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The role of shrimp miR-965 in virus infection

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ABSTRACT

RNAi, mediated by microRNAs (miRNAs), has attracted increasing attention for its important role in cross-talk between host and virus. However, the role of host miRNA in the virus infection *in vivo* has not been intensively investigated. In this study, the effects of a shrimp miRNA (miR-965) on the white spot syndrome virus (WSSV) infection were characterized. The results indicated that the expression of miR-965 was significantly upregulated in shrimp in response to the WSSV challenge, suggesting its involvement in the virus infection. The miR-965 silencing led to significant increases of WSSV copies and virus-infected shrimp mortality, while the miR-965 overexpression resulted in the decreased WSSV copies and virus-infected shrimp mortality, indicating that miR-965 played a negative role in the WSSV infection. The further data revealed that miR-965 inhibited the virus infection by targeting the viral *wsv240* gene, an important gene required for the WSSV infection in shrimp. The results demonstrated that miR-965 could promote the shrimp phagocytosis against virus infection by targeting the shrimp *ATG5* (autophagy related 5) gene. Therefore, our findings presented novel evidence to better understand the anfractuous host-virus interactions *in vivo*.

1. Introduction

Innate immunity, the first immune defense line, depends on cell-based immunity such as phagocytosis and apoptosis [1]. During immune responses, the host gene expressions, as well as the pathogen gene expressions, are accurately regulated. Growing evidences indicate that microRNAs, through targeting the genes associated with immunity or pathogens, play important roles in the regulation of immune response [2,3]. MicroRNAs, noncoding RNA molecules, are transcribed from the genome and subsequently processed by Drosha and Dicer nucleases [5]. With the combination of the 3'-untranslated regions (3′UTRs) of mRNAs, miRNAs can cleave targeted mRNAs or inhibit the mRNA translation, thus downregulating the corresponding gene expressions [4,5]. Since the first miRNA lin-4 was discovered in *Caenorhabditis elegans*, many microRNAs have been found to be involved in the regulation of biological processes including cell differentiation, development and immune response [6,7].

In the virus-host interactions, the host miRNAs and the virus miRNAs are required to regulate the gene expression. It is found that the expression profiles of host miRNAs are changed in response to the virus infection. For instances, in human immunodeficiency virus (HIV)-infected or influenza A H1N1-infected patient, the expression patterns of miRNAs of peripheral blood mononuclear cells have changed compared with normal subjects [8–10], suggesting the involvements of host miRNAs in the virus-host interactions. As reported, host miRNAs can be against the replication of virus [11–13]. When host cells are infected with herpes simplex virus (HSV), miR-101 is accumulated [11]. The accumulation of miR-101 attenuates the activity of AT5B, leading to the decrease of ATP production, thus inhibiting the virus replication. In the *Heliothis virescens* ascovirus-infected *Helicoverpa zea* fat body cells, the expression level of host miR-24 is altered [12]. The combination of miR-24 with 3′UTR of viral DNA-dependent RNA polymerase gene results in the inhibition of virus replication. The host miR-32 in HeLa cells has been reported to restrain the retrovirus primate foamy virus (PFV) infection, while decreasing the production of miR-32 can come about opposite results [13]. Fang et al. have compared the expression patterns of miRNAs of peripheral blood mononuclear cells in patients and normal people [14]. The results show that the expression of miR-29 is significantly upregulated in the virus-infected cells and miR-29 can increase the **c**yclo-oxygenase-2 (COX2) mRNA level by directly targeting the DNA

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methyltransferase gene. COX2 helps the binding of NF-κB to the promoter of IFN- λ1 to augment the IFN- λ1 expression, strengthening the antiviral function of the host finally [14]. It is reported that the synthetic miRNA mimcs of miR-196, miR-296, miR-351, miR-431 and miR-448, which target the virus genes, lead to the reduction of hepatitis C virus (HCV) RNA accumulation [15]. In shrimp, the host miR-7 can directly target the viral early gene *wsv477* and decrease the *wsv477* expression level, resulting in the inhibition of white spot syndrome virus (WSSV) infection [16]. On the other hand, the viral miRNAs play very important roles in the interactions between virus and host [17,18]. The viral miRNAs can target the host or virus genes to facilitate the virus infection.

In our previous study [19], the data indicated that miR-965 was involved in the virus infection and the shrimp phaogocytosis against the WSSV proliferation. To further reveal the mechanism of miR-965 in the virus infection, miR-965 was characterized in the present study. The results showed that miR-965 could function in the antiviral immunity of shrimp by targeting the virus gene *wsv240* and the host phagocytosis-related gene *ATG5*.

2. Materials and methods

2.1. Shrimp culture and virus infection

The shrimp *Marsupenaeus japonicus* (8–12 cm in length) were raised in tanks filled with seawater at 20 °C. For each treatment, 20 individuals were maintained. To make sure the shrimp were virus-free before experiments, randomly selected three shrimp from each treatment were subjected to PCR with WSSV-specific primers (5′-TATTGTCTCTCCTGACGTAC-3′ and 5′-CACATTCTTCACGAGTCTAC-3′). Subsequently 0.1 ml of WSSV solution at 10^5copies/ml was injected into the muscle tissue of shrimp. The WSSV solution was obtained from virus-infected shrimp [20]. At different time post-infection, the shrimp hemocytes were collected for later use.

2.2. Northern blotting

The small RNA was extracted from shrimp hemocytes with the mirVana miRNA isolation kit (Ambion, USA). After separation on a denaturing 15% polyacrylamide gel containing 7M urea, RNAs (30 μg/treatment) were transferred to a Hybond-N+ nylon membrane, followed by UV cross-linking. The membrane was prehybridized in DIG (digoxigenin) Easy Hyb granule buffer (Roche, Basel, Switzerland) for 0.5 h at 42 °C and then hybridized with DIG-labeled miR-965 (5′-GAGGGGAAAAGCCAT ACGCTTA-3′) or U6 (5′-GGGCCATGCTAATCTTCTCTGTATCGTT-3′) probes at 42 °C overnight. Subsequently the detection was performed with the DIG High Prime DNA labeling and detection starter kit II (Roche).

2.3. The silencing or overexpression of miR-965 in shrimp

The synthesized miR-965 (miR-965-mimic) (5′-TAAGCG-TATGGCTTTTCCCC TC-3′) and anti-miR-965 oligonucleotide (AMO-miR-965) (5′-AAAGCCATACGC TTA-3′) were dissolved in siRNA solution (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). As controls, the sequences of miR-965-mimic and AMO-miR-965 were randomly scrambled, generating miR-965-mimic-scrambled (5′-TATCGCA-TAGGCTTTTCCC CTC-3′) and AMO-miR-965-scrambled (5′-TTTCGGTATGCGAAT-3′) respectively. To get appropriate dosages of miR-965-mimic and AMO-miR-965 for injecting shrimp, miR-965-mimic or AMO-miR-965 at different concentrations (1 nM, 2 nM, 4 nM, 8 nM, 10 nM and 20 nM) was injected into shrimp, followed by the detection of miR-965 expression level with quantitative real-time PCR. It was revealed that the miR-965 expression could be inhibited by miR-965-mimic or overexpressed by AMO-miR-965 at 4 nM or more than 4 nM in shrimp. Therefore 4 nM/shrimp of miR-965-mimic or AMO-miR-965 was used in this study. Then shrimp were injected with WSSV (10^5 copies/ml) and miR-965-mimic (4 nM/shrimp) or AMO- miR-965 (4 nM/shrimp). MiR-965-mimic-scrambled and AMO- miR-965-scrambled were included in the injections. WSSV alone was used as a control. At 0 h, 24 h, 36 h and 48 h after infection, three shrimp were selected at random for each treatment. The shrimp hemocytes were collected for later use. At the same time, the cumulative mortality of shrimp was examined everyday. All the experiments were biologically repeated three times.

2.4. Analysis of WSSV copies with quantitative real-time PCR

The genomic DNA of WSSV was extracted with a SQ tissue DNA (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instruction. Subsequently the WSSV copies in shrimp were quantified with WSSV-specific primers (5′-TTGGT TTCATGCCCGA-GATT-3′ and 5′-CCTTGGTCAGCCCCTTGA-3′) and a TaqMan probe (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′). The PCR mixture (10 μl) containing 5 μl Premix Ex Taq (TaKaRa, Japan), 200 ng DNA template, 0.2 μl of 10 μM primers, and 0.2 μl of 10 μM TaqMan fluorogenic probe with a final concentration of 0.2 μM. The real-time PCR was conducted following the previous study [16]. The PCR condition was 95 °C for 1min, 45 cycles of 30 s at 95 °C and 30 s at 52 °C, and 30 s at 72 °C. A DNA fragment of 1400 bp from the WSSV genome constructed into a plasmid was used as the internal standard of real-time PCR. The quantification of WSSV copies by real-time PCR was biologically repeated three times.

2.5. Prediction of genes targeted by miR-965

In order to predict the viral gene targeted by miR-965, four algorithms including Targetscan, miRanda, Pictar and miRInspector [16] were used to predict the targeted sites in 3′UTRs (untranslated regions) of viral and shrimp genes.

2.6. Plasmid construction

To evaluate the direct interaction between miR-965 and *wsv240* gene, the *wsv240* 3′UTR was cloned into the pIZ/V5-His vector (Invitrogen, USA) which carried the sequence of enhanced green fluorescent protein (EGFP). As a control, the sequence of *wsv240* 3′UTR matching the seed sequence of miR-965 was mutated to yield the EGFP-Δwsv240-3′UTR construct. All the recombinant plasmids were confirmed by sequencing.

2.7. Cell culture, transfection and fluorescence assays

Insect High Five cells (Invitrogen) were cultured with Express Five serum-free medium (SFM) (Invitrogen) containing L-glutamine (Invitrogen) at 27 °C. To determine the dosage of miR-965-mimic, 10 pM, 50 pM, 100 pM, 200 pM, 500 pM or 1000 pM of miR-965-mimic was transfected into cells. Then the miR-965 expression in cells was detected with quantitative real-time PCR. It was indicated that miR-965-mimic at 100 pM or more could overexpress miR-965 in cells. MiR-965-mimic at 100 pM was used in this study. The cells were co-transfected with 2 μg of EGFP, EGFP-wsv240-3′UTR or EGFP-Δwsv240-3′UTR and 100 pM of miR-965-mimic or miR-965-mimic-scrambled. All the miRNAs were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). At 36 h after co-transfection, the fluorescence intensity of cells was evaluated with a Flex Station II microplate reader (Molecular Devices, USA) at 490/510 nm excitation/emission (Ex/Em). The experiments were biologically repeated three times.