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Short communication

Identification and characterization of a novel Toll-like receptor 2 homologue in the large yellow croaker *Larimichthys crocea*

Jingqun Ao [a, b, c], Yinnan Mu [a, b, c], Kunru Wang [a, b, c], Min Sun [a, b, c], Xianhui Wang [a, b, c], Xinhua Chen [a, b, c, *]

[a] *Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, PR China*
[b] *Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen 361005, PR China*
[c] *Collaborative Innovation Center of Deep Sea Biology, Third Institute of Oceanography, State Oceanic Administration, Daxue Road 178, Xiamen 361005, PR China*

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ABSTRACT

Toll-like receptors (TLRs) are key components of innate immunity that play significant roles in immune defence against pathogen invasion. In the present study, we identified a novel TLR2 homologue (LycTLR2b) in large yellow croaker (*Larimichthys crocea*) that shared low sequence identity with the previously reported large yellow croaker TLR2 (tentatively named LycTLR2a). The full-length cDNA of LycTLR2b was 2926 nucleotides (nt) long and encoded a protein consisting of 797 amino acids (aa). The deduced LycTLR2b protein exhibited a typical TLR domain architecture including a signal peptide, seven leucine-rich repeats (LRRs) in the extracellular region, a transmembrane domain, and a Toll-Interleukin 1 receptor (TIR) domain in the cytoplasmic region. Phylogenetic analysis showed that both LycTLR2a and LycTLR2b fall into a major clade formed by all TLR2 sequences, and are divided into two distinct branches. Genomic organization revealed that the LycTLR2b gene lacks intron, which is similar to zebrafish and human TLR2 genes, whereas the LycTLR2a gene contains multiple introns, as found in damselfish TLR2a and Fugu TLR2 genes. Syntenic analysis suggested that the occurrence of LycTLR2a and LycTLR2b may result from a relatively recent genome duplication event. LycTLR2b mRNA was constitutively expressed in all tissues examined although at different levels. Following bacterial vaccine challenge, LycTLR2b expression levels were significantly up-regulated in both spleen and head kidney tissues. Taken together, these results indicated that two different TLR2 homologues, which may play roles in antibacterial immunity, exist in large yellow croaker.

1. Introduction

The innate immune system is the first line of host defence against invading pathogens [1]. A crucial step for the initiation of immune defence mechanisms is the recognition of danger signals associated with pathogens, which subsequently activates a rapid immune response to fight the infection [2,3]. Pattern recognition receptors (PRRs), which sense conserved microbial features and endogenous structures released after tissue traumata, are key activators in innate immunity [4,5]. Toll-like receptors (TLRs), a family of conserved and germline-encoded PRRs, belong to type I membrane receptors that contain an extracellular leucine-rich repeats (LRR) domain mediating the recognition of pathogen-associated molecular patterns (PAMPs), and an intracellular Toll-Interleukin 1 receptor (TIR) domain required for downstream signal transduction [6]. At present, 13 different TLRs have been identified in mammals [7]. Among these mammalian TLRs, TLR1 family members, including TLR1, 2, 6, and 10, primarily recognise PAMPs from bacteria and play pivotal roles in sensing microbial products [8]. TLR2 is best known for recognising conserved components of Gram-positive bacteria such as lipoteichoic acid (LTA), peptidoglycans (PGN), and lipoproteins [9]. Zymosan from fungi, tGPI-mucin from *Trypanosoma cruzi*, and haemagglutinin protein from the measles virus are also recognised by TLR2 [10]. In addition to forming functional heterodimers with either TLR1, TLR6, or TLR10, TLR2 can likewise interact with a large number of non-TLR

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* Corresponding author. Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Daxue Road 178, Xiamen 361005, PR China.
E-mail address: chenxinhua@tio.org.cn (X. Chen).

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molecules, which allows for recognition of a large number and variety of PAMPs [11]. TLR2 binds to the corresponding PAMPs and triggers a signalling cascade through the MyD88 pathway, resulting in the activation of the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) and the subsequent induction of inflammatory cytokines [6].

At present, TLR2 homologues have been identified in several fish species, and their molecular characteristics and expression patterns have been analysed [12–18]. TLR2 mRNA was increased in peripheral blood leukocytes from Japanese flounder (*Paralichthys olivaceus*) after stimulation with PGN [12]. The induced expression of TLR2 was also observed in Indian major carp (*Labeo rohita*) following exposure to zymosan, PGN, and LTA [17]. Furthermore, the common carp TLR2 was confirmed to recognise LTA, PGN, and the synthetic triacylated lipopeptide Pam3CSK4 [14]. In a previous study, a TLR2 homologue (LycTLR2a) was reported in large yellow croaker (*Larimichthys crocea*), an economically important marine fish in China [19]. Recently, a high-quality genome assembly for large yellow croaker was produced, in which a novel TLR2 homologue (LycTLR2b) was found [20]. Here, we have performed the molecular and evolutionary analyses of LycTLR2b. We also investigated its tissue distribution and expression modulation after stimulation with a trivalent bacterial vaccine to better comprehend its role in immunity.

2. Materials and methods

2.1. Fish and challenge experiments

Large yellow croakers (length: 21 ± 1.5 cm; weight: 104 ± 13.6 g) were purchased from a mari-culture farm in Lianjiang, Fuzhou, China. Fish were maintained with a flow-through seawater supply at 25 °C. After acclimating for 5 days, the normal fish were used for the challenge experiments. A group of 20 fish received an intraperitoneal injection of the inactivated trivalent bacterial vaccine consisting of 5.0×10^8 colony forming units/mL^{-1} of gram-negative bacteria *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Aeromonas hydrophila* each at a dose of 0.2 mL/100 g fish. To serve as controls, a second group of 20 fish each received an injection of sterilised phosphate buffered saline (PBS, pH 7.4) at a dose of 0.2 mL/100 g. Various tissues were collected at different time points (0, 12, 24, 48, and 72 h) after induction, frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction. The tissues collected at 0 h were from non-challenged fish.

2.2. Cloning and characterization of LycTLR2b in large yellow croaker

To obtain the full-length cDNA of LycTLR2b, 3′- Rapid Amplification of cDNA Ends (RACE) and 5′-RACE were performed using the 3′-Full RACE and 5′-Full RACE Kits (TaKaRa, China). Primers (Supplementary Table 1) were designed based on predicted coding sequence obtained from the large yellow croaker genome (JRPU00000000) [20]. The 5′- and 3′-RACE assays were performed according to the manufacturer's instructions. The resulting PCR product was cloned into the pMD18-T simple vector and sequenced. The middle fragment between the 5′- and 3′-terminal sequences was then amplified using RT-PCR. All sequences were assembled to obtain the full-length cDNA sequence of LycTLR2b. Protein domain structures were predicted using the Simple Modular Architecture Research Tool (SMART), TMHMM v2.0, and SignalP 4.1. A phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.06) using the neighbour-joining (NJ) method [21], with the Poisson model for amino acid substitution and pairwise deletion for gap treatments. The tree was supported by 10,000 bootstrap repetitions. The Ensembl database and GenBank were used to identify related genes in other vertebrate genomes to initially derive the syntenic relationships between species. The genomic structure was analysed using the Spidey online software.

2.3. Expression analysis of LycTLR2b by real-time PCR

Each tissue, including spleen, head kidney, blood, liver, heart, gills, intestine, and skin, was collected and pooled from five normal large yellow croakers. To determine the tissue expression profile of LycTLR2b, real-time PCR was performed using a gene-specific primer set (Supplementary Table 1). The β-actin was amplified as an internal control using the primer set of Actin-F and Actin-R (Supplementary Table 1). Real-time PCR analyses were performed using the Mastercycler epgradient realplex 4 system (Eppendorf, Germany) with SYBR Green as the fluorescent dye, according to the manufacturer's protocol (Takara, China). The cycling conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s. After normalisation to β-actin, LycTLR2b expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [22,23]. To understand the changes in LycTLR2b expression following trivalent bacterial vaccine induction, total RNA was extracted from the pooled spleen and head kidney of five fish collected at 0, 12, 24, 48, and 72 h post-induction. Real-time PCR and data analysis were then performed using the conditions described above to detect the expression levels of LycTLR2b in spleen and head kidney tissues. The data obtained from three independent biological replicates were subjected to statistical analysis.

2.4. Statistical analysis

The real-time PCR data were analysed using GraphPad Prism 5 software and expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Independent samples t-test was used for the significance test of LycTLR2b expression levels between 0 h and each time point after induction using SPSS 18.0 ($n = 3$). $P < 0.05$ and $P < 0.01$ were considered statistically significant as indicated.

3. Results

3.1. Identification and characterization of LycTLR2b

Following a search of the large yellow croaker genome database, two TLR2 homologues were found in the genome (JRPU00000000), tentatively named LycTLR2a (KKF22682.1) and LycTLR2b (KKF15865.1). LycTLR2a was reported in a previous study [19]. Next, RACE-PCR was used for cloning the full-length LycTLR2b cDNA. The resulting full-length LycTLR2b cDNA was 2926 nucleotides (nt) in length, together with a 3'-untranslated regions (UTR) consisting of 535 nt. There were two mRNA instability signals (ATTTA) located at 459 and 277 nt upstream of the poly (A) tail, respectively (Supplementary Fig. 1). The 2391 nt open reading frame of LycTLR2b encoded a 797-amino acid (aa) protein that exhibited a typical TLR domain architecture, including a signal peptide (residues 1-19), 7 LRR domains (residues 92-510) in the extracellular region, a transmembrane domain (residues 581-603), and a TIR domain (residues 632-777) in the cytoplasmic region (Fig. 1 and Supplementary Fig. 1). The identification of 7 LRR domains in LycTLR2b was in accord with the number of domains detected in TLR2a in damselfish (*Segastes paritus*) and TLR2 in zebrafish, but was less than the number of LRR domains detected in LycTLR2a (10 LRRs), damselfish TLR2b (9 LRRs), Fugu (*Takifugu rubripes*) TLR2 (9 LRRs), and human (*Homo sapiens*) TLR2 (9 LRRs; Fig. 1). The various