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Eriocheir sinensis microRNA-7 targets crab *Myd88* to enhance white spot syndrome virus replication

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ABSTRACT

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression at the posttranscriptional level. In this study, the function of microRNA-7 (miR-7) in host-virus interaction was investigated. Replication of White spot syndrome virus (WSSV) was enhanced with the overexpression of miR-7 and inhibited with the downregulation of miR-7 by using anti-miRNA oligonucleotide AMO-miR-7. The target gene of miR-7 was predicted using bioinformatics methods. Results showed that crab myeloid differentiation factor 88 (*Myd88*) could be targeted by miR-7. When the expression of *Myd88* was knocked down by sequence-specific siRNA, WSSV copies in crabs were significantly increased. Further findings revealed that knockdown of *Myd88*, *Tube*, or *Pelle* inhibited the expressions of interleukin enhancer-binding factor 2 homolog (*ILF2*) and interleukin-16-like gene (*IL-16L*). While *ILF2* was silenced, *IL-16L* expression was inhibited. The overexpression of miR-7 inhibited the expressions of *ILF2* and *IL-16L*. Moreover, when *ILF2* or *IL-16L* was silenced, WSSV copies in crabs were increased. Thus, the up-regulated expression of miR-7 during WSSV challenge suppressed the host Myd88-ILF2-(IL-16L) signaling pathway in crabs and enhanced WSSV replication. Our study indicated that WSSV utilized crab miR-7 to enhance virus replication during infection.

1. Introduction

Chinese mitten crab (*Eriocheir sinensis*) is the most widely cultured crustacean species in China and other Asian countries due to its high economic value [1]. With intensive culture development and environmental deterioration in recent years, this aquatic species frequently suffers from various diseases caused by bacteria (especially spiroplasma), fungi, and viruses, which results in catastrophic economic losses to crab aquaculture [2]. White spot syndrome virus (WSSV) belongs to the genus *Whispovirus* of the family *Nimaviridae*; it is the pathogenic agent responsible for the massive mortalities of penaeid shrimp worldwide [3]. One recent study first reported that WSSV is one of the most devastating pathogens threatening the crab aquaculture industry, with a cumulative mortality of 100% observed within 10 days post-WSSV injection [4]. Therefore, the anti-viral immune defense of Chinese mitten crab needs to be studied.

In mammals, following the recognition of microbial derived pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), innate immunity is activated to trigger the release of inflammatory cytokines and type I interferons (IFNs) through the activation of NF-κB and interferon regulatory factor (IRF)3/7 [5,6]. As an adaptor molecule, myeloid differentiation factor 88 (Myd88) participates in the activation of the Toll signaling pathway [7]. It has a Toll-interleukin 1 receptor domain, which is essential for its interaction with TLRs [8,9]. Moreover, Myd88 interacts with interleukin-1 receptor-associated kinase (IRAK) family members through the amino terminal death domain (DD) [10,11]. It also has an important function in death signal transduction, regulation of apoptosis, and inflammatory response [12]. In *Drosophila*, Myd88 (dMyd88) is important for the resistance against fungal and Gram-positive bacterial infections [13]. In penaeid shrimp (*Fenneropenaeus chinensis*), the Myd88-dependent signaling pathway might be involved in defense against bacterial and WSSV infections [14]. In our previous study, we found that EsMyd88 is involved in the transcription of antimicrobial peptide genes by binding

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to EsTube in crabs challenged with *Vibrio parahaemolyticus* infection [15]. Therefore, Myd88 is a pivotal component of the animal's innate immune system [16].

Invertebrates rely on innate immunity for protection against microbial infections. However, to maintain the health of the organism, the immune signal pathway should be strictly regulated by multiple negative regulators [17]. Among these negative regulators, microRNAs (miRNAs) play important roles in negatively regulating the expression of genes of the innate immune signal pathway. Myd88 is a key component of the TLR/Toll signal pathway, and the *Myd88* gene is always targeted by miRNAs. In vertebrates, many researches have focused on miRNAs targeting Myd88. In fish, miR-3570 can negatively regulate the NF-κB signaling pathway by targeting the *Myd88* gene [18]. In addition to its negative regulation in Myd88-mediated anti-bacterial immunity, miRNA targeting Myd88 also induces the inhibition of tumor growth [19]. To escape from host immune system attack, viruses utilize host miRNAs to target Myd88 [20].

Studies on miRNAs regulating *Myd88* in invertebrates are limited, and only one study reports that *Myd88* is the target gene of miRNAs in *Drosophila* development [21]. Up to date, no report has focused on miRNAs targeting *Myd88* during virus infection in invertebrates. In an attempt to explore the involvement of miRNA in WSSV infection, the present study investigated the crab miRNA (microRNA-7, miR-7) targeting *Myd88*. The results showed that miR-7 targeting *Myd88* inhibited the host Myd88-ILF2 (interleukin enhancer-binding factor 2 homolog)-(interleukin-16-like gene [IL-16L]) signaling pathway and enhanced WSSV replication in crabs.

2. Materials and methods

2.1. Animals, WSSV challenge, and tissue collection

Crabs (*E. sinensis*) of approximately 40 g in body weight were purchased from an aquatic product market in Nanjing, Jiangsu Province, China. The samples were cultured in a group of 20 individuals at 25 °C. Three crabs of each group were randomly selected for PCR detection of WSSV with WSSV-specific primers (5′-TATTGTCTCTCCTGACGTAC-3′ and 5′-CACATTCTTCACGAGTCTAC-3′) to ensure that the crabs were free from viruses before the experiments. The virus-free crabs were then infected with 100 μl of WSSV solution, with each crab injected intramuscularly with 10^5 copies/ml. To serve as control, crabs were inoculated with the same volume of PBS. At different post-infection times (0, 2, 4, 6, 12, 24, 36, 48, 60, and 72 h), hemolymphs were collected from treated crabs by using a syringe with an equal volume of anticoagulant [22], and they were then centrifuged at 2000 rpm at 4 °C for 10 min to harvest the hemocytes. The heart, hepatopancreas, gills, muscles, intestines, nerves, and eyestalk from five untreated crabs were also collected. All samples were stored at −80 °C for later use.

2.2. Detection of miR-7 using real-time PCR

Total RNAs were extracted from aforementioned tissues by using the mirVana miRNA Isolation Kit according to the manufacturer's instructions (Ambion, USA). cDNA for miRNA qRT-PCR was then synthesized using the All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia™, USA), following the manufacturer's protocol. The reaction was carried out in a total volume of 12.5 μl, containing 100 ng small-molecule RNA, 0.5 μl of 2.5 U/μl poly(A) polymerase, 0.5 μl RTase Mix, 0.5 μl of 5 × PAP/RT buffer, and ddH_2O (RNase/DNase-free) up to 12.5 μl. Reverse transcription was conducted at 37 °C for 60 min and 85 °C for 5 min. Real-time PCR was carried out according to the protocol of the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia™, USA). The reaction was carried out in a final volume of 10 μl, which contained 5 μl of 2 × All-in-One qPCR Mix, 1 μl of All-in-One™ miRNA qPCR Primer (2 μM), 1 μl of Universal Adaptor PCR Primer (2 μM), 1 μl of first-strand cDNA (diluted 1:5), and 2 μl of ddH_2O. The thermal cycling profile used was 94 °C for 30 s followed by 40 cycles at 94 °C for 5 s and 60 °C for 30 s. A miR-7 special primer (5′-CGCGCGCAAGAAATCACTAATC-3′) was used in qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The first-strand cDNA synthesis was performed by using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara, Dalian, China). qRT-PCR amplification reactions were carried out in a final volume of 10 μl, which contained 2 μl 5 × PrimeScript Buffer, 0.5 μl PrimeScript^{RT} Enzyme Mix I, 0.5 μl Oligo dT Primer (50 μM), 0.5 μl Random 6 mers (100 μM), 500 ng total RNA, and RNase-free ddH_2O. The cDNA mix was incubated at 37 °C for 15 min, terminated by heating at 85 °C for 5 s, and diluted to 1:10 with diethyl pyrocarbonate-treated water. *GAPDH* was amplified with sequence-specific primers (5′-CTGC CCAAAACATCATCCCATC-3′ and 5′-CTCTCATCCCCAGTGAAAT CGC-3′). Three replicated experiments were used to ensure the validity and accuracy of the experimental results.

2.3. Overexpression or silencing of miR-7 in crabs

Based on the sequence of miR-7 (5′-AAGAAAUCACUAAUCUGC CUA-3′), miR-7 was synthesized using an *in vitro* transcription T7 kit for siRNA synthesis (Takara, Japan). The sequence of miR-7 was scrambled to generate the control miR-7-scrambled (5′-UCCGAAUGACUACUAAU CAAA-3′). The synthesized miRNA was dissolved in miRNA solution (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). To overexpress miR-7, miRNA (15 μg) and WSSV (10^5 copies/ml) were co-injected into virus-free crabs at a volume of 100 μl per crab. At 16 h after the co-injection, miRNA (15 μg) (100 μl/crab) was injected into the same crabs. As controls, miR-7-scrambled, WSSV alone (10^5 copies/ml), and PBS were included in the injections.

To knock down miR-7 expression, an anti-miRNA oligonucleotide (AMO) was injected into the WSSV-infected crabs. AMO-miR-7 (5′-TAGGCAGATTAGTGATTTCTT-3′) was synthesized (Sangon Biotech, Shanghai, China) with a phosphorothioate backbone and a 2′-O-methyl modification at the 6th, 16th, and 18th nucleotides. AMO (10 nM) and WSSV (10^5 copies/ml) were co-injected into virus-free crabs at a volume of 100 μl per crab. At 16 h after the co-injection, AMO (10 nM) (100 μl/crab) was injected into the same crabs. As controls, AMO-miR-7-scrambled (5′-GATATGGTGTATGTACTTATC-3′), WSSV alone (10^5 copies/ml), or physiological saline (0.85% NaCl) was injected into crabs.

For each treatment, 20 crabs were used. At different post-infection times (0, 24, 36, and 48 h), hemocytes from five crabs were randomly collected for each treatment and subjected to subsequent analysis. All the experiments were biologically repeated three times.

2.4. Quantitative analysis of WSSV copies by real-time PCR

The genomic DNA samples were extracted from hemocytes of five crabs/treatment using a SQ tissue DNA kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instruction. To quantify the WSSV copies in crabs, the extracted DNA was subjected to quantitative real-time PCR. qRT-PCR was performed using WSSV-specific primers (5′-TTGGTTTCAGCCCGAGATT-3′ and 5′-CCTTGGTCAGCCCCTTGA-3′) and WSSV-specific TaqMan probe (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′). The plasmid containing a DNA fragment of 1400 bp from the WSSV genome was used as the internal standard of real-time PCR [23]. The 25 μl PCR reaction solutions 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 PCR program started with the pre-denaturalization stage at 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.