Fish & Shellfish Immunology 59 (2016) 179–188

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular and functional characterization of Toll-like receptor 21 in large yellow croaker (*Larimichthys crocea*)

Min Sun [a, b, c], Yinnan Mu [b, c], Yang Ding [b, c], Jingqun Ao [b, c], Xinhua Chen [b, c, d, *]

[a] School of Marine Sciences, Ningbo University, Ningbo 315211, China
[b] Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, China
[c] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen 361005, China
[d] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China

ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form
7 October 2016
Accepted 13 October 2016
Available online 14 October 2016

Keywords:
Large yellow croaker *Larimichthys crocea*
Toll-like receptor 21
CpG-oligodeoxynucleotides
NF-κB activation
Proinflammatory cytokine

ABSTRACT

Toll-like receptor 21 (TLR21) is a non-mammalian TLR that functions similar to mammalian TLR9 in recognizing CpG DNA. In the present study, we identified a TLR21 homologue, LycTLR21, from large yellow croaker (*Larimichthys crocea*). The complete coding sequence of LycTLR21 is 2946 nucleotides long, encoding a protein of 981 amino acids. The deduced LycTLR21 protein has typical TLR domain architecture, including a signal peptide, 13 leucine-rich repeats (LRRs) in the extracellular region, a transmembrane region, and a cytoplasmic Toll-Interleukin-1 receptor (TIR) domain. Phylogenetic analysis showed that LycTLR21 falls into a major clade formed by all fish TLR21 sequences and is closely related to TLR21 in *Epinephelus coioides* and *Oplegnathus fasciatus*. LycTLR21 mRNA was constitutively expressed in all tissues tested, with higher levels in immune-related tissues, such as spleen, head kidney, and gills. Upon stimulation with inactivated trivalent bacterial vaccine, LycTLR21 mRNA was significantly increased in these three tissues. Overexpression of a chimeric plasmid containing the extracellular domain of human cluster of differentiation 4 (CD4) and the transmembrane and cytoplasmic domains of LycTLR21 could activate NF-κB, but not IFN-β in Chinese hamster ovary (CHO) cells, suggesting that LycTLR21 could mediate activation of NF-κB. LycTLR21 could specifically recognize three CpG-oligodeoxynucleotides (CpG-ODNs), CpG-ODN 1826, 2006, and 2007, but not other CpG-ODNs detected, poly(I:C), lipopolysaccharide (LPS), and lipoteichoic acid (LTA-SA). These three CpG-ODNs were found to significantly up-regulate the expression of LycTLR21 and downstream proinflammatory cytokines IL-1β and IL-6 of NF-κB pathway in large yellow croaker head kidney (LYCK) cells. In addition, the expression levels of LycTLR21, c-Rel subunit of NF-κB, IL-1β and IL-6 genes were quickly increased in the spleen and head kidney by bacterial infection, suggesting that LycTLR21 signaling pathway may play a role in immune response to bacterial infection.

1. Introduction

Innate immunity serves as the first line of host defense against various pathogenic infections [1–3]. Toll-like receptors (TLRs) are type I transmembrane proteins composed of several domains: the signal peptide, leucine-rich repeats (LRRs) that recognize ligands, the transmembrane (TM) domain, and the cytoplasmic Toll-Interleukin −1 receptor (TIR) domain that interacts with adaptor molecules [2,4]. TLRs play a significant role in host defense by recognizing pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoprotein, lipoteichoic acid (LTA-SA), flagellin, and DNA/RNA from a broad range of potentially pathogenic microbes [5]. At present, 13 different TLRs have been identified in mammals [6]. When activated by PAMPs, TLRs immediately initiate intracellular signal transduction pathways by a MyD88-dependent or MyD88-independent pathway and induce the production of multiple cytokines. These cytokines not only modulate innate immune responses but also instruct the development of antigen-specific acquired immunity [7].

Based on amino acid similarity, genomic structure, and ligand properties, at least 23 vertebrate TLRs have been found and classified into seven major families: TLR1, TLR3, TLR4, TLR5, TLR7, TLR11, and TLR21 [8,9]. In teleosts, the TLR21 family includes TLR13,

* Corresponding author. Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, 184 Daxue Road, Xiamen 361005, Fujian Province, China.
E-mail address: chenxinhua@tio.org.cn (X. Chen).

http://dx.doi.org/10.1016/j.fsi.2016.10.024
1050-4648/

TLR19, TLR20, TLR21, TLR22, and TLR23. TLR21 is a non-mammalian TLR found in birds [10,11], amphibians [12], and teleosts [13,14]. Chicken TLR21 has been shown to be a functional homologue to mammalian TLR9 in terms of response to CpG-ODN stimulation. Once recognized by a specific CpG-ODN or microbial DNA, TLR21 mediates a serial of signaling cascades, thus triggering downstream innate and adaptive immune responses [15,16]. To date, TLR21 has been identified from several teleost fish species, including pufferfish (*Takifugu rubripes*) [13], zebrafish (*Danio rerio*) [14], catfish (*Ictalurus punctatus*) [17], orange-spotted grouper (*Epinephelus coioides*) [18], olive flounder (*Paralichthys olivaceus*) [19], grass carp (*Ctenopharyngodon idella*) [20], Atlantic salmon (*Salmo salar*) [21], rock bream (*Oplegnathus fasciatus*) [22], and yellowtail (*Seriola lalandi*) [23]. Expression of TLR21 in some fish species was significantly increased by CpG-ODNs, poly(I:C) or aquatic pathogens [18–20,22,23], suggesting the immunological functions of fish TLR21. Further studies demonstrated that zebrafish TLR21 can sense a panel of CpG-ODNs with different sequences, but with a preferential recognition of CpG-ODN 2006 and CpG-ODN 2007 containing GTCGTT motifs [24]. Olive flounder TLR21 is found to be strongly induced by CpG-ODN 1668 with a GACGTT motif [19], while grass carp TLR21 is not responsive to the CpG-ODNs tested [25]. Thus, the ligand specificity and function of TLR21 from different fish species remain to be investigated.

In this study, we report the molecular characterization of a TLR21 homologue (LycTLR21) from large yellow croaker (*Larimichthys crocea*), an economically important marine fish in China. The tissue expression profile and expression modulation upon stimulation with inactivated bacterial vaccine of LycTLR21 were analyzed. We also demonstrated that LycTLR21 specifically recognized three CpG-ONDs, CpG-ODN 1826, 2006, and 2007, and mediated NF-κB activation. All these three CpG-ODNs were able to increase the expression of LycTLR21 and proinflammatory cytokines IL-1β and IL-6 in LYCK cells. In addition, the expression levels of LycTLR21, c-Rel, IL-1β and IL-6 genes were also found to increase by bacterial infection.

2. Materials and methods

2.1. Experimental fish and cell lines

Large yellow croakers (weight: 112 ± 19.6 g) were purchased from a mari-culture farm at Lianjiang county, Fuzhou city, China. After 3 days of acclimatizing in aerated seawater tanks, these fish were used for induction experiments. Two groups of 30 fish were intraperitoneally injected with the inactivated trivalent bacterial vaccine consisting of 1.0×10^8 colony forming units (CFU)/mL of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila* at a dose of 0.2 mL/100 g fish, or the mixed bacteria (1.0×10^8 CFU/mL of *V. alginolyticus*, *V. parahaemolyticus*, and *A. hydrophila*) at a dose of 0.2 mL/100 g fish. The inactivated trivalent bacterial vaccine was prepared in our laboratory as described previously [26]. A third group of 30 fish were injected with sterilized phosphate buffered saline (PBS, pH7.4) at a dose of 0.2 mL/100 g fish as a control. The spleen, head kidney, and gills were collected from 5 fish at different time points (0, 6, 12, 24, 48, and 72 h) after induction and stored at −80 °C for further use.

The large yellow croaker head kidney (LYCK) cells were derived from the head kidney of fish and the continuous cell lines were maintained at 28 °C in L-15 medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10% Fetal Bovine Serum (FBS) according to the previous study [27]. The CHO cells (China Center for Type Culture Collection, Wuhan, China) were cultured in DMEM-F/12 containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator.

2.2. Cloning of complete coding sequence of LycTLR21

The coding sequence of LycTLR21 was predicted from the genome sequence of large yellow croaker (JRPU00000000) [28], and a pair of gene-specific primers LycTLR21 ORF-F and -R (Supplementary Table 1) was designed. Using cDNA from the spleen of large yellow croaker as a template, PCR was performed using EasyPfu DNA Polymerase (TransGen Biotech) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min, followed by a final extension at 72 °C for 5 min. The resulting PCR product was then added a single adenosine (A) at its 3′ end using Taq DNA Polymerase (Takara, China) and cloned into pMD18-T simple vector (Takara, China). At least three clones containing the insert were sequenced. Sequence alignment was performed using DNAMAN program. Protein domains were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Signal peptide was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic tree was constructed with Molecular Evolution Genetics Analysis (MEGA) software version 6.06 using the neighbor-joining method [29].

2.3. Tissue expression analysis of LycTLR21

Various tissues including brain, spleen, heart, skin, head kidney, muscle, intestine, blood, liver, and gills were collected from 5 healthy large yellow croakers. Total RNA was isolated, treated with RNase-free DNase I, and reverse transcribed into the first strand cDNA using Oligo-dT Adaptor primer (TaKaRa, China). To determine the tissue expression profile of LycTLR21, real-time PCR was performed with gene-specific primers (Supplementary Table 1). The reference gene β-actin was amplified as an internal control with the primer set of Actin-F and -R (Supplementary Table 1). Real-time PCR was performed on the Mastercycler ep gradient realplex4 system (Eppendorf, Germany) using SYBR® Premix ExTaq™ (TaKaRa). Cycling conditions were 20 s at 95 °C, then 40 cycles of 95 °C for 5 s, 57 °C for 15 s, and 72 °C for 20 s. The fluorescence output for each cycle was analyzed upon the completion of the entire run. The expression levels of LycTLR21 were normalized by β-actin using the $2^{-\Delta\Delta CT}$ method and expressed as the ratio of the LycTLR21 expression levels in the brain [30]. Each real-time PCR assay was repeated three times. The data of real-time PCR were expressed as the standard error of the mean (SEM).

To understand the modulation of LycTLR21 expression upon the trivalent bacterial vaccine induction, total RNA was extracted from spleen, head kidney, and gills of 5 fish sampled above. The first strand cDNA was synthesized from 1 μg of each total RNA and used as template for real-time PCR analysis. Real-time PCR was performed to detect expression levels of LycTLR21 gene as above. The expression levels of LycTLR21 gene were normalized by β-actin. Fold change was expressed as the ratio of the normalized gene expression levels in fish injected with bacterial vaccine versus those in fish injected with PBS (defined as 1) at the corresponding time points. All data were obtained from three independent PCR assays with three replicates in each assay.

2.4. Luciferase reporter assay

To investigate whether the TIR domain of LycTLR21 was able to activate the signal transduction pathways that lead to the activation of NF-κB and/or IFN-β, a constitutively activated chimera CD4-LycTLR21 was generated by fusing cDNAs encoding the extracellular domain of human CD4 (amino acids 1–397) and the transmembrane and cytoplasmic domains of LycTLR21 (amino acids 795–944). The cDNA encoding the extracellular domain of human