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ORIGINAL ARTICLE

Analysis of Differentially Expressed Novel MicroRNAs as Potential Biomarkers in Dengue Virus Type 1 Infection

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SUMMARY

Background: **Host-derived miRNAs are reported to play diverse roles in dengue virus (DENV) infection, but DENV-derived miRNAs have been rarely studied.**
Methods: **Here, we used serum samples from three patients infected with dengue virus type 1 (DENV1) and three healthy volunteers to detect and profile novel microRNAs in dengue virus-infected human serum. MicroRNAs in serum samples were sequenced using an Illumina HiSeq 2000 system, and clean reads were trimmed, aligned, normalized, and analyzed to identify differentially expressed and novel microRNAs. Four microRNAs were selected as DENV1 infection biomarkers and verified through 1:2 paired case-control study, using serum from 15 DENV1-infected patients and 30 healthy volunteers.**
Results: **We identified 182 potential novel miRNAs, with 20 novel miRNAs found to have miRDeep2 score ≥ 4.0. Fifty-eight known and 11 novel miRNAs were upregulated at least 2-fold in DENV1-infected serum. Twenty-two known and 4 novel miRNAs were downregulated at least 0.5-fold. The AUCs of four selected miRNAs, hsa-miR-106b-3p, hsa-miR-122-5p, hsa-miR-novel-chr17_35150, and hsa-miR-novel-chr10_24390, were respectively: 0.962 (95% CI: 0.913 - 1.000, p > 0.05), 0.924 (95% CI: 0.851 - 0.998, p < 0.05), 0.941 (95% CI:0.877 - 1.000, p > 0.05), and 0.991 (95% CI: 0.973 - 1.000, p > 0.05).**
Conclusions: **In summary, our study demonstrates that serum miRNA levels are affected by DENV1 infection, identifies novel DENV1-associated miRNAs, and suggests that Hsa-miR-122-5p may be a potential biomarker for DENV1 infection.**
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KEY WORDS

dengue virus 1, microRNAs, biomarkers, hsa-miR-122-5p

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LIST OF ABBREVIATIONS

ALT - alanine aminotransferase
AMPK - adenosine 5'-monophosphate (AMP)-activated protein kinase
AUC - area under the curve
DENV1 - dengue virus type 1
HCV - hepatitis C virus
KEGG - Kyoto Encyclopedia of Genes and Genomes
miRNAs - microRNAs
PAGE - polyacrylamide gel electrophoresis
ORF - open reading frame
UTRs - untranslated regions
PCR - polymerase chain reaction
qRT-PCR - real time quantitative PCR
ROC - receiver operating characteristic curve
WNV - West Nile virus

INTRODUCTION

Dengue virus (DENV) belongs to the Flaviviridae family. DENV is a single-stranded positive-sense RNA virus, which is designated into four serotypes (DENV 1-4) [1]. The DENV genome consists of approximately 11,000 nucleotides, encoding a single open reading frame (ORF) flanked by conserved 5′ and 3′ untranslated regions (UTRs) [1,2]. DENV is the most prevalent mosquito-borne virus and is a growing challenge to public health, causing an estimated 390 million infections per year. Although there are currently dengue vaccines in clinical trials, and even a dengue vaccine named CYD-TDV (Dengvaxia) has been first licensed by WHO, dengue vaccines rate for population is low in epidemic areas [3,4]. MicroRNAs (miRNAs) may be derived from host or viral RNAs and are involved in various biological processes [5,6]. Host-derived miRNAs are reported to play diverse roles in DENV infection, and may be involved in inhibiting or promoting DENV replication [7,8]. miRNA expression profiles from DENV2-infected human peripheral blood mononuclear cells has been analyzed by microarray to study the association between dysregulated miRNAs and cytokines [9]. miRNA profiles from persistently DENV2-infected C6/36 cells revealed several miRNAs that participate in maintaining equilibrium between viral replication and the antiviral response [10]. However, miRNAs derived from DENV have been minimally studied, and this issue has gradually come into greater focus in recent years. A microRNA-like small RNA from DENV2 identified through deep sequencing was found to auto-regulate its replication in mosquito C6/36 cell lines [11]. This suggests that novel miRNAs derived from DENV might play important roles in its infection process and be potential biomarkers for DENV detection. These studies show that it is feasible and advantageous to study novel miRNAs in the serum of DENV-infected patients through miRNA deep sequencing. Novel miRNAs were identified, verified, and studied for their potential as DENV infection biomarkers.

MATERIALS AND METHODS

General description of the subjects

Fifteen DENV1-infected patients and 30 uninfected volunteers were matched in a 1:2 ratio by patient age, gender, and BMI. Peripheral blood samples from DENV1-infected patients were drawn at the time of admission, or one or two days after onset of fever. NS1 antigen (OneStep Dengue NS1 (Serum) RapiDipTM InstaTest, Cortez Diagnostics Inc, USA) was detected in the peripheral blood of all DENV1-infected patients. All patients were primarily infected with DENV1, which was diagnosed by presence of anti-DENV IgM, IgG (Dengue IgG&IgM Combo (Serum/WholeBlood/Plasma) RapiDipTM InstaTest, Cortez Diagnostics Inc, USA) and DENV1 nuclear detection (Dengue Fever Virus Genotyping I/II/III/IV Real Time RT-PCR Kit, Liferiver, China). The ages of the patients ranged from 12 to 86 years. The clinical course of DENV1 infection varied widely among the included patients (Table 1).

Sample collection and total serum RNA extraction

Peripheral blood samples from consenting participants (Dengue fever patients) were collected from the onset of DENV1 infection, as identified through NS1 antigen detection, polymerase chain reaction (PCR), and serology. Using vacuum tubes (Becton Dickinson and Company, Melbourne, Australia), 5 mL venous blood was drawn from each participant and after clotting, was centrifuged at 1,000 x g for 10 minutes. About 2 mL supernatant (serum) was separated, avoiding contamination with miRNAs from the cellular layer. The serum was stored at -80°C until RNA extraction. Total RNA from each sample was extracted using the following protocol: 200 µL serum was added into 800 µL Trizol reagent followed by vigorous vortexing. Then, 160 µL chloroform was added into the mixture and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase (400 µL) was then transferred to a new tube with 1 µL glycogen, and 480 µL isopropyl alcohol was added. The solution was chilled at -20°C for more than 2 hours. Total RNA was pelleted by centrifugation at 12,000 x g for 20 minutes at 4°C, and was then washed twice with 1 mL 75% alcohol and dried at room temperature. The RNA pellet was dissolved in 40 µL RNase-free water and the purity and concentration of the total RNA sample was determined using a NanoDrop ND-1000.

miRNA sequencing

Serum from 3 DENV1-infected patients and 3 uninfected controls matched by age (± 2 years), gender (4 male and 2 female), and body mass index (BMI ± 0.5 kg/m^2) were collected. In order to identify a higher abundance of novel miRNAs, the serum of the 3 DENV1-infected patients and 3 uninfected controls were pooled into a

DENV1 group and control group, respectively. Total RNA from each group's pooled serum samples was sequentially ligated to 3' and 5' small RNA adapters. cDNA was then synthesized and amplified using Illumina's proprietary RT primers and amplification primers. Subsequently, ~125 - 145 bp PCR-amplified fragments were extracted and purified from a PAGE gel. Finally, the completed libraries were quantified using an Agilent 2100 Bioanalyzer. The samples were diluted to a final concentration of 8 pM and cluster generation was performed on an Illumina cBot using TruSeq Rapid SR cluster kit (GD-402-4001, Illumina) following manufacturer's instructions. Sequencing was performed on an Illumina HiSeq2000 using TruSeq Rapid SBS kits (#FC-402-4002, Illumina), according to the manufacturer's instructions.

Validation of novel miRNAs by reverse transcription and real-time quantitative PCR

Total RNA from serum of 15 DENV1-infected patients (Table 2) and 30 uninfected controls was extracted and reverse-transcribed into cDNA in a 20 µL reaction system, consisting of 2 µL dNTPs (2.5 mM each), 2 µL 10 x RT Buffer, 0.3 µL RT primers (1 µM), 150 ng total RNA, 0.2 µL MMLV reverse transcriptase (200 U/µL), and 0.3 µL (40 U/µL) RNase enzyme inhibitor (Invitrogen, AM2682). miRNA validation assay was performed through real time quantitative PCR (qRT-PCR). The RT reaction was performed using the following reaction conditions: 30 minutes at 16°C, 40 minutes at 42°C, 5 minutes at 85°C on a Gene Amp PCR System 9700 (Applied Biosystems), and resulting cDNA was stored -20°C. Quantitative PCR analysis of selected miRNAs was performed using a real time quantitative PCR kit (TAKARA, Japan). qPCR reactions were conducted using a ViiA 7 Real-time PCR System (Applied Biosystems) and the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Melting curve analysis was performed after qPCR. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Sequences of the qRT-PCR primers are indicated in Table 2.

Analysis of pathways regulated by the selected 4 miRNAs and 6 novel miRNAs

miRNA target prediction was performed based on the combination of two algorithms from both miRanda [12] and TargetScan [13]. Pathway analysis is a functional analysis, mapping genes that were predicted to be targeted by certain miRNAs to KEGG pathways. The p-value denotes the significance of the pathway correlated to the conditions. The lower the p-value, the more significant the pathway is. (The recommend p-value cutoff is 0.05.) The bar plot shows the top ten enrichment score (-log10 (Pvalue)) values of the significant enrichment pathways.

Statistical analysis

Comparison of the fold changes of selected miRNAs between DENV1 patients and control patients was analyzed using the non-parametric Mann-Whitney U test. ROC curves were plotted using SPSS 20.0.

RESULTS

Serum miRNA levels and novel miRNAs

Mature serum miRNAs were sequenced and analyzed with comparison to donated DENV1-free serum. One hundred and ninety-six known miRNAs previously annotated in miRBase and a total of 182 novel and presently unreported miRNAs were identified, of which 20 potential novel miRNAs were found to have a miRDeep2 score ≥ 4.0 (Table 3). Compared with DENV1-free serum, 58 known and 11 novel miRNAs were up-regulated at least 2-fold in serum from DENV1 infected patients. Twenty-two known and 4 novel miRNAs were downregulated at least 0.5-fold (data not shown). miRNAs that were up- or downregulated greater than 3-fold are shown clustered in Figure 1. miRNAs that were up- or downregulated most significantly (top 20) are listed in Table 4. It is clear that DENV1 infection causes significant differential expression of miRNAs in human serum.

Differential expression of selected miRNAs in dengue patients

Four miRNAs are shown in Table 4, including two known miRNAs, hsa-miR-106b-3p and hsa-miR-122-5p, and two novel miRNAs, hsa-miR-novel-chr17_35 150 and hsa-miR-novel-chr10_24390. The levels of these four miRNAs were detected in subjects' serum to validate the significant differences between 15 DENV1-infected patients and 30 uninfected volunteers. Hsa-miR-106b-3p, hsa-miR-122-5p, and hsa-miR-novel-chr17_35150 were significantly up-regulated in serum of DENV1 infected patients, while hsa-miR-novel-chr10_24390 was significantly downregulated ($p < 0.01$) (Figure 2).

ROC curves of selected miRNAs

ROC curves were plotted to explore the diagnostic potential of selected miRNAs. The AUCs of the selected miRNAs hsa-miR-106b-3p, hsa-miR-122-5p, hsa-miR-novel-chr17_35150, and hsa-miR-novel-chr10_24390 were, respectively: 0.962 (95% CI: 0.913 - 1.000, $p > 0.05$), 0.924 (95% CI: 0.851 - 0.998, $p < 0.05$), 0.941 (95% CI: 0.877 - 1.000, $p > 0.05$), 0.991 (95%CI: 0.973 - 1.000, $p > 0.05$) (Figure 3). The AUC of hsa-miR-122-5p was statistically significant, suggesting that it may be a potential biomarker for DENV1 infection.

Table 1. Description of DENV1-infected patients

Case Number	Gender	Age (years)	BMI (kg/m^2)	Anti-DENV IgM/IgG	DENV1 infection	Nucleic acid detection [a]	NS1 antigen	Timing of blood draw relative to fever onset (days)	Disease duration (days)	Outcome
1	male	17	15.92	+/-	primary	+	+	2	8	recovered
2	male	12	14.59	+/-	primary	+	+	3	11	recovered
3	male	15	16.85	+/-	primary	+	+	2	10	recovered
4	male	24	26.77	+/-	primary	+	+	2	9	recovered
5	male	39	26.6	+/-	primary	+	+	1	7	recovered
6	female	23	23.53	+/-	primary	+	+	2	9	recovered
7	female	37	27.64	+/-	primary	+	+	2	7	recovered
8	male	46	25.98	+/-	primary	+	+	2	10	recovered
9	male	56	26.27	+/-	primary	+	+	1	11	recovered
10	female	49	24.81	+/-	primary	+	+	2	9	recovered
11	female	54	27.56	+/-	primary	+	+	3	13	recovered
12	male	86	20.27	+/-	primary	+	+	2	17	recovered
13	male	70	23.17	+/-	primary	+	+	1	16	recovered
14	female	82	22.19	+/-	primary	+	+	1	14	recovered
15	female	76	21.47	+/-	primary	+	+	1	21	recovered

[a] - Nucleic acid detection: acute infection by viral load by PCR and subtype identification by sequencing.

Table 2. Primer sequences used for reverse transcription (RT) reaction.

Mature miRNA	RT primer 5'-3' and reference miRNA sequence
hsa-miR-106b-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGC AGCA
hsa-miR-122-5p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCA AACA
hsa-miR-novel-chr17_35150	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGA ACCC
hsa-miR-novel-chr10_24390	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCT CCCT
Reference: hsa-miR-93-5p (MIMAT0000093)	CAAAGUGCUGUUCGUGCAGGUAG

Analysis of pathways regulated by the selected 4 miRNAs and 6 novel miRNAs

Pathway analysis was performed to predict the function of the four miRNAs which were validated in the serum of DENV1-infected patients. Hsa-miR-106b-3p and hsa-miR-122-5p are known miRNAs that are reported to be involved in several pathways, such as carbon metabolism, citrate cycle, AMPK and Wnt signaling pathways, and others (Figure 4A and B). Novel miRNA hsa-miR-chr17_35150 is predicted to regulate genes that are involved in mRNA surveillance, axon guidance, and T-cell receptor signaling, among others. Hsa-miR-chr10_24390 may be involved in the regulation of genes that are important for fluid shear stress and atherosclerosis, cell cycle, and Ras and Wnt signaling pathways (Figure 4C and D). It seems that the four selected miRNAs might play vital roles in the process of DENV1 infection. Although the symptoms of DENV1 infection could be as mild as fever and muscular pain, it had affected host cells' physiological functions extensively.

Table 3. T novel miRNAs with miRDeep2 score ≥ 4.0 and 2 novel miRNAs having coincident sequences with DENV-vsRNA-5.

Provisional id	Precursor coordinate	miR-Deep2 score	Mature read count	Consensus mature sequence	Coincident sequence with DENV-vsRNA-5
chr3_7160	chr3:20677482..20677531:+	131.7	247	CAGCUCACUGCAACCUCUGCCUCC	
chr11_25962	chr11:18546523..18546566:-	29.5	55	AGAAUCGCUUGAACCUGGGA	
chr10_24210	chr10:79491631..79491701:-	23.2	53	CUGCUGUUCUCACUUGAGAUGCUUC	
chrX_41406	chrX:41205038..41205109:-	9.7	15	AGGAGUUCUGGGCUGUAGUG	
chr7_18268	chr7:106916854..106916923:-	6.5	21	AGGCUGGUCUCGAACUCCU	
chr19_36576	chr19:14041128..14041182:+	5.5	33	UUGGGGGAUCACUGUGGUCGUAG	
chrX_40884	chrX:121474660..121474732:+	5.4	17	CAGGCUGGUGUUGAGUGACUG	
chr9_22556	chr9:138119393..138119473:-	5.4	12	GCUGUGUCUCUGGCUGACUCUCAG	
chr7_17790	chr7:41869448..41869488:-	5.3	59	GGCUGGAGAGCAUGUGUGAGC	
chr3_8344	chr3:13673755..13673798:-	5.3	42	AGGGGCCGAGGGAGCGAGA	
chr18_36054	chr18:19408975..19409036:-	4.9	164	UGGAAUGUAAAGAAGUAUGUAU	
chr6_14231	chr6:31453939..31454019:+	4.9	12	CAUGGAUGCCAUAUCUAGAAAACAC	
chr3_7665	chr3:108481396..108481456:+	4.8	30	GAGCAGAGGGCUUCAGCAUAGAAUG	
chrY_42187	chrY:4887192..4887232:+	4.7	9169	AUGGAUUUUUGGAGCAGGGAG	
chrX_40717	chrX:91053167..91053207:+	4.7	9169	AUGGAUUUUUGGAGCAGGGAG	
chr6_15556	chr6:51712825..51712879:-	4.6	45	GGCAGGGUGGUGUGGUGGAGUAGG	
chr17_34207	chr17:35813542..35813586:+	4.5	23	CAGGGACUACUUCUGGGAA	
chr16_33188	chr16:652347..652411:-	4.4	11	CGGGAGGAUCACUAGAACCC	
chr2_6521	chr2:162137885..162137943:-	4.1	66	GUGUGGUGUGAGGUAUGUGC	
chr19_36593	chr19:16198771..16198838:+	4	9	UGGGCCUUUCUGUCUCUGCAGG	
chr8_18834	chr8:22493164..22493236:+	1.9	5	ACUCGGAUUGGCGUUACC	UAAGUCAGCUGUCCAGAGCUG (consensus star sequence)
chr8_18995	chr8:43706794..43706841:+	0.6	80	GAUUGGAAACACUUUUUG	GAGAGUUAAGCCAUUCUGUGGAUUAAGCAGAUUGGAAACACUUUUUG (consensus precursor sequence)

Provisional id - novel miRNA ID.
Precursor coordinate - novel miRNA's precursor Genome Locus (HG19).
miRDeep2 score - miRDeep2's novel miRNA score, the higher the score, the more reliably the sequence represents identification of a novel miRNA
Mature read count - read count of novel miRNA mature form consensus mature sequence: novel miRNA mature sequence DENV-vsRNA-5 sequence [11]: NANAAGUCAGGNCGGAUUAAGCC.
Consensus star sequence - novel miRNA star sequence.
Consensus precursor sequence - novel miRNA precursor sequence.

DISCUSSION

Flaviviruses, including dengue viruses (DENV), Zika virus, West Nile virus (WNV), and yellow fever virus, are single-stranded positive-sense RNA viruses that are transmitted by mosquitoes or ticks [14]. DENV infection is a worldwide threat. In this study, we aimed to study the microRNA profile and detect novel microRNAs in dengue virus-infected human serum, in order to establish effective biomarkers for the diagnosis of DENV infection.

MicroRNA profiling from human samples including serum [15] and peripheral blood mononuclear cells (PBMCs) [9] has been reported. In DENV-infected PBMCs, miR-4290, miR-1290, miR-33a, and miRlet-7e were found to be upregulated, while miR-106b, miR-20a, and miR-30b were downregulated [9]. In the current study, miRNAs in serum from DENV1-infected pa-

Table 4. Top 20 up- and downregulated miRNAs in DENV1-infected human serum.

Up-regulated miRNAs		Tag Count (abundance)		Fold change
miRNA name	Mature sequence	Dengue virus infected	Uninfected control	Dengue virus infected vs. uninfected control
hsa-miR-novel-chr11_26268(3p)	AUGUUGGUGAAGGAAGAAGUGGGG	190	9	10.5
hsa-miR-novel-chr17_35150(3p)	GAAAGGGAGUCGGGUUC	91	1	9.2
hsa-miR-206(3p)	UGGAAUGUAAGGAAGUGUGUGG	64	0	7.4
hsa-miR-novel-chr6_15858(3p)	GGGGAGUAAGUGGUUCUGUCUCAC	61	0	7.1
hsa-miR-novel-chr19_37504(3p)	UGUGUGACUGUGAGUGUGUGUGC	60	0	7.0
hsa-miR-106b-3p	CCGCACUGUGGGUACUUGCUGC	57	0	6.7
hsa-miR-novel-chr10_24210(3p)	CUGCUGUUCUCACUUGAGAUGCUUC	55	0	6.5
hsa-miR-novel-chr11_25242(5p)	CUGAGUUUGCACAGUGGUC	55	0	6.5
hsa-miR-novel-chr3_7160(3p)	CAGCUCACUGCAACCUCUGCCUCC	55	0	6.5
hsa-let-7c(5p)	UGAGGUAGUAGGUUGUAUGGUU	283	43	5.5
hsa-mir-novel-chr19_37182(3p)	CUGUUUGGUGGGGAGUUGCUGG	45	0	5.5
hsa-mir-novel-chr1_1319(3p)	GUGGAUGGGGCAAGGGUUG	44	0	5.4
hsa-mir-novel-chr6_15035(3p)	CUGUUUGCUUGUCCUGUUAUUCCC	44	0	5.4
hsa-mir-novel-chr9_22058(3p)	CAGGAAGGCUGGCUGGUUUA	44	0	5.4
hsa-miR-novel-chr1_2304(3p)	CUCAGCUGAUCAUAGGCUUCCA	43	0	5.3
hsa-miR-novel-chr5_13589(5p)	UGGGAAAUGGUAGUGAAGGA	43	0	5.3
hsa-miR-novel-chr7_17790(3p)	GGCUGGAGAGCAUGUGUGAGC	64	4	5.3
hsa-mir-215(5p)	AUGACCUAUGAAUUGACAGAC	42	0	5.2
hsa-miR-novel-chr17_33895(5p)	CGCUGUGAGUGUAGUAUU	42	0	5.2
hsa-miR-novel-chr18_36009(5p)	UCUCUCUGUCGCCUUGGC	40	0	5.0
Downregulated miRNAs		**Tag Count (abundance)**		**Fold change**
miRNA name	Mature sequence	Dengue virus infected	Uninfected control	Dengue virus infected vs. uninfected control
hsa-miR-novel-chrX_40717(3p)	AUGGAUUUUUGGAGCAGGGAG	19	517	0.05
hsa-miR-novel-chrY_42187(3p)	AUGGAUUUUUGGAGCAGGGAG	19	517	0.05
hsa-miR-novel-chr5_12542(5p)	GACCCGCGGGCGCUCUCCAGUCCU	3	99	0.12
hsa-let-7d-3p	CUAUACGACCUGCUGCCUUUCU	43	337	0.15
hsa-miR-novel-chr10_24390(3p)	AAAUAGAUUUUUGGAGCAGGGAG	0	40	0.2
hsa-miR-1246(5p)	AAUGGAUUUUUGGAGCAGG	0	33	0.23
hsa-miR-4732-3p	GCCCUGACCUGUCCUGUUCUG	0	31	0.24
hsa-miR-486-3p	CGGGGCAGCUCAGUACAGGAU	0	29	0.26
hsa-miR-122a-5p	UCCCUGAGACCCUUUAACCUGUGA	8	53	0.28
hsa-miR-novel-chr8_19012(5p)	CCAGGCUGGAGUGCAGUGGUGC	0	23	0.30
hsa-miR-323a-3p	CACAUUACACGGUCGACCUCU	0	20	0.33
hsa-miR-144-5p	GGAUAUCAUCAUAUACUGUAAG	0	19	0.34
hsa-miR-151a-5p	UCGAGGAGCUCACAGUCUAGU	9	45	0.34
hsa-miR-654-5p	UGGUGGGCCGCAGAACAUGUGC	0	16	0.38
hsa-miR-433-3p	AUCAUGAUGGGCUCCUCGGUGU	0	15	0.4
hsa-miR-4446-3p	CAGGGCUGGCAGUGACAUGGGU	0	15	0.4
hsa-miR-1908-5p	CGGCGGGGACGGCGAUUGGUC	0	14	0.42
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU	0	12	0.45
hsa-miR-598-3p	UACGUCAUCGUUGUCAUCGUCA	0	12	0.45
hsa-miR-92a-1-5p	AGGUUGGGAUCGGUUGCAAUGCU	0	12	0.45
hsa-miR-novel-chr10_22939(3p)	CCUGGACUUUGGAAUGUCUACUU	0	12	0.45
hsa-miR-novel-chr11_25962(5p)	AGAAUCGCUUGAACCUGGGA	0	12	0.45
hsa-miR-novel-chr13_29506(3p)	CCUGGAGGUGACAGUGAGGCAUG	0	12	0.45

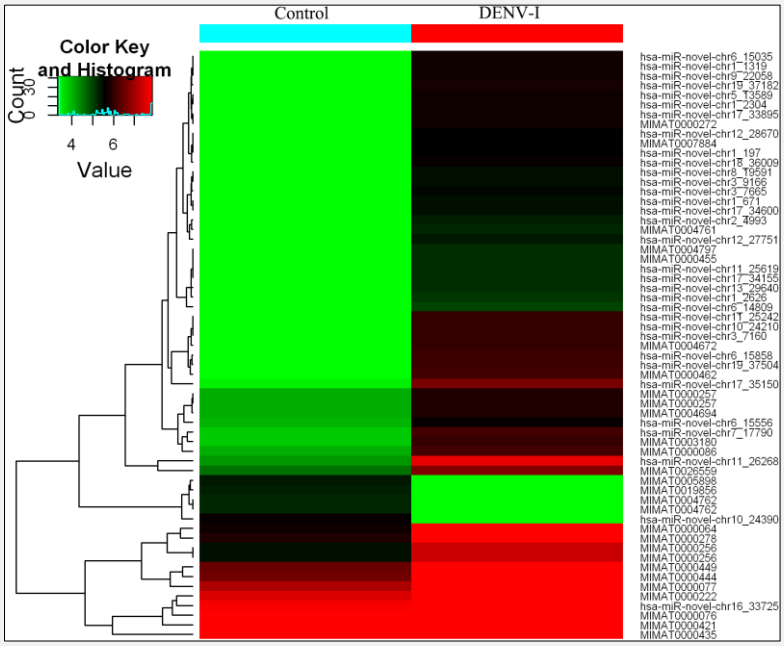

Figure 1. Cluster of miRNAs differentially expressed in the serum of DENV1-infected patients and healthy controls.

Sixty-one miRNAs were found to be differentially expressed more than 3-fold in DENV1-infected serum. Novel miRNAs: hsa-miR-novel-miRNAs, which have not been reported or registered in microRNA databases yet.

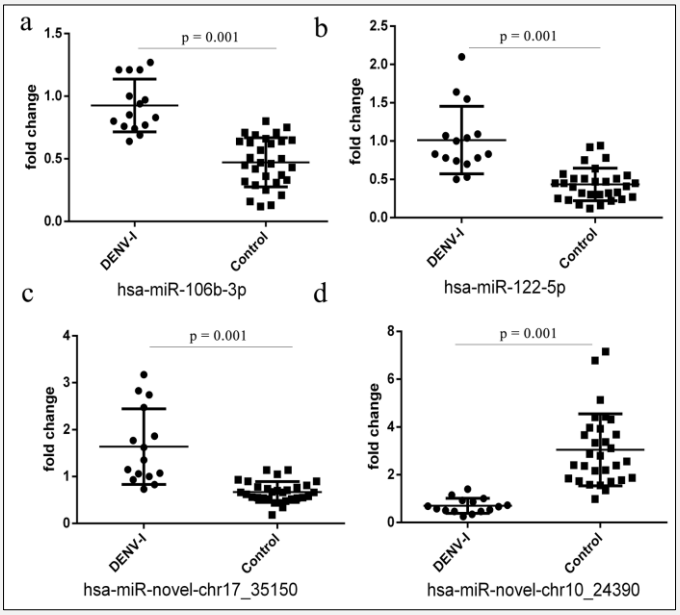

Figure 2. Differential expression of selected miRNAs in dengue patients.

*** p < 0.01, dengue-infected vs. control.**

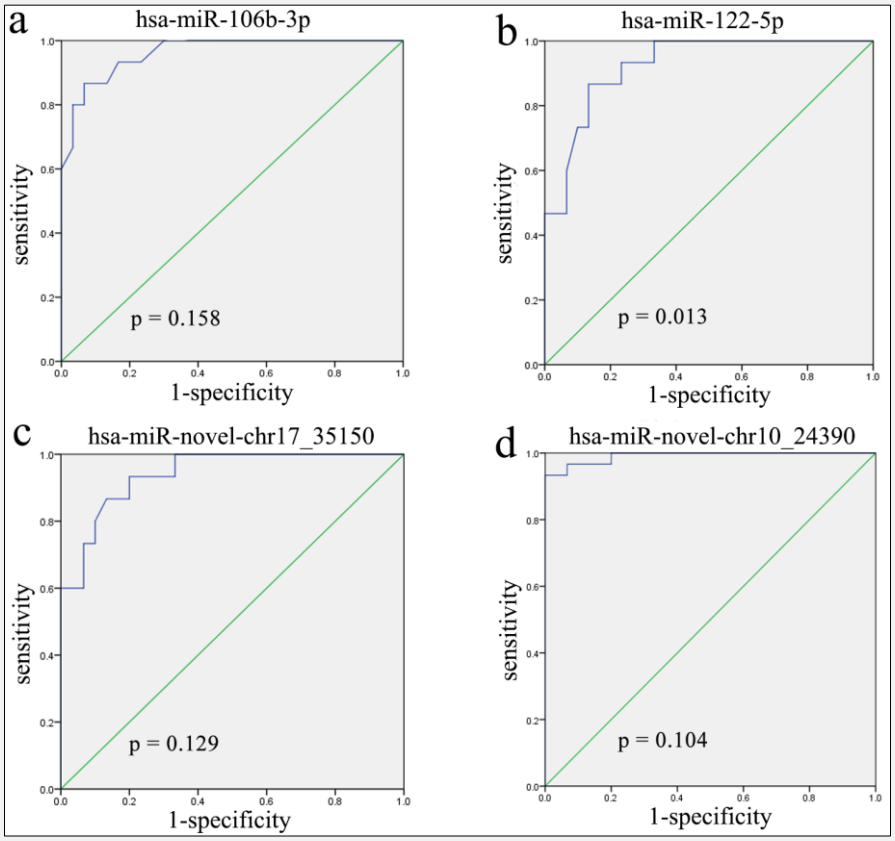

Figure 3. ROC curve of selected miRNAs.

A. ROC curve of hsa-miR-106b-3p; B. ROC curve of hsa-miR-122-5p; C. ROC curve of hsa-miR-novel-chr17_35150; D. ROC curve of hsa-miR-novel-chr10_24390.

tients were sequenced using an Illumina HiSeq 2000 system. We identified 20 novel miRNAs that to the best of our knowledge have never before been reported (Table 3). The top 20 up- and downregulated miRNAs are listed in Table 4. It is clear that there are more upregulated miRNAs than downregulated miRNAs. The magnitudes of fold changes of upregulated miRNAs are more substantial than that of downregulated.

We selected two known miRNAs, hsa-miR-106b-3p and hsa-miR-122-5p, and two novel miRNAs, hsa-miR-novel-chr17_35150 and hsa-miR-novel-chr10_24390, for validation. Hsa-miR-106b-3p fold changes are more concentrated compared with the other 3 selected miRNAs (Figure 2). The fold changes of hsa-miR-novel-chr10_24390 between DENV1 and control is 0.70 ± 0.30 vs. 3.05 ± 1.15 (average ± standard deviation), respectively, which is more significant than that of hsa-miR-122-5p (1.01 ± 0.42 vs. 0.43 ± 0.20), hsa-miR-novel-chr17_35150 (1.64 ± 0.77 vs. 0.67 ± 0.22), and hsa-miR-106b-3p (0.93 ± 0.21 vs. 0.47 ± 0.19). However, the AUC of hsa-miR-122-5p, 0.924 (95% CI: 0.851 - 0.998, $p < 0.05$) alone is statistically significant among the selected 4 miRNAs (Figure 3), which suggests that hsa-miR-122-5p might be able to distinguish DENV1-infected patients from controls. MicroRNA-like viral small RNA (DENV-vsRNA-5) from Dengue virus 2 was reported to negatively regulate its replication in C6/36 cells [11]. The consensus sequence of DENV-vsRNA-5 is NANAAGUCAGGNCGGANCG-GAUU AAGCC. Parts of this sequence, AAGUCAG and GGAUUAAGC, are found in the novel microRNAs chr8_18834 and chr8_18995 reported in the current study (Table 3). This suggests that viral small RNAs may exist in the serum of DENV1-infected patients with a relatively high abundance, such that they may be detectable. Although miRNAs in DNA virus-host interactions have been researched extensively, the roles of miRNAs in the pathogenesis of RNA virus infection is less understood. It was reported that hsa-miR-122 plays important roles in the hepatitis C virus (HCV) life cy-

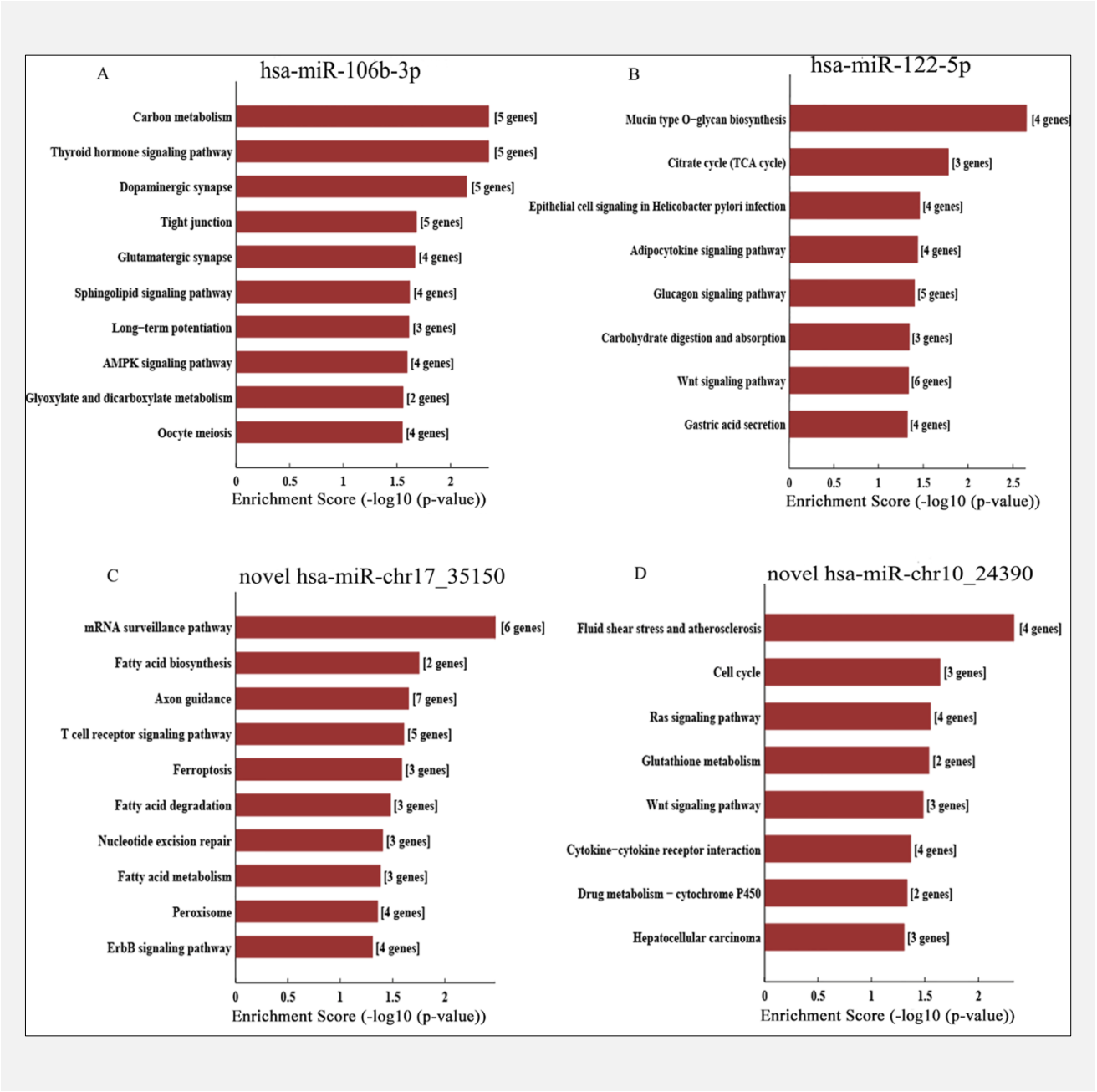

Figure 4. KEGG Signaling pathways enrichment of the selected 4 miRNAs.

The p-value denotes the significance of the pathway correlated to the DENV1 infection. The bar plots show the top ten enrichment score (-log10 (p-value)) values of the significantly enriched pathways.

cle, which presented an opportunity for miRNA-targeted treatment during HCV infection using a specific inhibitor of miR-122 [16]. In the current study, hsa-miR-122-5p was found to be upregulated in DENV1-infected patients. Both dengue virus and hepatitis C virus belong to the Flaviviridae family. Therefore, it is tempting to speculate that hsa-miR-122-5p may enhance its replication through manipulating the host RNA interference (RNAi) pathway. However, hsa-miR-122-5p levels were also found to be significantly upregulated in the serum of chronic hepatitis B patients with persistently normal alanine aminotransferase (ALT) levels [17]. It is reasonable to speculate that hsa-miR-122-5p expression was elevated during virus infection, which suggests the involvement of hsa-miR-122-5p in the innate cellular response to virus infection. In the current study, hsa-mi-

R-122-5p was found to be able to distinguish DENV1-infected patients from controls, but its diagnostic potential still needs to be validated. In fact, level changes of a group of miRNAs were thought to be more reliable as to be biomarkers of diseases or viral infection [18,19]. The host-pathogen interface during flavivirus infection remains vague, as flaviviruses depend highly on the host cell to complete their replication [20,21]. It has been speculated that novel miRNAs expressed during DENV infection may shed light on the mechanisms underlying the host-pathogen interface and the pathogenesis of dengue fever, and the existence of additional viral miRNAs expressed by DENV. We anticipate that the novel miRNAs reported in the current study may inspire further studies on the role of miRNAs in DENV infection, as well as more broadly in flavivirus infection.

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Declaration of Interest:
None declared.

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