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Up-regulated of miR-8159-5p and miR-217-5p by LPS stimulation negatively co-regulate TLR1 in miiuy croaker

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ABSTRACT

Toll-like receptors (TLRs) are a group of pattern-recognition receptors which play vital roles in ligand recognition and activation of the innate immune response. As an important member of TLRs family, TLR1 is mainly responsible for PAMPs from bacteria and play a pivotal role in sensing microbial products. Recent studies revealed that TLR1 could perceive LPS stimulation and transfer signals to activate the NF-κB pathway, whereas ligands and signaling pathway of TLR1 are still unclear in fish. Growing evidence has shown that miRNAs (microRNAs) play as negative regulators in controlling the diverse of biophysical and biochemical processes at the post-transcriptional level. In this study, we used a combination of bioinformatics and experimental techniques to exhibit that both miR-8159-5p and miR-217-5p were the direct negative regulators of TLR1 in miiuy croaker. Furthermore, dual-luciferase reporter assays showed that combining miR-8159-5p and miR-217-5p exhibited a greater negative regulatory effect on TLR1 than only miR-8159-5p or miR-217-5p. Additionally, we also demonstrated that the expression of both the two miRNAs could be up-regulated by LPS stimulation in either LPS-stimulation spleen tissue or LPS-treated cultured macrophage, which indicating that miR-8159-5p and miR-217-5p could be induced by LPS and may be as the negative regulators of TLR1 involved in the immune response to LPS stimulation. These results would enhance our understanding of the miRNA regulation in fish TLR signaling pathways.

1. Introduction

Innate immunity is the first line of host defense mechanisms against invading microbial pathogens. Recognition microbial pathogen is an essential step in innate immune responses, which conducted by germline-encoded pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are the most characterized PRRs, and total 13 TLRs (TLR1-13) have been identified in mammals since the first mammalian homologue of Toll (now known as TLR4) was cloned in humans in 1997 (Medzhitov et al., 1997; Hopkins and Sriskandan, 2005). TLRs initiate innate immune responses by sensing pathogen-associated molecular patterns (PAMPs), such as lipoproteins, lipopeptides, LPS, flagellin, dsRNA, ssRNA, and CpG DNA motifs and activating signaling pathways (Janeway and Medzhitov, 2002; Akira and Takeda, 2004; Akira et al., 2006). TLRs signaling occur through lots of adaptor proteins, liking MyD88 or TRIF, which consequently induce pro-inflammatory cytokine and type I interferon (IFN) production (Takeda and Akira, 2004). During this signaling cascade process, many mechanisms for the negative regulation of TLR signaling have been described. For example, TRIM38 negatively regulates TLR3-mediated type I interferon signaling by targeting TRIF inducing IFN-β (Xue et al., 2012), and Integrin CD11b negatively regulates TLR induced inflammatory responses by targeting MyD88 and TRIF (Han et al., 2010). Additionally, recent studies have revealed that IRF4 could also interact with MyD88 and act as a negative regulator of TLR signaling (Negishi et al., 2005).

MicroRNAs (miRNAs), an abundant class of non-coding RNAs, have recently showed as important regulators of gene expression at the post-transcriptional level. MiRNAs are endogenous, 18 to 25 nucleotides (nt) long, single-stranded RNAs and they are generally highly conserved in eukaryotes. Growing evidences indicate that these small RNAs could induce mRNA degradation or protein translation repression mainly through binding to the 3'-untranslated region (UTR) of target mRNAs (Bartel, 2009). Since the first miRNA *lin-4* was discovered in *Caenorhabditis elegans* (Wightman et al., 1993), lots of miRNAs have been found to involve in the

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regulation of diverse biological processes, including development, differentiation, immune activation, in organisms such as animals, plants, and even some DNA viruses (Bartel, 2009; Fabian et al., 2010; Yates et al., 2013). It is well known that TLR-signaling pathways is required for hosts to eliminate invading pathogens, so identifying miRNAs that can regulate TLR expression is of vital importance. Presently, many of miRNAs have been shown to regulate TLR expression in mammal. Let-7i, the let-7 miRNA family, targets the 3′UTR of TLR4 in human biliary epithelial cells (O'Hara et al., 2010), and down-regulates TLR4 expression. Another TLR-induced miRNA, the myeloid-specific miR-223, can regulate the expression of both TLR4 and TLR3 in granulocytes (Johnnidis et al., 2008). Additionally, recent report shows that miR-26a can negatively regulate the TLR3 signaling pathway through targeting TLR3 expression in rat macrophages (Jiang et al., 2014). Moreover, level of miR-105 has been found in primary human oral keratinocytes and TLR2 mRNA has been shown to be regulated by miR-105 (Benakanakere et al., 2009). However, the miRNAs involved in regulating TLR-signaling pathways is rarely reported in fish (Cui et al., 2016; Wang et al., 2016b), and these findings in mammal open up a broad perspective of the regulation of TLR-signaling pathway for fish.

Miiuy croaker (*Miichthys miiuy*), a member of the Sciaenidae family, is an economically important marine fish. As its fast growth, strong resistance and economic value, the artificial breeding of miiuy croakers is thriving in China. Evidences indicate that the miiuy croaker has been studied in-depth from transcriptome (Che et al., 2014; Chu et al., 2015), genome (Xu et al., 2016c) to functional genes (Zhu et al., 2013; Wang et al., 2016a; Shu et al., 2016), which left miiuy croaker to be a new model for studying the immune system or immune response in fish. In mammals, TLR1 dimerizes with TLR2 to recognize triacylated lipopeptides and lipoprotein, while TLR2 dimerizes with TLR6 to recognize diacylated lipopeptides. The recognization can activate the transcription factor NF-κB to induce inflammatory cytokines (e.g., TNF-α and IL-6) through recruitment of TIRAP and MyD88 (Jin et al., 2007; Alexopoulou et al., 2002). The TLR1 subfamily in mammals includes four members, TLR1, TLR2, TLR6, and TLR10, whereas TLR6 and TLR10 were missed in fish (Rebl et al., 2010). This condition leaves TLR1 the most probable co-worker of TLR2 in fish. Recently, TLR1 firstly characterized in miiuy croaker was demonstrated to play vital roles in LPS infection and transmit this stimulation signal to activate the NF-κB pathway via MyD88 and TIRAP in miiuy croaker (Xu et al., 2016b). In this study, miR-8159-5p and miR-217-5p involved in the regulation of TLR1 after lipopolysaccharide (LPS) stimulation were identified and characterized with dual luciferase reporter assay. Meanwhile, the expression profile of the two miRNAs responding to LPS stimulation was conducted *in vitro* and *in vivo*. These results would enrich the knowledge of the miRNA regulation TLR-signaling pathways in fish.

2. Material and methods

2.1. Sample and challenge

Healthy miiuy croakers (~750 g) were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China) and raised in aerated seawater tanks at 25 °C for at least one week. For the stimulation experiment, briefly, these healthy fishes were randomly divided into two groups in which the experimental group was challenged with 1 ml suspension of LPS (1 mg/ml) through intraperitoneal and the other group kept in separate tanks was corresponding challenged with 1 ml physiological water as the control. Fishes were respectively killed after 6 h, 12 h, 24 h, 36 h, 48 h of treatment and the tissues of spleen were collected and then stored at −80 °C for later use.

2.2. Macrophage isolation and LPS exposure

For the macrophage isolation, the head kidneys from three juvenile miiuy croakers were collected aseptically. These tissues were minced thoroughly with scissors and pushed carefully through a 100-μm nylon mesh in L-15 medium containing penicillin (100 IU/ml), streptomycin (100 mg/ml), 2% foetal bovine serum (FBS) and heparin (20 U/ml) to give a single cell suspension. The filtered cell suspension was loaded onto 34%/51% Percoll (Pharmacia, USA) density gradient, and then centrifuged at 400 g for 40 min at 4 °C. Then, the supernatant was removed and cell at the interfaces were obtained with care and washed twice in L-15 medium at 300 g for 10 min at 4 °C. Whereafter, macrophage were seeded in 6-well plates at a density of 4×10^7 per well at 26 °C, 4% CO_2. Cell count and viability were determined with a haemocytometer by trypan blue exclusion assay. The next day, the cell pellet was replaced with the fresh complete L- 15 medium supplemented with 20% FBS. For the LPS exposure, the cells were challenged with 10 μg/ml (Sigma) lipopolysaccharides and harvested at 3 h and 6 h for RNA extraction. Cells with no stimulation were collected as the control, and each experiment had three biological replicates.

2.3. Real-time quantitative PCR for miRNA and mRNA

Total RNA was isolated from spleen tissues or macrophage using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA then underwent reverse transcription using the FastQuant RT Kit (Tiangen) which including DNase treatment of RNA to eliminate genomic contamination. Harvested cDNA was stored at −20 °C for later use. Primers of miiuy croaker TLR1 were designed by Primer Premier 5 software and primers of β-actin were applied to as internal control (Table S1). Real-time quantitative PCR, using SYBR® Premix Ex Taq™ (Takara), was performed on a 7300 real-time PCR system (Applied Biosystems, USA). The amplification reaction volume of 20 μl contained 10 μl SYBR® Premix Ex Taq™, 2 μl cDNA sample, 0.4 μl ROX Reference Dye, 0.4 μl of each primer (10 μM) and 6.8 μl ddH_2O. The thermal cycling conditions were 30 s at 95 °C, followed by 40 cycles 5 s at 95 °C and 34 s at 60 °C. Dissociation curve was conducted after each assay to determine target specificity. For each gene, the triplicate fluorescence intensities of the control and challenged groups were measured.

For sRNA (<200 nt) harvested, miRcute miRNA Isolation Kit (Tiangen) was utilized followed the manufacturer's instructions. Afterward, 2 mg RNA of each sample was used for cDNA synthesis, and miRcute miRNA FirstStrand cDNA Synthesis Kit (Tiangen) was applied to reverse transcription of miRNAs. The amplifications reaction were carried out using the miRcute miRNA qPCR Detection Kit (Tiangen), following conditions: 95 °C for 15 min, 40 cycles of two steps (95 °C for 5 s, 60 °C for 30 s) within triplicate well of each sample. Sequences of miRNA primers were lists in Table S1 and 5.8s RNA was used as internal control (Xu et al., 2016a).

2.4. Prediction of candidate miRNA binging to TLR1-3′UTR

To further analysis the function of TLR1, we used three software programs, miRanda (John et al., 2004), TargetScan (Lewis et al., 2005) and miRInspector (Huang et al., 2012), to predict candidate miRNAs binging to 3′-UTR region of TLR1. Those miRNAs who were predicted binging to TLR1-3′UTR by all of the three programs were listed and selected for the further study.

2.5. Plasmid construction

To construct TLR1-3′UTR vector, the full length 3′-UTR region of TLR1 was amplified from cDNA derived from healthy miiuy croaker.