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Short communication

MicroRNA-146a promotes red spotted grouper nervous necrosis virus (RGNNV) replication by targeting TRAF6 in orange spotted grouper, *Epinephelus coioides*

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ABSTRACT

MicroRNA-146a (miR-146a) has been demonstrated to function as a negative regulator of cellular immune responses against pathogens in mammals, however, little information focused on its functions in lower vertebrates. In this study, we investigated the regulatory roles of orange spotted grouper, *Epinephelus coioides* miR-146a during red spotted grouper nervous necrosis virus (RGNNV) infection. During RGNNV infection in grouper spleen (GS) cells, the endogenous expression level of miR-146a and tumor necrosis factor receptor-associated factor 6 (TRAF6) significantly increased along with the infection time. Overexpression of miR-146a significantly facilitated viral infection, evidenced by the increased transcription of viral CP and RdRp genes, while miR-146a knockdown by specific inhibitors decreased RGNNV replication. Using pMIR-REPORT Luciferase system, we found that the 3′ untranslated region (UTR) of grouper TRAF6 could be specifically targeted by miR-146a. Further studies showed that its downstream target gene pro-inflammatory cytokines, including TNF-α, IL-8 and IL-1β, were all significantly decreased in miR-146a mimic transfected cells, but increased in miR-146a inhibitors transfected cells during RGNNV infection. Thus, our results suggested and verified that holding the level of miR-146a exerted crucial roles in RGNNV infection through TRAF6-mediated inflammatory response.

1. Introduction

MicroRNA (miRNA) represents a class of ~22 nt non-coding small RNA, and functionally regulates genes expression through binding to the untranslated regions (UTRs) of target mRNAs to introduce degradation or suppression of translation [1,2]. It has been demonstrated that miRNA participated in various physiological processes, including proliferation, differentiation, infection, immune response and tumorigenesis [3–5]. Currently, there have been nearly thirty thousand entries of mature miRNAs registered in miRBase, in-depth exploration of these non-coding RNAs, especially the functional identification and targets screening, has been urgent tasks to be settled. As one of the most reported miRNA, miR-146a participated in the regulation of multiple cellular processes. During Japanese encephalitis virus infection, miR-146a suppressed NF-κB activation and abrogated Jak-STAT pathway by targeting TRAF6, IRAK and STAT1 genes, and finally facilitated viral infection [6]. In human macrophages responding to mycobacterium infection, induction of miR-146a negatively regulated TNF-α release, further exploration found that cellular TARF6, IRAK1 and PTGS2 could be targeted by miR-146a [7]. Although functions of miR-146a in mammalian virus infection were well studied, few literature focused on the roles of miR-146a in fish virus infection and pathogenesis.

Grouper, *Epinephelus* sp, as one of the most important maricultured fish species in tropical and subtropical regions, always suffered deeply from the invasion of infectious pathogens recently, such as bacteria including *Vibrio vulnificus, streptococcus* [8,9], parasites including *Cryptocaryon irritans, trypanosoma* [10,11], and viral pathogens including iridovirus and nervous necrosis virus [12–14]. Our previous

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studies have identified orange spotted grouper, *E. coioides* encoded miRNAs by Solexa deep sequencing method, and validated the miRNA expression profiles after Singapore grouper iridovirus (SGIV) infection by miRNA microarray assays [15]. Based on these information, we confirmed that grouper derived miR-146a could be induced by SGIV infection and in turn suppressed virus induced cells apoptosis, thus maintaining a favorable intracellular environment to maximize the viral replication [16]. While whether miR-146a could affect fish RNA virus replication still remained uncertain.

In the current study, we investigated the immune regulatory mechanism of grouper miR-146a during red spotted grouper nervous necrosis virus (RGNNV) infection, and demonstrated that grouper miR-146a facilitated RGNNV infection through attenuating TRAF6 mediated cellular antiviral immunity. Our data will contribute greatly to understanding the immune regulation roles of fish miRNAs during virus infection.

2. Materials and methods

2.1. Cell and virus

Grouper spleen (GS) cells were maintained in our laboratory and grown in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco, USA) at 25 °C [17]. Red spotted grouper nervous necrosis virus (RGNNV) was prepared as described previously [18] and stored at -80 °C in our laboratory until use. RGNNV infection of GS cells was operated at an MOI of ~0.1.

2.2. miRNA transfection

Grouper miR-146a mimics, miR-146a specific inhibitors (in-146a) and a negative control of scrambled miRNA were purchased from Ribobio (Guangzhou). To overexpress or knock down miR-146a in vitro, 100 pmol miR-146a mimics or 100 pmol miR-146a inhibitors was transfected into GS cells by using Lipofectamine® RNAiMAX transfection reagent (Invitrogen, USA). Scrambled miRNA was transfected as control.

2.3. Bulge-LoopTM miRNA qRT-PCR

Total RNA of RGNNV-infected GS cells was purified using TRIzol reagent (Ambion, USA). miR-146a specific reverse transcription was performed by a customized miRNA RT kit (RiboBio, Guangzhou). Briefly, 1 μg total RNA was incubated at 70 °C for 10 min to be denatured and then cooled on ice. A 20 μl reaction containing 4 μl 5x RT buffer, 5 pmol miR-146a specific RT primer, 1 μl RT polymerase and the denatured RNA was carried out by incubation at 42 °C for 60 min, followed by 70 °C for 10 min and 4 °C cooling down. miR-146a levels were determined by real-time quantitative PCR using Bulge-LoopTM miRNA qRT-PCR starter kit (RiboBio, Guangzhou) and performed on the LightCycler® 480 Detection System (Roche, Switzerland). PCR amplification was performed inquadruplicate wells using cycling parameter: 95 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 56 °C and 15 s at 72 °C. 18S rRNA was detected as internal reference.

2.4. qPCR analysis of viral genes and cytokines

To assess the expression conditions of RGNNV Cp (coat protein), RdRp (RNA-dependent RNA polymerase) and cytokines TNF-α, IL-8 and IL-1β after transfection of miR-146a mimics or inhibitors, total RNA of RGNNV-infected GS cells was purified by using SV Total RNA Isolation System (Promega, USA) and reversely transcripted with random primer (TOYOBO, Japan) at 24 h and 48 h after RGNNV infection. SYBR Green I Master was used to carry out qRT-PCR in a LightCycler® 480 Detection System (Roche, Switzerland). In brief, a 10 μl reaction contained 5 μl SYBR qPCR Mix, 0.4 μl forward primer and 0.4 μl reverse primer of detecting gene, 3.2 μl PCR-grade water and 1 μl diluted cDNA template. PCR amplication was performed in quadruplicate wells using the following parameter: 95 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 56 °C and 15 s at 72 °C. 18S rRNA was detected as internal reference.

2.5. miR-146a target prediction analysis

3′ UTR regions of grouper immune-related genes were collected from NCBI database and the grouper ESTs sequence database in our laboratory. RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) was used to predict the potential targets of miR-146a and energy threshold was set ≤ -15 kcal/mol.

2.6. Construction of target-contained luciferase plasmids

Wild-type untranslated region (UTR) of TRAF6 that contained the predicted target of miR-146a was amplificated with forward primer 5′-GACTAGTTAACCAAACTCATATCTATAGG-3′ and reverse primer 5′-CAAGCTTTTAAAACAAAAATCACAC-3'. Mutant UTR (mUTR) with missense mutation of predicted target sites was amplificated with forward primer 5′- GACTAGTTAACCAAACTCATTAGATAAGGAGGATCG-3′ and reverse primer 5′- CAAGCTTTTAAAACAAAAATCACAC-3'. These two UTR fragments were respectively cloned into the pMIR-REPORT Luciferase vector (Ambion, USA) between the *Spe*I and *Hind*III restriction enzyme sites. Recombinant plasmids were verified by DNA sequencing (Invitrogen, Guangzhou).

2.7. Luciferase activity assay

To clarify whether miR-146a could affect gene expression through bonding to the predicted target, recombinant luciferase plasmid was cotransfected with scrambled miRNA or miR-146a mimics into GS cells, empty pMIR-REPORT vector was cotransfected as negative control with scrambled miRNA or miR-146a mimics. Ranilla luciferase vector (Promega, USA) was cotransfected as normalization in each group. 24 h after transfection, cells were collected and luciferase activity was measured using the Luciferase Assay System (Promega, USA) and fluorescence microplate reader (PerkinElmer, USA).

2.8. Statistical analysis

All experiments were performed at least three times independently, and data were expressed as mean ± SD. Statistical differences between groups were analyzed by the Student's *t*-test. $p < 0.05$* was considered statistically significant.

3. Results

3.1. RGNNV infection increased the expression of miR-146a and TRAF6 in GS cells

To clarify whether RGNNV infection affected the endogenous expression of miR-146a, GS cells were infected with RGNNV, and the expression patterns of miR-146a were evaluated by real-time PCR. As shown in Fig. 1A, miR-146a expression increased significantly during RGNNV infection. In detail, the expression level of miR-146a increased about 2-fold than that of the control as early as 2 h after RGNNV infection, and reached a peak about 5-fold at 48 h p.i., suggested that miR-146a might be involved in RGNNV infection. Meanwhile, the expression levels of TRAF6 were also significantly increased during RGNNV infection (Fig. 1B).

3.2. miR-146a positively regulated RGNNV infection in GS cells

To explore the effect of miR-146a on RGNNV infection, miR-146a