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.

Identification of differentially expressed microRNAs in acute Kawasaki disease

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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 to the manufacturer's protocol of the miScript II Reverse Transcripase 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 CAGCAGCTTCTATATGGCCG3'. The upstream primer of hsa-miR-765 was 5'TGGAGGAGAGGAAGGATGATG3'. The upstream primer of hsa-miR-33b-3p was 5'CATGCCCCTCGGC AGTGCAGG3'. The upstream primer of has-miR-223-3p was 5'TGTCATGTTGTGAAATACCCCA3'. 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-ΔΔCt 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).
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.
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 circu-
lating 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 differ-
entially 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 endo-
thelial 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

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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    | AACCACACACCTACTACC                 | 22  | MIMAT000003|
| hsa-miR-223-3p| 3.71x10^{-2} | 8.468529   | Up    | TGGGTAATGTGACAAACTGAC              | X   | MIMAT000020|
| hsa-miR-33b-3p| 4.90x10^{-2} | 3.495833   | Down  | GGGCTGACGTGCCG                     | 17  | MIMAT000481|
| hsa-miR-4443  | 4.26x10^{-2} | 2.347132   | Down  | AAAACCCACGCCTCC                    | 3   | MIMAT001891|
| hsa-miR-4485  | 1.05x10^{-2} | 1.699564   | Up    | TTAGGTACCCGGGC                     | 11  | MIMAT001909|
| hsa-miR-4515  | 2.21x10^{-2} | 2.975258   | Down  | GGGCTGCCGGGA                      | 15  | MIMAT001902|
| hsa-miR-4644  | 1.00x10^{-7} | 34.0109    | Up    | CTCTCTCTCTCTCTCT                   | 6   | MIMAT001974|
| hsa-miR-4800-5p| 4.78x10^{-2} | 7.431324   | Up    | TCTCTCTCTCTCTCGG                   | 4   | MIMAT001998|
| hsa-miR-6510-5p| 5.53x10^{-3} | 2.450577   | Up    | GACTCTCTCTCTCC                    | 17  | MIMAT002546|
| hsa-miR-765   | 4.13x10^{-2} | 8.229987   | Up    | CATCACCTCTCTCTCTCT                | 1   | MIMAT000395|

Chr, chromosome; 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). 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.

### 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   |
| 9      | 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  | PHF20L |
| 2308   | FOXO1  | 8939   | FUBP3  | 5617   | PRL    |
| 149018 | LELP1  | 54842  | MFSD6  |        |        |

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 KD, and determination of its functions and mechanisms in KD require further verification.

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