Abstract. MicroRNAs (miRNA) are considered to be potential therapeutic targets for the treatment of various cardiovascular diseases (CVDs). To understand the underlying mechanism of miRNAs and target genes associated with CVD, deep sequencing of blood samples from three patients with CVD and three controls was performed using the Illumina HiSeq 2000 system. The results of the present study revealed that 65 abnormal hsa-miRNAs targeted 2,784 putative genes in patients with CVD; 59 upregulated miRNAs targeted 2,401 genes and six downregulated miRNAs targeted 383 genes. In addition, a total of 49 Gene Ontology (GO) biological processes and were enriched, and the target genes of downregulated miRNAs were enriched in 12 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Most of these pathways are responsible for lipid and glycan metabolism. In particular, three downregulated miRNAs, hsa-miR-1268b, hsa-miR-1273d, hsa-miR-3187-5p, were involved in a-linolenic acid metabolism. The target genes of upregulated miRNAs were enriched in 15 KEGG pathways, mainly in the ‘neurodegenerative diseases and cancers’ class. In the present study five novel upregulated miRNAs, including m0499-5p, m0970-5p, m1042-5p, m1061-5p and m1953-5p, and a downregulated miRNA, novel-m1627-5p, were identified in patients with CVD. Novel-m1627-5p was demonstrated to target 146 human genes. Additionally, Novel-m1061-5p targeted four genes, including fumarylacetoacetate hydrolase domain containing 2A, potassium voltage-gated channel, Shaw-related subfamily, member 4, coiled-coil domain containing 85C and solute carrier family 35 member E3 (SLC35E3). The GO term, ‘carbohydrate derivative transport involving in biological process’, was associated with SLC35E3. Novel-m1061-5p in patients with CVD may repress the expression levels of SLC35E3, a member of the nucleoside sugar transporter subfamily E, which is known to cause defective glycol-conjugation in the Golgi complex and/or the endoplasmic reticulum. Further investigation is required to understand the underlying mechanisms of the novel miRNAs. Novel-m1061-5p may serve as a marker for prognosis or a potential target for the treatment of CVD.

Introduction

Cardiovascular disease (CVD) is the largest cause of mortality worldwide that involves the heart or blood vessels, and is associated with high blood pressure, diabetes, obesity, high blood cholesterol, poor diet and excessive alcohol consumption (1-6). microRNAs (miRNAs) are the most abundant class of regulatory noncoding RNA (ncRNA) involved in cell differentiation, expansion and apoptosis, and other biological processes by regulating over half of all human protein-coding genes. The dysfunction of miRNA may cause abnormal gene expression, affecting human health.

Previously, miRNAs have been regarded as a potential therapeutic target for a variety of CVDs, including atherosclerosis, myocardial infarction and hypertrophy (1-4). Additionally, miRNAs have been considered as key regulators in vascular biology (5-9), miRNA-21 (miR-21), miR-146a, miR-155, miR-221, miR-222 and miR-34a are reportedly associated with angiogenesis in patients with CVD (10-12); however, some miRNAs have been associated with the regulation of low-density lipoprotein and high-density lipoprotein (HDL) in atherosclerosis and other CVDs (13,14).

However, the etiology of CVD is complex and variations in miRNA expression patterns have been observed in patients with CVD. For example, miR-22 targets monocyte chemoattractant protein-1 and contributes to the pathogenesis of coronary artery disease (15). Therefore, a comprehensive understanding of miRNAs and target genes associated with various types of CVD is required. In the present study, miRNA profiles of blood samples from patients with CVD were investigated to improve understanding of the underlying mechanism of miRNA in the pathogenesis of CVD, and may therefore contribute to the effective treatment of CVDs.
Patients and methods

Patients. The present study included 6 patients diagnosed in the Shandong Provincial Hospital (Jinan, China) between May and September 2014. A total of 3 patients with CVD were diagnosed as atherosclerotic, 3 healthy volunteers were included as the control (CK). All patients provided written informed consent. The present study was approved by the ethics committee of Shandong Provincial Hospital.

Sample preparation and sequencing. Blood samples were prepared for isolating the RNA. Total RNA of all six samples were isolated and purified using TRIZol reagent (Invitrogen; Thermo Fischer Scientific, Inc., Waltham, MA) according to the manufacturer's protocol. RNA quality was assessed using a BioAnalyzer 2100 kit (Agilent Technologies, Inc. Santa Clara, CA) and a RNA 6000 Nano kit (Agilent Technologies, Inc.). Subsequently, RNA libraries were prepared using the Small RNA Sample Prep kit (Illumina, Inc., San Diego, CA) according to the manufacturer's protocol. Deep sequencing was performed via the HiSeq™ 2000 system (Illumina, Inc.).

Sequencing analysis. Removal of adaptor sequences was conducted using Cutadapt v.1.9 software [http://cutadapt.readthedocs.io/1.9] (16). Low quality reads of >95% base length with Phred quality scores <20 were filtered using the FASTX-Toolkit [http://hannonlab.cshl.edu/fastx_toolkit/] (17). Additionally, reads with polyA and polyT were also removed, and reads of <15 nucleotides or >34 nucleotides in length were discarded via miRDeep [https://www.mdc-berlin.de/8551903/en/] (18). Clean sequencing reads from small RNA (sRNA) libraries were summarized for length distribution and sRNA annotation. The sRNAs were mapped to the ncRNAs deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and Rfam database (http://rfam.janelia.org/) using the BLAST algorithm ([http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences that matched ncRNAs constituted rRNAs, tRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs, were annotated. Furthermore, the unique sRNA sequences were analysed via BLAST against miRBase v.20 ([ftp://mirbase.org/pub/mirbase/CURRENT/). Sequences in the libraries were filtered with the standard of ≤1 mismatch and ≥15 matches to miRNA database were considered as mature miRNAs of a known miRNA family. The identified mature miRNA sequences were aligned against a human genomic sequence using Bowtie v.2.2.4 [http://bowtie-bio.sourceforge.net/2.2.4] (19). The novel miRNA prediction pipeline was performed with Perl scripts combined using miREAP [http://mirrep.sourceforge.net/0.2] (20).

Differential expression analysis. Alterations in the expression levels of mature and novel miRNAs within CVD and CK groups were investigated in present study. Expression levels of all miRNAs were normalized to the transcript expression level per million reads. The fold change of the miRNA expression levels between CVD and CK groups were calculated to present the fold change. The differential expression analysis was adjusted with a q-value adjusted P-value. Fold change \[\log_2 \frac{\text{CVD}}{\text{CK}} > 1\] and P<0.05 were combined to identify the differentially expressed miRNAs associated with disease. The visual differential expression patterns of the 65 miRNAs were collected from the heatmap program in R (21).

Target gene prediction and analysis. Target gene prediction of miRNAs was performed using miRanda 3.3a [http://www.microrna.org/microrna/3.3a] (22); differentially expressed miRNAs were mapped to the human transcriptome. Sequences matching perfectly were identified as the predicted target genes. Predictions with less than five mismatches and the cleavage site from the 10th to 11th nucleotides perfectly matched were admitted and scored. Targets with \(P \leq 0.05\) were retained. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of target genes regulated by differentially expressed miRNAs were performed to predict miRNA function.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the TRizol reagent method. The cDNA first strand was synthesized using the miRcute miRNA First-Strand cDNA Synthesis kit according to the manufacturer's protocol (Tiangen Biochem Co., Ltd., Beijing, China). PolyA was added to 3'-end of miRNA and RT was performed according to the manufacturer's protocol of the kit (Tiangen Biochem Co., Ltd.). qPCR was performed using the miRcute miRNA qPCR Detection kit (containing SYBR Green; Tiangen Biochem Co., Ltd.) using the LightCycler 96 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). A 20 µl PCR reaction volume contained 1 µl cDNA, 10 µl 2X miRcute miRNA Premix (with SYBR and ROX), 0.4 µl forward primer, 0.4 µl reverse primer, 8.2 µl ddH₂O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA). The primer sequences were: hsa-mir-1061-5p, 5'-TCAGTTTGTTCATGTCTGC AG-3' and solute carrier family 35 member E3 (SLC35E3), forward 5'-ACG ACA GGT ACA CA-3' and reverse primer, 8.2 µl ddH₂O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA). The primer sequences were: hsa-mir-1061-5p, 5'-TCAGTTTGTTCATGTCTGC AG-3' and solute carrier family 35 member E3 (SLC35E3), forward 5'-ACG ACA GGT ACA CA-3' and reverse primer, 8.2 µl ddH₂O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA). The primer sequences were: hsa-mir-1061-5p, 5'-TCAGTTTGTTCATGTCTGC AG-3' and solute carrier family 35 member E3 (SLC35E3), forward 5'-ACG ACA GGT ACA CA-3' and reverse primer, 8.2 µl ddH₂O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA). The primer sequences were: hsa-mir-1061-5p, 5'-TCAGTTTGTTCATGTCTGC AG-3' and solute carrier family 35 member E3 (SLC35E3), forward 5'-ACG ACA GGT ACA CA-3' and reverse primer, 8.2 µl ddH₂O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA).

Results and Discussion

Deep sequencing of miRNA libraries and identification of conserved and novel miRNAs. In the present study, six sRNA profiles of three CK and three CVD individuals were sequenced. A total of >64.6 million clean reads were calculated.
generated, in the range of 2.1-21.2 million for individual samples. Small sequences were filtered as described, which were annotated to the NCBI GenBank and Rfam databases. The sRNAs annotated to rRNA, tRNA and snRNA were removed; the remaining small sequences of the six sRNA profiles were then aligned to the miRBase v.20 database. A total of 4,771 conserved miRNAs were identified, including 2,764, 1,319, 2,730, 2,191, 2,191, 2,050 unique conserved miRNAs from CK1, CK2, CK3, CVD1, CVD2, and CVD3 samples, respectively (Table I). In addition, 1,520 miRNAs were predicted as novel miRNAs in all samples, the number in each sample ranged from 374-1,059.

Transcript expression levels of the 6,291 identified miRNAs, including previously reported and novel miRNAs, in the 6 samples were calculated; 5,035 miRNAs were expressed within the CK group and 4,521 miRNAs within the CVD group. As presented in Fig. 1A, 1,419 commonly expressed miRNA were identified, with a proportion of 28.2%, among the CK group samples. Conversely, 1,909 commonly expressed miRNA were identified with a proportion of 42.2% among the CVD group samples. Conversely, 1,909 commonly expressed miRNA were identified with a proportion of 42.2% among the CVD group samples.

Table I. miRNAs identified within the CK and CVD groups.

| Category       | CK1  | CK2  | CK3  | CVD1 | CVD2 | CVD3 | Total |
|----------------|------|------|------|------|------|------|-------|
| Known_miRNA    | 2,764| 1,319| 2,730| 2,191| 2,191| 2,050| 4,771 |
| Novel_miRNA    | 666  | 374  | 1,059| 733  | 733  | 742  | 1,520 |
| Total          | 3,430| 1,693| 3,789| 2,924| 2,924| 2,792| 6,291 |

CK, control; CVD, cardiovascular disease; miRNA, microRNA.

Figure 1. microRNA distribution in each sample. CK, control; CVD, cardiovascular disease. Red colour indicated upregulated genes and green indicated downregulated genes.
The results of the present study indicated that CVD may be associated with a higher proportion of commonly expressed miRNAs in a variety of individuals.

**Differentially expressed miRNAs.** In order to identify the miRNAs associated with CVD, the fold change of CVD group vs. the CK group was determined. A set of 65 abnormal miRNAs, that were included in the commonly expressed 1,909 miRNAs demonstrated ≥2-fold change and P<0.05 in the CVD group compared with the CK group (Fig. 1C). A >2-fold upregulation of 59 miRNAs and downregulation of 6 miRNAs was detected (Table II; Fig. 2). In addition, differential expression levels of six novel miRNAs were identified, in which five were upregulated and one downregulated.

In the present study, the sRNA libraries were used to identify abnormally expressed miRNAs. Consequently, a total of 65 miRNAs were identified, among which miR-33 with the function of HDL synthesis and cholesterol transport, was upregulated in the CVD group. A previous study reported that miR-33a/b is embedded within the introns of human sterol regulatory element-binding protein (hsREBP)-1 and -2, which encodes the transcriptional regulator of cholesterol synthesis (23). miR-33a/b binds the mRNA of ATP-binding cassette A1 (ABCA1), a key transporter of intracellular cholesterol efflux. Upregulated expression levels of miR-33a reduces cholesterol efflux activity of apolipoprotein A1 and HDL, raising intracellular cholesterol levels (24). Upregulated miR-144 has also...

### Table II. Differential expression of miRs within the CK and CVD groups.

| A, Upregulated | miR name          | \( \log_2(\text{CVD/CK}) \) | P-value |
|----------------|-------------------|-----------------------------|---------|
| ccr-miR-457b_R1-15L21 | 1.33              | 0.02                        |
| cfa-let-7j_R1-15L23 | 1.17              | 0.02                        |
| chi-miR-20b_R1-22L23 | 1.44              | 0.01                        |
| dre-miR-2191_R20-6L21 | 1.31              | 0.00                        |
| hsa-let-7f-2-3p_R1-21L22 | 1.17              | 0.02                        |
| hsa-let-7f-5p_R5-22L22 | 1.10              | 0.02                        |
| hsa-miR-106b-5p_R1-16L21 | 1.17              | 0.00                        |
| hsa-miR-1227-3p_R1-20L20 | 1.67              | 0.03                        |
| hsa-miR-126-5p_R1-21L21 | 1.10              | 0.00                        |
| hsa-miR-144-5p_R2-22L22 | 1.23              | 0.02                        |
| hsa-miR-18a-5p_R1-17C-T | 1.01              | 0.01                        |
| hsa-miR-190a-5p_R1-21L22 | 2.05              | 0.01                        |
| hsa-miR-190b_R1-21L21 | 1.10              | 0.02                        |
| hsa-miR-20a-3p_R1-19L22 | 1.61              | 0.01                        |
| hsa-miR-20a-5p_R5-22L23 | 1.12              | 0.02                        |
| hsa-miR-2355-5p_R1-21L21 | 1.57              | 0.02                        |
| hsa-miR-26b-5p_R1-17L21 | 1.11              | 0.03                        |
| hsa-miR-30b-5p_R6-22L22 | 1.01              | 0.02                        |
| hsa-miR-32-3p_R1-21L22 | 1.43              | 0.01                        |
| hsa-miR-32-5p_R1-20L22 | 1.10              | 0.02                        |
| hsa-miR-33a-3p_R1-20L22 | 1.21              | 0.05                        |
| hsa-miR-340-5p_R1-19L22 | 1.57              | 0.01                        |
| hsa-miR-3688-3p_R3-22L22 | 1.06              | 0.03                        |
| hsa-miR-374a-3p_R1-21L22 | 1.30              | 0.02                        |
| hsa-miR-374a-5p_R1-18L22 | 1.42              | 0.01                        |
| hsa-miR-374b-3p_R1-21L22 | 1.27              | 0.04                        |
| hsa-miR-374c-3p_R22-3L22 | 1.53              | 0.01                        |
| hsa-miR-454-3p_R1-21L23 | 1.04              | 0.04                        |
| hsa-miR-4802-3p_R1-22L23 | 1.40              | 0.04                        |
| hsa-miR-542-5p | 1.09              | 0.04                        |
| hsa-miR-548ar-3p_R1-20L21 | 1.76              | 0.04                        |
| hsa-miR-548au-3p_R6-20L21 | 2.44              | 0.01                        |
| hsa-miR-548av-3p_R3-19L20 | 1.17              | 0.01                        |
| hsa-miR-548ay-3p_R1-20L22 | 1.18              | 0.01                        |
| hsa-miR-548e-3p_R3-22L22 | 1.02              | 0.01                        |
| hsa-miR-548i_R1-21L22 | 1.10              | 0.04                        |
| hsa-miR-548p_R1-20L22 | 2.21              | 0.02                        |
| hsa-miR-548u_R1-21L23 | 1.11              | 0.05                        |
| hsa-miR-550b-3p_R19-1L20 | 1.34              | 0.04                        |
| hsa-miR-556-3p_R1-19L22 | 2.71              | 0.00                        |
| hsa-miR-576-3p_R2-22L22 | 1.01              | 0.01                        |
| hsa-miR-582-5p_R19C-T | 1.45              | 0.04                        |
| hsa-miR-590-3p_R1-21L21 | 1.54              | 0.01                        |
| hsa-miR-599_R5-19L20 | 1.05              | 0.04                        |
| hsa-miR-627-3p_R1-20L20 | 1.41              | 0.04                        |
| hsa-miR-651-5p_R1-20L22 | 1.04              | 0.01                        |
| hsa-miR-8057_R19-5L21 | 1.82              | 0.02                        |
| hsa-miR-98-3p_R2-21L22 | 1.53              | 0.03                        |
| mml-miR-6134_R1-16L19 | 1.02              | 0.01                        |

| B, Downregulated | miR name          | \( \log_2(\text{CVD/CK}) \) | P-value |
|-------------------|-------------------|-----------------------------|---------|
| hsa-miR-1268b_R1-18L20 | -1.42             | 0.04                        |
| hsa-miR-1273d_R9-25L25 | -1.61             | 0.01                        |
| hsa-miR-3187-5p_R1-22L23 | -1.14             | 0.05                        |
| hsa-miR-4492_R1-17L17 | -2.16             | 0.04                        |
| hsa-miR-7641_R2-19L19 | -1.32             | 0.03                        |
| novel-miR-1627-5p | -2.06             | 0.05                        |

CK, control; CVD, cardiovascular disease; miR, microRNA.

among the CVD group samples (Fig. 1B). The results of the present study indicated that CVD may be associated with a higher proportion of commonly expressed miRNAs in a variety of individuals.

**Table II. Continued.**

| miR name          | \( \log_2(\text{CVD/CK}) \) | P-value |
|-------------------|-----------------------------|---------|
| novel-m0499-5p | 1.65                        | 0.02    |
| novel-m0970-5p | 1.47                        | 0.03    |
| novel-m1042-5p | 1.22                        | 0.02    |
| novel-m1061-5p | 1.79                        | 0.03    |
| novel-m1953-5p | 1.07                        | 0.03    |
| pma-miR-20b_R2-23L23 | 1.60              | 0.00    |
| pma-miR-30g_R1-22L23 | 1.04              | 0.04    |
| mo-miR-17-2-3p_R18-2L23 | 1.25              | 0.02    |
| sha-miR-340_R17-1L21 | 1.46              | 0.03    |
| tgu-miR-20a-3p_R18-2L23 | 1.64              | 0.02    |
been demonstrated to mediate the expression levels of ABCA1; the 3'-untranslated region of ABCA1 has been reported to be conservatively targeted by miR-144, thus reducing ABCA1 expression levels and cholesterol efflux of HDL (25).

miR-126 was upregulated in the current study. miR-126 has been reported to be an endothelium-enriched miRNA that regulates the response of endothelial cells to vascular endothelial growth factor, and modulates vascular integrity and angiogenesis (26). A previous study reported that miR-126 regulates the expression levels of Sprouty-related protein and phosphoinositol-3 kinase regulatory subunit 2, which are responsible for the inhibition of angiogenic signalling (27).

Functional annotation for target genes. The biological functions of CVD-associated miRNAs were investigated using the 2,784 putative genes targeted by 65 differentially expressed miRNAs. The results of the present study revealed that the expression of 2,401 target genes were repressed by 59 upregulated miRNAs; expression of 383 target genes were reduced by the downregulation of the other 6 miRNAs. GO classification analysis for the 2,784 differently expressed miRNA target genes was performed (Table III). The P-value was combined with Bonferroni correction for multiple testing; 49 GO biological processes were enriched (Table III), including ‘regulation of axonogenesis’, ‘cell-cell adhesion’, ‘intracellular signal transduction’, ‘cellular localization’, ‘regulation of signal transduction’, ‘cellular protein modification process’, ‘positive regulation of cellular process’, ‘cellular component organization’, ‘regulation of biological quality’ and ‘regulation of transcription’.

Figure 2. Clustering heatmap of differentially expressed microRNAs. CVD, cardiovascular disease; CK, control. Red colour indicated upregulated and green indicated downregulated. E, F and B represent CK2, CK3 and CK1, respectively. E2, B2 and F2 represent CVD2, CVD1 and CVD3, respectively.
Table III. GO biological processes of target genes of differentially expressed microRNAs.

| GO term                | Biological process          | Expected | +/- | Fold enrichment | P-value |
|------------------------|----------------------------|----------|-----|-----------------|---------|
| GO:0016049             | Cell growth                | 0.3      | +   | 1.70E+01        | 3.45E-03|
| GO:0007254             | JNK cascade                | 1.36     | +   | 1.40E+01        | 1.41E-13|
| GO:0016079             | Synaptic vesicle exocytosis| 2.04     | +   | 7.34E+00        | 1.04E-06|
| GO:0007269             | Neurotransmitter secretion | 2.97     | +   | 7.06E+00        | 1.73E-09|
| GO:0007249             | I-kb kinase/NF-kb cascade  | 1.63     | +   | 6.12E+00        | 1.93E-03|
| GO:0040011             | Locomotion                 | 3.54     | +   | 5.65E+00        | 2.51E-07|
| GO:0051726             | Regulation of cell cycle   | 2.47     | +   | 4.85E+00        | 2.51E-03|
| GO:0000165             | MAPK cascade               | 7.17     | +   | 4.46E+00        | 1.13E-09|
| GO:0007268             | Synaptic transmission      | 7.35     | +   | 4.22E+00        | 9.82E-09|
| GO:0050790             | Regulation of catalytic activity | 4.83 | +   | 3.93E+00        | 1.70E-04|
| GO:0019220             | Regulation of phosphate metabolic process | 8.6 | + | 3.49E+00 | 1.55E-06 |
| GO:0008104             | Protein localization       | 5.47     | +   | 3.47E+00        | 9.96E-04|
| GO:0065009             | Regulation of molecular function | 5.92 | +   | 3.38E+00        | 8.48E-04|
| GO:0006915             | Apoptotic process          | 9.42     | +   | 3.29E+00        | 3.21E-06|
| GO:0009605             | Response to external stimulus | 7.06 | +   | 3.26E+00        | 2.88E-04|
| GO:0006928             | Cellular component movement| 9.37     | +   | 3.20E+00        | 1.01E-05|
| GO:0016265             | Death                      | 9.71     | +   | 3.19E+00        | 6.38E-06|
| GO:0008219             | Cell death                 | 9.71     | +   | 3.19E+00        | 6.38E-06|
| GO:0032989             | Cellular component morphogenesis | 12.37 | +     | 3.15E+00        | 1.35E-07|
| GO:0007267             | Cell-cell signalling        | 10.89    | +   | 3.12E+00        | 2.28E-06|
| GO:0006796             | Phosphate-containing compound metabolic process | 28.28 | + | 3.01E+00 | 3.84E-17 |
| GO:0035556             | Intracellular signal transduction | 22.49 | + | 2.93E+00 | 2.31E-12 |
| GO:0009056             | Catabolic process          | 17.91    | +   | 2.85E+00        | 9.49E-09|
| GO:0016337             | Cell-cell adhesion         | 6.92     | +   | 2.74E+00        | 2.34E-02|
| GO:0030154             | Cell differentiation       | 10.03    | +   | 2.59E+00        | 3.44E-03|
| GO:0016043             | Cellular component organization | 35.95 | +   | 2.59E+00        | 8.48E-15|
| GO:0007154             | Cell communication         | 60.69    | +   | 2.52E+00        | 9.01E-26|
| GO:0006950             | Response to stress         | 19.52    | +   | 2.51E+00        | 1.41E-06|
| GO:0071840             | Cellular component organization or biogenesis | 39.13 | + | 2.45E+00 | 6.68E-14 |
| GO:0006897             | Endocytosis                | 9.49     | +   | 2.42E+00        | 2.83E-02|
| GO:0044085             | Cellular component biogenesis | 11.19 | +   | 2.41E+00        | 8.02E-03|
| GO:0007399             | Nervous system development | 15.16    | +   | 2.37E+00        | 5.67E-04|
| GO:0006996             | Organelle organization     | 17.07    | +   | 2.34E+00        | 2.12E-04|
| GO:0007165             | Signal transduction        | 54.25    | +   | 2.12E+00        | 1.23E-12|
| GO:0007166             | Cell surface receptor signalling pathway | 29.48 | + | 2.07E+00 | 1.99E-05 |
| GO:0044707             | Single-multicellular organism process | 41.76 | + | 2.04E+00 | 7.64E-08 |
| GO:0051179             | Localization               | 48.66    | +   | 2.03E+00        | 1.82E-09|
| GO:0032501             | Multicellular organismal process | 42.13 | + | 2.02E+00 | 1.16E-07 |
| GO:0050877             | Neurological system process | 25.99 | + | 1.96E+00 | 1.11E-03 |
| GO:0050896             | Response to stimulus       | 57.88    | +   | 1.95E+00        | 5.21E-10|
| GO:0032502             | Developmental process      | 43.99    | +   | 1.93E+00        | 8.83E-07|
| GO:0048731             | System development         | 24.17    | +   | 1.90E+00        | 7.07E-03|
| GO:0015031             | Protein transport          | 23.79    | +   | 1.89E+00        | 9.93E-03|
| GO:0003008             | System process             | 31       | +   | 1.87E+00        | 1.00E-03|
| GO:0006810             | Transport                  | 44.21    | +   | 1.85E+00        | 1.06E-05|
| GO:0065007             | Biological regulation      | 51.93    | +   | 1.85E+00        | 6.46E-07|
| GO:0006886             | Intracellular protein transport | 23.26 | + | 1.81E+00 | 4.87E-02 |
| GO:0050789             | Regulation of biological process | 43.08 | + | 1.72E+00 | 9.06E-04 |
| GO:0009987             | Cellular process           | 186.09   | +   | 1.57E+00        | 2.38E-20|

GO, gene ontology; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; I-kb kinase, inhibitor of xk kinase; NF-kb, nuclear factor-xk.
The target genes of downregulated miRNAs were enriched in 12 KEGG pathways. Most of these pathways are responsible for lipid and glycan metabolism (Table IV). In particular, three downregulated miRNAs, hsa-miR-1268b, hsa-miR-1273d,
hsa-miR-3187-5p, were associated with a-linolenic acid metabolism. hsa-miR-4492 was predicted to target phospholipase A2 group (ENSG00000100078; ENSG00000158786; ENSG00000168907; ENSG00000184381; ENSG00000187980) and fatty acid desaturase 2 (ENSG00000134824) which are key enzymes in a-linolenic acid metabolism. The target gene of hsa-miR-3187 was predicted to be phospholipase A2 group IVE gene (ENSG00000188089), and hsa-miR-1273d was predicted to target acyl-CoA oxidase 1 (ENSG00000161533).

Additionally, the target genes of upregulated miRNAs were enriched in 15 KEGG pathways, mainly in the ‘human diseases’ class (Table V). These KEGG pathways did not match the CVDs directly, but were mainly involved in ‘neurodegenerative diseases and cancers’ class.

**Novel miRNA annotations.** In the present study, six novel miRNAs were upregulated, including novel-m0499-5p, novel-m0970-5p, novel-m1042-5p, novel-m1061-5p and novel-m1953-5p, and novel-m1627-5p was downregulated. Target gene prediction of novel miRNAs, m0499-5p, m0970-5p, m1042-5p and m1953-5p was unsuccessful, which indicated that the functions of these miRNAs remain unidentified. Novel-m1627-5p was predicted to target 146 human genes; however, further investigations into these target genes are required.

With the analysis using miRanda 3.3a (http://www.microrna.org/microrna/3.3a), novel-m1061-5p was predicted to target four genes, including ENSG00000115042 [fumaryl-lactoacetate hydrolase domain containing 2A (FAHD2A)], ENSG0000016396 [potassium voltage-gated channel subfamily C member 4 (KCNAC4)], ENSG00000205476 [coiled-coil domain containing 85C (CCDC85C)] and ENSG00000175782 (SLC35E3; GO term, GO:01901264 ‘carbohydrate derivative transport’; GO class, ‘biological process’), and these genes were observed on the website: http://asia.ensembl.org/index.html. FAHD2A, KCNC4 and CCDC85C were not enriched in the KEGG pathways or GO terms of differential genes. Differential ENSG00000175782 (SLC35E3) had the GO term of carbohydrate derivative transport involving in biological process. SLC35E3 is a member of the nucleoside sugar transporter subfamily E (28,29). The nucleoside sugar transporters are localized at the Golgi complex and the endoplasmic reticulum (ER). SLC35E3 transports cytosolic nucleotide sugars into the lumen of Golgi complex and ER, where nucleotide sugars are substrates for the glycosylation of proteins, lipids and proteoglycans (28). Deficiency in nucleotide sugar transporters has been associated with tumour metastasis, cellular immunity, organogenesis and morphogenesis (29). For instance, congenital disorder of glycosylation type IIc (also known as leukocyte adhesion deficiency-2) is caused by deficiency in nucleotide sugar transporters. The nucleoside sugar transporters are involved in the transport of nucleotide sugars into the lumen of Golgi complex and ER, SLC35E3 transports cytosolic nucleotide sugars into the lumen of Golgi complex and ER, which nucleotide sugars are substrates for the glycosylation of proteins, lipids and proteoglycans (28).

RT-qPCR analysis was performed to confirm the expression levels of novel-m1061-5p and SLC35E3. Expression levels of novel-m1061-5p within the three patients with CVD were significantly increased to the 3.71207-fold, 3.26909-fold and 2.40420-fold greater than in the CK group, respectively. Expression levels of SLC35E3 were significantly decreased by the 0.33-fold, 0.28-fold, 0.41-fold within patients with CVD, respectively, compared with in the CK group. The results of the present study revealed that upregulation of novel-m1061-5p expression levels was associated with the repression of SLC35E3 expression levels within the 3 patients with CVD.

The sequencing data of the present study revealed the miRNA profiles and associated target genes in patients with CVD; however, more patients for large-scale data collection and further investigation to confirm gene function are required. Molecular detection may contribute to the prognosis and treatment of CVDs.

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