Fish & Shellfish Immunology 56 (2016) 397–401

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

The Dorsal/miR-1959/Cactus feedback loop facilitates the infection of WSSV in *Litopenaeus vannamei*

Xiaopeng Xu [a, b, d], Jia Yuan [a, b], Linwei Yang [a, b], Shaoping Weng [a, b, d], Jianguo He [a, b, c, d, **], Hongliang Zuo [a, c, d, *]

[a] State Key Laboratory for Biocontrol/MOE Key Laboratory of Aquatic Product Safety, School of Life Sciences, Sun Yat-sen University, Guangzhou, PR China
[b] Institute of Aquatic Economic Animals and Guangdong Provice Key Laboratory for Aquatic Economic Animals, Sun Yat-sen University, Guangzhou, PR China
[c] School of Marine Sciences, Sun Yat-sen University, Guangzhou, PR China
[d] South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, Guangzhou, PR China

ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
14 July 2016
Accepted 31 July 2016
Available online 2 August 2016

Keywords:
Litopenaeus vannamei
microRNA
miR-1959
Dorsal
Cactus
NF-κB pathway
Feedback loop

ABSTRACT

miR-1959, a novel microRNA identified from *Litopenaeus vannamei*, mediates a positive feedback loop between Dorsal and Cactus that can continuously maintain the activation of the NF-κB pathway. It has been known that miR-1959 is involved in antibacterial immunity in shrimp, but its function in antiviral responses is still unknown. In this study, we focused on the role of miR-1959 in infection of white spot syndrome virus (WSSV), the major viral pathogen in shrimp worldwide. The expression of miR-1959 in shrimp hemocytes, gill, and hepatopancreas was significantly up-regulated upon WSSV infection. Dual-luciferase reporter assays demonstrated that miR-1959 could enhance the activity of the promoter of WSSV immediate early gene *ie1*. *In vivo* experiments also showed that inhibition of miR-1959 led to decrease of the mortality of WSSV-infected shrimp and the genome copies of WSSV in tissues, meanwhile the expression of WSSV *ie1* and VP28 genes was down-regulated. In contrast, increase of the miR-1959 level in shrimp by injection of miR-1959 mimics produced opposite results. These suggested that the Dorsal/miR-1959/Cactus feedback loop could favor the infection of WSSV in shrimp. Thus, our study helps further reveal the interaction between WSSV and shrimp immune system.

1. Introduction

As the major aquaculture shrimp in the world, Pacific white shrimp, *Litopenaeus vannamei,* faces huge threats from various devastating pathogens, which bring great economic losses to shrimp farming industry. White spot syndrome virus (WSSV) is the major viral pathogen in shrimp, which causes up to 100% mortality within 3–10 days post infection [1,2]. It has been known that WSSV can utilize the host immune signaling pathway to favor its own infection [3]. Many immediate early genes of WSSV, in particular the *ie1* (WSV069), can be directly induced by the shrimp NF-κB family protein Dorsal. In addition, the *WSSV449* gene shares 15.7–19.4% sequence identity to Tube, an important signaling adaptor of the Toll/NF-κB pathway in shrimp [4], and could be involved in the activation of the NF-κB pathway. On the other hand, the NF-κB pathway is an essential regulator of the shrimp immunity [4,5]. Many effector genes of the NF-κB pathway, such as antimicrobial peptides (AMPs) and C-type lectins (CTLs), play important roles in antiviral responses [6]. These suggest the complex relation between WSSV and the shrimp immune system.

microRNAs are a group of 18–25 nt small non-coding RNAs that regulate gene expression at the post-transcriptional level by interfering with mRNA translation and stability through a mechanism involving specific binding to mRNA based on perfect or near-perfect complementary base pairing [7,8]. Functional studies of miRNAs have shown their regulatory functions in various biological processes including growth, development, apoptosis, and immunity [9]. Previously, we identified a novel microRNA from *L. vannamei*, named miR-1959, which targets Cactus, the *L. vannamei* homologue of the inhibitor of NF-κB (IκB) gene [10]. As a cytoplasmic inhibitor of Dorsal, Cactus interacts with Dorsal to

* Corresponding author. School of Marine Sciences, Sun Yat-sen University, Guangzhou, 510275, PR China.
** Corresponding author. School of Life Sciences, School of Marine Sciences, Sun Yat-sen University, Guangzhou, 510275, PR China.
E-mail addresses: lsshjg@mail.sysu.edu.cn (J. He), zuohliang@mail.sysu.edu.cn (H. Zuo).

http://dx.doi.org/10.1016/j.fsi.2016.07.039
1050-4648/

form cytoplasmic complexes to prevent the nuclear translocation of Dorsal, leading to inhibition of the NF-κB pathway [11]. miR-1959 can indirectly activate Dorsal through inhibiting Cactus, and up-regulate the expression of downstream target genes of the NF-κB pathway. Interestingly, the activated Dorsal can in turn bind the promoter of miR-1959 to induce its expression. These suggest that miR-1959 mediates a positive feedback loop between Dorsal and Cactus, which can enhance the activation of shrimp NF-κB pathway. It has been known that the Dorsal/miR-1959/Cactus feedback loop positively regulates the antibacterial immunity in shrimp [10]. However, its roles in antiviral responses of shrimp are still unknown. In the current study, we focused our attention on the function of the miR-1959 mediated feedback loop in virus infection and demonstrated that it could facilitate the infection of WSSV. This may provide an in-depth insight into the miRNA-mediated immune mechanism in shrimp, and help further reveal the interactions between WSSV and *L. vannamei*.

2. Materials and methods

2.1. Shrimp and immune challenge

Healthy *L. vannamei* (~5 g) were raised in a shrimp farm in Zhanjiang City, Guangdong Province, China, 5% of which was randomly detected by PCR to ensure shrimps were free of WSSV. Shrimps were acclimated in 0.8-m^3 tanks filled with air-pumped seawater (2.5% salinity) at ~27 °C and cultured at least a week for acclimation before experiments.

The WSSV stock used in this study was prepared as mentioned in previous study [12]. For miRNA expression analysis, each shrimp was injected with 50 μL PBS (control) or PBS containing 10^6 copies newly extracted WSSV. The hemocytes, gill, and hepatopancreas were sampled at 4, 12, 24, 36, 48, 72, 96, and 120 h post injection, each from 12 shrimps.

The cholesterol modified miRNA mimics agomiR-1959 and inhibitor antagomiR-1959 and their controls (agomiR-NC and antagomiR-NC) were chemosynthesized in GenePharma (China). Shrimps ($n = 55$) were individually injected with 0.4 nmol mimics, inhibitors or their controls, and 48 h later were challenged with 10^6 copies WSSV diluted in 50 μL PBS containing 0.4 nmol mimics, inhibitors or their controls. Gills were sampled from 12 shrimps at 24, 48, 72, 96, 120, and 168 h post infection (hpi). Simultaneously, cumulative mortality was recorded every 4 h in a parallel experiment.

2.2. Real-time PCR for miRNA, mRNA, and DNA

To analyze the expression of miR-1959, total RNA was extracted from hemocytes, gill, and hepatopancreas after WSSV injection, and cDNA was then synthesized with primers of 1959-RT and U6-RT (Table 1) using PrimeScript RT reagent kit (Takara, Japan), respectively. Stem-loop real-time PCR was performed with primers of 1959-qRTF/1959-qRTR or U6-qRTF/U6-qRTR on a LightCycler 480 System (Roche, Swiss) as previously described [10]. To detect the expression of *ie1* and VP28, cDNA was synthesized with primers of Oligo d(T)18 together with Random 6 (Takara, Japan). Real-time PCR was performed with primers of *ie1*-qRTF/*ie1*-qRTR and VP28-qRTF/Vp28-qRTR with parameters as previously described [4]. The EF-1α gene was detected using primers EF-1α-qRTF/EF-1α-qRTR as internal control. Primers used were listed in Table 1. To investigate the genome copies of WSSV, total DNA was extracted from muscle after WSSV injection and real-time PCR was performed following a previous method [4].

2.3. Dual-luciferase reporter assays

To analyze the effect of miR-1959 on the promoters of *ie1* and PEN4 genes in the presence of Dorsal and Cactus, dual-luciferase reporter assays were performed following method modified from that of Zuo et al. [10]. Briefly, *Drosophila* S2 cells plated on a 96-well plate were co-transfected with pAC-Dorsal, pAC5.1-Cactus-wt/pAC5.1-Cactus-Mut, agomiR-1959/agomiR-NC, and PGL3-ie1/PGL3-PEN4. The Renilla luciferase plasmid pRL-TK (Promega, USA) (50 ng) was used as the internal control. Cells co-transfected with pAC5.1-GFP, pAC-Dorsal and PGL3-ie1/PGL3-PEN4 were also used as a positive control. At 48 h post transfection, the activity of luciferases was detected using a dual-luciferase reporter assay system (Promega, USA). The results shown were representative of three independent experiments, and for each assay, data were obtained from 8 repeated wells.

2.4. Statistical analysis

The relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method [13]. For statistical analysis, all data were presented as mean ± SD. Student *t*-test was used to calculate the comparisons between groups of numerical data. For mortality analysis, data were subjected to statistical analysis using MedCalc statistical software (Mariakerke, Belgium) to generate the Kaplan-Meier plot (log rank $\chi 2$ test).

3. Results

Emerging evidence has unveiled that miRNAs play a critical role in the regulation of innate immunity in both vertebrates and invertebrates [14,15]. As an important regulator of gene expression, many miRNAs that are involved in immune responses are highly responsive to pathogen stimulations [16]. In the previous study, it has been demonstrated that the expression of miR-1959 could be significantly up-regulated after the stimulation of *Vibrio parahaemolyticus*, *Staphylococcus aureus*, LPS, and poly (I:C) [10]. In this study, using real-time PCR, we investigated the miR-1959 expression during WSSV infection (Fig. 1). In hemocytes, compared with the PBS-mock challenged control, the miR-1959 level was obviously suppressed at 4–24 hpi, while after 36 hpi it was significantly up-regulated with a peak at 96 hpi (Fig. 1A). This may indicated that the miR1959 expression could be inhibited during the first stage of WSSV infection in hemocytes. As hemocytes are an important component of the shrimp immune system, the inhibitory effect might be caused by the host immune responses against WSSV infection, which needs further evidence. In contrast, in both gill and hepatopancreas, the expression of miR-1959 was periodically up-regulated after 4 dpi (Fig. 1B and C), suggesting that the Dorsal/miR-1959/Cactus feedback loop was continuously activated during WSSV infection in these tissues.

It has been known that Dorsal could directly interact with the promoter of WSSV *ie1* gene to regulates its transcription [3]. miR-1959 could enhance the activity of Dorsal through inhibiting the expression of Cactus. Next, we investigated the effect of the miR-1959 mimics on the expression of *ie1* gene in the presence of Dorsal and 3′UTR tagged Cactus gene using dual-luciferase reporter assays (Fig. 2). The results demonstrated that Dorsal could efficiently activate the promoters of *ie1* gene and the positive control antimicrobial peptide gene PEN4. Compared with the control GFP, both the 3′UTR tagged and the mutated 3′UTR tagged Cactus (Cactus-wt and Cactus-mut) could significantly reduce the activation effect of Dorsal. miR-1959 attenuated the inhibitory effect of Cactus-wt on the activity of both *ie1* and PEN4 promoters, whereas it did not show significant effects on that of Cactus-mut. This