SLC35E3 identified as a target of novel-m1061-5p via microRNA profiling of patients with cardiovascular disease

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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.

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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.org/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 (https://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://mireap.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 was employed to collate the differentially expressed miRNA. The average abundance of miRNA expression within the CVD and CK groups were calculated. Log_2 (CVD/CK) values were calculated to present the fold change. The differential expression analysis was adjusted with a q-value adjusted P-value. Fold change [log_2 (CVD/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≤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 manufacture'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 with 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 ddH_2O. Primers were designed using DNAMAN version 6.0 software (Lynnon Biosoft, San Ramon, CA, USA)**.** The primer sequences were: hsa-novel-m1061-5p, 5'-TCAGTTGTTCCATGTCCTGC AG-3' and solute carrier family 35 member E3 (SLC35E3), forward 5'-ACGACAGGT GATCCACCTGC-3', reverse: 5'-TATGAACCAACAAAT ACACC-3'. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The following thermocycling conditions were used for RT-qPCR: 95˚C 5 min for pre-denature; 95˚C 15 sec, 60˚C 45 sec, 72˚C 15 sec, 40 cycles for amplification; default dissociation condition. U6 served as the internal reference. The primer sequences of U6 were forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCT TCACGAATTTGCGT-3'. The cycle threshold values were obtained; the relative interest/reference expression quantity of genes was calculated with the formula $2^{-\Delta Cq(miR\text{-}U6)}$. Relative test/normal CK quantity was calculated with the formula $2^{-\Delta\Delta Cq[(test\ miR\text{-}U6)-(CK\ miR\text{-}U6)]}$ (10-12).

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

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

	CK			CVD			
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.

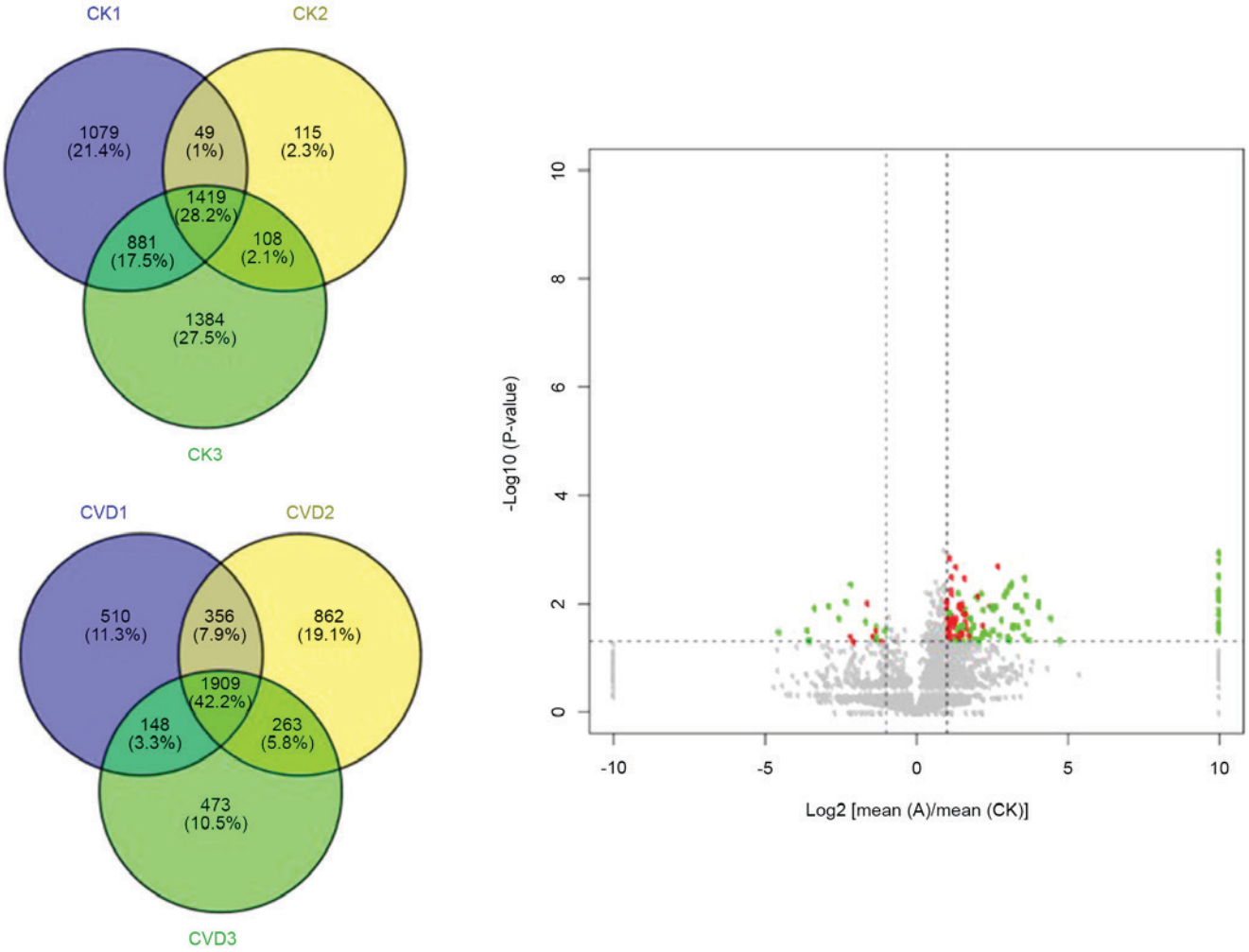

Figure 1. microRNA distribution in each sample. CK, control; CVD, cardiovascular disease. Red colour indicated upregulated genes and green indicated downregulated genes.

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, 2,528, and 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 miRNAs 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%

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

A, Upregulated

miR name	log_2(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_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_R1-23L23	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-548l_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_19T-C	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
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
rno-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

B, Downregulated

miR name	log_2(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-m1627-5p	-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.

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

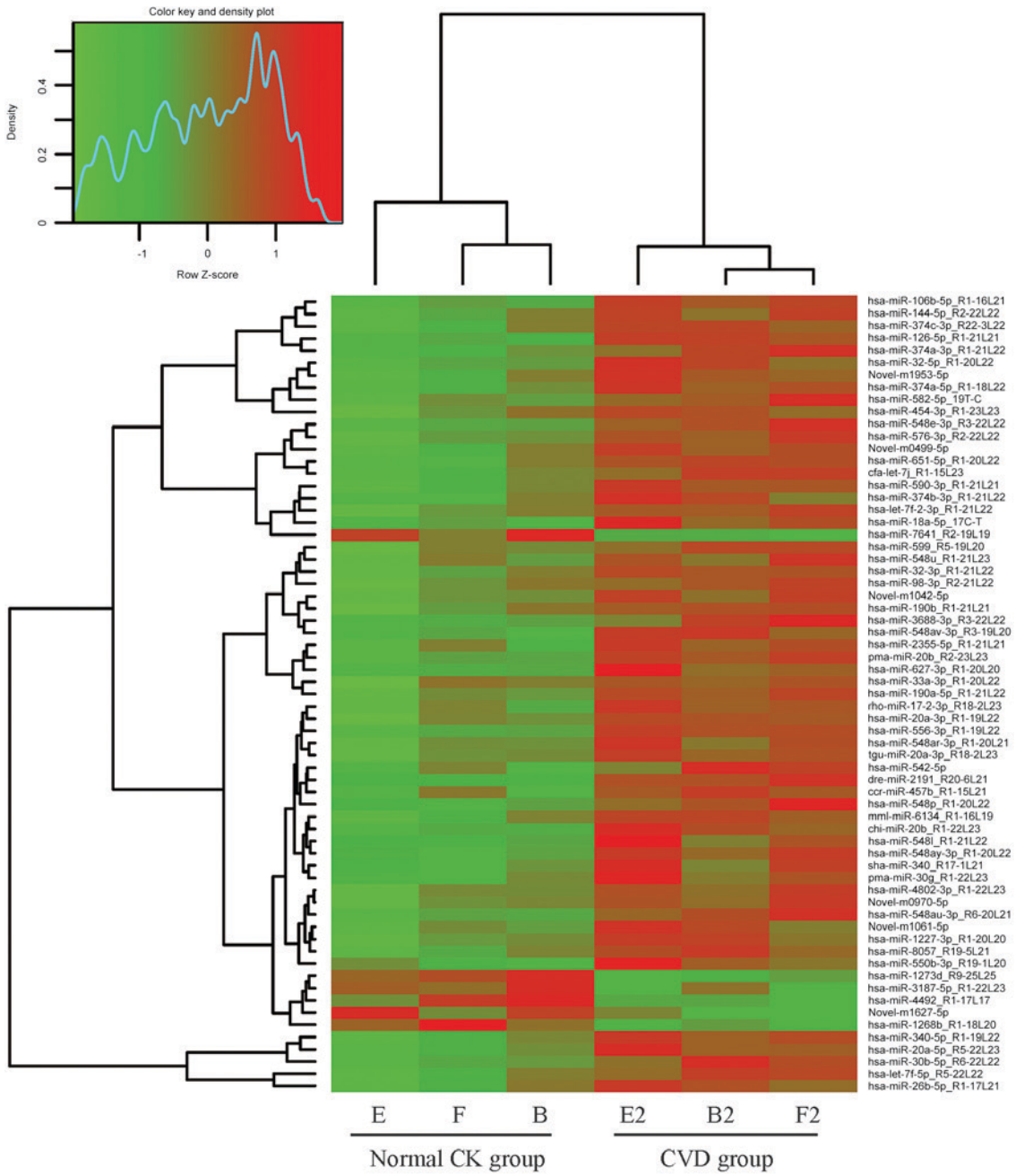

Figure 2. Clustering heatmap of differentially expressed microRNAs. CVD, cardiovascular disease; CK, control. Red colour indicated upregulated and green indicated downregulated. E, F and B represent CK2, CK3 and CK1, respectively. E2, B2 and F2 represent CVD2, CVD1 and CVD3, respectively.

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 downreguation 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'.

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-κb kinase/NF-κb 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; Iκb kinase, inhibitor of κb kinase; NF-κb, nuclear factor-κb.

Table IV. KEGG pathway enrichment of the target genes of downregulated microRNAs.

KEGG pathway	Class name	Category	P-value	q-value
α-linolenic acid metabolism	Metabolism; lipid metabolism	ko00592	2.20E-03	1.83E-01
Ras signalling pathway	Environmental information processing; Signal transduction	ko04014	2.20E-03	1.83E-01
Circadian entrainment	Organismal systems; environmental adaptation	ko04713	2.42E-03	1.83E-01
Axon guidance	Organismal systems; development	ko04360	2.59E-03	1.83E-01
Glycosphingolipid biosynthesis	Metabolism; glycan biosynthesis and metabolism	ko00603	2.64E-03	1.83E-01
Other glycan degradation	Metabolism; glycan biosynthesis and metabolism	ko00511	3.56E-03	2.03E-01
Glycosphingolipid biosynthesis-ganglion	Metabolism; glycan biosynthesis and metabolism	ko00604	4.10E-03	2.03E-01
Glycerophospholipid metabolism	Metabolism; lipid metabolism	ko00564	5.10E-03	2.20E-01
Focal adhesion	Cellular processes; cellular community	ko04510	7.50E-03	2.62E-01
Regulation of actin cytoskeleton	Cellular processes; cell motility	ko04810	8.01E-03	2.62E-01
MAPK signalling pathway	Environmental information processing; signal transduction	ko04010	8.34E-03	2.62E-01
Endocytosis	Cellular processes; transport and catabolism	ko04144	9.25E-03	2.67E-01

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

Table V. KEGG pathway enrichment of the target genes of upregulated microRNAs.

KEGG pathway	Class name	Category	P-value	q-value
Genetic information Processing; folding, sorting and degradation	Genetic information processing; folding, sorting and degradation	ko04141	4.10E-03	6.55E-01
Human diseases; cancers	Human diseases; cancers	ko05219	6.12E-03	6.55E-01
Human diseases; Neurodegenerative diseases	Human diseases; Neurodegenerative diseases	ko05016	7.58E-03	6.55E-01
Human diseases; Neurodegenerative diseases	Human diseases; Neurodegenerative diseases	ko05010	1.03E-02	6.55E-01
Metabolism; amino acid metabolism	Metabolism; amino acid metabolism	ko00280	1.04E-02	6.55E-01
Organismal systems; development	Organismal systems; development	ko04320	1.14E-02	6.55E-01
Metabolism; carbohydrate metabolism	Metabolism; Carbohydrate metabolism	ko00053	1.53E-02	7.54E-01
Organismal systems; environmental adaptation	Organismal systems; environmental adaptation	ko04713	2.07E-02	8.24E-01
Human diseases; substance dependence	Human diseases; substance dependence	ko05032	2.14E-02	8.24E-01
Human diseases; neurodegenerative diseases	Human diseases; neurodegenerative diseases	ko05012	2.75E-02	9.50E-01
Metabolism; carbohydrate metabolism	Metabolism; carbohydrate metabolism	ko00040	3.51E-02	1.00E+00
Human diseases; cancers	Human diseases; cancers	ko05206	3.89E-02	1.00E+00
Organismal systems; nervous system	Organismal systems; nervous system	ko04727	4.56E-02	1.00E+00
Cellular processes; Transport and catabolism	Cellular processes; transport and catabolism	ko04144	4.62E-02	1.00E+00
Cellular processes; cell growth and death	Cellular processes; cell growth and death	ko04112	4.81E-02	1.00E+00

KEGG, Kyoto Encyclopedia of Genes and Genomes.

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 [fumarylacetoacetate hydrolase domain containing 2A (FAHD2A)], ENSG00000116396 [potassium voltage-gated channel subfamily C member 4 (KCNC4)], ENSG00000205476 [coiled-coil domain containing 85C (CCDC85C)] and ENSG00000175782 (SLC35E3; GO term, GO:1901264 '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 termed leukocyte adhesion deficiency-2) is caused by defective guanosine 5'-diphosphate transport (29). In the present study, upregulation of novel-m1061-5p in patients with CVD may reduce SLC35E3 expression levels, resulting in defects in glycol-conjugation. In addition, novel-m1061-5p may serve a marker or potential target in the prognosis or treatment of CVD; however, the underlying mechanism of this miRNA requires further investigation.

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 be 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 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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