Developmental and Comparative Immunology 40 (2013) 227–231

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Molecular cloning, characterization and immunological response analysis of Toll-like receptor 21 (TLR21) gene in grass carp, *Ctenopharyngodon idella*

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ARTICLE INFO

Article history:
Received 22 January 2013
Revised 6 March 2013
Accepted 6 March 2013
Available online 13 March 2013

Keywords:
Grass carp
TLR21
Aquareovirus
Aeromonas hydrophila
Gene cloning
Characterization

ABSTRACT

TLR21, a non-mammalian Toll-like receptor, has been recently identified in fishes, frogs and birds. In the present study, the full-length cDNA sequence of TLR21 (*Ci*TLR21) from *Ctenopharyngodon idella* has been isolated and characterized. The *Ci*TLR21 full-length cDNA sequence consists of 3578 bp, with an open reading frame (ORF) of 2958 bp encoding 985 amino acid residues. The putative *Ci*TLR21 protein contains a signal peptide sequence, 17 leucine-rich (LRR) motifs, a transmembrane region and a Toll/interleukin-1 receptor (TIR) domain. The *Ci*TLR21 gene is expressed in a wide range of tissues with the highest expression in skin. Upon induction by *Aquareovirus*, *Ci*TLR21 expression is significantly down-regulated in liver and spleen, whereas is significantly up-regulated in liver and spleen after *Aeromonas hydrophila* infection. These results suggest that *Ci*TLR21 plays an important role in *Aquareovirus* and *A. hydrophila*-related diseases. This work may provide the basis for further investigations into the immune system of grass carp and other teleost fishes.

1. Introduction

Grass carp is one of the most important aquaculture species in China. According to FAO, the production of grass carp had reached to 420 tons in 2011 (FAO, 2012). However, this fish is highly susceptible to viral and bacterial pathogens, including grass carp reovirus (GCRV) and *Aeromonas hydrophila*. Preventing these infectious diseases is still a great challenge in grass carp farming. Better understanding of the immune defense mechanisms and identification of crucial genes are the key steps to the development of management strategies for disease control and long-term sustainability of grass carp aquaculture.

The innate immune system is a fundamental defense mechanism of fish. Compared with adaptive immunity, innate immunity, which is the first line of defense against infection, is a less specific but a faster process (Akira et al., 2006; Magnadottir, 2006). As part of the ancient innate arm of the immune system, receptors recognize conserved pathogen molecules. Three major classes of pattern recognition receptors (PRRs) have been identified: Toll-like receptors (TLRs) that recognize ligand on either the extracellular surface or within the endosome; NOD-like receptors (NLRs) that function as cytoplasmic sensors and RIGI-like receptors (RLRs) that recognize viruses (Akira et al., 2006; Chen et al., 2009; Franchi et al., 2009). Toll-like receptors (TLRs) play an important role in the innate immune system by recognizing different but overlapping pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2009). TLRs have also been implicated in the maintenance of normal homeostasis (Rakoff-Nahoum et al., 2004). In general, TLRs consist of three parts (LRR, transmembrane and TIR). To date, TLR genes described from mammals contain 19–25 LRR repeats (Choe et al., 2005). The LRR region is involved in pathogen recognition, while the TIR domain is involved in binding to TIR-containing adaptor molecules that regulate downstream signaling (Akira and Takeda, 2004). Until now, 17 TLR types have been identified in various teleost (Rebl et al., 2010), including several fish-specific novel TLRs such as TLR5 soluble, TLR14, TLR21 and TLR22 (Hirono et al., 2004; Rebl et al., 2007; Tsukada et al., 2005).

TLR21 is a non-mammalian Toll-like receptor, which was initially identified in pufferfish (*Takifugu rubripes*) and zebrafish (*Danio rerio*) by analyzing their genome database using in silico analysis (Jault et al., 2004; Meijer et al., 2004; Oshiumi et al., 2003). It has been identified in some species of fish, frog and bird, including channel catfish (*Ictalurus punctatus*) (Baoprasertkul et al., 2007), koi carp (*Cyprinus carpio*) (Uma et al., 2010), orange-spotted grouper (*Epinephelus coioides*) (Li et al., 2012), Atlantic cod (*Gadus*

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http://dx.doi.org/10.1016/j.dci.2013.03.003

morhua), African clawed frog (*Xenopus laevis*) (Ishii et al., 2007) and chicken (*Gallus gallus*) (Temperley et al., 2008). Meanwhile, the function of TLR21 was being elucidated. Previous studies have shown that the chicken TLR21 plays similar roles to the mammalian TLR9, which works as a receptor to recognize CpG-ODN and its activation required endosomal acidification (Brownlie et al., 2009; Keestra et al., 2010). In orange-spotted grouper, the expression of TLR21 gene was shown to be modulated after stimulation with *Cryptocaryon irritans* (Li et al., 2012). However, there are no information about the recognition of TLR21 molecule and its function in grass carp. To better understand the functional mechanism of the family of Toll-like receptor, we cloned and characterized the TLR21 gene from grass carp and analyzed its gene expression patterns in various tissues. In addition, to understand the crucial innate immune response of grass carp, we analyzed expression of *Ci*TLR21 gene against *Aquareovirus* and *A. hydrophila* infection.

2. Materials and methods

2.1. Animal treatment and RNA extraction

Adult grass carp weighing about 2500 g and juvenile grass carp weighing about 100 g were obtained from Nanhui fish farm in Shanghai, China. All fish were healthy and acclimatized at 26 °C in 400 L aerated aquaria for one week. For full-length cDNA cloning and expression pattern analysis, various tissues were collected from three adult grass carps, including blood, gill, fin, skin, muscle, liver, spleen, trunk kidney, intestine, head kidney, heart and brain.

For the viral challenge, 24 healthy juvenile grass carps were used. The fish were divided into two equal groups: (1) control (PBS injected) and (2) challenged (GCRV injected). The challenged group was injected intraperitoneally with *Aquareovirus* (GCRV-JX01) (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of 1.0×10^7 cells suspended in 200 μL PBS per fish, whereas the control group was similarly injected with 200 μL sterile PBS per fish.

For the bacterial challenge, 24 healthy juvenile grass carps were used. The fish were divided into two equal groups: (1) control (PBS injected) and (2) challenged (*A. hydrophila* injected). The challenged group was injected intraperitoneally with formalin-killed *A. hydrophila* S2 (Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of 5.0×10^7 cells (suspended in 100 μL PBS) per fish, whereas fish from the control group were injected with 100 μL sterile PBS per fish. For both challenge experiments, three fish were sampled at 4 h, 1, 3 and 7 d post-injection from each group, respectively. Gill, liver and spleen were collected from each fish for total RNA extraction. The viral and bacterial infections were confirmed by measuring the expression of another host immune gene (TNF13b) (Pandit et al., 2013).

All tissues were quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The total RNAs were isolated using TRIZOL Reagent (invitrogen) following the manufacturer's instruction. All RNA samples were treated with RNase-free DNase (TaKaRa, Japan), and stored at −80 °C.

2.2. Cloning the full-length cDNA of CiTLR21

cDNA from a healthy grass carp spleen was used as the template for amplification of the *Ci*TLR21 partial sequence with gene specific primers PTLR21F1/R1 (Table 1 of supporting information), which were designed based on the conserved regions of TLR21 from zebrafish (GenBank accession No. NM-001199335) using Primer Premier 5 program. The PCR program was 1 cycle of 94 °C for 3 min; 31 cycles of 94 °C for 30 s, 51.5 °C for 30 s, 72 °C for 1 min; 1 cycle of 72 °C for 10 min. The PCR products were purified from gel by a Gel Extraction Kit (Tiangen, China), and then ligated into a pGEM-T easy vector (Promega), transformed into competent *Escherichia coli* DH5α cells, plated on a LB-agar Petri dish and incubated overnight at 37 °C. Positive clones containing the insert with the expected size were identified by colony PCR. Five of the positive clones were sequenced on an ABI PRISM 3730 Automated Sequencer (USA), with the sequence primers M13F/M13R.

To obtain the full-length cDNA, 5′ RACE and 3′ RACE were performed with gene specific primers and adaptor primers (UPM) (Table 1 of supporting information) using a SMART RACE cDNA Amplification Kit (Clontech, USA). The PCR conditions for the 3′ RACE was 1 cycle of 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; and 1 cycle of 72 °C for 10 min. Similarly, the PCR conditions for the 5′ RACE was five cycles of (94 °C for 30 s; 72 °C for 3 min), five cycles of (94 °C for 30 s; 70 °C for 30 s; 72 °C for 3 min), followed by 25 cycles (94 °C for 30 s; 68 °C for 30 s; 72 °C for 3 min). PCR products were cloned and sequenced as described above.

2.3. Sequence analysis

BLASTn and BLASTp from National Center for Biotechnology Information (NCBI) were used to search homologous sequences in Genbank. The cDNA of TLR21 was translated into its potential open reading frame (ORF) by the ORF Finder algorithm (http://www.ncbi.nlm.nih.gov/gorf). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/). The protein domain features were predicted by Simple Modular Architecture Reach Tool (SMART) (http://smart.embl-heidelberg.de/) (Letunic et al., 2006). Amino acid sequence identity analysis was performed using bl2seq (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed based on the deduced full-length amino acid sequences using the neighbor-joining (NJ) algorithm in MEGA version 5.0 (Tamura et al., 2011), and analysis reliability was assessed by 1000 bootstrap replicates.

2.4. Real-time PCR analysis

First-strand cDNA was synthesized from about 1 μg RNA of each sample template using the iScript™ cDNA Synthesis Kit (BIO-RAD, USA). The RT-qPCR was conducted using the CFX96 real-time PCR Detection System (BIO-RAD, USA) in a 20 μL reaction system, which contained the following components: 10 μL iQ™ SYBR Green Supermix (BIO-RAD, USA), 0.8 μL of each primer (10 μmol/L), 1.6 μL cDNA and 6.8 μL RNase-free water. The sequences of *Ci*TLR21 specific primers used in RT-PCR are shown in Table 1 of supporting information. A 10-fold dilution series of cDNAs were used to construct standard curves. The amplification efficiency was measured from the regression slope of the standard curve. Only the primer pair that had a single peak in melting curve analysis and displayed amplification efficiency close to the theoretical 100% was retained for further use. Grass carp β-actin gene (GenBank Accession No. DQ211096.1) was amplified as an internal standard reference gene. The RT-PCR program was 95 °C for 3 min, 39 cycles at a melting temperature of 95 °C for 5 s and an annealing temperature of 60 °C for 30 s, then followed the melting curve analysis.

2.5. Statistical analysis

The triplicate fluorescence intensities of the control and treatment products, as measured by crossing-point (*Ct*) values, were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384v.1 (REST) (Pfaffl et al., 2002). Expression differences between