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Short communication

Genomic structure and molecular characterization of Toll-like receptors 1 and 2 from golden pompano *Trachinotus ovatus* (Linnaeus, 1758) and their expression response to three types of pathogen-associated molecular patterns

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ABSTRACT

Toll-like receptors (TLRs) play an essential role in the immune response. Here two Toll-like receptors from golden pompano (*Trachinotus ovatus*), ToTLR1 and ToTLR2, were characterized, the full-length cDNAs were 3126 bp and 7430 bp, and the deduced proteins consisted of 801 and 825 amino acids, respectively. ToTLR1 and ToTLR2 both contained the typical TLR domain architecture including signal peptide, leucine rich repeat (LRR), C-terminal LRR domain at the extracellular region and Toll/interleukin (IL)-1 receptor (TIR) domain in the cytoplasmic region. ToTLR1 only had one intron and two exons, but ToTLR2 consisted of twelve introns and thirteen exons. The promoters of ToTLR1 and ToTLR2 contained several putative transcription factor binding sites. Phylogenetic analysis showed that ToTLR1 and ToTLR2 were clustered into the clade of TLR1 and TLR2, respectively. Tissues distribution analysis indicated that both genes were ubiquitously expressed in all examined tissues, with higher expression levels observed in blood, head-kidney and spleen. After injection with poly inosinic:cytidylic [poly(I:C)], flagellin and lipopolysaccharides (LPS), ToTLR1 and ToTLR2 mRNAs were significantly up-regulated in the immune related tissues, indicating the possible the role of ToTLR1 and ToTLR2 in defense against pathogenic microbes. Further research should be carried out to identify ligands of fish TLR1 and TLR2 in order to understand the function of these receptors.

1. Introduction

The host can identify pathogenic microorganisms through innate immunity in vertebrates, invertebrates and plants, which is the effective first line of defense against pathogen invasion. Toll-like receptors (TLRs) are important pattern recognition receptors (PRRs) in innate immune system, which can specifically recognize pathogen associated molecular patterns (PAMPs), including lipopolysaccharides (LPS), lipoprotein, lipoteichoic acid, fiagellin, CpG-DNA, double strand RNA (dsRNA) and single strand RNA (ssRNA) (Akira et al., 2006). TLRs are type I transmembrane proteins consisting of three typical functional domains: an extracellular leucine-rich repeat (LRR) domain that can specifically recognize PAMPs, a transmembrane (TM) region that is a cysteine-rich domain and an Toll/interleukin (IL)-1 receptor domain that activates the signaling pathway to modulate the host immune response (Tong et al., 2015).

28 types of TLRs have been identified in vertebrates, and those TLRs are classified into six major subfamilies: TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11 (Meijer et al., 2004; Wang et al., 2016a). TLR1-subfamily contains TLR1, TLR2, TLR6, TLR10, TLR14, TLR18, TLR25, TLR27 (Wang et al., 2015) and TLR28 (Wang et al., 2016b),

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which mainly recognize the bacterial peptidoglycans and lipoproteins. TLR3-subfamily includes only TLR3 gene, which can recognize double-stranded viral RNA (Liu et al., 2008). TLR4-subfamily containing only TLR4 gene can detect the LPS (Park et al., 2009). TLR5-subfamily including TLR5M and TLR5S mainly recognize flagella (Umasuthan et al., 2017). TLR7-subfamily comprises TLR7, TLR8 and TLR9, of which TLR7 and TLR8 (Palti et al., 2010) recognize single-stranded viral RNA while TLR9 (Wei et al., 2016) recognizes viral or bacterial DNA and unmethylated CpG motifs. TLR11-subfamily consists of TLR 11–13, TLR 19–23 and TLR26, which can recognize protozoan parasite, dsRNA and unmethylated CpG-DNA (Pietretti et al., 2014; Rauta et al., 2014; Sundaram et al., 2012).

TLR2 is reported to recognize microbial hydrophobic ligands in mammals, which forms heterodimers with TLR1, TLR6, or TLR10 (Guan et al., 2010; Jin et al., 2007; Kang et al., 2009). In fish, TLR1 and TLR2 have been identified in *Epinephelus coioides* that the expression levels of TLR1 and TLR2 in immune tissues after injected with poly(I:C) and LPS were markedly up-regulated (Wei et al., 2011), *Pseudosciaena crocea* which exhibited an obvious increase of TLR1 mRNA expression in the head kidney after LPS stimulation (Wang et al., 2013). TLR1 and TLR2 also has been identified in various fish, including *Oplegnathus fasciatus* (Elvitigala et al., 2015), *Pampus argenteus* (Gao et al., 2016), *Salmo salar* (Salazar et al., 2016), *Cyprinus carpio* (Fink et al., 2016), *Megalobrama amblycephala* (Lai et al., 2017), and their expression analysis has been performed in these fish species.

Golden pompano (*Trachinotus ovatus*) is one of the most important marine fish commercially cultured in South China. The aquaculture is heavily suffered from serious diseases caused by bacterial and parasite infection. TLRs can recognize pathogens and play an essential role in the immune response. However, so far there only has been reported TLR7, TLR8 and TLR9 in golden pompano (Wei et al., 2017). To further understand the function of TLRs of golden pompano, we identified and characterized TLR1 and TLR2 genes of golden pompano (ToTLR1 and ToTLR2) and analyzed the basal expression level in different tissues and their expression responses to the different PAMPs.

2. Materials and methods

2.1. Fish and challenge experiments

Golden pompano with body weight of 40±5 g, were obtained from marine fish culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 26–30 °C in tanks with the recirculating seawater for one week before experiment. The tissues covering head-kidney, liver, stomach, spleen, intestine, brain, skin, gill, muscle and blood were collected from three healthy fish and stored in liquid nitrogen for RNA extraction.

The experiment contained four groups: the control group, the poly(I:C), the LPS and the flagellin stimulation group. The poly(I:C) stimulation group was performed by injecting 200 μL of poly(I:C) (Sigma, USA) dissolved in phosphate buffered saline (PBS) (poly(I:C) 200 μg/ml) into abdomina of each fish. The LPS stimulation group was performed by injecting 200 μL of LPS (Sigma, USA) dissolved in PBS (LPS 50 μg/ml) into abdomina of each fish. The flagellin stimulation group was performed by injecting 200 μL of flagellin from *Salmonella typhimurium* (Sigma, USA) dissolved in PBS (flagellin 1 μg/ml) into abdomina of each fish. The control group was performed by injecting 200 μL of PBS. Three individuals of each group were randomly sampled at the same time point. At each time point (0, 6, 12, 24, 36, 48, 72 and 96 h), tissues (blood, liver, head-kidney, intestine, skin and muscle) were surgically isolated, placed in liquid nitrogen and used for gene expression assay.

2.2. RNA extraction and cDNA preparation

Total RNAs were extracted from tissues using the HiPure Fibrous RNA Plus Kit (Magen, Guangzhou, China). Total RNA concentration was measured by the ratio of UV absorbance at 260/280 nm (NanoDrop-2000, Thermo Fisher, USA) and the quality was assessed by 1.2% agarose electrophoresis. The cDNA was synthesized from total RNA by using a PrimeScript™ Reverse Transcriptase kit (TaKaRa, Dalian, China) following the manufacturers' protocol.

2.3. Gene cloning and sequencing

The full-length sequence of ToTLR1 and ToTLR2 gene were obtained based on genomic data of *T. ovatus* (Accession No. PRJEB22654 under ENA; Sequence Read Archive under BioProject PRJNA406847, unpublished). To verify the accuracy of the sequence, gene-specific primers were designed with Primer Premier 5.0 software (Supplementary Table 1). The PCR reaction was performed using a GradientMaster cycler (Eppendorf, Hamburg, Germany) system with a total volume of 25 μL of PCR mixture containing 2.5 μL 10×reaction buffer with 15 mmol/L^{-1} $MgCl_2$, 2 μL of 10 mmol/L^{-1} dNTP mix, 1.5 μL of 25 μmol/L^{-1} of each primer, 1 μL template cDNA, 16.5 μL Milli-Q water, and 0.5 μL BioReady ExTaq DNA Polymerase (5 U/μL^{-1}) (TaKaRa, Dalian, China). The PCR products were analyzed through electrophoresis in 1.2% agarose gel and subsequently purified using an agarose gel DNA purification kit (Sangon, Shanghai, China). Afterward, the products were ligated into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced (Invitrogen, Guangzhou, China). The full-length gene sequences of ToTLR1 and ToTLR2 were assembled by DNAstar software.

To clone ToTLR1 and ToTLR2 cDNA sequence, primers (Supplementary Table 1) were designed based on the genome sequence, which were located in the 5′-untranslated region (UTR) and 3′-UTR of ToTLR1 and ToTLR2, respectively. PCR products were then sequenced.

2.4. Sequence and phylogenetic analysis

The search for nucleotide sequence similarity was conducted with the BLAST program at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). The exon/intron boundaries of ToTLR1 and ToTLR2 were identified on the basis of the known TLRs using the GSDS (http://gsds.cbi.pku.edu.cn/). The protein structures of ToTLR1 and ToTLR2 were predicted by SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignment analysis was conducted by the Clustalx software with default parameters. The promoter structures of ToTLR1 and ToTLR2 were predicted by BDGP (http://www.fruitfly.org/seq_tools/promoter.html) and AliBaba2.1(http://gene-regulation.com/pub/programs/alibaba2/index.html). Phylogenetic tree was constructed with MEGA7 using the neighbor-joining method with bootstrap test of 1000 replicates.

2.5. Expression patterns of ToTLR1 and ToTLR2 mRNAs

The expression patterns of ToTLR1 and ToTLR2 mRNAs were analyzed by quantitative real-time (qRT)-PCR performed on an Applied Light Cycler (Roche Diagnostics, Shanghai, China). Specific primer pairs for ToTLR1, ToTLR2 and the reference gene EF-1α (elongation factor 1, 172 alpha) were shown in Supplementary Table 1. A 12.5 μL reaction volume contained 6.25 μL 2 × Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics, Shanghai, China), 1 μL first-strand cDNA template, 0.5 μL each primer and 4.25 μL MilliQ water. The thermal profile for qRT-PCR was 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s, 60 °C for 20 s. In all cases, each PCR was performed with triplicate samples.