Fish and Shellfish Immunology 94 (2019) 258–263

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

miR-21 targets *jnk* and *ccr7* to modulate the inflammatory response of grass carp following bacterial infection

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ARTICLE INFO

Keywords:
miR-21
Inflammation
Grass carp

ABSTRACT

Grass carp septicemia is a systemic inflammatory response that develops following a bacterial infection. The hyperinflammatory state develops could lead to septic shock and lethality. There is increasing evidence that microRNAs are involved in the regulation of the inflammatory response. In the present study, miR-21 was confirmed to be involved in the inflammatory response following infection with *Aeromonas hydrophila* and LPS stimulation. Both *jnk* and *ccr7* were identified as target gene of miR-21 by overexpression, inhibition, and dual luciferase reporter assays experiments. Meanwhile, miR-21 targets the *jnk* and *ccr7* to modulate downstream pro-inflammatory factors *tnf-α, il-1β, il-6,* and *il-12*. Our results provide a theoretical basis for exploring the molecular mechanism of grass carp miR-21 regulating inflammation.

1. Introduction

MicroRNA (miRNA), first discovered in *Caenorhabditis elegansin*, is a type of endogenous non-coding small RNA (sRNA) and 21–25 nucleotides (nt) long (typically 22 nt) [1,2]. miRNAs are regulatory proteins bind to 3′ untranslated regions (UTRs) of target mRNAs to control their translation and stability [3,4]. Moreover, there is increasing evidence that miRNAs are involved in the regulation of the inflammatory response [5]. Previous studies indicated that miR-21 may play a key role in regulating the innate immune response in grass carp. Furthermore, miR-21 was shown to target multiple genes associated with the inflammatory against *Aeromonas hydrophila* infection [6].

A large number of experiments have shown that miR-21 has an important influence on the formation and development of various tumors, cardiovascular disease, and inflammatory reactions [7,8]. The ability of miR-21 to enhance the vitamin D-dependent antimicrobial pathway provides a potential therapeutic strategy to intervene in infectious disease [9]. miR-21 expression is significantly up-regulated during macrophage activation, which inhibits LPS-induced production of inflammatory factors [10]. The previous study suggested that c-Jun N-terminal kinase (*jnk*) and CC-chemokine receptor 7 (*ccr7*) genes may be targets of miR-21 [11]. JNK is a type of serine/threonine protein kinase involved in apoptosis, inflammatory conditions, and cytokine production [12–14]. The inactivated JNK leads to the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin1 beta (IL-1β), IL-6, and IL-12 [15]. CCR7is a lymph node homing receptor that belongs to the G protein-coupled receptor family originally found to be primarily expressed in memory T cells, B cells, and the surface of mature dendritic cells [16]. CCR7 mediates the cellular inflammatory response through specific binding to its ligand, CCL19, and further promotes the release of inflammatory factors [17].

Grass carp (*Ctenopharyngodon idella*) is a species of fish with the largest reported global production in aquaculture at over six million tons per year [18]. However, the large-scale intensive culture of grass carp is more easily susceptible to death and disease due to pathogen infections. In particular, disease outbreaks associated with bacterial infections (e.g., *Aeromonas hydrophila*) have caused high mortality rates among populations of grass carp, resulting in reduced production and considerable economic losses [19].

Based on previous study, miR-21 is potential regulator of anti-bacterial activity in grass carp kidney. In *vitro*, *Ctenopharyngodon idella* kidney (CIK) cells have constantly served as an infection model to study infection mechanisms, grass carp antibacterial, and antiviral innate immune systems [20,21]. In the present study, we demonstrated that the miR-21 significantly increased in CIK cells following *A. hydrophila* infection and LPS stimulation. Furthermore, *jnk* and *ccr7* were

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https://doi.org/10.1016/j.fsi.2019.09.022
Received 13 March 2019; Received in revised form 4 September 2019; Accepted 8 September 2019
Available online 09 September 2019
1050-4648/

predicted to be the target genes of miR-21, which was confirmed with a dual-luciferase reporter assay. The regulatory role of miR-21 on the pro-inflammatory cytokines via the targeting the *jnk* and *ccr7*genes should enhance our understanding of the role of miRNAs on regulating the immune response in fish.

2. Materials and methods

2.1. Culture and infection of C. idella kidney cells

CIK cells were provided by the China Center for Type Culture Collection (CCTCC, Wuhan, China) and maintained in a 50-mL culture dish containing 6 mL nutrient solution consisting of M199 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 100 U/ml penicillin-streptomycin (GIBCO, USA). The cells were incubated at 28°Cand 5% CO_2. Before the experiment, CIK cells (2×10^6 cells/mL) were seeded in 6- or 24-well plates for 24 h.

For experiment, all wells were grouped into three classes with as equal number of cells as possible. One class was infected with 100 μL *A. hydrophila* (10^3 cfu/mL). The second cell class was exposed to 10 μg/mL of lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 (Sigma-Aldrich, USA) and the last class was left uninfected (negative control). After 30 min of exposure, all cells then washed with PBS and maintained in M199 supplemented with 10% FBS, 100 U/ml penicillin-streptomycin. Infected cells were cultured for the indicated time (4 h, 12 h, 24 h, and 36 h) and then lysed in TRIzol (Thermo Fisher Scientific, USA) for total RNA isolated. All treatment groups were analyzed in quadruplicate.

2.2. RNA extraction and cDNA acquisition

Total RNA from each sample was extracted using TRIzol reagent. RNA was quantified by Nanodrop 2000 (Thermo Fisher Scientific) and visualized using agarose gel electrophoresis to ensure purity. 1 μg of the total RNA was reverse transcribed using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). For miRNA, cDNA was prepared using aMir-X miRNA First Strand Synthesis Kit (Clontech Palo Alto, USA). This kit adds poly (A) to the 3′ end of miRNAs and performs reverse transcription. cDNA synthesis was directed by oligo-dT ligated to specific 5' sequence.

2.3. Real-time PCR

The qPCR analysis was conducted on CFX96 (Bio-Rad Laboratories, Hercules, CA, USA) using TB Green Advantage qPCR Premix (Clontech). qPCR was performed in a final volume of 25 μL and each reaction included 9.5 μL ddH_2O, 12.5 μL of TB Green Advantage Premix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer for mRNA or mRQ 3' primer (Clontech) for miRNA (10 μM), and 2 μL of cDNA preparation. The following qPCR cycling conditions were 1 cycle at 95 °C for 10 s; 40 cycles at 95 °C for 5 s, 60 °C for 20 s, and dissociation curve analysis was performed after each assay to determine target specificity. miR-101 for miRNA and *β-actin* for mRNA were used to normalize the relative expression of miRNA and mRNA, respectively [22]. The primers used for qPCR are listed in Table 1. Each experimental group was performed in quadruplicate.

2.4. Dual-luciferase reporter assays

The 3′ UTR of *jnk* or *ccr7* fragment containing presumptive miR-21 target sequences was amplified by PCR. The amplicon was cloned into the SacI/Xbal sites of the dual luciferase pmirGLO vector (Promega Wisconsin, USA) to generate pmirGLO-jnk or pmirGLO-ccr7. The recombinant plasmid was confirmed by Sanger sequencing.

To test whether miR-21 directly targeted *jnk* or *ccr7*, pmirGLO-jnk or pmirGLO-ccr7 and miR-21 agomir (a miR-21 agonist) or miR-21 antagomir (a miR-21 inhibitor) (GenePharma, Shanghai, China) were transfected into CIK cells using Lipofectamine 3000 reagent (Invitrogen Carlsbad, USA), according to the manufacturer's instructions.

After 24–48 h, reporter luciferase activity was measured with a Dual-Luciferase reporter assay system (Promega). Relative luciferase activity values were obtained using firefly luciferase activity/renilla luciferase activity.

2.5. Statistical analysis

Data regarding the relative gene expression were obtained using the $2^{-\Delta\Delta CT}$ method, and comparisons between the various groups were analyzed by a one-way ANOVA followed by a Duncan multiple comparison test. All data are presented as the mean ± SE; significant differences between groups were determined using a two-tailed Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$ was considered to indicate statistical significance.

3. Results

3.1. The characteristics of miR-21

The precursor and mature miR-21 sequences were obtained from our small RNA transcriptome data of grass carp [21]. In this study, the miR-21 sequences were used to construct a phylogenetic tree, which revealed that miR-21 is divided into three distinct branches: 1) fish; 2) reptiles; and 3) mammals (Fig. 1A). Both the pre-miR-21 hairpin structure presented in Fig. 1B and mature miR-21 was found to be conserved across multiple species (Fig. 1C). However, the bases located at positions 16 and 22 of the mature miR-21 sequence are “G" in fish and “A" in mammals (Fig. 1B–C).

3.2. miR-21 regulation in response to A. hydrophila and LPS

To further explore the potential involvement of miR-21 in response to pathogen exposure, we investigated the expression of miR-21 in CIK cells following *A. hydrophila* infection and LPS stimulation. The qRT-PCR analysis showed that the miR-21 expression pattern was similar between the *A. hydrophila* and LPS challenged CIK cells. The expression of miR-21 was decreased after infected or stimulated, and lowest expression was found at 36 h or 48 h time point (Fig. 2).

3.3. Identification of the miR-21 target genes

Based on previous data, *jnk* and *ccr7* were predicted to be the potential target genes of miR-21 [6]. The putative binding site for miR-21 was predicted on the 3′ UTR of *jnk* and *ccr7* (Fig. 3B and C). The minimum free energy (mef) of the miR-21 with the target genes was-26.7 or −25.2 kcal/mol for *jnk* and *ccr7*, respectively.

Our results showed that the miR-21, *jnk* and *ccr7* were time-dependent expression pattern upon *A. hydrophila* challenge. miR-21 (Fig. 2A) showed significantly decreased after bacterial infection, *jnk* (Fig. 3D) and *ccr7* (Fig. 3E) showed significantly increased. Our data showed an inverse correlation between the expression of miR-21 and target genes (*jnk* and *ccr7*). The miR-21 with *jnk* and *ccr7* observed an inverse expression correlation. The miR-21 overexpression and inhibition effect were presented in Figs. S1A and S1B. The *jnk* and *ccr7* was inhibited by miR-21. Moreover, when miR-21 was inhibited, the level of *jnk* and *ccr7* expression increased (Fig. 3F and G). These results indicate that both *jnk* and *ccr7* were regulated by miR-21.

The direct interaction between miR-21 with *jnk* and *ccr7* was confirmed using a luciferase reporter system (Fig. 3A). The miR-21 agomir and pmirGLO-jnk or pmirGLO-ccr7 were co-transfected into CIK cells. The luciferase activity of pmirGLO-jnk and pmirGLO-ccr7 was significantly reduced (Fig. 3H and I). Overall, the above data fully

Table 1
Primer sequences used in the present study.

Gene Name	Primer sequence		Application
	Forward primer (5′-3′)	Reverse primer(5′-3′)	
miR-21	TAGCTTATCAGACTGGTGTTGGC	mRQ 3′ primer	qPCR
miR-101a	TACAGTACTGTGATAACTGAAG	mRQ 3′ primer	qPCR
jnk	ACAGGTTCGCATCCATCAGC	GTCTGGAGCACAAGGCATTG	qPCR
pmirGLO-jnk	*CGAGCTCG*GCTGCACCTGTGCTAGTGAG	*CTCTAGAG*CTCTTCCCTGCAGACTCGGA	Vector construction
ccr7	CTCTGCCCATCGCTGTCGCT	AGGCGAATGTTGTCCTTGTC	qPCR
pmirGLO-ccr7	*CGAGCTCG*CTGGTCTTGGCTGCGAAGTT	*CTCTAGAG*AGGTACGGTTGGAGCCCTTT	Vector construction
tnf-α	ACCCTGAAGTCTCTAATAAAACCC	GTGGCTCATATGCACAATGTCT	qPCR
il-1β	TCTCCTCGTCTGCTGGGTGT	CAAGACCAGGTGAGGGGAAG	qPCR
il-6	ATTAGAAACTGAACGGCGAG	TCTGAATGTTGTGCTGGGAT	qPCR
il-12	GAAAAGGAGGGGAAGATGAA	ACACTGGGCTGGTAGGAGTT	qPCR
β-actin	CATGCCATCCTCCGTCTGGA	GGATGGCTGGAACAGAGCCT	qPCR

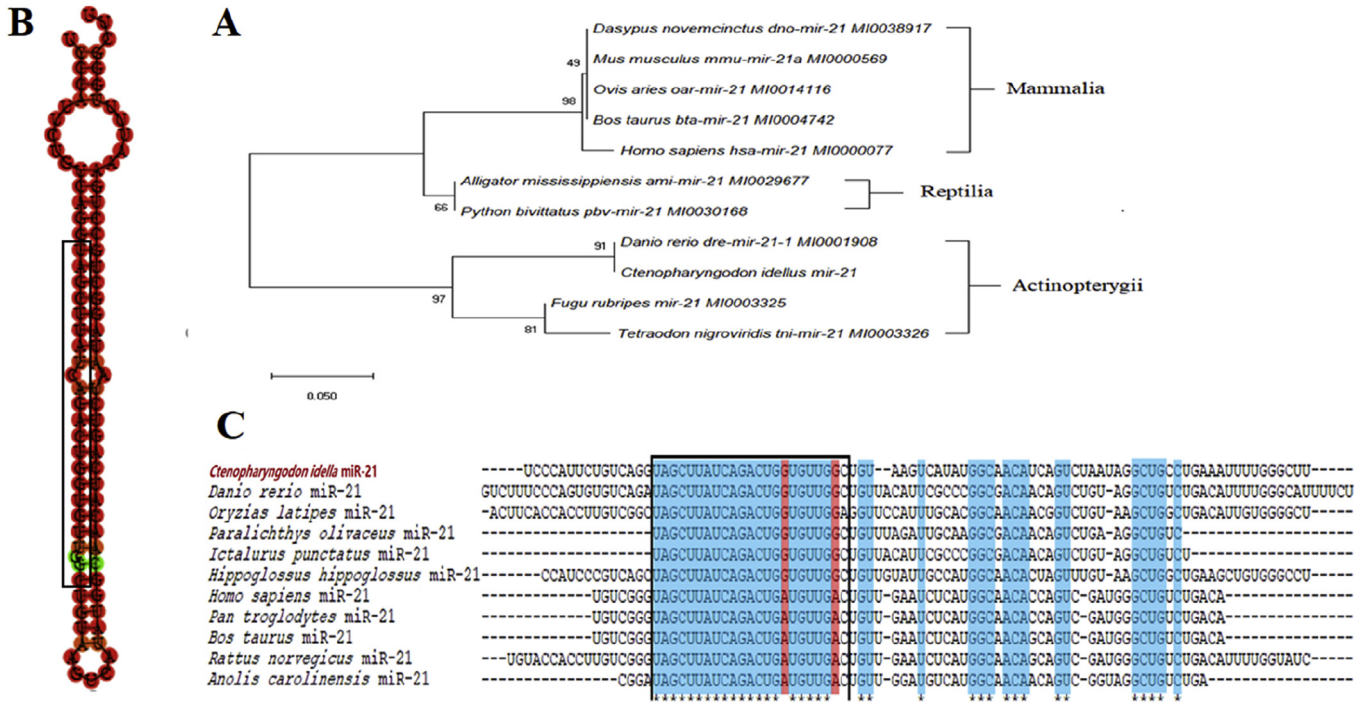

Fig. 1. Characteristic analysis of miR-21. (A) The phylogenetic tree of precursor miR-21 were constructed by the neighbor-joining (NJ) algorithm by using the Poisson model in the MEGA5 package. The accession numbers of the 10 sequences used in this analysis are listed in figure. (B) The hairpin structures of pre-miR-21 from grass carp and mature miR-21 sequences are indicated in the black box. (C) miR-21 precursor sequences from different species.

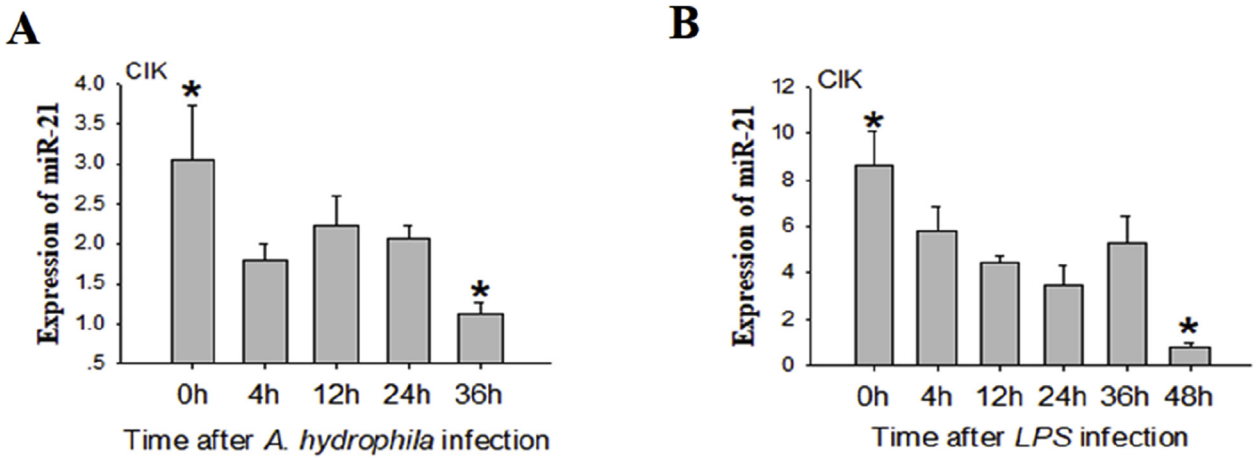

Fig. 2. miR-21 is involved in the inflammatory response. miR-21 expression profiles in CIK cells after *A. hydrophila* infection (A) and LPS stimulation (B). In all panels, the data are representative of three independent experiments (*$p < 0.05$; **$p < 0.01$).

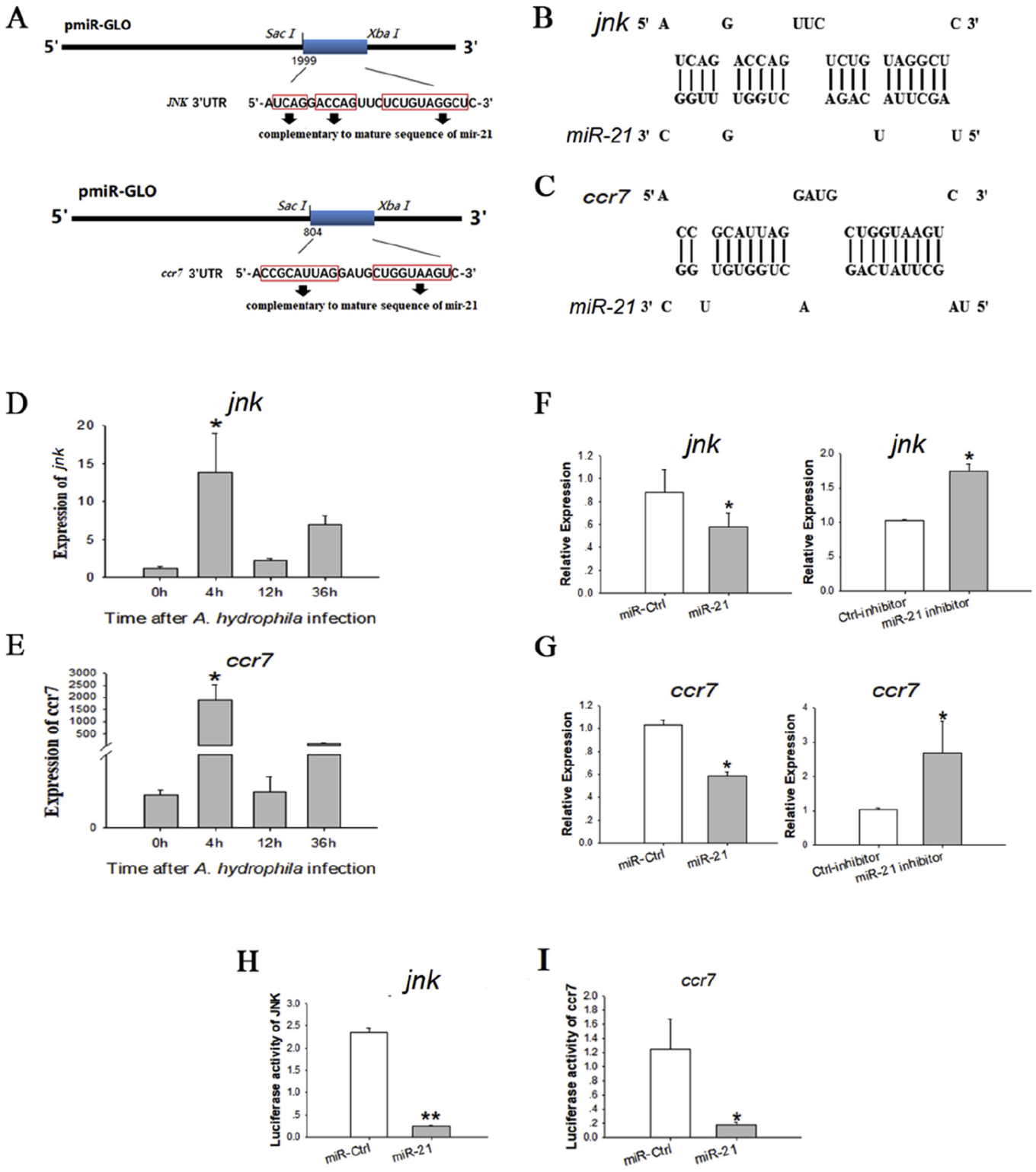

Fig. 3. Prediction and validation of miR-21 target genes. Schematic diagram of the SacI/Xbal sites (A) and binding site of miR-21 to 3′ UTR of *jnk* (B) and *ccr7* (C). Expression profiles of *jnk*(D) and *ccr7* (E) in CIK cells following *A. hydrophila* infection. The expression of *jnk* (F) and *ccr7* (G) were changed by miR-21 overexpression or inhibitor. CIK cells were transfected with miR-21 agomir or Ctrl, along with the pmirGLO-jnk (H) or pmirGLO-ccr7 (I) for 24 h, and the luciferase activity was determined. The data were normalized to *β-actin*. In all panels, the data are representative of three independent experiments (*$p < 0.05$; **$p < 0.01$).

demonstrate that *jnk* and *ccr7* are the target genes of miR-21.

3.4. miR-21 regulates downstream inflammatory factors

We next assessed whether miR-21-mediated targeting of *jnk* and *ccr7* could down-regulate inflammatory factors (*tnf-α, il-1β, il-6,* and *il-12*) in CIK cells. The level of *tnf-α, il-1β, il-6,* and *il*-12 mRNA was found to be significantly increased ($P < 0.05$) in the miR-21 knockdown group compared with the control (Fig. 4).

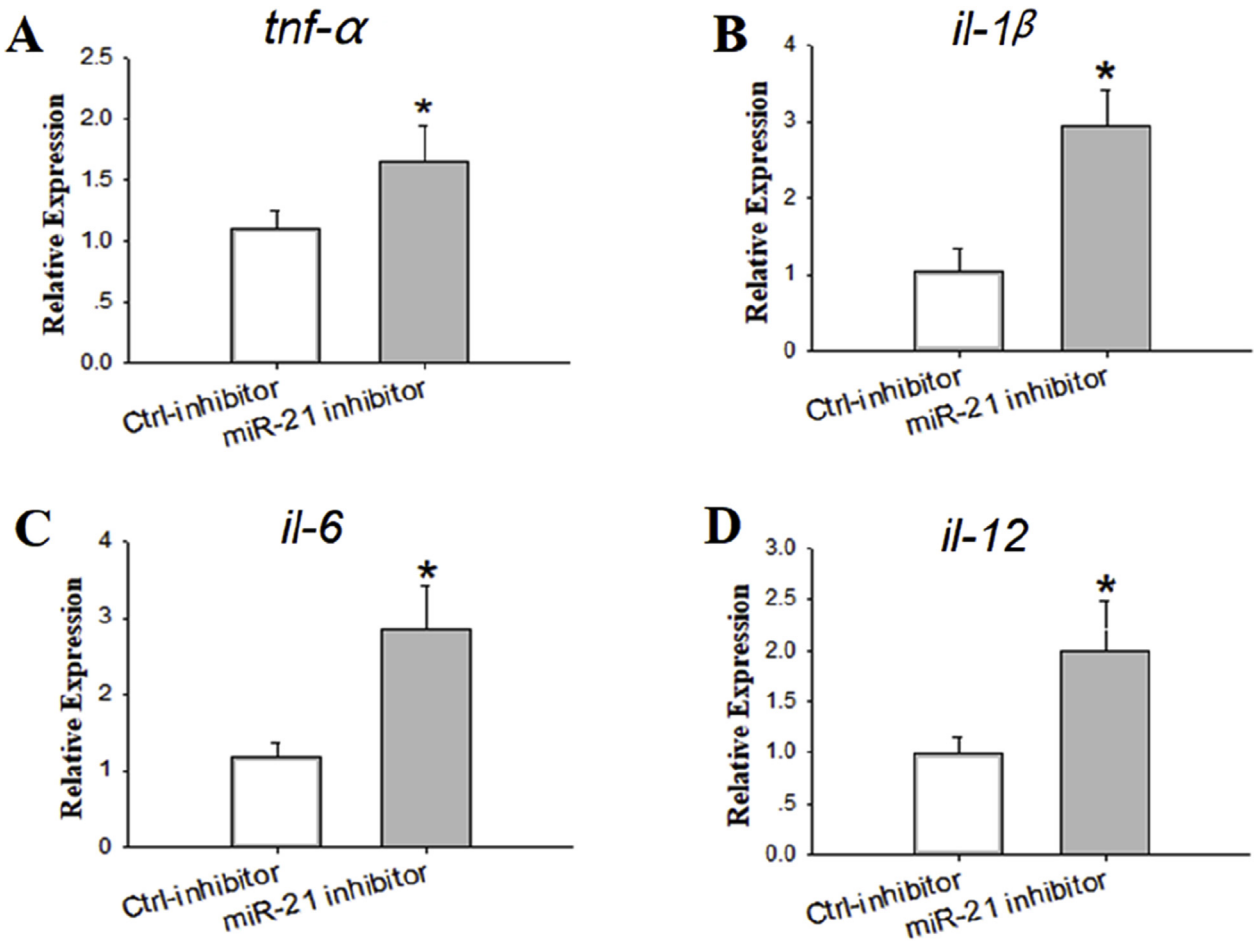

Fig. 4. miR-21-mediated regulation of downstream inflammatory factors. CIK cells were transfected with either the miR-21 antagomir or Ctrl. After 48 h, the level of *tnf-α* (A), *il-1β* (B), *il-6* (C), and *il-12* (D) expression was determined by qPCR. The data were normalized to *β-actin*. In all panels, the data are representative of three independent experiments (*$p < 0.05$; **$p < 0.01$).

4. Discussion

A. hydrophila is a Gram-negative motile bacillus widely distributed throughout aquatic environments that can cause motile aeromonad septicemia (MAS), which results in substantial economic losses to freshwater fish farming worldwide [6]. Thus, the identification of genes that can enhance the tolerance of fish to bacterial infection is of critical importance to the aquaculture industry. Studies have shown that miRNAs are involved in various physiological processes in fish, including growth, development, and reproduction [23,24]. Moreover, the dysregulation of miRNAs might impact the expression of genes that are involved in chemokine/cytokine signaling, TLR signaling, inflammatory response, complement and coagulation cascades, as well as B- and T-cell mediated immune responses [25–27]. In our previous study, miR-21 was predicted to target several immune-related genes and was significantly downregulated following *A. hydrophila* infection in resistant grass carp [11]. In the present study, we found that miR-21 plays an important role in infected grass carp and decreased endogenous miR-21 expression could strengthen the inflammatory response. These findings provide further evidence that miRNAs are important players in the immune response of bacterially infected grass carp.

miR-21 is one of miRNAs that was initially discovered in mammals. Similar to other miRNAs, mature miR-21 is completely conserved in mammals and encoded by a single gene [28]. Previous studies have demonstrated that the sequence complementary to the miR-21 seed sequence (UAGCUUAU) is completely conserved in the 3′UTR of the *PDCD4* gene of humans, mice, and zebrafish. The conservation of miR-21 and *PDCD4* suggests that the regulation of *PDCD4* expression by miR-21 may be evolutionarily conserved among vertebrates [29]. The conservation of the miR-21 sequence can also be observed in our present experimental results, as only a difference in one or two bases was found in fish, reptiles and mammals. Moreover, based on the biochemical analysis of the miR-21 5′-flanking sequence, a highly conserved region containing three activator protein 1 (AP-1) sites was revealed [30].

In the present study, we found that the expression of miR-21 was significantly altered in CIK cells infected with *A. hydrophila* and stimulated with LPS. Moreover, the same trend was observed in response to both *A. hydrophila* and LPS, indicating that miR-21 is involved in the regulation of the inflammatory response. miR-21 can be activated by inflammatory mediators and has been found to be expressed both in DSS-induced colitis and macrophages [31]. Related studies in human colitis have found that an miR-21 deficiency has different effects on the colonic inflammatory symptoms induced by a variety of factors, suggesting that miR-21 may play multiple roles within the same disease [32].

Our data indicate that miR-21 regulates the inflammatory response by targeting *jnk* and *ccr7*. Inflammation is induced following the activation of a complex series of signaling cascades that are induced in response to pathogen infection and tissue damage [33,34]. In the inflammatory response caused by bacterial sepsis, a number of pro-inflammatory factors regulated by AP-1 are abnormally elevated (i.e., *tnf-α, il-1β, il-6,* and *il-12*). Moreover, the binding of *CCR7* to its ligand

(*CCL19/CCL21*) leads to the initiation of mitogen-activated protein kinase (MAPK), which regulates inflammation [35].

In summary, our findings indicate that miR-21 regulates the proinflammatory response of *A. hydrophila*-infected grass carp, as the downregulation of miR-21 promoted proinflammatory cytokine expression. Furthermore, we identified *jnk* and *ccr7* to be possible targets of miR-21 involved in regulating the inflammatory response of grass carp. Therefore, our results provide critical insight into the immunogenic role of miR-21 in teleost; however, the precise mechanisms associated with the role of miR-21 require further characterization.

Acknowledgements

This study was supported by National Natural Science Foundation of China Youth Project (Grant No. 31802285), China's Agricultural Research System (Grant No. CARS-45-03), and Project of Shanghai Engineering and Technology Center for Promoting Ability (Grant No. 16DZ2281200).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.022.

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