Identification of differentially expressed microRNAs in acute Kawasaki disease

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Abstract. The present study used microarray analysis to screen the plasma expression of microRNAs (miRNAs) in patients with acute Kawasaki disease (KD) and aimed to explore the pathogenesis of KD. Plasma was collected from children with acute KD (n=6) and from healthy control children (n=6). Total RNA was extracted and differential miRNA expression between the two groups was determined. Differentially expressed miRNAs were validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in an independent cohort (n=8). Target genes of the differentially expressed miRNAs were predicted and analyzed for gene ontology term enrichment and Kyoto Encyclopedia of Genes and Genomes pathways. miRNA microarray analysis revealed that seven miRNAs (miRs) were significantly upregulated (hsa-let-7b-5p, hsa-miR-223-3p, hsa-miR-4485, hsa-miR-4644, hsa-miR-4800-5p, hsa-miR-6510-5p and hsa-miR-765) and three were significantly downregulated (hsa-miR-33b-3p, hsa-miR-4443 and hsa-miR-4515) in acute KD compared with the healthy controls. hsa-miR-223-3p expression levels detected by RT-qPCR were consistent with the microarray results. A total of 62 target genes of hsa-miR-223-3p were predicted. In total, 10 differentially expressed miRNAs were identified in acute KD, of which hsa-miR-223-3p was verified by RT-qPCR.

Introduction

Kawasaki disease (KD) is a systemic vasculitis syndrome of unknown etiology. The vasculitis mainly attacks coronary arteries, and cardiac sequelae, such as coronary aneurysms and coronary insufficiencies, are some of the most serious manifestations of this disease (1-3). It is particularly prevalent in infants and young children (4). Although the clinical features, diagnosis and treatment of KD are well established, its pathogenesis has not been identified yet. Several lines of evidence suggest that an interplay between microbial infection and genetic predisposition serve a role in the development of the disease (5-7).

MicroRNAs (miRNAs) are endogenous single-strand, non-coding RNAs of 18-25 nucleotides in length, that post-transcriptionally regulate gene expression through sequence-specific interaction with target messenger RNAs (mRNAs) (8,9). miRNAs are highly conserved, and their expression is time specific (10). miRNAs exhibit powerful regulatory roles in many biological processes, including cell metabolism, proliferation, differentiation and apoptosis (11). Aberrant expression of miRNAs has been confirmed to be associated with various human diseases including cancers, cardiovascular diseases and inflammatory conditions (12-14). Blood circulating miRNA levels are stable (15) and their unique expression patterns may be used as a novel, non-invasive biomarker for disease diagnosis (16). Recent studies identified circulating miRNAs as biomarkers for many disorders, such as cardiovascular disease (17,18) and inflammatory diseases (19). However, previous reports on the expression of circulating miRNAs in KD are limited. Additional studies are required to determine whether there is differential miRNA expression in the circulating plasma in patients with KD and the functions of target genes.

The present study aimed to identify a panel of plasma miRNAs that are differentially expressed in patients with KD and to provide a possible direction for studying the pathophysiological mechanisms of KD.

Materials and methods

Specimen source. Plasma specimens for miRNA microarray hybridization were obtained from children with KD (n=6) and from healthy control children (n=6) between May 2013 and August 2013; plasma specimens for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were obtained

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from children with KD (n=8) and from healthy control children (n=8) between September 2013 and October 2013 at the Children's Hospital of Soochow University (Suzhou, China). Patients in the control group underwent regular health examinations and had no infections. KD was defined according to the criteria established by the American Heart Association in 2004 (20). Venous blood (4 ml) was collected from the patients in EDTA-containing tubes on the day of diagnosis for KD. The blood samples were first centrifuged at 820 x g for 10 min at 4˚C, and then at 16,000 x g for 10 min at 4˚C. Plasma was collected in 1.5 ml eppendorf tubes and stored at -80˚C. The 12 biologically independent plasma samples were analyzed individually, rather than pooling the samples. The plasma samples were labeled K or C for KD and control, respectively, followed by a coding number, to protect the privacy of the participants during all molecular studies. All parents of participants provided written informed consent for participation in this study, and the samples were processed under the approval of the Ethics Committee of Children's Hospital of Soochow University (Suzhou China).

RNA extraction and quantification. Total RNA was extracted from plasma (400 μl/sample) using a mirVana PARIS RNA and Native Protein Purification kit (cat. no. AM1556; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Briefly, 10 volumes of lysis/binding buffer and 1/10 volume of miRNA homogenate was added to the plasma and mixed well. A 1:1 ratio of acid phenol:chloroform equal to the lysate volume was added to the miRNA homogenate additive. The mixture was centrifuged for 5 min at 1,000 x g at room temperature, the aqueous upper phase was removed and transferred to a fresh tube. Following the addition of 1.25 volumes 100% ethanol, the lysate/ethanol mixture was passed through a filter cartridge, which was subsequently washed with 700 μl miRNA wash solution 1 and 500 μl wash solution 2/3. RNA was eluted from the filters with 100 μl elution solution that was warmed to 95˚C. Subsequently, the eluate, which contained the RNA, was collected and stored at -80˚C. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA sample quality was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). An RNA ≥7.0 was accepted for microarray analysis and RT-qPCR.

miRNA microarray hybridization. miRNA profile analysis of the plasma samples was performed using a miRNA Microarray Chip V2.4 (Agilent Technologies, Inc.), which contains probes for 2,549 human miRNAs with a sample input of 100 ng total RNA. Briefly, dephosphorylation was performed by gently mixing the total RNA with 2 μl calf intestinal alkaline phosphatase master mix (Agilent Technologies, Inc.) and incubating the mixture at 37˚C in a circulating water bath for 30 min. Subsequently, 2.8 μl 100% dimethyl sulfoxide was added to each sample and incubated at 100˚C in a circulating water bath for 5-10 min for denaturation. The samples were labeled using a miRNA Complete Labeling and Hybridization kit and hybridized on an Agilent SureHyb Microarray Hybridization Chamber (both from Agilent Technologies, Inc.). Following hybridization, the chip was washed using GE wash buffer 1 and GE wash buffer 2 (Gene Expression Wash Buffer kit; cat. no. 5188-5327; Agilent Technologies, Inc.). The chip was scanned and the data were extracted using Agilent Feature Extraction Software version 10.7.1.1 (Agilent Technologies, Inc.). Data were standardized using GeneSpring Software version 13.1 (Agilent Technologies, Inc.). Fold change ≥2 and $P<0.05$ were used to indicate significant differences in gene expression, and cluster analysis was performed using Genespring software version 14.8 (Agilent Technologies, Inc.).

RT-qPCR. The total RNA extracted from the samples met the quality control requirements and qualified for RT-qPCR analysis. Each 20 μl RT reaction was performed according the manufacturer's protocol of the miScript II Reverse Transcriptase kit (Qiagen GmbH, Hilden, Germany) in a GeneAmp PCR system 9700 (Applied Biosystems) for 60 min at 37˚C, followed by heat inactivation of the RT for 5 min at 95˚C. qPCR was performed using a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland) with the 10 μl reaction mixtures comprising cDNA (1 μl), 2X LightCycler 480 SYBR-Green I Master mix (5 μl), universal primer (0.2 μl), miRNA-specific primer (0.2 μl) and nuclease-free water (3.6 μl). The upstream primer of hsa-miR-16 was 5'TAG CAGCACGTAAATATTGGCG3'. The upstream primer of hsa-miR-765 was 5'TGGAGGAGAAGGAAGGTGATG3'. The upstream primer of hsa-miR-33b-3p was 5'CAGTGCCTCGGC AGTGCAGCCC3'. The upstream primer of has-miR-223-3p was 5'TGTCAGTTTGTCAAATACCCCA3'. Reactions were incubated in a 384-well plate at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec and at 60˚C for 30 sec. Each sample was run in triplicate. miRNA expression levels were normalized to the internal reference hsa-miR-16 and external reference cel-miR-39 and were determined using the comparative threshold cycle $2^{-\Delta\Delta Cq}$ method (21).

miRNA target gene prediction. miRNA target genes were predicted using GeneSpring version 13.1 software (Agilent Technologies, Inc.). TargetScan (www.targetscan.org), PITA (genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and microRNA.org (www.microrna.org/microrna/home.do) databases were used to predict the intersectional miRNA target genes. The data were analyzed using Venny software version2.1 (bioinfogp.cnb.csic.es/tools/venny). Common target genes were analyzed for gene ontology (GO) functional term enrichment, such as biological process (BP), cellular component (CC) and molecular function (MF); GeneSpring and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used in the pathway analysis.

Statistical analysis. The patients with KD and control patient sample data were compared using the Wilcoxon rank sum test. Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical features. Patient samples used for both microarray and RT-qPCR analyses exhibited no differences in sex and age distributions among the patients and controls (Tables I and II).

Table I. Clinical features of patients with KD and control patients used in the microarray analysis.

	Age (months)		Sex	
Sample no.	KD	Control	KD	Control
1	25	19	Female	Male
2	46	17	Female	Female
3	24	17	Female	Female
4	8	7	Male	Male
5	36	37	Female	Female
6	7	8	Male	Female

KD, Kawasaki disease.

Table II. Clinical features of patients with KD and control patients used for reverse transcription-quantitative polymerase chain reaction analysis.

	Age (months)		Sex	
Sample no.	KD	Control	KD	Control
1	13	15	Male	Male
2	24	22	Male	Male
3	34	32	Male	Female
4	36	31	Male	Female
5	22	17	Female	Male
6	11	15	Female	Male
7	56	49	Male	Male
8	22	39	Female	Male

KD, Kawasaki disease.

Differential miRNA expression. Microarray analysis of the plasma samples from the KD and control groups revealed that seven miRNAs were significantly upregulated (hsa-let-7b-5p, hsa-miR-223-3p, hsa-miR-4485, hsa-miR-4644, hsa-miR-4800-5p, hsa-miR-6510-5p and hsa-miR-765) and three were significantly downregulated (hsa-miR-33b-3p, hsa-miR-4443 and hsa-miR-4515) in the KD plasma samples compared with the control group (Fig. 1; Table III).

RT-qPCR. The selected miRNAs hsa-miR-765, hsa-miR-223-3p and hsa-miR-33b-3p underwent RT-qPCR which have been reported in previous studies (22-25) and their target genes can be found in miRNA databases, such as TargetScan (www.targetscan.org) and PITA (genie.weizmann.ac.il/pubs/mir07/mir07_data.html). Automated RT-qPCR determination of the three miRNAs was performed using hsa-miR-16 as the internal reference. The melting curves indicated good PCR amplification specificity, with one perfect single peak for each miRNA. The relative expression levels of hsa-miR-223-3p and hsa-miR-33b-3p were significantly higher in the KD group compared to the control group ($P<0.05$; Fig. 2). The relative expression level of hsa-miR-765 between the two groups was not significantly different ($P>0.5$).

Target genes. A total of 62 common target genes of hsa-miR-223-3p were identified by comparing three different target gene predictions and was detected by both RT-qPCR and microarray analysis (Fig. 3 and Table IV).

GO analysis. The 62 predicted target genes of hsa-miR-223-3p were enriched in BPs (including regulation of translation, norepinephrine metabolic process and regulation of neural precursor cell proliferation; Fig. 4A), CCs (including, basolateral plasma membrane, recycling endosome and cytoplasmic vesicle membrane; Fig. 4B) and MFs (including, protein binding, PDZ-domain binding and calmodulin binding; Fig. 4C).

KEGG pathway analysis. The biological pathway enrichment analysis of the 62 predicted target genes showed that hsa-miR-223-3p was significantly enriched in the AMP-activated protein kinase (AMPK) signaling pathway, mineral absorption pathway and signaling pathways regulating pluripotency of stem cell (Fig. 4D).

Discussion

KD is a childhood multisystemic vasculitis; the mechanisms involved in the pathogenesis of vasculitis are poorly understood. Necrotizing arteritis, subacute chronic vasculitis and luminal myofibroblastic proliferation have been previously identified as the three basic processes of KD pathogenesis (26). Necrotizing arteritis is an acute process that may be responsible for saccular aneurysms. Following the onset of KD, both subacute chronic vasculitis and luminal myofibroblastic proliferation persist for months to years.

The present study identified 10 differentially expressed miRNAs, a number of which have been reported previously, such as hsa-miR-765, hsa-miR-33b-3p and hsa-miR-223-3p. hsa-miR-765 has been reported in coronary disease (22) and cancer (23). hsa-miR-33b-3p has also been reported in cancer (24). And hsa-miR-223-3p has been reported in diabetes mellitus (25), and KD (27). Therefore, RT-qPCR was performed to verify these three miRNAs. As most patients with KD are newborns, the amount of blood that can be withdrawn is limited and that is why different plasma samples were used in microarray analysis and RT-qPCR, which is a limitation of the current study. Given miRNAs strong regulatory roles in cellular metabolism, proliferation, differentiation, apoptosis and stress (reviewed in 11), they may provide clues for understanding the pathophysiology of KD and may be potentially useful in future diagnostic and therapeutic strategies. As they exist in a very stable state in the serum or plasma (28,29), miRNAs are suitable as biological markers for KD diagnosis and follow-up (30). As the results of microarray are not always stable, the present study used RT-qPCR to validate the results of microarray. RT-qPCR validation revealed no difference in hsa-miR-765 expression levels and increased hsa-miR-33b-3p levels in the plasma of acute KD, which was inconsistent with the microarray results. The difference in results between the microarray and RT-qPCR may be due to detection sensitivity

Table III. Differentially expressed microRNAs of the two groups.

miRNA	P-value	FC	Trend	Sequence	Chr	miRBase ID
hsa-let-7b-5p	$3.15x10^{-2}$	8.448197	Up	AACCACACAACCTACTACC	22	MIMAT000003
hsa-miR-223-3p	$3.71x10^{-2}$	8.468529	Up	TGGGGTATTTGACAAACTGAC	X	MIMAT000020
hsa-miR-33b-3p	$4.90x10^{-2}$	3.495833	Down	GGGCTGCACTGCCG	17	MIMAT000481
hsa-miR-4443	$4.26x10^{-2}$	2.347132	Down	AAAACCCACGCCTCC	3	MIMAT001891
hsa-miR-4485	$1.05x10^{-2}$	1.699564	Up	TTAGGGTACCGCGGC	11	MIMAT001909
hsa-miR-4515	$2.21x10^{-2}$	2.975258	Down	GGGCTGCCGGGA	15	MIMAT001902
hsa-miR-4644	$1.00x10^{-7}$	34.0109	Up	CTTCTGTCTCTTTTCTCTC	6	MIMAT001974
hsa-miR-4800-5p	$4.78x10^{-2}$	7.431324	Up	TCCTTCCTTCCTCGG	4	MIMAT001998
hsa-miR-6510-5p	$5.53x10^{-3}$	2.450577	Up	GACTCCTCTCTCTCCC	17	MIMAT002546
hsa-miR-765	$4.13x10^{-2}$	8.229987	Up	CATCACCTTCCTTCTCCT	1	MIMAT000395

Chr, chromosome; miR, microRNA.

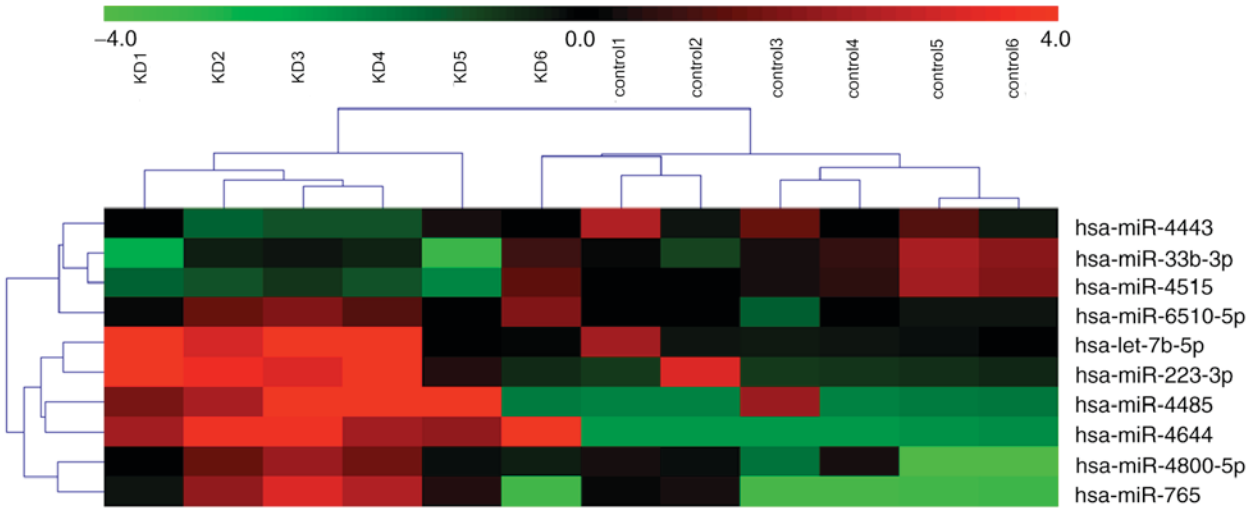

Figure 1. Distinguishable microRNA expression patterns were identified among the samples by hierarchical clustering and displaying by heatmap. In the heatmap, the chromatism represents the level of genes expression, with green corresponding to downregulated and red corresponding to upregulated expression.

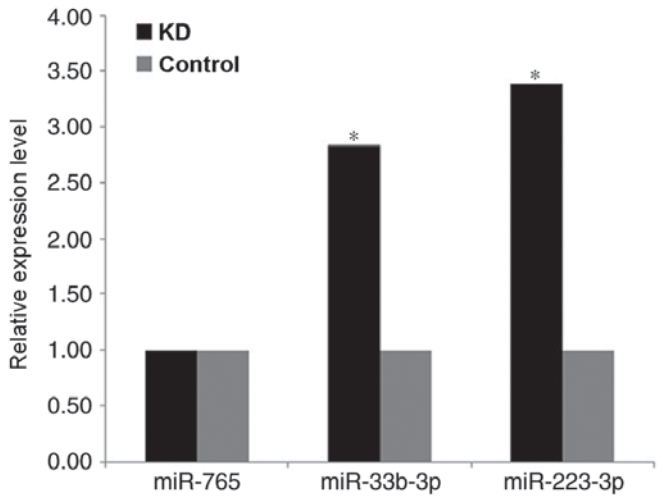

Figure 2. Comparison of the relative expression levels of hsa-miR-765, hsa-miR-223-3p and hsa-miR-33b-3p with the internal reference hsa-miR-16 in the KD and control groups, as measured by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. Control. KD, Kawasaki disease; miR, microRNA.

differences and sample heterogeneity. In addition, one of the differentially expressed miRNAs, hsa-miR-223-3p, for which both microarray and RT-qPCR revealed increased expression, was selected for target gene prediction.

Currently, few studies (27,31,32) have focused on circulating miRNAs in patients with KD. One previous study used high-throughput sequencing in the peripheral blood in patients with acute and convalescent KD to identify six differentially expressed miRNAs, including miR-143, miR-199b-5p, miR-618, miR-223, miR-145 and miR-145* (27). Using a group of febrile patients with KD as the control, another study reported elevated serum levels of miRNA-200c and miR-371-5p in patients with KD (31). High levels of miR-182 and miR-296-5p have been reported during the acute febrile phase, whereas high levels of miR-93, miR-145*, miR-145 and miR-150-3p were detected in the defervescence stage (32). It has been suggested that miR-93 may regulate vascular endothelial growth factor (VEGF-A) expression and may contribute to the understanding of the pathogenesis of arteritis in acute KD. A recent study demonstrated significantly higher serum

Table IV. Predicted target genes of hsa-miR-223-3p.

GeneID	Symbol	GeneID	Symbol	GeneID	Symbol
6477	SIAH1	2872	MKNK2	10600	USP16
84133	ZNRF3	5997	RGS2	8763	CD164
2034	EPAS1	4848	CNOT2	9962	SLC23A2
538	ATP7A	1080	CFTR	57835	SLC4A5
10890	RAB10	160518	DENND5B	84312	BRMS1L
6925	TCF4	3836	KPNA1	26118	WSB1
84255	SLC37A3	10492	SYNCRIP	143098	MPP7
255488	RNF144B	1010	CDH12	4774	NFIA
9852	EPM2AIP1	23250	ATP11A	463	ZFHX3
64145	RBSN	6383	SDC2	23220	DTX4
92	ACVR2A	91860	CALML4	284403	WDR62
9472	AKAP6	23435	TARDBP	3131	HLF
55156	ARMC1	29789	OLA1	4628	MYH10
27154	BRPF3	214	ALCAM	3572	IL6ST
9882	TBC1D4	55602	CDKN2AIP	2309	FOXO3
22883	CLSTN1	5898	RALA	9868	TOMM70
55588	MED29	490	ATP2B1	26269	FBXO8
5581	PRKCE	125950	RAVER1	5814	PURB
154796	AMOT	11221	DUSP10	51105	PHF20L1
2308	FOXO1	8939	FUBP3	5617	PRL
149018	LELP1	54842	MFSD6		

miR, microRNA.

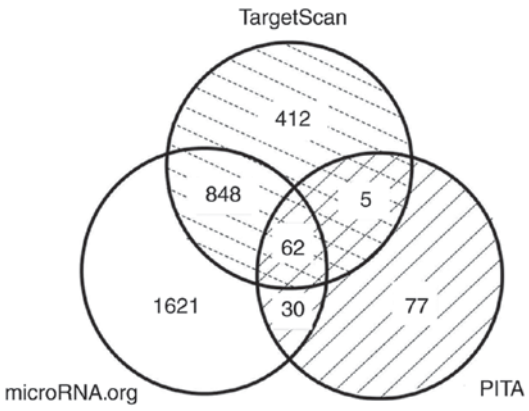

Figure 3. Venn diagram comparing three target-gene predictions by TargetScan, PITA and microRNA.org databases for hsa-miR-223-3p. miR, microRNA.

miR-92a-3p expression levels were detected in children with KD compared with febrile children (33); however, a different study reported that no miRNAs in coronary artery tissues were diagnostic for KD (34). The present study hypothesized that the different sample sources of *in vivo* circulating blood and *in vitro* coronary artery tissues contributed to the wholly opposite results. For example, the study by Rowley *et al* examined miRNA expression in coronary artery tissue from patients who had succumbed to KD (death within weeks after onset), which differed from the study by Rong *et al* that used circulating blood from living patients with KD (33,34). He *et al* revealed that KD sera suppressed the Krüppel-like factor 4/miR-483 axis in human umbilical vein endothelial cells, and increased the expression of connective tissue growth factor and induction of endothelial-to-mesenchymal transition. This detrimental process in the endothelium may contribute to coronary artery abnormalities in KD patients (35).

Previous studies have demonstrated that miR-223 is expressed in monocytes and macrophages, and may be the key to regulating inflammation (36). miR-223 was also reported to be transported in plasma and delivered to recipient cells by high-density lipoproteins (HDLs) in patients or mice with hypercholesterolemia (37), and it was demonstrated that the anti-inflammatory properties of HDL may be conferred, in part, through HDL-miR-223 delivery and the repression of intercellular adhesion molecule-1 translation in endothelial cells (38). miR-223 was previously demonstrated to target β1 integrin to antagonize angiogenesis and prevent growth factor signaling in endothelial cells (39). miR-223 was suggested to be a potential biomarker of type 2 diabetes (25). In addition, platelets were demonstrated to remotely modulate vascular endothelial cell apoptosis by releasing microvesicles that contain miR-223, which targets insulin-like growth factor 1 receptor and promotes advanced glycation end product-induced vascular endothelial cell apoptosis (40). One recent study revealed that high miR-223 expression levels in vascular endothelial cells may function as a novel endocrine genetic signal and participate in vascular injury of KD (41); however, the exact mechanism was not determined.

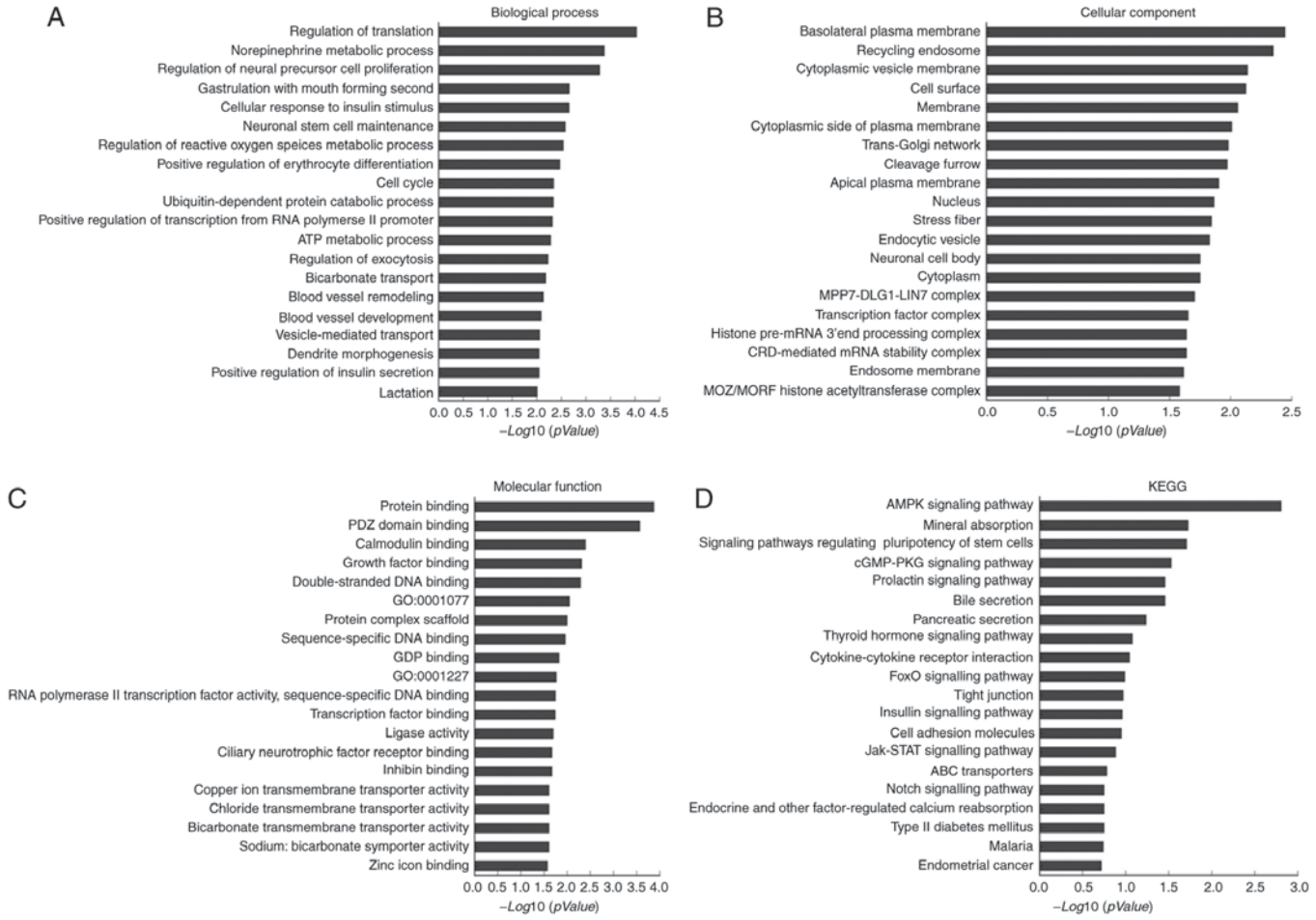

Figure 4. Gene ontology term enrichment and KEGG pathway analysis of the predicted target genes of hsa-miR-223-3p. (A) Enrichment of the biological process of the predicted target genes. (B) Enrichment of the cellular components of the predicted target genes. (C) Enrichment of the molecular functions of the predicted target genes. (D) Enrichment of KEGG pathways of the predicted target genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; miR, microRNA.

The present study identified 62 putative target genes of hsa-miR-223-3p. GO term enrichment analysis identified a number of biological processes, cellular components and molecular functions that may be related to KD; KEGG pathway analysis indicated that the target genes were enriched in AMPK signaling, mineral absorption and signaling pathways regulating stem cell pluripotency, whose role in KD needs to be defined. Previous studies have indicated the existence of specific signals or pathways in KD. For example, it was predicted that, along with other differentially expressed miRNAs, miR-145 may participate in regulating the expression of genes in the transforming growth factor β (TGF-β) pathway of arterial wall myofibroblasts (27), and miR-93 may participate in regulating VEGF-A expression in the pathogenesis of arteritis in acute KD (32); signaling pathways regulating stem cell pluripotency and cytokine-cytokine receptor interaction pathways may individually include TGF-β and VEGF-A.

In conclusion, 10 differentially expressed miRNAs were detected by microarray chip in the plasma of patients with acute KD, of which 3 miRNAs were verified by RT-qPCR, but only hsa-miR-223-3p was found to be consistently detected by both. A total of 62 potential target genes of hsa-miR-223-3p were identified. Previous studies have reported that miR-223 may regulate inflammation of vascular endothelial cells. Therefore, hsa-miR-223-3p may participate in the pathogenesis of KD, and determination of its functions and mechanisms in KD require further verification.

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