Title page

Viral miRNAs targeting virus genes promote virus infection in shrimp in vivo

Yaodong He, Kai Yang, Xiaobo Zhang*

Key Laboratory of Animal Virology of Ministry of Agriculture and College of Life Sciences, Zhejiang University, Hangzhou 310058, The People’s Republic of China

* Corresponding author: Prof Xiaobo Zhang

Tel: 86-571-88981129

Fax: 86-571-88981151

Email: zxb0812@zju.edu.cn
Abstract

Viral miRNAs, most of which are characterized in cell lines, are found to play important roles in virus lifecycle to avoid the attack of the host immune system or keep virus in the latency state. Viral miRNAs targeting virus genes can inhibit the virus infection. In this study, the in vivo findings in *Marsupenaeus japonicus* shrimp revealed that the viral miRNAs could target virus genes and further promoted the virus infection. The results showed that white spot syndrome virus (WSSV)-encoded miRNAs, WSSV-miR-66 and WSSV-miR-68, were transcribed at the early stage of WSSV infection. When the expressions of WSSV-miR-66 and WSSV-miR-68 were silenced with sequence-specific anti-miRNA oligonucleotides (AMOs), the copies of WSSV and the WSSV-infected shrimp mortality were significantly decreased, indicating that the two viral miRNAs took great effects on virus infection. It was revealed that the WSSV-encoded *wsv094* and *wsv177* genes were the targets of WSSV-miR-66, and the *wsv248* and *wsv309* genes were the targets of WSSV-miR-68. The data demonstrated that the four target genes played negative roles in the WSSV infection. The targeting of the four virus genes by WSSV-miR-66 and WSSV-miR-68 led to the promotion of virus infection. Therefore our in vivo findings presented a novel aspect of viral miRNAs in the virus-host interactions.

**Keywords:** white spot syndrome virus, viral miRNA, virus infection

**Running title:** Viral miRNAs promote virus infection
Introduction

MicroRNAs (miRNAs), small non-coding RNAs, play regulatory roles posttranscriptionally in a lot of eukaryotic cellular processes such as proliferation, differentiation, apoptosis, immune response and tumorigenesis (1-3). In the host-virus interactions, miRNAs are considered to be essential. When suffering virus infection, the expression profile of host miRNAs is changed as a result of viral modulation of cellular miRNA expression (4). At the same time, the miRNAs encoded by the invading viruses participate in the virus-host interactions. Epstein Barr virus (EBV), a member of the gamma sub-family of Herpesviridae, is the first virus discovered to encode viral miRNAs (5). So far, 44 mature miRNAs encoded by EBV have been reported (5-8). Besides EBV, many other herpesviruses are also found to encode large numbers of viral miRNAs, such as Kaposi sarcoma-associated herpesvirus (KSHV) (9-11). However, most mammal viruses including simian virus 40 (SV40) and adenovirus encode a single viral miRNA (12). The tendency of herpesviruses to encode large numbers of viral miRNAs suggests the important roles of viral miRNAs in the life cycles of viruses. Unlike the miRNAs of eukaryotes, the viral miRNAs are not conserved among different species.

Although most of viral miRNAs need to be further studied so that clear insights into their functions can be gained, it is clear that viral miRNAs can target both viral and cellular transcripts. As reported, viral miRNAs take great effects on virus infection by regulating virus or host gene expression to avoid the host defenses and/or to maintain latent and persistent infection (13-19). In mammal virus SV40, the
viral miRNA miR-S1 does not directly enhance the replication of SV40, but it can protect virus-infected cells from elimination by the host immune system (20). The SV40 miRNA targets the host mRNA encoding the SV40 T antigens, which are viral transcription factors that induce the expression of late viral genes (20). Modulated by a plurality of host cell environment, viral miRNAs can regulate expressions of viral genes and promote cell environment conducive to virus life cycle. Many viral miRNAs target mRNAs of virus early genes or DNA polymerase genes (21). The expressions of some virus genes need to be inhibited in certain stages of the virus life cycle to avoid the cell toxicity or hide from host immune system. It is found that the expression of EBV DNA polymerase BALF5 is inhibited by virus-encoded miR-BART2 (BamHI A rightward transcript 2) (22). In insect DNA virus Heliothis virescens ascovirus (HvAV), the virus encodes a miRNA (virus-encoded miR-1) to downregulate the expression of the viral DNA polymerase gene (23). During the latency of herpes simplex virus 1 (HSV-1) in mammal, the virus can generate viral miR-H2-3p to downregulate the expression of the viral immediate early gene ICP0 (24). The documented data show that viral miRNAs are essential for virus latency in hosts by suppressing the expressions of virus genes, indicating that viral miRNAs play negative roles in virus infection. However, the positive role of viral miRNA in virus infection has not been addressed at present.

In our previous study, 40 distinct white spot syndrome virus (WSSV)-encoded miRNAs involved in virus infection in shrimp are identified (25). Among them, two viral miRNAs, WSSV-miR-66 (GenBank ID JN113104) and WSSV-miR-68
(GenBank ID JN113105), were further characterized in this study. The results revealed that the two viral miRNAs could promote the WSSV infection in shrimp by targeting the virus genes. Our study presented a novel aspect of viral miRNAs in virus infection in vivo.

Materials and methods

Shrimp culture and WSSV challenge

The *Marsupenaeus japonicus* shrimp were cultured in groups of 20 individuals in tanks containing 80 L of aerated sea water at room temperature. The shrimp individuals were about 10 g in weight and 10-12 cm in length. To ensure the absence of WSSV prior to experimental infection, the shrimp were detected by PCR using WSSV-specific primers (5'-TATTGTCTCTCCTGACGTAC-3' and 5'-CACATTCTTGACGTAC-3'). The DNA template was extracted from shrimp with SQ tissue DNA kit (Omega-Bio-Tek, USA) according to the manufacturer’s instructions. The shrimp were infected with WSSV (10^5 copies/mL) by injection (100 μl WSSV inoculum/shrimp) into the lateral area of the fourth abdominal segment of virus-free shrimp. At different times postinfection (0, 2, 4, 6, 12, 24, 36, 48 and 72h), three shrimp were randomly collected for each treatment. Subsequently the shrimp hemocytes were collected for later use.

Detection of miRNA with Northern blot

Total RNAs were extracted from shrimp hemocytes with mirVana™ miRNA isolation kit according to the manufacturer’s protocol (Ambion, USA). After separation on a denaturing 15% polyacrylamide gel with 8 M urea, the RNAs were
transferred to a Hybond-N+ membrane (Amersham Biosciences, United Kingdom).

Subsequently the RNAs were crosslinked under ultraviolet. The membrane was
prehybridized in DIG Easy Hyb Granules buffer (Roche, Switzerland) for 30 min and
then hybridized with DIG-labeled probes completely complementary to WSSV-miR-
66 (5'-UCUGUUGCUCCAGAAACGUACA-3') or WSSV-miR-68 (5'-UGGACACAC
ACAGUACAGAAC-3') at 42°C overnight. The DIG-labeled U6 probe (5'-GGGC
CATGCTAATCTTCTGTATCGTT-3') was used as a control. Immunological
detection was performed using the DIG High Prime DNA Labeling and Detection
Starter Kit II (Roche, Switzerland).

Detection of viral miRNA using real-time PCR

Shrimp hemocytes were collected from WSSV-infected shrimp at different time
points after WSSV infection (0, 2, 4, 6, 12, 24, 36, 48 and 72 h). The total RNAs were
extracted from shrimp hemocytes using mirVana™ miRNA isolation kit according to
the manufacturer’s instructions (Ambion, USA). Then cDNA was synthesized with
TaqMan® microRNA reverse transcription kit (Applied Biosystems, USA) following
the manufacturer’s protocol. The reverse transcription was conducted at 16°C for 30
min, 42°C for 30 min and 85°C for 5 min using the WSSV-miR-66 reverse
transcription primer (5'-GTCGTATCCAGTGCAGGGTGCCAGGTATTGCACC
GACTG-3') and WSSV-miR-68 reverse transcription primer (5'-GTC
GTATCCAGTGCAGGTATTGCACGGATACGCGGTTCTG-3') (33, 34). U6 was used as a control with the reverse transcription primer Random6
(Applied Biosystems, USA). Real-time PCR was carried out according to the protocol
of SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Japan) using WSSV-miR-66-specific primers (5’-TCTGGTCTCCAGAAACGTACA-3’ and 5’-GTGCAGGGTGAGGTATTC-3’), WSSV-miR-68-specific primers (5’-TGGACACAACAGTACCCAGAAC-3’ and 5’-GTGCAGGGTGAGGTATTC-3’) or U6-specific primers (5’-GTCATCCTTGCGGCGGCCA-3’ and 5’-CTCGCTTCGCAGCACATATA-3’). The real-time PCR procedure was 95°C for 5 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s.

Silencing of WSSV-miR-66 and WSSV-miR-68 expressions in WSSV-infected shrimp

To knock down the viral miRNA expression, the anti-miRNA oligonucleotide (AMO) was injected into WSSV-infected shrimp. The AMO-WSSV-miR-66 (5’-TGTACGTTTCTGGAGC-3’) and AMO-WSSV-miR-68 (5’-GGTACTGTTGTCTGATCCACA-3’) were synthesized (Sangon Biotech, Shanghai, China) with a phosphorothioate backbone and a 2’-O-methyl modification at the 12th nucleotide. The AMO (10 nM) and WSSV (10⁵ copies/mL) were co-injected into virus-free shrimp at a volume of 100 μl per shrimp. At 16 h after the co-injection, the AMO (10 nM) (100 μl/shrimp) was injected into the same shrimp. As controls, AMO-WSSV-miR-66-scrambled (5’-TTGCATGCTGCTGTCGAG-3’) and AMO-WSSV-miR-68-scrambled (5’-GTGTACTGTTGTCTGAG-3’) were included in the injections. WSSV only (10⁵ copies/mL) and physiological saline (0.85% NaCl) were also injected into shrimp. At different times postinfection (0, 2, 4, 6, 12, 24, 36, 48 and 72 h), three shrimp were randomly collected for each treatment and subjected to Northern blot and detection of WSSV.
copies. The shrimp mortality was monitored every 12 hours. All the assays were biologically repeated for three times.

Detection of WSSV copies by quantitative real-time PCR

Quantitative real-time PCR was performed to examine the WSSV copies in hemocytes of WSSV-infected shrimp. The viral DNA was extracted from shrimp hemocytes using SQ Tissue DNA kit (Omega-Bio-Tek, USA). Then the WSSV copies were detected real-time PCR with WSSV-specific primers (5’- TTGGTTTCAG CCCGAGATT-3’ and 5’- CCTTGGTCAGCCCCCTTGA-3’) and WSSV-specific TaqMan probe (5’-FAM-TGCTGCCGTCTCCAA-TAMRA-3’). The 25-μl PCR reaction solution contained 12.5 μl of Premix Ex Taq (Takara, Japan), 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer, 1 μl of 10 μM TaqMan fluorogenic probe, 1 μl of DNA template and 9.5 μl distilled water. The pre-denaturalization stage of the PCR program was 95 °C for 1 min, followed by the amplification stage consisting of 40 cycles of 95 °C for 30 s, 52 °C of 30 s and 72 °C for 30 s.

Shrimp mortality analysis

The shrimp with different treatments were cultured at 20 shrimp/group. Shrimp mortality was examined at different time points postinfection with WSSV (12, 24, 36, 48 and 72 h). The experiments were repeated for three times.

Prediction of target genes

To predict the target genes of viral miRNAs, the WSSV genome sequence (GenBank accession no AF332093) was employed with three independent computational algorithms including TargetScan 5.1 (http://www.targetscan.org),
miRanda (http://www.microrna.org/) and pictar (http://pictar.mdc-berlin.de/). The TargetScan was used to search for miRNA seed matches (nucleotides 2–8 from the 5’-end of miRNA), and the miRanda tool was utilized to match the entire miRNA sequences with the parameters free energy < -20 kcal/mol and score > 50. Pictar was employed to search the combined effects of microRNA target microRNA-based or other characteristics and score > 20.

**Plasmid construction**

The 3’ UTRs of WSSV genes (wsv094, wsv177, wsv248 and wsv309) and enhanced green fluorescent protein (EGFP) gene were cloned into a pIZ/EGFP V5-His vector (Invitrogen, USA), respectively. Every 3’ UTR was cloned into the pIZ vector downstream of EGFP using the XbaI and SacII restriction sites to generate the EGFP-wsv094-3’UTR (primers 5’-AACTCTAGAAAGGTACGAGGGGTGTTGGTGTC GG-3’ and 5’-AACCGCCGACAACCCACTCCTCCGCACAGGACTT-3’), EGFP-wsv177-3’UTR (primers 5’-AACTCTAGAAAACATAGTATGGTATTTGTTA-3’ and 5’-AAC CGGCCGTCTTGGCATAAGCTCTCGTTTTTGG-3’), EGFP-wsv248-3’UTR (primers 5’-AACT CTAGAACCCAGCGGTCCACCCTCCGAATATGTGTA-3’ and 5’-AACCGCGGGGTCAAGGATGGA CGGGCTTTGTC-3’), and EGFP-wsv309-3’UTR (primers 5’-AACTCTAGAAAGCGAGCCGCTACTACGACA-3’ and 5’-AACCGCGGAGCAACAGAAGAGCCGGA-3’). Mutation constructs of 3’ UTRs were obtained by DpnI-mediated site-directed mutagenesis (New England BioLabs, USA). Briefly, two nucleotides of a UTR sequence were randomly mutated by PCR with two complementary primers. The primers contained the two mutated nucleotides with two
15-bp flanking 3’UTR sequences. The 3’UTR mutant was amplified by PCR from the plasmid containing the wild-type 3’ UTR. Subsequently the amplified DNA (plasmid) was incubated with DpnI at 37°C for 1h to degrade the methylated DNA. The DNA was then transformed into *E. coli*. The mutant plasmid was extracted. All the recombinant plasmids were confirmed by sequencing.

**Cell culture, transfection and fluorescence assays**

Insect High Five cells (Invitrogen, USA) were cultured at 27°C in Express Five serum-free medium (Invitrogen) containing l-glutamine (Invitrogen). When the cells were at about 70% confluence, they were co-transfected with the synthesized viral miRNA (300 nM) and the plasmid consisting of *EGFP* and 3’ UTR of WSSV gene (6 μg/ml). The viral miRNAs were synthesized with D6140 in vitro transcription T7 kit (Takara, Japan). All transfections were carried out in triplicate with Cellfectin transfection reagent (Invitrogen) according to the manufacturer’s protocol. After being cultured for 12 h, the transfected cells were seeded to 96-well plates at concentrations of 2.0 × 10⁴ cells per well. At 48 h after transfection, the fluorescence of cells was examined with a Flex Station II microplate reader (Molecular Devices, USA) at 490/510 nm of excitation/emission (Ex/Em). The fluorescence values were corrected by subtracting the autofluorescence of cells not expressing EGFP. All the experiments were repeated biologically three times.

**Synthesis of siRNAs and RNAi assay in shrimp in vivo**

Based on the sequences of WSSV genes, the siRNAs specifically targeting WSSV genes were synthesized in vitro using a commercial kit according to the
manufacturer’s instructions (TaKaRa, Japan). The siRNAs used were wsv094-siRNA (5’-AGAGUGUAGUUCAAAAAAUUUG U
GCAUCA-3’), wsv177-siRNA (5’-GGAGGAAAUUG U222 GCAGAUU-3’) and wsv309-
siRNA (5’-GGACAAACUCUCCUGUAUAU-3’). The sequences of siRNAs were
scrambled to generate the controls (wsv094-siRNA-scrambled, 5’-GACAUUAAGA
UAUAUAUGG-3’; wsv177-siRNA-scrambled, 5’-CUGUGUAAAGUCGGAAGA-
3’; wsv248-siRNA-scrambled, 5’-UAUACGGACCGGAAAAACUA-3’; wsv309-
siRNA-scrambled, 5’-CACAACUUUACGGCAGAAU-3’). The formation of double-
stranded RNAs was monitored by determining the size in agarose gel electrophoresis.
The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, 100 mM
NaCl, pH 7.5) and quantified by spectrophotometry.
RNAi assay was conducted in shrimp by the injection of a siRNA into the lateral
area of the fourth abdominal segment at 30 μg/shrimp using a syringe with a 29-gauge
needle. The siRNA (15μg) and WSSV (10^5 copies/mL) were co-injected into
virus-free shrimp at a volume of 100 μl per shrimp. At 12 h after the co-injection, the
siRNA (15μg) (100 μl/shrimp) was injected into the same shrimp. At the same time,
the AMO-WSSV-miR-66 (10 nM) or AMO-WSSV-miR-68 (10 nM) and WSSV (10^5
copies/mL) were co-injected into virus-free shrimp. At 12 h after the co-injection, the
AMO (10 nM) (100 μl/shrimp) was injected into the same shrimp. WSSV (10^5
copies/mL) (100 μl/shrimp) only was included in the injections as a positive control.
For each treatment, 20 shrimps were used. At different time after the last injection (0,
24, 36 and 48h), the shrimp hemocytes were collected. Three shrimp specimens from
each treatment, selected at random, were collected for later use. The above assays were biologically repeated for three times.

Detection of viral mRNA with quantitative real-time PCR

Shrimp hemocytes were collected from WSSV-infected shrimp with different treatments at different time points after WSSV infection (24, 36 and 48 h). The total RNAs were extracted from shrimp hemocytes using mirVana™ miRNA isolation kit according to the manufacturer’s instructions using the total RNA isolation procedure (Ambion, USA). The cDNA was synthesized using PrimeScript reverse transcription kit (Takara, Japan) according to manufacturer’s instructions. Real-time PCR was carried out with the protocol of Premix Ex Taq™ (Probe qPCR) (Takara, Japan). Actin was used as a control. The gene-specific primers (wsv094, 5' - CATTTCTTCCTGTGCTGGT-3' and 5' - TGGA GGAAGAGGAAGAGGAA-3'; wsv177, 5' - CCAATGGAAATCCTGGTTG-3' and 5' - TGAACCCCTCTAGTGCA-3'; wsv248, 5' - GGTGTTGGTGGTGATGTA-3' and 5' - TCCTGGTTGGA-3'; wsv309, 5' - CCGGACAAACTCCCTGTAA-3' and 5' - AATGTC TAC-3') and gene-specific probes (wsv094, 5' - FAM-TCCTCCTCCTTGC-3'; wsv177, 5' - FAM-TTCGCCCTATTGCCTC-3'; wsv248, 5' - FAM-CTTATGCTCGGCTG-3'; wsv309, 5' - FAM-ATCCTAGGTGCGCCAGTG-3'; actin, 5' - FAM-ATCGGTGGCTCCATCCTGGG-3'; actin, 5' - FAM-ATCGGTGGCTCCATCCTGGG-3') were used. PCR was conducted at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s.
Detection of viral mRNA with Northern blot

The expression of a known early gene of WSSV (wsv477) was detected with Northern blot (26). The total RNAs were extracted from shrimp hemocytes with mirVana™ miRNA isolation kit using the total RNA isolation procedure (Ambion, USA). The RNAs were separated on a denaturing 15% polyacrylamide gel with 8 M urea, and then transferred to Hybond-N+ membrane (Amersham Biosciences, United Kingdom). After crosslinking under ultraviolet, the membrane was prehybridized in DIG Easy Hyb Granules buffer (Roche, Switzerland) for 30 min. Subsequently the membrane was hybridized with DIG-labeled wsv477 probe (5'-CGATTTCGGCAGGCCAGTTGTCAGA-3') at 42°C overnight. DIG-labeled actin probe (5'-CTCGCTCGGCGGTGGTCGTGAAGG-3') was used as a control. Immunological detection was performed as described in the manual of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland).

Statistical analysis

The numerical data collected from three independent experiments were processed using one-way analysis of variation (ANOVA). Students’ t-test was employed to test the significant difference.

Results

Time-course expression profiles of WSSV-miR-66 and WSSV-miR-68 in hemocytes of WSSV-infected shrimp

To reveal the time-course expression patterns of viral miRNAs, Northern blot and
quantitative real-time PCR were conducted using hemocytes of WSSV-infected shrimp at different time points (0, 2, 4, 6, 12, 24, 36, 48 and 72 h postinfection). The results indicated that the viral miRNAs WSSV-miR-66 and WSSV-miR-68 shared similar expression profiles during virus infection (Fig 1A and B). Both of the viral miRNAs could be detected at 6 h postinfection. As shown in Fig 1C, the transcript of a known early gene of WSSV (wsv477) was detected at 4 h postinfection. In this context, the data suggested that WSSV-miR-66 and WSSV-miR-68 were the early viral miRNAs of WSSV. It was found that WSSV-miR-66 and WSSV-miR-68 were expressed in WSSV-infected shrimp at all time points examined (Fig 1A and B). The findings showed that WSSV-miR-66 and WSSV-miR-68 might play important roles in virus infection.

**Effects of WSSV-miR-66 and WSSV-miR-68 on virus infection in shrimp in vivo**

In an attempt to reveal the roles of viral miRNAs in virus infection, the expressions of WSSV-miR-66 and WSSV-miR-68 were silenced by sequence-specific AMOs. The results of Northern blots and quantitative real-time PCR analyses showed that the expressions of WSSV-miR-66 and WSSV-miR-68 were inhibited by AMO-WSSV-miR-66 and AMO-WSSV-miR-68 in vivo, respectively (Fig 2A). However, the AMO-WSSV-miR-66-scrambled and AMO-WSSV-miR-68-scrambled had no effect on viral miRNA expression (Fig 2A).

Under the conditions that the WSSV-miR-66 or WSSV-miR-68 expression was knocked down, the WSSV copies were examined. As shown in Fig 2B, both AMO-WSSV-miR-66 and AMO-WSSV-miR-68 led to significant decreases of WSSV.
copies from 12 h to 36 h postinfection. As controls, AMO-WSSV-miR-66-scrambled and AMO-WSSV-miR-68-scrambled had no effect on virus replication (Fig 2B). The findings indicated that the two viral miRNAs played positive roles in the WSSV replication.

The data of mortality analysis showed that the mortality of WSSV-infected shrimp with AMO treatment (AMO-WSSV-miR-66 or AMO-WSSV-miR-68) was significantly decreased \( (p<0.01) \) compared with the controls (WSSV only and WSSV-infected shrimp treated with AMO-WSSV-miR-66-scrambled or AMO-WSSV-miR-68-scrambled) (Fig 2C). The results indicated that WSSV-miR-66 and WSSV-miR-68 were involved in WSSV infection in vivo. Taken together, the findings revealed that WSSV-miR-66 and WSSV-miR-68 had positive effects on WSSV infection in shrimp in vivo.

**Interactions between viral miRNAs and viral genes**

To reveal the pathways mediated by viral miRNAs, the target genes of viral miRNAs were analyzed. Based on the target prediction using Targetscan, miRanda and Pictar algorithms, it was found that the WSSV-encoded \textit{wsv094} and \textit{wsv177} genes were the targets of WSSV-miR-66, and the \textit{wsv248} and \textit{wsv309} genes were the targets of WSSV-miR-68 (Fig 3A and D).

To validate this bioinformatic predictions, the synthesized viral miRNAs and the plasmid \textit{EGFP-wsv094-3'}UTR, \textit{EGFP-wsv177-3'}UTR, \textit{EGFP-wsv248-3'}UTR or \textit{EGFP-wsv309-3'}UTR were co-transfected into insect High Five cells (Fig 3B and E). The results indicated that the fluorescence intensity in the cells co-transfected with
WSSV-miR-66 and EGFP-wsv094-3′UTR or EGFP-wsv177-3′UTR was significantly decreased compared with those in the controls (Fig 3B and C), showing that WSSV-miR-66 was interacted with wsv094 and wsv177 genes. The co-transfection of WSSV-miR-68 and EGFP-wsv248-3′UTR or EGFP-wsv309-3′UTR yielded the similar results (Fig 3E and F), indicating that WSSV-miR-68 targeted the wsv248 3′UTR and wsv309 3′UTR.

**Pathways mediated by viral miRNAs in virus infection**

The above data showed that WSSV-encoded wsv094, wsv177, wsv248 and wsv309 genes were the targets of WSSV-miR-66 and WSSV-miR-68, respectively. In an attempt to reveal the roles of the four virus genes in the WSSV infection, the expressions of the four genes were respectively silenced by gene-specific siRNAs in shrimp in vivo, followed by detections of virus copies. The results indicated that the expressions of wsv094, wsv177, wsv248 and wsv309 genes were significantly knocked down at 24 and 36 h postinfection compared with the positive control WSSV only, while the control siRNA-scrambled had no effect on the viral gene expression (Fig 4A), showing that the siRNA was highly specific. By comparison with the shrimp treated with AMO-WSSV-miR-66 or AMO-WSSV-miR-68, the sequence-specific siRNA significantly inhibited the expression of the corresponding viral gene in shrimp (Fig 4A).

Under the conditions that the expression of wsv094, wsv177, wsv248 or wsv309 gene was knocked down, the WSSV copies in shrimp hemocytes and the WSSV-infected shrimp mortalities were detected. It was revealed that the silencing of...
the expression of *wsv094, wsv177, wsv248* or *wsv309* gene led to a significant increase of WSSV copies compared with the control (WSSV only) (Fig 4B). At the same time, the results showed that the viral gene silencing resulted in significant increases of the WSSV-infected shrimp mortalities by comparison with the positive control (Fig 4C). These findings showed that the four virus genes played negative roles in the WSSV infection in vivo.

As reported, *wsv177* and *wsv309* genes are identified to be early genes of WSSV (27, 28). However, the *wsv094* and *wsv248* genes have not been characterized yet. In this context, the viral miRNAs WSSV-miR-66 and WSSV-miR-68 could promote the virus infection by targeting the virus genes (*wsv094, wsv177, wsv248* and *wsv309*) which had negative roles in the WSSV infection (Fig 4D).

**Discussion**

RNAi plays very important roles in animal immunity. DNA viruses take advantage of the host immune system by participating in the RNAi system. It is well known that DNA viruses can encode their own miRNAs to regulate the expressions of both host and virus genes. The viral miRNAs target mRNAs and further result in either mRNA degradation or translational repression. As reported, viral miRNAs share the same biogenesis and function pathways as its host. In recent years, more and more viral miRNAs have been demonstrated to take great effects in virus infection with well-defined targets (13-19). It is reported that viral miRNAs can downregulate host cellular mRNAs and/or viral mRNAs during virus infection. Most cellular targets of viral miRNAs are required in the host immune system, thus viral miRNAs can inhibit
the host immune responses to facilitate virus infection. Viral miRNAs can also target virus early genes or some important virus genes for virus infection to keep the virus in the latency state or avoid the attack of the host immune system. Up to date, the viral miRNAs targeting virus genes are found to inhibit the virus infection. However, the findings revealed in this study showed that the viral miRNAs WSSV-miR-66 and WSSV-miR-68 could target the WSSV genes and further promoted the WSSV infection. Therefore our study presented a novel aspect of viral miRNAs in the virus-host interactions.

So far, it is reported that virus-encoded proteins are conducive to virus infection. However, our study demonstrated that the virus could encode viral proteins to inhibit the virus infection. The results of this study indicated that the WSSV-encoded wsv094 and wsv177 genes were the targets of WSSV-miR-66, and the wsv248 and wsv309 genes were the targets of WSSV-miR-68. Based on homology analysis, it is found that the wsv094, wsv177, wsv248 and wsv309 genes share no similarity with any known proteins or motifs (27). Among the four genes, the wsv177 and wsv309 genes are transcribed at the early stage of WSSV infection in shrimp (27, 28). The data in this study presented that the wsv094, wsv177, wsv248 and wsv309 genes played negative roles in the WSSV infection in shrimp, indicating that the virus could encode some viral proteins to precisely balance the virus invasion and virus latency in animals. As a result, WSSV-miR-66 and WSSV-miR-68 promoted the WSSV infection by targeting the WSSV genes. In this context, the viral miRNAs could serve as positive or negative regulators in the virus lifecycle to keep the virus survive forever in its host.
Although functions of most viral miRNAs remain unknown, some specific viral miRNAs have been shown to autoregulate viral mRNAs or downregulate cellular mRNAs (24, 29). As reported, most of investigations on viral miRNAs are performed in cell lines (8, 30-32). The assays conducted in cell lines can be artificially controlled. However, the cells cultured in an artificial environment are not the cells in body, in which the immune system, the nervous system and the endocrine system do not exist. The findings in cell lines may not fully reflect the in vivo issues. In this study, the viral miRNAs were characterized in shrimp in vivo. The roles of viral miRNAs revealed in our study would be helpful to get insights into the molecular mechanisms in virus-host interactions in vivo.

Acknowledgements

This work was financially supported by the National Basic Research Program of China (2012CB114403), Project of Ministry of Agriculture, China (201103034) and Hi-Tech Research and Development Program of China (863 program of China) (2012AA092205-2).
References


24. BART microRNAs are produced from a large intron prior to splicing. Journal of Virology, 82 (18), 9094-9106.


Figure legends

Fig 1. The time-course expression profiles of viral miRNAs in virus-infected shrimp. The shrimp were infected with WSSV. At different times postinfection, the WSSV-miR-66 (A) and WSSV-miR-68 (B) were detected in hemocytes of WSSV-infected shrimp with Northern blots and quantitative real-time PCR. In Northern blots, the probes used were indicated at the right. U6 was used as a control. The numbers indicated the time points after WSSV infection in shrimp. As a control, an early gene of WSSV (wsv477) was included in the time-course Northern blots (C). The shrimp β-actin was used as a control.

Fig 2. Roles of viral miRNAs in virus infection in shrimp in vivo. AMO-WSSV-miR-66 and AMO-WSSV-miR-68 were respectively injected into WSSV-infected shrimp to silence the viral miRNA expression. As controls, AMO-WSSV-miR-66-scrambled and AMO-WSSV-miR-68-scrambled were included in the injection. At different times postinfection, the shrimp were subjected to Northern blot and real-time PCR analysis (A), detection of WSSV copies (B) and shrimp mortality analysis (C). U6 was used as a control. The numbers showed the time points after WSSV infection in shrimp. Each column or point represented the mean ± standard deviation from three independent assays. The significant differences between treatments were indicated with asterisks (*, P<0.05; **, P<0.01).

Fig 3. Interactions between viral miRNAs and viral genes. (A) Predicted target genes of WSSV-miR-66. The 3’UTRs of the wsv094 and wsv177 genes were targeted by WSSV-miR-66. (B) Interactions between viral WSSV-miR-66 and virus genes in...
insect cells. The insect High Five cells were co-transfected with WSSV-miR-66 and
EGFP, EGFP-wsv094-3’UTR, EGFP-wsv177-3’UTR, EGFP-wsv094-3’UTR-
mutation or EGFP-wsv177-3’UTR-mutation. At 48 h after co-transfection, the
fluorescence of cells was examined. The sequences targeted by viral miRNA were
underlined. (C) The effects of WSSV-miR-66 on viral gene expressions. The relative
fluorescence intensity of cells was determined. (D) Predicted target genes of
WSSV-miR-68. The 3’UTRs of the wsv248 and wsv309 genes were targeted by
WSSV-miR-68. (E) Interactions between WSSV-miR-66 and virus genes in insect
cells. The insect High Five cells were co-transfected with WSSV-miR-68 and EGFP,
EGFP-wsv248-3’UTR, EGFP-wsv309-3’UTR, EGFP-wsv248-3’UTR- mutation or
EGFP-wsv309-3’UTR-mutation. At 48 h after co-transfection, the fluorescence of
cells was detected. The sequences targeted by viral miRNA were underlined. (F) The
effects of WSSV-miR-68 on WSSV gene expressions. Statistically significant
differences between treatments were indicated by asterisks (*P<0.05).

Fig 4. Pathways mediated by viral miRNAs in virus infection. (A) Silencing of
expressions of WSSV genes targeted by viral miRNAs. The sequence-specific siRNA
was injected into shrimp to knock down the expression of WSSV-encoded wsv094,
wsv177, wsv248 or wsv309 gene. As a negative control, the siRNA-scrambled was
included in the injections. WSSV only was used as a positive control. To reveal the
effects of viral miRNAs on the expressions of viral genes, AMO-WSSV-miR-66 and
AMO-WSSV-miR-68 were respectively injected into shrimp. At different times
postinfection, the mRNA of wsv094, wsv177, wsv248 or wsv309 in shrimp hemocytes
was detected by quantitative real-time PCR with gene-specific primers and probe. The numbers showed the times postinfection. The treatments were indicated at the top. In all panels, plotted data referred to the means ± standard deviations of triplicate assays and asterisks represented statistically significant differences (*, p<0.05; **, p<0.01).

(B) Detection of WSSV copies in shrimp hemocytes with quantitative real-time PCR. The hemocytes of shrimp treated with siRNA were subjected to real-time PCR analysis. The significant differences between treatments were indicated with asterisks (*, p<0.05; **, p<0.01). WSSV only was used as a positive control. (C) Effects of WSSV gene silencing on the mortality of WSSV-infected shrimp. The shrimp were injected with various siRNA. Then the shrimp mortality was monitored everyday. WSSV only was used as a control. Each point represented the mean ± standard deviation from three independent assays. (D) Mode for the viral miRNA-mediated pathways in virus infection in vivo.