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MicroRNAs profiles of Chinese Perch Brain (CPB) cells infected with *Siniperca chuatsi* rhabdovirus (SCRV)

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ABSTRACT

MicroRNAs are non-coding RNAs, which widely participate in biological processes. In recent years, *Siniperca chuatsi* rhabdovirus (SCRV) has caused mass mortality in Chinese perch (*Siniperca chuatsi*). To identify specific miRNAs involved in SCRV infection, deep sequencing of microRNA on Chinese perch brain cell line (CPB) with or without SCRV infection were performed at 6 and 12 h post of infection (hpi). Totally 382 miRNAs were identified, including 217 known miRNA aligned with zebrafish miRNAs and 165 novel miRNAs by MiRDeep2 program. Of which 15 and 35 differentially-expressed miRNAs were determined respectively to 6 and 12 hpi. Nine miRNAs were selected randomly from the differentially-expressed miRNAs and validated by quantitative real-time PCR (qRT-PCR). These results were consistent with the microRNA sequencing results. Besides, target genes of 98 differentially-expressed miRNAs were predicted. Three of miRNAs (miR-122, miR-214, miR-135a) were selected, and its effects were analyzed in CPC cells transfected with appropriate miRNA mimics/inhibitors to evaluate its regulation effects by qRT-PCR and western blot. The results demonstrated that miR-214 inhibited the replication of SCRV, while miR-122 promoted the replication of SCRV and there was no correlation between the miR-135a and SCRV replication. These results will pave a new way for the development of effective strategies against the SCRV infection.

1. Introduction

MicroRNA (miRNA) is a class of small noncoding RNA found in various organisms. It regulates gene expression by degrading and/or suppressing their protein-coding genes, mRNA degradation or both by binding complementary sequences in 3′ untranslated region [1–3]. MiRNA is initially transcribed as long pri-miRNA in the nucleus and processed by the RNase III enzyme DROSHA in the nucleus and Dicer in the cytoplasm to become mature miRNA [4]. Previous studies suggested that miRNA regulated virus replication in two main aspects. Firstly, miRNA could inhibit host's gene expression via binding to host's specific target genes; e.g., miRNA mir-214 inhibited snakehead vesiculovirus replication by promoting IFN-α expression by targeting the host gene of adenosine 5′-monophosphate-activated protein kinase in SSN-1 (striped snakehead) cells [5]. Secondly, some miRNAs could target viral genes and inhibit virus replication; e.g. microRNA-203 inhibits influenza A virus replication by targeting down-regulator of transcription 1 (DR1) in A549-Human lung epithelial cells [6]. MicroRNA miR-122 facilitates an efficient replication in non-hepatic cells upon infection with the hepatitis C virus by targeting virus genome [7]. Moreover, Singapore grouper iridovirus (SGIV) even encoded its miRNA (SGIV-miR-13) targeting its major capsid protein genes to attenuate viral infection [8]. The study of miRNA mediated host-virus interaction contributes to understand the mechanisms of virus infection and host counteraction better.

In the past decades, more than 20 rhabdoviruses have been identified in fish, including species from *Cypinifromes, Perciformes,* and *Pleuronectiformes* [9]. SCRV (*Siniperca chuatsi rhabdovirus*) is a member of the Perhabdovirus that belongs to Rhabdoviridae, which recently caused serious disease outbreaks in a wide variety of aquaculture

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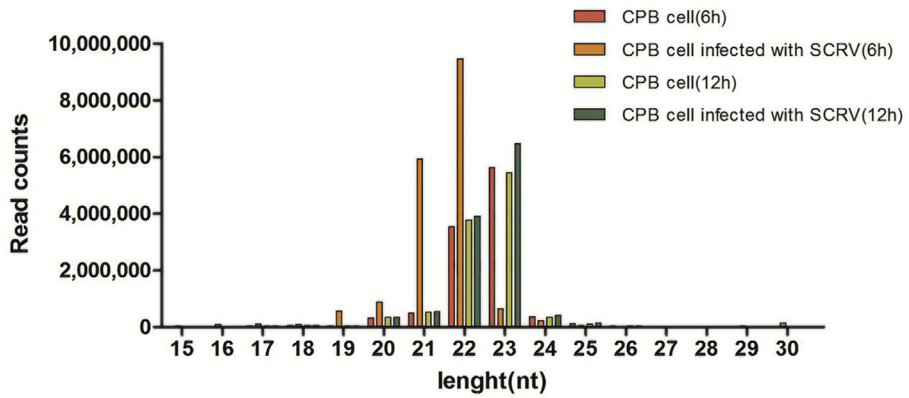

Fig. 1. Length distribution of small RNAs derived from CPB cells with or without SCRV infection at 6 and 12 hpi.

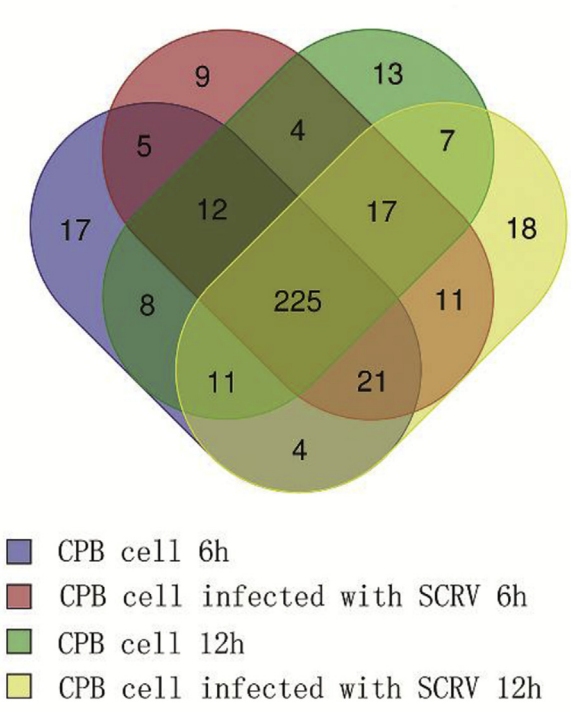

Fig. 2. Identification of miRNAs compared to zebrafish miRNAs. MiRNAs from four samples were compared to the known zebrafish miRNAs in miRBase. The Venn diagram shows the distribution of miRNAs of four samples. The overlapping section represents the number of co-expressed miRNAs.

species and threatens mandarin fish aquaculture [10–12]. It is a single-stranded, non-segmented negative-sense RNA virus with a bullet-shaped particle, 100–430 nm in length and 45–100 nm in diameter [13]. SCRV genome is approximately 11 kb in length that encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase protein (large protein, L) [14,15]. The diseased fish does not show any specific external signs except punctate hemorrhages on their body surface and fins, and the same signs were observed in the visceral organs, including gill, liver, and gut of Chinese perch (*Siniperca chuatsi*) [16].

Recent studies have demonstrated that the expression of host miR-3570 and miR-210 was upregulated consequent to SCRV infection in HEK293 cells and Miiuy croaker kidney cells (MKC), respectively [17,18]. However, there is scarce information about the miRNA profile during SCRV infection that modulates the host immune response or viral replication in the early and late stage of infection. In the present study, we explored the miRNA expression profiles of Chinese Perch Brain (CPB) cells upon SCRV infection using Illumina sequencing platform, and the roles of three specific miRNAs of interest were further investigated.

2. Materials and methods

2.1. Cell and virus

The CPB cells were previously isolated from the Chinese perch brain and stored in our lab [19]. The CPB cells were grown at 28 °C in L15 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS). SCRV-QY20140714 was isolated from a diseased Chinese perch in Qingyuan, Guangdong, China and stored in our lab [12].

2.2. SCRV infection and samples collection

About 80% confluence of CPB cells were incubated with SCRV at a multiplicity of infection (MOI) of 0.001, or with an equal volume of PBS as a negative control for 1 h at 28 °C, and then the inoculum was replaced with L15 medium with 5% FBS after washing twice with PBS. Mock-infected cells or SCRV-infected for 6 and 12 h post infection (hpi) were harvested by using cell scraper. The cells were washed with cold PBS and pelletized by centrifugation at 500 × *g* for 10 min, then stored in −80 °C with TRIzol reagent (Takara). Three parallel replicates at each time point were pooled as biological replicates.

2.3. RNA extraction, small RNA library construction, and sequencing

Total RNA containing small RNA was extracted from SCRV-infected or mock-infected CPB cells at 6 and 12 hpi using the mirVanaTM miRNA Isolation Kit (Ambion), following the manufacturer's protocol. The small RNA library construction and sequencing were performed by OE Biotech (China). The quality, quantity, and integrity of the total RNAs were evaluated using NanoDrop 2000 (Thermo Scientific, USA), Qubit 2.0 Fluorometer (Life Technologies, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The small RNA libraries were constructed using TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Subsequently, RNA-Seq libraries were sequenced on the Illumina sequencing platform (HiSeq™ 2500, USA). Raw Illumina paired-end reads were filtered using the internal software.

2.4. Sequence analysis and identification of miRNAs

Small RNA reads were generated by Illumina analysis (OE biotech,

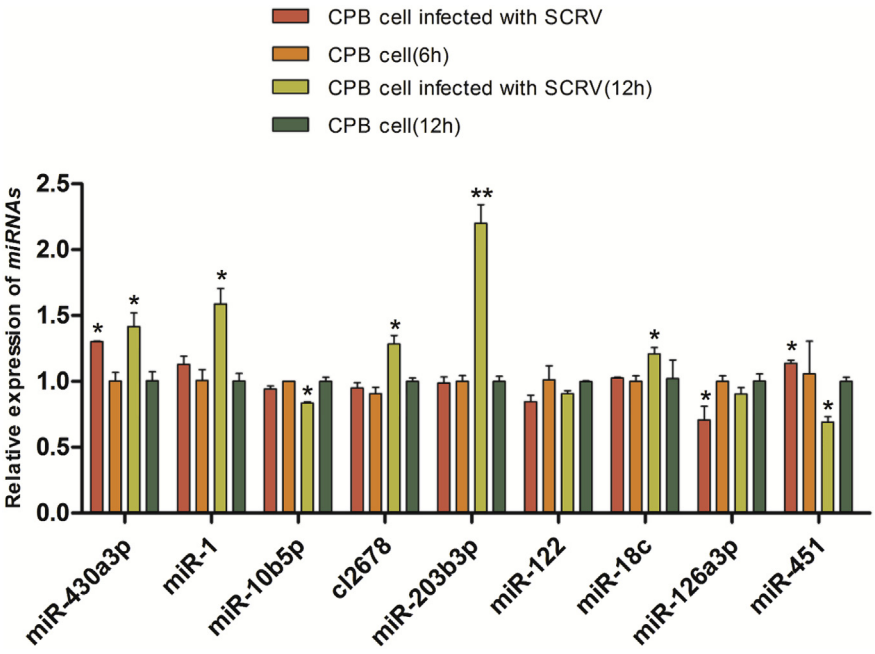

Fig. 3. Expression analysis of nine selected miRNAs. Nine differentially expression miRNAs those differently expressed at a higher level were selected randomly and quantify their expression by qRT-PCR. All expression levels were normalized to the arithmetic mean of the selected U6 gene. Significance was validated by T test, * and ** respectively indicate statistically-significant differences between infected and uninfected samples. All data are represented of at least three independent experiments (* $p < 0.05$; ** $p < 0.01$).

Shanghai, China) and basic reads were converted into sequence data by base calling. The forward and reverse reads with low-quality reads without 3' adapter and insert tag were removed. Clean reads with high quality and the specified length distribution (15–41 nt) in the reference genome was determined then used for the downstream analysis. Reference miRNA sequences were downloaded from the latest version of miRBase database (v.21, http://www.mirbase.org), and the known miRNA expression patterns in different samples were analyzed. Resulting sequences were subjected for secondary structure prediction using Rfam release 10.1 [20] against RFAM non-coding RNA (ncRNA) sequences to remove tRNAs and rRNAs, and the remaining reads were matched against miRNA precursor sequences in miRBase. The unique small RNA sequences were analyzed by localized NCBI BLASTP algorithm [21] program using miRDeep2 [22] statistical controls software package to predict novel miRNAs. Since the whole-genome sequence of Chinese perch is unknown, the remaining unannotated reads were mapped to the zebrafish (*Danio rerio*) genome using miRDeep2.

2.5. Analysis of differentially expressed miRNAs

Identification of differentially expressed miRNAs were carried out using the IDEG6 web tool [23]. The p-value was calculated and adjusted by using false discovery rate (FDR) [24]. FDR < 0.05 and $\log_2$ (fold change) ≥1 were set as the threshold for significant differential expression.

2.6. qRT-PCR validation of differentially expressed miRNAs

To further validate our deep sequencing data, nine randomly selected RNA samples used for miRNA sequencing were analyzed by quantitative real-time (qRT-PCR). The cDNA was synthesized from the miRNA using the Mir-X™ miRNA First-Strand Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. The PCR primer were designed based on mature miRNA sequences, optimized and validated before conducting the reactions. Quantitative PCR reactions were performed in the ABI Q6 (Applied Biosystems). The reaction mixture (20 μL) consisted of 2 μL of diluted cDNA, 300 nM of each primer, and 10 μL of the SYBR Master Mix (Vazyme Biotech, Nanjing, China) with the following cycling conditions: 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 60 °C for 20 s, and ended with 95 °C at 5 °C/s. All reactions were done in triplicates along with the U6 RNA gene as an internal control and normalized. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on the cycle threshold (CT) values were used to analyze expression levels [25].

2.7. Target prediction and functional annotation of differentially expressed miRNAs

The functions and possible roles of differentially expressed miRNAs and the candidate target genes were predicted by using the software miRanda v3.3a [26] with the following parameters: $S \geq 150$ $\Delta G \leq -30$ kcal/mol and demand strict 5′ seed pairing. The putative gene function was annotated by the succeeding pipelines: non-redundant nucleotide (nr/nt; NCBI) [27] database, Protein family (PFAM) database [28], KOG/COG database (Clusters of Orthologous Groups of proteins) [29], and Swiss-Prot (http://www.uniprot.org/). The highest sequence similarity and protein function annotation information about miRNA was obtained.

2.8. GO and KEGG pathway enrichment analysis

To investigate the potential metabolic pathways regulated by Chinese perch miRNAs, the predicted miRNA target genes were subjected to GO and KEGG analysis [30,31] D480–D484]. Both the enrichment analysis of differentially expressed miRNA-target-Gene were respectively performed using GOseq, R based on the hyper-geometric distribution [30].

2.9. Effects of differently expressed miRNAs on virus replication

To explore the effects of differently expressed miRNA, three specific miRNAs (miR-122, miR-214, and miR-135a) differently expressed at higher levels were randomly selected and analyzed with appropriate mimics and inhibitors on viral replication as previously described [32]. HPLC-purified miR-122, miR-214, and miR-135a mimics, inhibitors and seed mutants were generated and provided by Shanghai GenePharma Co., Ltd, China. Briefly, the CPB cells in 12-well plates were transfected with 100 nM mimics or 50 nM inhibitors of specific miRNAs, as well as mimic control or inhibitor control using Xfect RNA Transfection Reagent (Takara, Japan). After 24 h post-transfection (hpt), the cells were either collected for miRNA expression analysis or infected with 0.001 MOI of SCRV and incubated for another 24 h. Later, the cells were

Table 1
Summary of targeted virus gene prediction of the 98 miRNAs.

Name	SCRV target gene	Sequence	Length
dre-let-7a	P	TGAGGTAGTAGGTTGTATAGTT	22
dre-let-7b	P	TGAGGTAGTAGGTTGTGTGGTT	22
dre-let-7c-5p	P	TGAGGTAGTAGGTTGTATGGTT	22
dre-let-7d-5p	P	TGAGGTAGTTGGTTGTATGGTT	22
dre-let-7e	P	TGAGGTAGTAGATTGAATAGTT	22
dre-let-7f	P	TGAGGTAGTAGATTGTATAGTT	22
dre-let-7g	P	TGAGGTAGTAGTTTGTATAGTT	22
dre-let-7h	P	TGAGGTAGTAAGTTGTGTTGTT	22
dre-let-7i	P	TGAGGTAGTAGTTTGTGCTGTT	22
dre-let-7j	P	TGAGGTAGTTGTTTGTACAGTT	22
dre-miR-100-2-3p	N,M	CAAGCTCGTGTCTATAGGTATG	22
dre-miR-101a	L	TACAGTACTGTGATAACTGAAG	22
dre-miR-101b	L	TACAGTACTATGATAACTGAAG	22
dre-miR-103	L	AGCAGCATTGTACAGGGCTATGA	23
dre-miR-107a-3p	L	AGCAGCATTGTACAGGGCTATCA	23
dre-miR-107b	L	AGCAGCATTGTACAGGGCTTT	21
dre-miR-10b-3p	N,L	ACAGATTCGATTCTAGGGGAGT	22
dre-miR-10d-3p	L	CAGATTCGGTTTTAGGGGAGTA	22
dre-miR-122	M,L	TGGAGTGTGACAATGGTGTTTG	22
dre-miR-124-4-5p	G,L	TGTGTTCACAGTGGACCTTGAT	22
dre-miR-124-5p	G,L	CGTGTTCACAGCGGACCTTGAT	22
dre-miR-124-6-5p	G,L	CGTGTTCACGGCGGACCTTGAT	22
dre-miR-125b-1-3p	M	ACGGGTTAGGTTCTTGGGAGCT	22
dre-miR-129-3p	L	AAGCCCTTACCCCAAAAAGCAT	22
dre-miR-129-5p	L	CTTTTTGCGGTCTGGGCTTGCT	22
dre-miR-130c-5p	G,L	GCCCTTTTTCTGTTGTACTACT	22
dre-miR-133a-3p	L	TTTGGTCCCCTTCAACCAGCTG	22
dre-miR-133a-5p	L	AGCTGGTAAAATGGAACCAAAT	22
dre-miR-133b-3p	L	TTTGGTCCCCTTCAACCAGCTA	22
dre-miR-135a	P,M	TATGGCTTTTTATTCCTATGTGA	23
dre-miR-135b-5p	P,M	TATGGCTTTTTATTCCTATCTG	22
dre-miR-135c	P,M	TATGGCTTTCTATTCCTATGTG	22
dre-miR-138-5p	G,L	AGCTGGTGTTGTGAATCAGGCC	22
dre-miR-1388-5p	L	AGGACTGTCCAACCTGAGAATG	22
dre-miR-140-3p	L	TACCACAGGGTAGAACCACGGAC	23
dre-miR-143	L	TGAGATGAAGCACTGTAGCTC	21
dre-miR-144-5p	L	GGATATCATCGTATACTGTAAGT	23
dre-miR-145-3p	N,L	GGATTCCTGGAAATACTGTTCT	22
dre-miR-145-5p	G,L	GTCCAGTTTTCCCAGGAATCCC	22
dre-miR-146a	L	TGAGAACTGAATTCCATAGATGG	23
dre-miR-146b	L	TGAGAACTGAATTCCAAGGGTG	22
dre-miR-148	L	TCAGTGCATTACAGAACTTTGT	22
dre-miR-150	L	TCTCCCAATCCTTGTACCAGTG	22
dre-miR-152	L	TCAGTGCATGACAGAACTTTGG	22
dre-miR-153a-5p	L	GTCATTTTTGTGATGTTGCAGCT	23
dre-miR-153b-5p	L	GTCATTTTTGTGGTTTGCAGCT	22
dre-miR-155	N	TTAATGCTAATCGTGATAGGGG	22
dre-miR-181a-5p	L	AACATTCAACGCTGTCGGTGAGT	23
dre-miR-181b-3p	G	CTCACTGATCAATGAATGCAAA	22
dre-miR-181b-5p	L	AACATTCATTGCTGTCGGTGGG	22
dre-miR-181c-5p	L	CACATTCATTGCTGTCGGTGGG	22
dre-miR-184	G,L	TGGACGGAGAACTGATAAGGGC	22
dre-miR-193a-5p	L	TGGGTCTTTGCGGGCAAGGTGA	22
dre-miR-196a-5p	P	TAGGTAGTTTCATGTTGTTGGG	22
dre-miR-196b	P	TAGGTAGTTTCAAGTTGTTGGG	22
dre-miR-199-3p	L	TACAGTAGTCTGCACATTGGTT	22
dre-miR-199-5p	N,L	CCCAGTGTTCAGACTACCTGTTC	23
dre-miR-19b-5p	P	AGTTTTGCTGGTTTGCATTCAG	22
dre-miR-200b-3p	L	TAATACTGCCTGGTAATGATGA	22
dre-miR-200c-3p	L	TAATACTGCCTGGTAATGATGC	22
dre-miR-203a-3p	L	GTGAAATGTTTAGGACCACTTG	22
dre-miR-203b-3p	L	GTGAAATGTTCAGGACCACTTG	22
dre-miR-204-5p	G,L	TTCCCTTTGTCATCCTATGCCT	22
dre-miR-205-3p	N,P	GATTTCAGTGGTGTGAAGTGTA	22
dre-miR-210-5p	N	AGCCACTGACTAACGCACATTG	22
dre-miR-214	N,P	ACAGCAGGCACAGACAGGCAG	21
dre-miR-216a	L	TAATCTCAGCTGGCAACTGTGA	22
dre-miR-216b	N,G,L	TAATCTCTGCAGGCAACTGTGA	22
dre-miR-218a	G	TTGTGCTTGATCTAACCATGTG	22
dre-miR-218b	G	TTGTGCTTGATCTAACCATGCA	22
dre-miR-23a-3-5p	N,L	GGATTCCTGGCAGAGTGATTT	21
dre-miR-23a-5p	G,L	GAATTCCTGGCAGAGTGATTT	21
dre-miR-26a-2-3p	L	CCTATTCATGATTACTTGCACT	22
dre-miR-26a-5p	L	TTCAAGTAATCCAGGATAGGCT	22
dre-miR-26b	L	TTCAAGTAATCCAGGATAGGTT	22
dre-miR-27b-5p	L	AGAGCTTAGCTGATTGGTGAACA	23
dre-miR-301b-5p	G,L	GCTTTGACGATGTTGCACTAC	21
dre-miR-30e-3p	L	CTTTCAGTCGGATGTTTGCAGC	22
dre-miR-338	N,L	TCCAGCATCAGTGATTTTGTTG	22
dre-miR-34a	L	TGGCAGTGTCTTAGCTGGTTGT	22
dre-miR-365	L	TAATGCCCCTAAAAATCCTTAT	22
dre-miR-375	L	TTTGTTCGTTCGGCTCGCGTTA	22
dre-miR-429a	L	TAATACTGTCTGGTAATGCCGT	22
dre-miR-430a-3p	L	TAAGTGCTATTTGTTGGGGTAG	22
dre-miR-430b-3p	L	AAAGTGCTATCAAGTTGGGGTAG	23
dre-miR-430b-5p	L	CAACTCTAACTTTAGCATCTTTC	23
dre-miR-430c-3p	L	TAAGTGCTTCTCTTTGGGGTAG	22
dre-miR-455-5p	L	TATGTGCCCTTGGACTACATCG	22
dre-miR-459-3p	M,L	CAGGGAATCTCTGTTACTGGGG	22
dre-miR-460-5p	M	CCTGCATTGTACACACTGTGCG	22
dre-miR-489	G	AGTGACATCATATGTACGGCTGC	23
dre-miR-499-5p	M,G,L	TTAAGACTTGCAGTGATGTTTA	22
dre-miR-7147	G	TGTACCATGCTGGTAGCCAGT	21
dre-miR-722	L	TTTTTTGCAGAAACGTTTCAGATT	24
dre-miR-724	L	TTAAAGGGAATTTGCGACTGTT	22
dre-miR-730	G	TCCTCATTGTGCATGCTGTGTGT	23
dre-miR-734	L	GTAAATGCTGCAGAATCGTACCG	23
dre-miR-9-5p	L	TCTTTGGTTATCTAGCTGTATGA	23

Dre represents zebra-fish.

harvested to determine the effects of three different miRNA mimics or inhibitors on viral gene expression by fluorescent real-time PCR (TaqMan) and protein expression by western blotting.

Western blot analysis was performed as previously described [33]. Briefly, total protein from the cells infected with SCRV were extracted by cell lysis buffer, separated by SDS-PAGE gel and transferred onto a PVDF membrane (Millipore). After blocking with 5% skim milk powder in Tris-buffered saline (TBS) with 0.1% Tween 20, the membrane was incubated with primary antibodies (at 1:1000 dilutions in) which raised against the SCRV viral recombinant protein in rabbits (N and M) and mouse (P) in our lab or β-actin (1:1000) at 4 °C overnight in 5% skimmed milk plus 0.1% Tween 20 in TBS. Subsequently, the membranes were incubated with the HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Abbkine) at 1:5000 dilutions for 1 h at room temperature. Photos and signal intensity were detected by LI-COR Odyssey instrument (for fluorescence detection), and quantitation of raw digital images was implemented with the ImageJ gel analysis plug-in.

After extraction of total RNA (TRIzol Reagent, Invitrogen) and cDNA Synthesis (First-Strand cDNA Synthesis SuperMix, Transgen, China). The SCRV viral copies were measured by fluorescent real-time PCR (TaqMan), approximately 50 ng of total RNA sample and 2 pmol of specific primer (vRNAtag, SCRV87R) were heated for 5 min at 65 °C ad snap chilled on ice for 2 min. Subsequently, fluorescent real-time PCR (TaqMan) reaction mixtures was carried out in a final volume of 20 μL containing 2 × Premix Ex Taq (Takara, Japan) 10 μL, 10 μmol/L each primer 0.4 μL, 10 μmol/L probe 0.4 μL, 50 × ROX Reference Dye II 0.4 μL, DNA or cDNA 2 μL. Finally, double distilled water (ddH2O) was added to the mixture until the total volume. TaqMan real-time PCR thermocycling was performed in an ABI 7500 (Applied Biosystems, USA) as follows: denaturation at 95 °C for 5 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Fluorescence signals were collected at the 60 °C step of each cycle. Fluorescent real-time PCR (TaqMan) were performed in triplicates.

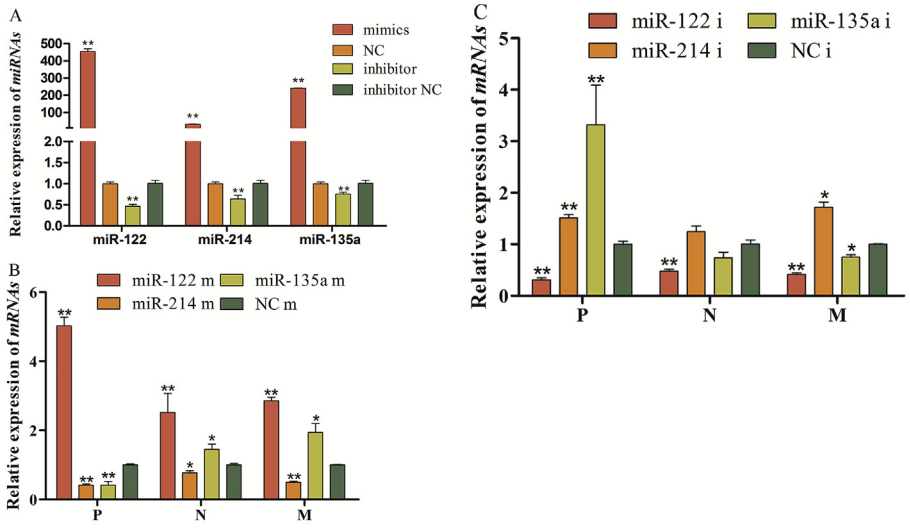

Fig. 4. Effects of three differentially-expressed miRNAs on SCRV replication. The mimics or inhibitors and corresponding NC were transfected into CPB cells followed by SCRV infection. (A) The qRT-PCR was performed for the expression of three miRNAs after transfection; NC and inhibitor NC was set as control. All expression levels were normalized to the arithmetic mean of the selected U6 gene. * and ** respectively indicate statistically-significant differences between infected and uninfected samples (* $p < 0.05$; ** $p < 0.01$). (B) Quantification of mRNA levels of SCRV P, N, and M genes in post-transfected cells with miRNAs mimics-m by qRT-PCR. (C) Quantification of mRNA level of SCRV P, N, and M genes post-transfected cells with miRNAs inhibitor-i by RT-PCR.

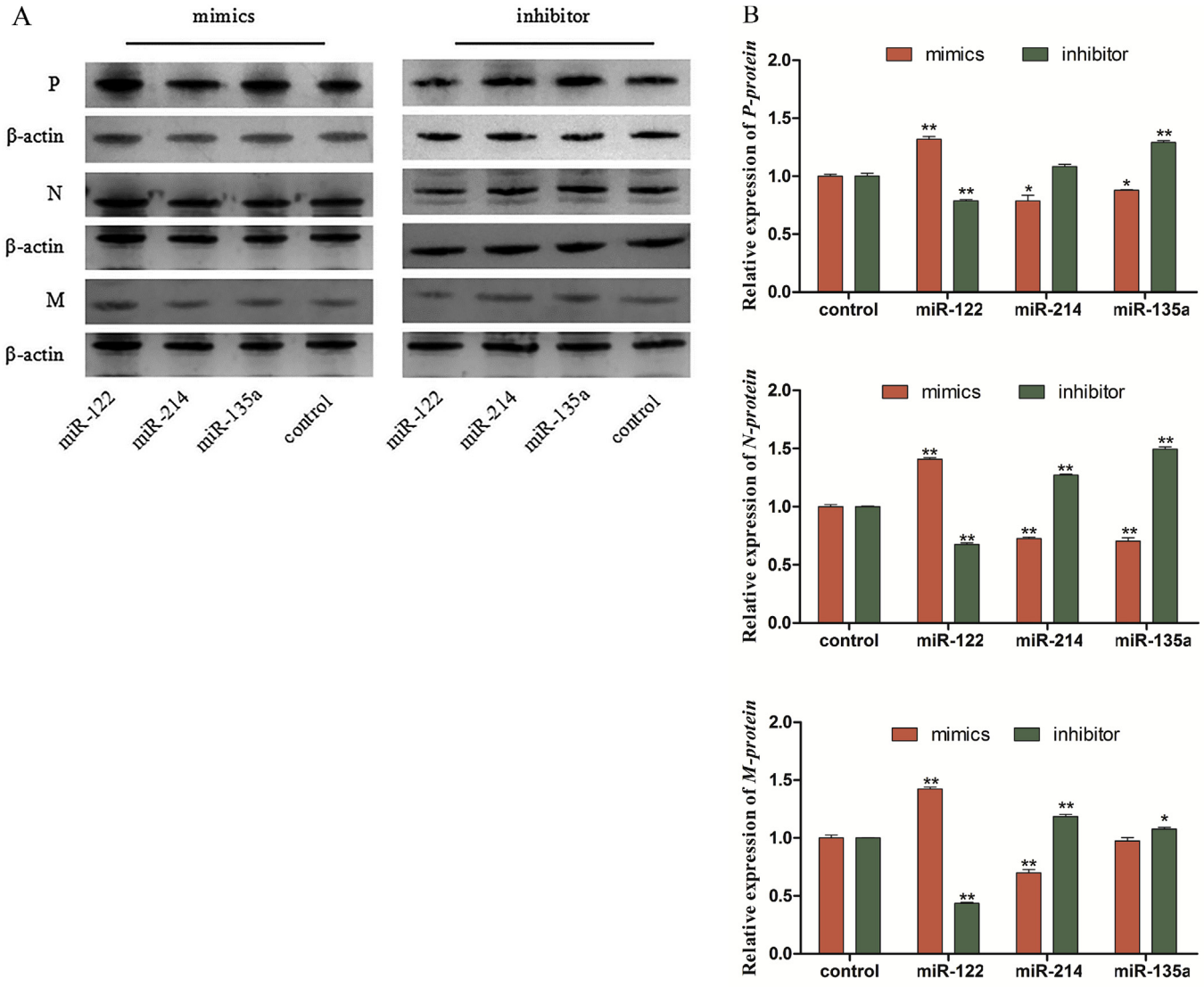

Fig. 5. (A) Viral protein levels of P, N, and M was detected by western blotting. NC and inhibitor NC was set as control. All expression levels were normalized to β-actin. (B) The integrated optical densities of the protein bands were measured using Image J. The value of the P, N, and M protein bands are normalized to β-actin. The value of the P, N, and M protein bands of the control were set as 1 while the values of the P, N, and M protein bands of the three miRNAs were compared to that of the control. * and ** respectively indicate statistically-significant differences between transfected and control samples (* $p < 0.05$; ** $p < 0.01$).

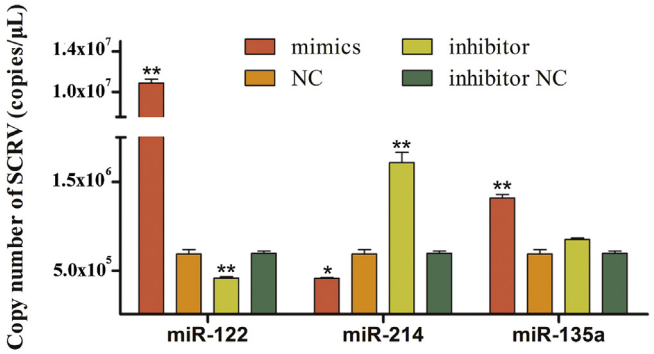

Fig. 6. The viral copy number was validated via fluorescent real-time PCR (TaqMan). NC and inhibitor NC was set as control. * and ** respectively indicate statistically-significant differences between transfected and control samples (* $p < 0.05$; ** $p < 0.01$).

3. Results

3.1. Overview on small RNAs sequencing

To interpret and to identify miRNAs expression profiles from the CPB cells, the supernatants were obtained with or without SCRV at 6 and 12 hpi and sequenced. The Illumina Hiseq 2500 sequencing resulted in 11–21 million raw reads obtained per sample. A preliminary statistical analysis of a total number of raw reads and reads after trimming of adaptors and length filtering were summarized in Table S1. After data filtering based on quality score ($Q \geq 20$), high-quality clean reads were obtained from the four samples with no significant differences in length distribution of small RNAs that had 21-23 nt length (Fig. 1) which used for further analysis. BLASTN was used to search against RFAM and GenBank databases amongst the pooled data for the four samples 2.59%–4.36% of the clean reads were annotated as rRNA, snRNA, snoRNA, scRNA or tRNA (Table S2). The clean reads were compared with zebrafish (*Danio rerio*) genome, the results showed about 59.03%–61.51% of the clean reads were mapped to zebrafish genome (Table S2).

3.2. Identification of conserved miRNAs in CPB cells

The overall reads from the four samples were aligned to zebrafish miRNAs database in miRBase for characterization and then counted. According to the database results, 382 miRNAs were identified, among them 217 known miRNAs belonging to zebrafish miRNA and remaining 165 are putative novel miRNAs identified by MiRDeep2 (Supp. file S1). Comparison between four samples sequenced in this study and zebrafish miRNA database showed about 303 miRNAs from the CPB cells (6 h), 304 from the CPB cells infected with SCRV (6 h); 297 and 315, respectively for the 12 h with and without SCRV, while 225 miRNAs represented as common libraries (Fig. 2). Among the 382 conserved miRNAs, dre-miR-21, dre-miR-100-5p, dre-miR-146a, dre-let-7a, and dre-miR-22a-3p were the most abundant miRNAs (TPM > 100,000 reads) in the four samples (Supp. file S1). However, the expression levels of dre-miR-737-3p, dre-miR-205-5p, dre-miR-145-5p, dre-miR-125b-2-3p, dre-miR-100-2-3p, and some other miRNAs were rather low (Supp. file S1). These results indicated that the miRNAs exhibited a wide range of expression levels in normal CPB cells.

3.3. Differentially-expressed miRNAs and validation of miRNAs sequencing

The differentially-expressed miRNAs, including known and novel miRNAs between SCRV infected and non-infected CPB cells, were analyzed by qRT-PCR. In total, 15 miRNAs were significantly differentially expressed, of which 12 were up-regulated, and 3 were down-regulated that including five novel and 10 known miRNAs at 6 hpi (Supp. file S2). Whereas, from the total of 35 differentially expressed miRNAs 25 were up-regulated and 10 down-regulated that including 11 novel and 24 known miRNAs at 12 hpi (Supp. file S3). To confirm the results from the deep sequencing, 9 miRNAs were randomly selected to quantify their expression by qRT-PCR, showing their expression was consistent (Fig. 3, Supp. file S2 and S3).

3.4. miRNA targets prediction

Finding viral mRNA targets by cellular miRNAs can reduce protein expression either by inhibiting translation or by promoting target mRNA degradation. Prediction of target genes based on differentially-expressed miRNAs on SCRV genome was predicted using miRanda software. The result indicated 98 miRNAs had been predicted, among them, 73 miRNAs could bind to the single viral gene, 23 of them bind to two viral genes, dre-miR-216b and dre-miR-499-5p bind on three genes (Table 1). Also, seven known differential expression miRNAs were predicted at 6 and 12 hpi (Table 1, Supp. file S2 and S3).

To gain insight into the functions of the dysregulated genes and the biological processes involved in the SCRV infection, the dysregulated genes were mapped in terms of GO and KEGG databases. The GO enrichment analysis identified 356 and 548 GO terms and enriched biological processes at 6 and 12 hpi, respectively. The three most enriched biological process at 6 hpi were related to “transcription, DNA − templated”, “regulation of transcription, DNA − templated”, and “regulation of cell shape” (Fig. S1); at 12 hpi the most three enriched GO terms of biological process were “transcription, DNA − templated”, “regulation of transcription, DNA − templated”, and “protein transport” (Fig. S2).

Moreover, the KEGG pathway analysis indicated 115 and 134 enriched pathways of dysregulated genes at 6 and 12 hpi, respectively. The three most enriched pathways were “Regulation of actin cytoskeleton”, “Endocytosis”, and “Pathways in cancer” at 6 hpi (Fig. S3), “Regulation of actin cytoskeleton”, “Axon guidance”, and “Pathways in cancer” were the top three enriched pathway at 12 hpi (Fig. S4).

3.5. Effects of three selected miRNAs on SCRV replication

First, we analyzed the effect of three miRNA (miR-122, miR-214 and miR135a) mimics and inhibitors with its appropriate NC controls in CPB cells, and their expression levels were measured in 24 hpt cells by qRT-PCR. The results showed that all the three miRNAs mimics significantly promoted the corresponding miRNA expression and the inhibitors exhibited a strong inhibitory effect in the cells at 24 hpt (Fig. 4A). MiR-122 was predicted to bind to M and L genes, while miR-214 was predicted to bind to N and P genes, and miR-135a was predicted to bind to M and P genes (Table 1). Following the infection with SCRV in the 24 hpt, three differentially-expressed miRNAs exhibited a dysregulated effects on viral mRNA (P, N, and M) expression (Fig. 4A). While in the cells treated with the mimics, miR-135a and miR-214 m had significant inhibitory effects on all the three viral genes, however, miR-122 could significantly stimulate the expression of all the three viral genes when compared to NC (Fig. 4B). Contrasting results could be observed in the cells post infected with SCRV for 24 h, earlier transfected with respective inhibitors (Fig. 4C).

To confirm further, total proteins from the post SCRV infected cells treated with miRNAs mimic and inhibitors were analyzed by western blot, to evaluate the effects of these three miRNAs on viral protein expression. Similar to the effects on mRNA expression level, miR-214 exhibited a significant inhibitory effect on the protein expression of viral P, N, and M genes; miR-122 could also stimulate the protein level expression of these three genes (Fig. 5 A and B). The viral burden assessment showed that SCRV-specific RNA was detected from the total RNA after 24 hpi. In the three miRNA mimics treated cells, miR-122 was identified to be significantly increased the viral load, contrarily,

miR-214 inhibitor decreased the SCRV replication. There was no correlation between the expression of miR-135a and SCRV replication (Fig. 6). Together, these obtained results demonstrated that the three miRNAs could modulate SCRV replication in host cells.

4. Discussion

The miRNAs are involved in a wide spectrum of physiological and pathological cellular events that includes development, immunity, and apoptosis [34]. Growing pieces of evidence revealed that host or viral microRNAs play an important role in the regulation of various host-pathogen interactions, and the dysregulation of miRNA expression was often associated with disease [35–37] Due to the complex interactions entailed between the miRNAs and gene targets, the activity concerning the miRNAs expression should be carefully considered before attempting miRNA functional assays. Thus, in the present study, we employed Illumina sequencing platform to identify and to study the small RNA sequencing profile in CPB cells following SCRV infection.

Until now, there are about 26 miRNA gene clusters have been considered as conserved miRNA genes that are closely located in the genome evolution that including, Atlantic salmon, Atlantic cod, zebrafish and humans (http://www.mirbase.org/), [38–40]. Due to lack of whole-genome sequence for Chinese perch, we have utilized the miRBase (v.21), till now which has only nine teleost fish species miRNA databases that including, zebrafish miRNAs and miRNA genes [41]. From the miRNA profiling data, a total of 382 miRNAs were identified, and numerous host genes could be dysregulated upon SCRV infection via the miRNA mechanism at two different time points (6 and 12 hpi). Intriguingly, a total of 37 upregulated and 13 downregulated miRNAs were reported that including, 12 upregulated and 3 downregulated miRNAs at 6 hpi, while 25 miRNAs were upregulated and 10 miRNAs were downregulated at 12 hpi. Comparatively, number of miRNAs were expressed at 12 hpi than 6 h, it may be due to the prolonging effects of the viral pathogenesis. On the other hand, it may be the effect of the SCRV genome that modulating the host miRNAs at a specific period. To further confirming the functions of those genes is the key to better understand the role of miRNAs during SCRV infection.

Many researchers previously have reported that miRNA targeting elements of innate immunity are a major way to regulate the disease. The adult zebrafish exclusively depend on adaptive immunity; conversely, the embryos rely solely on innate immunity, unification of both the infection models permits defining the influence of immune system [42,43]. The highly conserved miR-146 family between fish and human has emerged as an infection-inducible miRNAs of embryonic and adult zebrafish, have previously been linked to the to the innate immune system in mammalian organisms [44]. Viral hemorrhagic septicemia virus (VHSV) in rainbow trout had strongly induced the two clustered miRNAs, miR-462 and miR-731 in the liver of the infected fish as well as in RTL-W1 fish cells when stimulated with formulated poly I:C, indicating their involvement in fish-virus interactions [37]. Recombinant plasmids encoding the sequence of IFNa1 or IFN-γ which use as rhabdovirus G-based DNA vaccine against *Viral hemorrhagic septicemia virus* (VHSV) has the potential to increase the miR-462, and miR-731 in the skeletal muscle and in the liver of the vaccinated fish representing the miRNA involvement in host-virus interactions and immune responses in teleost fish [45]. The infection of influenza A virus (H5N1) specifically activated the alteration of MiR-136, a host antiviral innate immune gene that acted as an immune agonist of retinoic acid-inducible gene 1 (RIG-1) in human A549 cells [46]. MiRNA-146 was identified as negative feedback regulated gene that negatively targeted to signaling proteins appertaining to innate immune responses of host IRAK1and TRAF6 genes to microbial infections [44]. MiRNAs also regulate virus replication by targeting viral transcripts: e.g., miRNA-122 stimulates translation of hepatitis C virus RNA through interaction with the 3'UTRs of the viral genome [47]. MiR-181 can directly impair the porcine reproductive and respiratory syndrome virus replication, by binding specifically to a conserved region in the downstream ORF 4 of the viral genome [48]. MiR-let-7c can inhibit H1N1 influenza A virus replication by targeting the 3′ UTRs of the M1 genome and suppressed the M1 protein expression [49]. In the present study, 98 miRNAs were predicted to bind to the SCRV viral genome, at intervals three of them were randomly chosen to assess their effects on SCRV replication (Table 1).

Recent studies had revealed that miR-214 could efficiently repress the SCRV replication by targeting N and P gene of SCRV genome that further decreased IFN-α production in SSN-1 cells [50,51]. A similar conclusion was obtained in our study with miRNA-122. However, it was astounding to find in what way miRNA-122 could significantly stimulate the SCRV replication. Viral M (Matrix) protein was identified as the virus' most potent anti-host protein that can exert different functions during the course of the infection [52], if miR-122 bound on M and L gene as predicted, the enhanced expression of miR-122 would have exogenously decreased the replication of SCRV. So, we realized that miR-122 could affect the SCRV replication, but might not through binding to the viral genome.

There have been reported that microRNA-122 could stimulated translation of viral RNAs from hepatitis C virus RNA or Hepatitis E Virus by stimulating the association of the small ribosomal subunit with the viral RNAs [47,53]. Since MiR-122 is a very conservative miRNA, it might have similar function in regulation of the replication of SCRV. However, the exact mechanism underlying the stimulation of SCRV replication by miR-122 remained to be elucidated.

In conclusion, miRNA-214 was able to inhibit the replication of SCRV, while miRNA-122 could significantly stimulate the replication of SCRV. These results suggest that regulating miRNA-122 and miRNA-214 levels might help in the management of SCRV infection. The inhibitor of miR-122 and the mimics of miR-214 might be used to control the SCRV infection of Chinese perch. These results support valuable evidence for an enriched understanding of the piece and underlying mechanisms of miRNAs cellular immunity and SCRV pathogenesis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.020.

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