Dai *et al.* have conducted a serum miRNA signature in MMD, and it identified 94 differential expressed miRNAs[41]. Non-coding RNAs and mRNAs compete for binding to miRNAs by sharing one or more miRNA response elements (MREs) to regulate gene
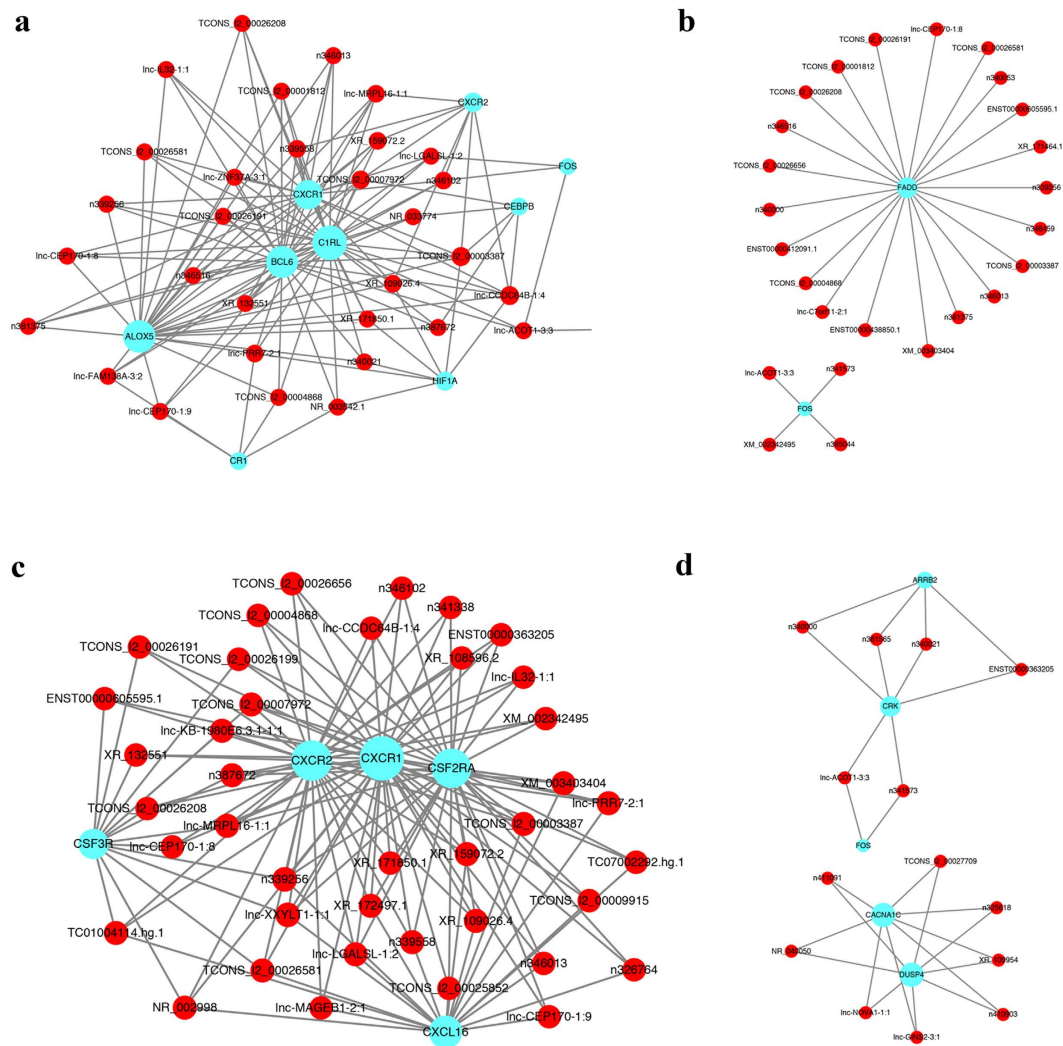
Figure 5. LncRNA-mRNA co-expression network. (**a**) 32 lncRNAs interacted with 11 mRNAs in the GO term of inflammatory response. (**b**) 26 lncRNAs interacted with 2 mRNAs in the Toll-like signaling pathway. (**c**) 41 lncRNAs interacted with 6 mRNAs in the Cytokine-cytokine receptor interaction. (**d**) 15 lncRNAs interacted with 6 mRNAs in the MAPK signaling pathway.

expression[42], and the differentially expressed lncRNAs and mRNAs in our study partly correlate with these miR-NAs. Therefore, future studies could focus on finding a competing endogenous RNAs (ceRNA) network with a key role in the pathogenesis of MMDs

Next, we investigated whether mRNAs tend to be neighbors with lncRNAs in MMD. We have performed all lncRNA-mRNA (63431 lncRNAs, 39887 mRNAs) co-expression analyses and lncRNA/mRNA reciprocal expression pattern analyses for MMD patients. Surprisingly, the genomic position and orientation of lncRNAs and mRNAs do have relativity with their correlation coefficient.

We further performed GO and KEGG pathway analyses, which could help us understand the pathogenesis of MMD and could provide new potential therapeutic targets. Moreover, the lncRNA-mRNA co-expression analysis showed that 32 lncRNAs interacted with 11 mRNAs in the GO of inflammatory response and 15 lncRNAs interacted with 6 mRNAs in the MAPK signaling pathway (Fig. 5a,d). Association of differentially expressed lncRNAs and mRNAs with pathways relevant to MMD pathogenesis may partly explain the etiology of MMD. Inflammatory response leads to the hyperplasia of intimal vascular smooth muscle cells (VSMCs), which causes lumen stenosis of MMDs[43,44]. The MAPK signaling pathway played important roles in vascular pathological processes[45–47] and vascular inflammation[48]. It was reported that many cytokines induce the proliferation of VSMCs via MAPK signaling pathway[49–51]. MAPK signaling pathway inhibitors have been successfully used to treat atherosclerosis *in vivo*[52]. Our results can elucidate key lncRNAs and provide leads to further understand the pathogenesis of MMD.

There are limitations to our study. There were only 15 MMD patients and 10 healthy controls included in our analysis, and we could still identify valuable genes and pathways from our database. Further studies would enlarge the sample size. Moreover, the results were obtained from the bioinformatic analysis and microarray analysis.

Therefore, further studies are needed to confirm these differentially expressed genes and pathway mechanisms with experiments. This manuscript is our preliminary work, and further work remains to be done.

In conclusion, we identified 880 differentially expressed lncRNAs (3649 probes) and 2624 differentially expressed mRNAs (2880 probes) from the microarray of MMD patients and healthy controls. The upregulated differentially expressed mRNAs were enriched for inflammatory response, the Toll-like receptor signaling pathway, chemokine signaling pathway and the MAPK signaling pathway. The integrated analysis also indicated inter-regulation in MMD patients. These findings may reveal the pathogenesis of MMD, and future studies should focus on the inflammatory response and MAPK signaling pathway for MMD patients.

Methods

Patients and Samples.   We enrolled 15 MMD patients who had been diagnosed with MMD according to the characteristic angiographic findings with strict inclusion criteria in Beijing Tiantan Hospital and 10 healthy controls in our study. Informed consent was obtained at enrolment and the basic characteristics of all MMD patients and healthy controls are summarized in Table 1. Our study was approved by the Ethics Committee in Beijing Tiantan hospital. The study was carried out in accordance with the Declaration of Helsinki, and all methods were performed in accordance with the relevant guidelines and regulations.

We obtained the peripheral blood anticoagulated with ethylene diamine tetraacetic acid (EDTA) from all patients and healthy controls. Further, we extracted the RNA by using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions and quality evaluations were performed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

LncRNA and mRNA Microarrays.   The Agilent Human 4×180 K lncRNA and mRNA Microarrays (Agilent, Santa Clara, CA, USA) were performed using a Gene Expression Hybridization Kit (Agilent, Santa Clara, CA, US) 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 (Agilent, Santa Clara, CA, USA) with default settings according to the manufacturer's instructions. Raw data were normalized by Quantile algorithm using Gene Spring Software 12.6 (Agilent Technologies). The differentially expressed lncRNAs and mRNAs were identified by using R software (version 3.2.3) with the samr package[53].

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR).   We selected several differentially expressed genes to detect their expression in MMD. Total RNAs were isolated from all samples using PAXgene Blood RNA Kit (Qiagen, Germany) and then reverse transcribed using an iScript cDNA synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Select Master Mix (Applied Biosystems, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, and all the primer sequences are shown below:

NR_015395-F: 5′-CTAATTTGCCACCACCCTGT-3′
NR_015395-R: 5′-AAGACCCAGATGCCGTTTTA-3′
NR_024420-F: 5′-CTGCAACGAATCCCAAAAGT-3′
NR_024420-R: 5′-ACCACTTTCCAGAGGCTGAA-3′
NR_033908-F: 5′-CGAGCTGTAAAAGCCAAAGG-3′
NR_033908-R: 5′-CCTGGGCGATAAGAGTGAAA-3′
NM_004994-F: 5′-TGTACCGCTATGGTTACACTCG-3′
NM_004994-R: 5′-GGCAGGGACAGTTGCTTCT-3′
NM_005621-F: 5′-ATTGAGGGGTTAACATTAGGCTG-3′
NM_005621-R: 5′-GATATTCTTGATGGTGTTTGCAAGC-3′
GAPDH-F: 5′-AGGGCTGCTTTTAACTCTGGT-3′
GAPDH-R: 5′-CCCCACTTGATTTTGGAGGGA-3′

Statistical Analysis.   All statistical data were analyzed by using SPSS (version 22; SPSS Inc., 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. The P value was false discovery rate (FDR) corrected. LncRNAs-mRNAs co-expression networks were constructed by Cytoscape software[54] (version 3.4.0; The Cytoscape Consortium, San Diego, CA, USA).

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Author Contributions

W.W. and F.L.G. wrote the main manuscript text. Z.Z., H.Y.W., L.Z., D.Z. and Y.Z. prepared Figures 1–5. Q.L. and J.F.W. collected clinical samples. J.Z.Z. designed the protocol of the study. All authors reviewed the manuscript.

Additional Information

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