Gene 527 (2013) 174–182

Contents lists available at SciVerse ScienceDirect

Gene

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

Molecular characterization and functional analysis of Toll-like receptor 3 gene in orange-spotted grouper (*Epinephelus coioides*)

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ARTICLE INFO

Article history:
Accepted 7 June 2013
Available online 19 June 2013

Keywords:
TLR3
Innate immunity
Gene expression
Epinephelus coioides
RNA virus

ABSTRACT

Toll-like receptor 3 (TLR3) plays an important role in activating innate immune responses during viral infection. In this report, TLR3 (EcTLR3) was characterized and analyzed for the first time in *Epinephelus coioides*. The full-length EcTLR3 cDNA is predicted to encode a 909 amino acid polypeptide that contains a signal peptide sequence, 18 leucine-rich repeat (LRR) motifs, a transmembrane region and a Toll/interleukin-1 receptor (TIR) domain. Quantitative real-time PCR revealed that the EcTLR3 mRNA was much more abundant in the liver than in other immune organs, and that the expression levels were very low in hemocyte and muscle. During development of the grouper, the levels of EcTLR3 transcripts increased with age, with very low expression levels at the early stages of development. EcTLR3 mRNA levels were examined in the liver at different times after treatment with polyriboinosinic polyribocytidylic acid (Poly I:C), and in nervous necrosis virus (NNV)-infected larval groupers. The results suggested that EcTLR3 plays an important role in a fish's defense against viral infection.

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1. Introduction

Innate immunity, also known as non-specific immunity, is the first line of defense against microbial assaults. It is an efficient and complex system that uses a set of evolutionary conserved receptors termed as pattern recognition receptors (PRRs) to recognize conserved microbial structures known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are a family of best-characterized PRRs. They recognize PAMPs expressed by pathogenic microorganisms, and activate a variety of signaling pathways, including MAP kinase, Jun N-terminal kinase(JNK), p38, NF-κB, and IFN regulatory factor (IRF)3, leading to the induction of proinflammatory cytokines and chemokines (Kawai et al., 2001; Shinobu et al., 2002; Takeuchi and Akira, 2001). In addition to triggering innate immunity, TLRs play a pivotal role in activating adaptive immune responses (Akira et al., 2001; Kaisho and Akira, 2001; Kaisho et al., 2002). TLRs are type I transmembrane receptors containing an extracellular N-terminus with a leucine-rich repeat region (LRR), and an intracellular C-terminus called the Toll-interleukin-1 receptor (TIR) domain (Akira et al., 2001). The LRRs are involved in pathogen recognition (Bell et al., 2003), while the conserved cytoplasmic TIR domain has been shown to be critical in the signaling as well as in the localization of the TLRs (Funami et al., 2004; Sarkar et al., 2003). The Toll gene was originally described as being essential for embryonic dorsoventral patterning in *Drosophila* and was subsequently shown to be involved in response to fungal infection (Anderson et al., 1985; Lemaitre et al., 1996). So far, about 13 TLRs have been identified in mammals, and most of them have been shown to recognize a unique set of PAMPs (Rebl et al., 2010). For example, TLR2, TLR4, TLR7/8 and TLR9 recognize peptidoglycan, LPS, single-stranded (ss)RNA and unmethylated bacterial CpG DNA, respectively (Heil et al., 2004; Hemmi et al., 2000; Poltorak et al., 1998; Schwandner et al., 1999; Tabeta et al., 2004).

TLR3, was the first identified antiviral TLR member (Schroder and Bowie, 2005). TLR3 binds double-stranded (ds)RNA, including the replication intermediates of single-stranded (ss)RNA viruses, dsRNA viruses and Poly I:C (Alexopoulou et al., 2001; Kariko et al., 2004; Sen and Sarkar, 2005; Wang et al., 2004). Activation of TLR3 leads to the expression of chemokines, especially type I interferon (IFNα/β) (Yamamoto et al., 2003), which are responsible for producing an antiviral state (Jamaluddin et al., 2001; Spann et al., 2004). In acute viral infections, IFN could further induce the expression of myxovirus-resistant protein (Mx) and oligoadenylate synthetase to suppress viral replication (Samuel, 2001). Several studies have demonstrated that TLR3 participates in host defense against Punta Toro virus (PTV, ssRNA virus), Encephalomyocarditis virus (EMCV, ssRNA virus) and Respiratory syncytial virus (RSV, ssRNA virus) in humans and mice (Gowen et al., 2006; Groskreutz et al., 2006; Hardarson et al., 2007; Liu et al., 2007; Rudd et al., 2005). However, its antiviral role is controversial and the precise mechanism is not completely defined. Therefore, further analysis of TLR3 is needed to elucidate the mechanism(s) responsible for the

Abbreviations: TLRs, Toll-like receptors; TIR, Toll/interleukin-1 receptor; LRR, leucine-rich repeat; Poly I:C, polyriboinosinic polyribocytidylic acid; NNV, nervous necrosis virus; PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; JNK, Jun N-terminal kinase; VNN, viral nervous necrosis; IHNV, infectious hematopoietic necrosis virus.

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http://dx.doi.org/10.1016/j.gene.2013.06.014

immune responses to viral infection. In fish, TLR3 genes have been identified in *Danio rerio* (Meijer et al., 2004), *Ictalurus punctatus* (Bilodeau and Waldbieser, 2005), *Takifugu rubripes* (Oshiumi et al., 2003), *Oncorhynchus mykiss* (Rodriguez et al., 2005), *Ctenopharyngodon idella* (Su et al., 2009), *Gobiocypris rarus* (Su et al., 2008), *Cyprinus carpio* (Yang and Su, 2010), *Larimichthys crocea* (Huang et al., 2011) and *Paralichthys olivaceus* (Hwang et al., 2011). In *O. mykiss*, *G. rarus*, *C. idella*, *D. rerio*, *L. crocea* and *P. olivaceus*, TLR3 transcripts were shown to be up-regulated after stimulation with RNA viruses or Poly I:C (Huang et al., 2011; Hwang et al., 2011; Meijer et al., 2004; Novoa et al., 2006; Rodriguez et al., 2005; Su et al., 2008, 2009). However, TLR3 transcripts have been shown to be down-regulated in *C. carpio* after injection with grass carp reovirus (GCRV, dsRNA virus) (Yang and Su, 2010). Additionally, TLR3 has also been found to respond to bacterial infection in mice (Kadowaki et al., 2001), zebrafish (Meijer et al., 2004), channel catfish (Bilodeau and Waldbieser, 2005), channel-blue backcross hybrids (Peterson et al., 2005) and the large yellow croaker (Huang et al., 2011).

Grouper (*Epinephelus coioides*) is an important commercial mariculture fish species in Asia and around the world because of its excellent meat quality and rapid growth. At present, this species is especially vulnerable to infections by viral nervous necrosis (VNN) viruses at the larval and juvenile stages, resulting in high mortality rates (80 to 100%) (Skliris et al., 2001). The disease VNN is caused by a fish nodavirus belonging to the family *Nodaviridae* (genus *Betanodavirus*), whose genome consists of two single-stranded positive sense RNAs (Nishizawa et al., 1995; Pakingking et al., 2010). Nodaviruse produces dsRNA in its life cycle and therefore it is possible that they are detected by TLR3. Understanding the immune mechanisms of TLR3 in grouper may contribute to the development of strategies for disease control and for long-term sustainability of grouper farming. Poly I:C is structurally similar to dsRNA, which is present in some viruses and is a "natural" stimulant of TLR3 (Fortier et al., 2004). Thus, Poly I:C can be considered a synthetic analog of dsRNA and is a very useful tool for scientific research on the immune system.

In our study, expression of *EcTLR3* was detected in both the embryonic-larval and the tissue mRNA samples of the grouper. To understand the roles of EcTLR3 in the grouper immune system, temporal expression of *EcTLR3* was studied after groupers were injected with Poly I:C. Furthermore, the expression profile of *EcTLR3* was analyzed in groupers suffering from NNV infection and compared to that of healthy larval groupers.

2. Materials and methods

2.1. Animals, immune challenge, sample collection and preparation

All embryos, larvae and juvenile groupers were obtained from a fish farm in Zhaoan (Fujian, China) in October 2011. Juvenile fish (length 14 ± 2.3 cm, body weight 45 ± 15.1 g) were acclimatized at 25 ± 2 °C for two weeks in 80 × 80 × 50 cm polyethylene net cages in water of salinity (23‰). Groupers were challenged, by intraperitoneal injection, with 200 μL Poly I:C (Sigma-Aldrich Corporation, 1 mg/mL) in 0.9% NaCl, or 200 μL of 0.9% NaCl (as control). Livers, spleen tissues and blood cells were collected from five individuals at 0, 3, 6, 12, 24 and 48 h after injection. Tissues from a healthy group, including gill, liver, spleen, intestine, head kidney, heart, muscle, brain, stomach and hemocyte were also sampled. Embryos and larvae collected at different developmental stages included fertilized egg (0 h post-fertilization (pf)), multi-cell stage (2 h 30 min pf), morula (3 h 25 min pf), blastula (5 h 30 min pf), gastrula (9 h 30 min pf), embryo body stage (12 h pf), muscle burl stage (15 h pf), crystal stage (23 h 30 min pf), heart-beating stage (24 h pf), hatching stage (28 h pf), 2-day-old larva, 3-day-old larva, 4-day-old larva, 5-day-old larva, 10-day-old larva, 15-day-old larva, 20-day-old larva, 25-day-old larva and 30-day-old larva. Additionally, VNN-infected 25-day-old larval groupers which had abnormal swimming behavior (whirling and spiraling), and the corresponding normal larval fish (as control) were also collected. All of the samples were homogenized in TriPure Isolation Reagent (Roche Diagnostics, Switzerland), and total RNA was isolated according to the manufacturer's instruction. The total RNA samples were then incubated with RNase-free DNase I (Promega Corporation, USA) to remove any contaminating genomic DNA. First strand cDNA was synthesized from total RNA with random primers using M-MLV reverse transcriptase (Promega Corporation, USA), following the manufacturer's protocol.

2.2. Identification and confirmation of EcTLR3 cDNA

An *E. coioides* TLR3 open reading frame (ORF) sequence, here referred to as *EcTLR3*, was obtained from NCBI (GenBank: HQ857748). Gene-specific primers were used to confirm the DNA sequence of the putative gene. The 5′ and 3′ unknown sequences of TLR3 were then amplified using a SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instruction. Gene-specific primers and adapter primers (Table 1) were used for the nested PCR to amplify the 5′ and 3′ region. PCR products were purified from agarose gel using a Qiaquick Gel Extraction Kit (Qiagen), and ligated into the T/A cloning vector pMD19-T (TaKaRa Biotechnology Co., Ltd.) followed by transformation into *Escherichia coli* JM109 competent cells. The positive clones were confirmed by DNA sequencing.

2.3. Sequence characterization and phylogenetic analysis

Isoelectric point and molecular weight prediction was carried out at (http://cn.expasy.org/tools/pi_tool.html). Conserved tyrosine phosphorylation sites were predicted by http://www.cbs.dtu.dk/services/NetPhos/. Protein domain features were predicted by Simple Modular Architecture Reach Tool (SMART) (http://smart.embl-heidelberg.de/) (Letunic et al., 2006) and the LRR domain was additionally analyzed according to the method described by Matsushima et al. (2007). Multiple-alignment of protein sequences was performed with the BioEdit program, and phylogenies of protein sequences were estimated using the neighbor-joining (NJ) method and MEGA4.0 software. The bootstrap values were replicated 1000 times to obtain the confidence value for the analysis.

2.4. Quantitative real-time PCR analysis of EcTLR3 mRNA level

Real-time PCR was carried out in a 20 μL reaction volume containing 9 μL of 1:10 diluted original cDNA, 10 μL of 2 × SYBR Green Master Mix, and 0.5 μL of each primer (10 pmol mL^{-1}). Reactions were performed with the SYBR Green PCR Master Mix (Applied Biosystems), and analyzed in the ABI 7500 real-time System. For the absolute quantification method, standard curve was generated according to the cycle threshold (CT) value and the logarithm of vector dilutions using serial dilutions of quantified pMD19-T Easy vector (TaKaRa Biotechnology Co., Ltd., Japan) containing the *EcTLR3* fragment. For the relative quantification method, β-actin gene was used as a reference gene. Reactions were conducted as follows: initiation at 95 °C for 3 min, then 40 cycles of denaturation at 94 °C for 20 s, annealing and

Table 1
Oligo nucleotide primers used in this article.

Primer name	Nucleotide sequence (5′ → 3′)	Purpose
TLR3-5out	5′ AGTGCAGTGGCTCAGTTCCTCCTTC 3′	5′ RACE
TLR3-5inner	5′ TGACAATCACCGCCGAGACGAG 3′	
TLR3-3out	5′ CTCCACTGGCTTACTACAACCACCCC 3′	
TLR3-3inner-1	5′ CACATTAGGATTCAGCGACGCCACA 3′	3′ RACE
TLR3-3inner-2	5′ GCCAGCAGGGACTCTGTGGTTC 3′	
TLR3-RT-F	5′ CTGGCTTACTACAACCACCCC 3′	Target gene expression
TLR3-RT-R	5′ CAAACTCCCTGCCCTCTTCA 3′	analysis
β-Actin-F	5′ CACTGTGCCCATCTACGAG 3′	Reference gene
β-Actin-R	5′ CCATCTCCTGCTCGAACTC 3′	

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