Circulating microRNA profile in patients with membranous obstruction of the inferior vena cava

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Abstract. Membranous obstruction of the inferior vena cava (MOVC) is a common type of Budd-Chiari syndrome. However, the pathogenesis of MOVC has not been fully elucidated. Recent studies demonstrated that microRNAs (miRNAs or miRs) are involved in multiple diseases. To the best of our knowledge, specific changes in the expression of miRNAs in MOVC patients have not been previously assessed. The present study used a microarray analysis, followed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation, with the aim to access the miRNA expression levels in the plasma of 34 MOVC patients, compared with those in healthy controls. The results revealed a total of 16 differentially expressed miRNAs in MOVC patients. Subsequently, RT-qPCR analysis verified the statistically consistent expression of 5 selected miRNAs (miR-125a-5p, miR-133b, miR-423-5p, miR-1228-5p and miR-1266), in line with the results of the microarray analysis. These 5 miRNAs, which were described as crucial regulators in numerous biological processes and vascular diseases, may play an important role in the pathogenesis of MOVC. Bioinformatics analysis of target genes of the differentially expressed miRNAs revealed that these predicted targets were significantly enriched and involved in several key signaling pathways important for MOVC, including the ErbB, Wnt, MAPK and VEGF signaling pathway. In conclusion, miRNAs may involve in multiple signaling pathways contributing to the pathological processes of MOVC. The present study offers an intriguing new perspective on the involvement of miRNAs in MOVC; however, the precise underlying mechanisms require further validation.

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Introduction

Budd-Chiari syndrome (BCS) is a heterogeneous disorder that occurs due to an obstruction present between the hepatic venules and the junction of the inferior vena cava, resulting in significant mortality and morbidity (1). It is a complex disease with a wide spectrum of etiologies and presentations (2). BCS remains rare with incidence rates of 0.2-0.8 million people affected per year (3,4)

Numerous studies have suggested that the clinical features and etiology distribution of BCS vary according to the geographical area (5-7). In western countries, pure hepatic venule obstruction accounts for >50% of BCS cases, whereas thrombosis is the predominant pathological lesion of BCS (1). The majority of cases are closely associated with underlying inherited or acquired thrombotic risk factors, with ~80% of BCS patients presenting at least one thrombotic risk factor (8). Myeloproliferative neoplasms (MPNs) are considered to be the leading cause in the development of BCS, and have been reported in ~50% of BCS patients (9). In developing countries, including Nepal and China, the most common type of BCS is membranous obstruction of the inferior vena cava (MOVC) (7,10), which has been shown to account for 62.7% of BCS cases in China (11). Recent data from several centers have consistently shown that underlying thrombotic disorders in Chinese BCS patients are rarely detected (6). In addition, certain risk factors that have been confirmed in western countries, such as MPNs, do not seem to be etiological factors in Chinese BCS patients (12,13). This observation suggests that the etiological distribution of BCS may differ between western countries and China. Accordingly, due to the potentially different pathogenesis factors in BCS patients of different ethnicities (14), the present study focused on Chinese patients with BCS.

MicroRNAs (miRNAs or miR) are a class of naturally small noncoding RNAs with a length of 21-23 nucleotides, which bind to the 3'-untranslated regions of their target mRNAs and regulate gene expression at the post-transcriptional level (15). Previous studies have revealed that miRNAs serve a crucial role in numerous pathological and physiological processes,

including organ development, cell differentiation, immune function and vascular diseases (16,17). In addition, a number of miRNAs have been identified as important modulators of vascular pathologies, including apoptosis, angiogenesis, inflammation, hypertension and atherosclerosis (18). Furthermore, the genetic abnormality or dysregulation of miRNAs may contribute to the pathogenesis of vascular diseases (19,20); thus, these findings demonstrate that miRNAs play an important role in vascular disorders.

Certain studies have demonstrated that the amount of circulating miRNAs may be used as clinical biomarkers (21,22). However, to the best of our knowledge, cell-free miRNAs in the plasma of BCS patients with MOVC have not been previously investigated. Therefore, the aim of the present study was to identify a panel of plasma miRNAs that are differentially expressed in patients with MOVC and to investigate the potential biological function of these candidate miRNAs.

Materials and methods

Study population. The study was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical College (Xuzhou, China), and informed consent was obtained from each subject.

A total of 34 patients aged 27-75 years who were diagnosed with incident MOVC at the Affiliated Hospital of Xuzhou Medical College between February 2013 and September 2013 were enrolled into the present study. Patients were diagnosed based on the BCS characteristics, as previously described (REFold1). All diagnoses were confirmed using radiographic imaging, including Doppler ultrasound and magnetic resonance imaging. In addition, venography images suggested the existence of a membrane in the inferior vena cava. Patients with other coexisting diseases, including hypertension, coronary heart disease, diabetes, blood diseases and cancer, were excluded. Additionally, 30 healthy age- and gender-matched subjects were recruited as the controls. The microarray cohort of subjects included 9 MOVC patients and 5 healthy volunteers (MOVC-1/controls-1). The present study also investigated a second group composed of 25 MOVC patients and 25 healthy controls (MOVC-2/controls-2) for independent validation using reverse transcription-quantitative reverse transcription (RT-qPCR) analysis. No statistically significant differences were detected in the age and gender distribution of patients and controls (Table I).

Plasma collection and RNA isolation. A 5-ml sample of venous blood was collected from each patient or healthy volunteer before breakfast on the morning after hospital admission. Blood samples were drawn into EDTA-containing tubes and the plasma was immediately separated by a two-step centrifugation protocol at room temperature (centrifugation at 1,800 x g for 10 min, followed by centrifugation at 15,000 x g for 10 min) (23). The supernatant was then transferred into RNase-free microcentrifuge tubes and stored at -80˚C until use.

Total plasma RNA was harvested with the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 200 μl plasma was diluted with 1 ml QIAzol lysis reagent. After a 5-min incubation, 5 μl of 5 nmol/l synthetic *Caenorhabditis elegans* miR-39 (Shanghai GenePharma Co., Ltd., Shanghai, China) was added to each sample as a spike control (24). Next, phenol extraction and various filter cartridge steps were performed according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific Inc., Wilmington, DE, USA) was used to measure the quality and concentration of RNA in each plasma sample.

miRNAs expression profiling. In order to assess the levels of specific miRNAs in the patients with MOVC, miRNA expression profiling of the plasma samples from 9 MOVC-1 patients and 5 healthy control-1 participants was performed using miRNA microarray analysis. As BCS is a rare disease, the maximum number of patients with MOVC were selected and the number of controls were reduced accordingly due to economic constraints. Microarray analysis and RT-qPCR are two common miRNA detection methods (25). In order to detect the miRNA expression levels of the samples, high-throughput microarray experiments were performed by KangChen Bio-tech Inc. (Shanghai, China), as this technique is highly sensitive and time-efficient. However, as the detection results may contain certain errors, it was necessary to conduct further validation using RT-qPCR, as this technique is considered the gold standard for detecting miRNAs (26). Therefore, independent expanding samples were chosen for RT-qPCR validation in order to make the results more reliable, as through microarray analysis and subsequent RT-qPCR validation the dysregulated miRNAs in patients with MOVC can be reflected with increased accuracy.

Briefly, 3 μg RNA samples were labeled with the Exiqon Hy3/Hy5 power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on the miRCURY LNA™ array (version 18.0; Exiqon), which contains 3,100 capture probes, covering all human miRNAs annotated in the miRBase database (http://www.mirbase.org/). Subsequently, the slides were scanned using a GenePix 4000B microarray laser scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA) and microarray images were analyzed using GenePix Pro 6.0 software. The green signal intensity was calculated following background subtraction, and the average of replicated spots on the same slide was calculated to determine the median intensity. The median normalization method was used to acquire normalized data (foreground - background / median). The threshold value for statistical significance used to define upregulated or downregulated miRNAs was a fold change of >2, with a value of $P<0.05$ calculated by the Student's *t* test.

RT-qPCR platform for relative quantification of miRNAs. In order to confirm the miRNA array results, stem-loop RT-qPCR was performed (Table II). Briefly, 5 μl total RNA was reverse-transcribed into cDNA in a volume of 15 μl with the TaqMan MicroRNAs Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Subsequently, PCR was performed in 15 μl reaction mixtures using the GeneAmp PCR system 9700 (Applied Biosystems; Thermo Fisher Scientific Inc.). The samples were subjected to thermal cycling parameters of 30 min at 16˚C, 40 min at 42˚C, and 5 min at 85˚C, and then kept at 4˚C. qPCR reactions were performed in triplicate using the TaqMan PCR

Table I. Characteristics of the study samples.

Characteristic	miRNA microarray			RT-qPCR		
	MOVC (n=9)	Control (n=5)	P-value	MOVC (n=25)	Control (n=25)	P-value
Male gender, n (%)	3 (33)	2 (40)	0.80	15 (60)	15 (60)	1.00
Age, years[a]	42.6 (27.5-60.3)	42.5 (28.1-1.7)	0.65	48.1 (27.3-74.6)	48.0 (26.9-74.8)	0.98

[a]Presented as the median (range). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MOVC, membranous obstruction of the inferior vena cava.

Table II. Characterization of miRNAs selected for reverse transcription-quantitative polymerase chain reaction validation.

miRNA	Primer sequence	miRBase accession number
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	MIMAT0000443
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	MIMAT0004748
hsa-miR-133b	UUUGGUCCCCUUCAACCAGCUA	MIMAT0000770
hsa-miR-1228-5p	GUGGGCGGGGGCAGGUGUGUG	MIMAT0005582
hsa-miR-1266	CCUCAGGGCUGUAGAACAGGGCU	MIMAT0005920

Table III. Differentially expressed miRNAs in MOVC-1 patients, as compared with healthy control-1 participants.

miRNAs	Fold change	Regulation	P-value
hsa-miR-423-5p	4.22	Up	0.0032
hsa-miR-133b	3.42	Up	0.0012
hsa-miR-125a-5p	2.89	Up	0.0140
hsa-miR-1299	2.71	Up	0.0099
hsa-miR-1265	2.56	Up	0.0380
hsa-miR-296-3p	2.01	Up	0.0370
hsa-miR-1266	0.09	Down	0.0040
hsa-miR-1228-5p	0.12	Down	0.0006
hsa-miR-659-5p	0.15	Down	0.0060
hsa-miR-3133	0.22	Down	0.0002
hsa-miR-523-3p	0.32	Down	0.0065
hsa-miR-301a-5p	0.37	Down	0.0080
hsa-miR-299-5p	0.44	Down	0.0110
hsa-miR-513a-5p	0.45	Down	0.0280
hsa-miR-149-3p	0.45	Down	0.0240
hsa-miR-337-3p	0.47	Down	0.0030

MOVC, membranous obstruction of the inferior vena cava.

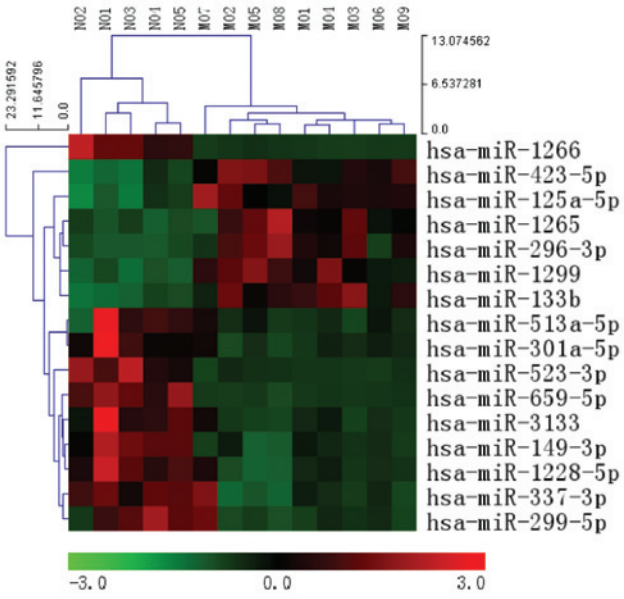

Figure 1. Heat map showing 16 differentially expressed miRNAs (fold change of >2) in the plasma of MOVC-1 patients (n=9) and healthy control-1 participants (n=5). Each row represents one miRNA, and each column represents a plasma sample. The relative miRNA expression is depicted according to the color scale. Red indicates upregulation and green indicates downregulation. Denotations beginning with M indicate the MOVC patients, while those beginning with N indicate the healthy controls. MOVC, membranous obstruction of the inferior vena cava.

Master Mix on an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific Inc.). Each amplification reaction was performed in a volume of 20 μl containing 1.33 μl cDNA and 1 μl of gene-specific primers. The cycle threshold (Cq) values were calculated with the SDS version 2.0.6 software (Applied Biosystems; Thermo Fisher Scientific Inc.), and the fold change for each miRNA was calculated using the comparative method ($2^{-\Delta\Delta Cq}$) with cel-miR-39 as the endogenous control (27).

Prediction of target genes and bioinformatics analysis of gene functions. In order to predict the target genes of differentially expressed miRNAs that were significantly dysregulated in the plasma of MOVC patients, the online databases Miranda (http://www.microrna.org), miRDB (http://mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org) were used. In the

Table IV. KEGG pathway analysis of putative target genes regulated by differentially expressed miRNAs.

A, Upregulated miRNAs

Analysis for predicted target genes	P-value
Pathways in cancer	$2.55x10^{-10}$
Axon guidance	$6.90x10^{-8}$
ErbB signaling pathway	$6.52x10^{-6}$
Insulin signaling pathway	$1.15x10^{-5}$
Focal adhesion	$3.41x10^{-5}$
Wnt signaling pathway	$1.15x10^{-4}$
MAPK signaling pathway	$1.30x10^{-4}$
VEGF signaling pathway	$3.39x10^{-4}$
p53 signaling pathway	$1.23x10^{-3}$

B, Downregulated miRNAs

Analysis for predicted target genes	P-value
Pathways in cancer	$2.96x10^{-9}$
ErbB signaling pathway	$1.45x10^{-6}$
Wnt signaling pathway	$1.67x10^{-5}$
p53 signaling pathway	$2.93x10^{-5}$
T cell receptor signaling pathway	$5.47x10^{-5}$
Apoptosis	$1.70x10^{-4}$
Vascular smooth muscle contraction	$4.99x10^{-4}$
MAPK signaling pathway	$7.90x10^{-4}$
VEGF signaling pathway	$3.69x10^{-3}$

KEGG, Kyoto Encyclopedia of Genes and Genomes; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase.

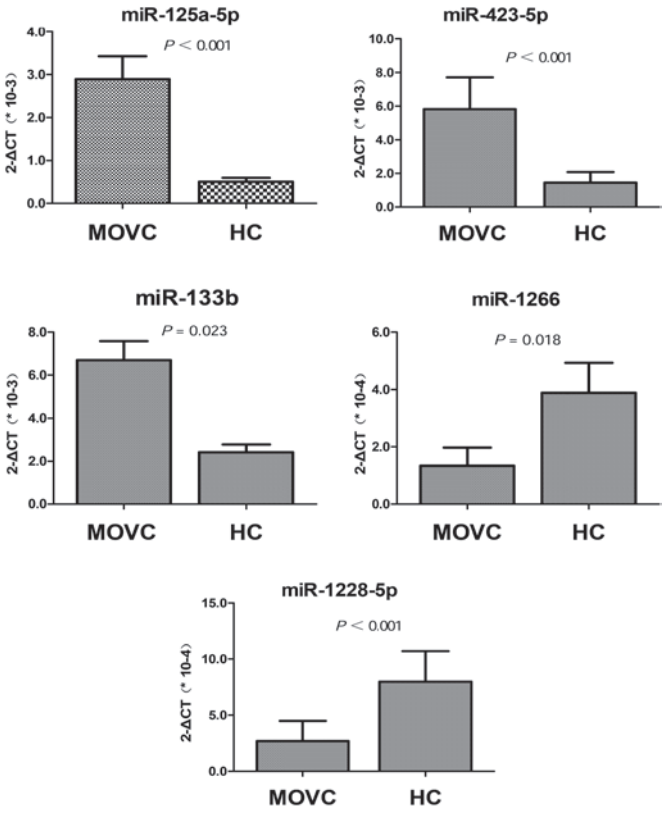

Figure 2. Circulating miRNAs in patients with MOVC-2, as compared with healthy control-2 participants. The expression of 5 selected miRNAs in EDTA-plasma obtained from patients with MOVC (n=25) and HC (n=25), were determined using reverse transcription-quantitative polymerase chain reaction. The relative expression of each miRNA in MOVC patients compared with the healthy control subjects was normalized to the expression of cel-miR-39. P-values were calculated by two-sided Student's t test. MOVC, membranous obstruction of the inferior vena cava; HC, healthy control.

present study, a predicted gene was considered as a putative target candidate when it was predicted by at least two of the aforementioned databases.

The predicted miRNA target genes were analyzed for Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment using the online DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). Fisher's exact test and χ^2 test were used to classify the GO category and the pathway analysis. Only GO annotations or pathways with a P-value of <0.01 and false discovery rate of <0.05 were selected.

Statistical analysis. SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the dataset from miRNA microarray experiments. Descriptive statistics were applied to determine differential individual characteristics between MOVC patients and healthy controls by the Mann-Whitney U test for continuous variables and the χ^2 test or Fisher exact test (two-tailed) for categorical variables. Statistical graph analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences with P<0.05 (two-tailed) were considered to be statistically significant.

Results

MicroRNA microarray expression profiling. Following normalization of the raw data, a total of 16 microRNAs were found to be significantly differentially expressed in the MOVC-1 patients, as compared with the controls-1 (>2-fold change; P<0.05). Among them, 6 upregulated miRNAs were identified, while 10 downregulated miRNAs were identified between the two groups (Table III). Unsupervised hierarchical clustering analysis was conducted using the expression levels of these 16 differentially expressed miRNAs, resulting in clear differentiation of MOVC samples from normal samples into two different clusters (Fig. 1).

Validation of profiling data using RT-qPCR. To verify the findings obtained through the miRNA profile analysis, RT-qPCR was performed in the independent cohort of 25 MOVC-2 patients and 25 healthy control-2 participants. The investigation was focused on 5 candidate miRNAs (miR-125a-5p, miR-133b, miR-423-5p, miR-1266 and miR-1228-5p), based on their differential expression levels observed in the present microarray analysis (2.89, 3.42, 4.22, 0.09 and 0.12-fold changes, respectively) (Table III) and their roles as crucial regulators of various biological processes and vascular diseases (28-32). The RT-qPCR results were consistent with

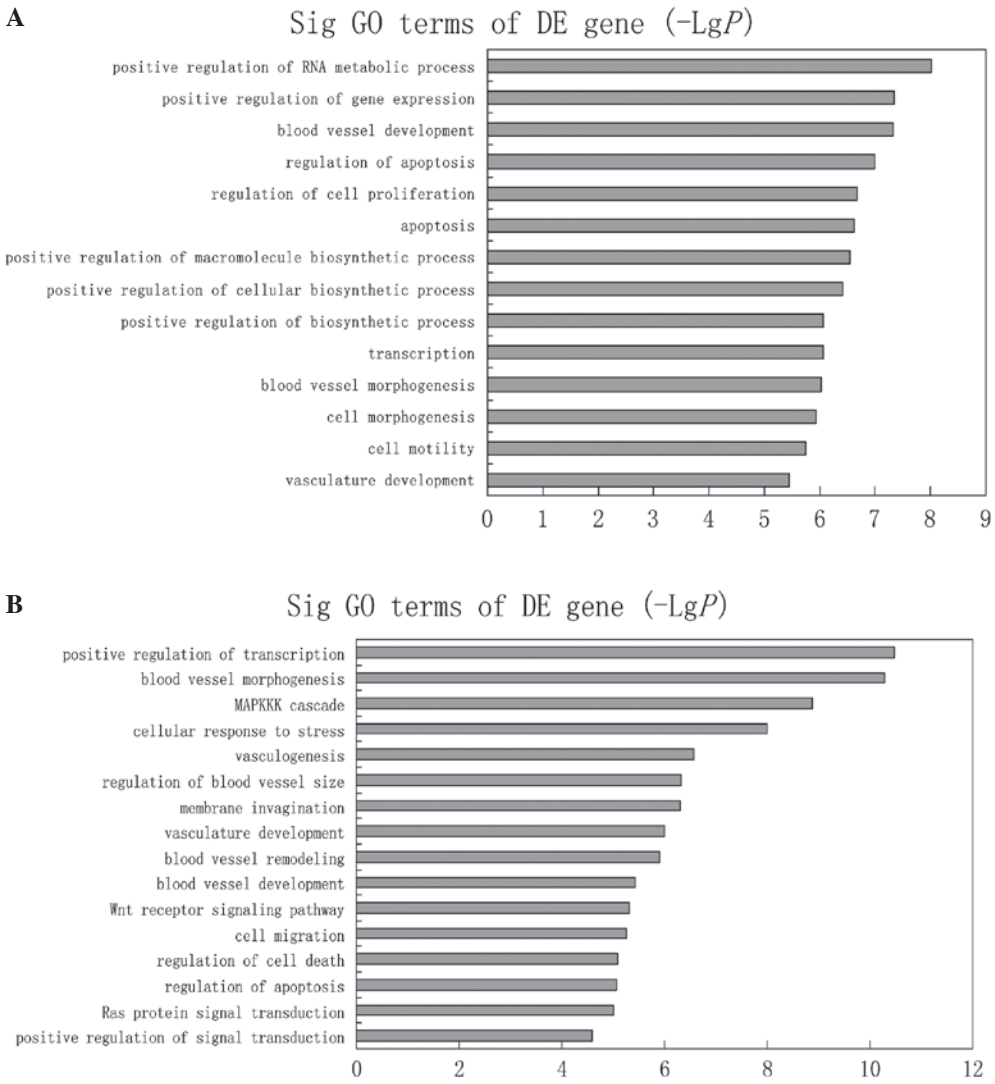

Figure 3. GO annotations for the predicted target genes. The charts compose the GO terms targeted by (A) upregulated and (B) downregulated miRNAs. The y-axis shows the GO category and the x-axis shows the enrichment of GOs. Sig, significance; GO, gene ontology; DE, differentially expressed; Lg*P*, Lg P-value; MAPKKK, mitogen-activated protein kinase kinase kinase.

the microarray data (Fig. 2). Specifically, the expression levels of miR-125a-5p, miR-133b and miR-423-5p were significantly increased, whereas those of miR-1228-5p and miR-1266 were significantly decreased in the MOVC patients. Therefore, the RT-qPCR results validated the observed microarray data, suggesting that the data obtained from the miRNA microarrays accurately reflected the miRNA expression levels.

Bioinformatics analysis of miRNAs. Three available databases (Miranda, miRBase and TargetScan) were used to predict targets genes of the 16 miRNAs that were differentially expressed in the MOVC-1 and control-1 groups. In order to reduce the possibility of a false positive, only targets estimated by at least two of these databases were considered as putative candidates. To assess the potential biological impact of the differentially expressed miRNAs, the present study performed GO annotations and KEGG pathway enrichment analysis for the predicted target genes. Employing that criterion, 20,009 target genes were predicted for the upregulated miRNAs, including Janus kinase 2 and vascular endothelial growth factor B (VEGFB); whereas 25,831 target genes were predicted for the downregulated miRNAs, including mitogen-activated protein kinase (MAPK) and patelet-derived growth factor. The primary GO terms targeted by upregulated miRNAs were the response to blood vessel development and morphogenesis, apoptosis and cell motility. By contrast, significant GO terms corresponding to downregulated miRNAs appeared to be the regulation of blood vessel morphogenesis, the MAPK kinase kinase cascade, blood vessel remodeling and Wnt receptor signaling pathway (Fig. 3).

KEGG pathway analysis showed that the target genes for upregulated miRNAs were associated with pathways in cancer, axon guidance, focal adhesion and the ErbB and Wnt signaling pathways. The target genes for downregulated miRNAs were associated with pathways in cancer, as well as with the ErbB, Wnt, p53 and T cell receptor signaling pathways. Notably, certain signaling pathways, such as pathways in cancer and the ErbB, Wnt, MAPK and VEGF signaling pathways, were targeted by the upregulated and downregulated miRNAs, suggesting that extensive miRNA regulation of these pathways is involved in MOVC (Table IV).

Discussion

The levels of circulating miRNAs potentially reflect altered pathological and physiological conditions (33). As previously discussed, numerous studies have shown that miRNAs serve a crucial role in vascular disorders (19,20). However, the gene regulation of miRNAs in the pathogenesis of MOVC has not been extensively investigated. Thus, the present study aimed to assess the miRNA expression profiles in the plasma of patients with MOVC in comparison with those in age- and gender-matched healthy individuals.

The profiling data in the present study demonstrated a distinct miRNA expression profile in MOVC patients, including 6 miRNAs that were significantly upregulated and 10 miRNAs that were significantly downregulated. Furthermore, 5 significantly altered miRNAs (miR-125a-5p, miR-133b, miR-423-5p, miR-1266 and miR-1228-5p) were verified in a second cohort of patients using RT-qPCR, and the results were found to be in line with those obtained by the miRNA microarray analysis, thus suggesting that the profiling results were reliable. Among the dysregulated miRNAs (Table III), miR-125a-5p has previously been reported to have a higher expression level in vascular endothelial cells (VECs) and brain endothelial cells (34,35), and thus potentially contributing various vascular diseases, including atherosclerosis, hypertension and stroke. It has been reported that the upregulation of miR-125a-5p may be involved in angiogenesis defects in mature endothelial cells by targeting the associated transcriptional enhancer factor-1 (36). Additionally, Li *et al* (34) identified that miR-125a-5p was able to inhibit endothelin-1 (ET-1) expression in VECs, while Hao *et al* (37) found that miR-125a-5p was able to suppress the expression of ET-1 in the coronary arteries. ET-1, as a potent vasoconstrictive peptide, plays critical roles in the progression of atherosclerosis, vascular inflammation and remodeling (38,39).

miR-133b, a myogenic miRNA, has been shown to have a close association with multiple tumors and is generally considered to have tumor-suppressive functions (40,41). It is also overproduced in early myocardial injury subsequent to heart transplantation (42), suggesting its association with cardiovascular system disorders. Furthermore, miR-423-5p, which has been reported to be upregulated in human failing myocardium, may be a novel blood-based biomarker for heart failure (43). Thus far, studies concerning miR-1266 and miR-1228-5p are limited, with only a small number of reports investigating their role in cancer (44,45). Combining the aforementioned findings and our results, these dysregulated miRNAs (miR-125a, miR-133b, miR-423-5p, miR-1266 and miR-1228-5p) are suggested to be closely associated with the pathogenesis of MOVC; however, the detailed underlying mechanism requires further investigation.

To further clarify the role of miRNAs in the pathogenesis of MOVC, GO annotations and KEGG pathway analysis were performed on the target genes known to be regulated by the differentially expressed miRNAs. KEGG pathway analysis showed significant changes associated with the ErbB, Wnt, MAPK and VEGF signaling pathways in the MOVC patients, as compared with the healthy controls. ErbB signaling is implicated in the regulation of the normal function of the embryonic and adult heart (46). The Wnt signaling pathway plays an important role in morphogenesis, cell survival, differentiation and proliferation (47). Additionally, the MAPK and VEGF signaling pathways are closely associated with the function of VECs. Siddiqui *et al* (48) found that MAPK had a considerable effect on the endothelial monolayer integrity by signaling proliferation and survival of endothelial cells. VEGF, which is crucial in angiogenesis, is an endothelial cell-specific mitogen (49) that regulates endothelial cell proliferation, migration, vascular permeability, secretion and other endothelial functions (50). Bioinformatics analysis performed in future studies may improve the understanding on the pathogenesis of MOVC.

To the best of our knowledge, the present study is the first to evaluate the plasma miRNA expression pattern in MOVC patients by microarray-based miRNA analysis. In conclusion, a total of 16 differentially expressed miRNAs were identified in the plasma between MOVC patients and healthy controls. In total, 5 of these miRNAs were verified by RT-qPCR, which provided results in line with those obtained by the miRNA microarray analysis. Functional bioinformatics analysis demonstrated that the target genes regulated by these miRNAs were involved in several biological processes and signaling pathways. The study of these miRNAs may provide a clearer understanding on the pathogenesis of MOVC and help identify novel methods for the diagnosis, treatment and prevention of MOVC. However, further investigation using more samples is required to verify the differential expression of the miRNAs observed in the present study, and their cellular origin and function in MOVC should be analyzed.

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