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Short communication

Identification and characterization of a novel Toll-like receptor 4 homologue in blunt snout bream, *Megalobrama amblycephala*

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ABSTRACT

Toll-like receptors (TLRs) are central players in the innate immune system in response to a wide range of pathogen infection. Among various TLRs, TLR4 plays a key role in recognition of bacterial lipopolysaccharides (LPS). In the present study, we identified and characterized a novel TLR4 homologue (*maTLR4b*) in blunt snout bream (*Megalobrama amblycephala*) which was significantly distinct from previously reported *M. amblycephala* TLR4 (tentatively named *maTLR4a*). The results showed that the complete cDNA sequence of *maTLR4b* was 3261 bp with an open reading frame encoding a polypeptide of 820 amino acids, and that its genomic sequence was 3793 bp, which had 3 exons. Structurally, the deduced *maTLR4b* protein showed a typical TLR domain architecture, including a signal peptide, eight 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 revealed that all TLR4s from teleost fish formed a monophyletic clade. Both *maTLR4a* and *maTLR4b* were divided into two distinct branches, and showed the highest level of similarity with the grass carp *TLR4.2* and *TLR4.4* homologue, respectively. *MaTLR4b* was constitutively expressed in all healthy tissues tested although at different levels. After LPS stimulation, the expression levels were significantly up-regulated in spleen, and peaked at 4 h between *maTLR4a* and *maTLR4b*, but with a distinct and complementary expression patterns. Taken together, these results suggested that *maTLR4b* is indeed a functional homologue of TLR4 in other species, which may play vital role in innate immune.

1. Introduction

The innate immune system, an evolutionary conserved defense mechanism, is an efficient first line of host defense against invading microbes [1]. Fish protect themselves from various microbial pathogens mostly with the help of innate or non-specific immunity [2]. Innate immune response is initiated by pattern recognition receptors (PRRs) which target the conserved molecular structure of pathogens, known as pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS), polyinosinic: polycytidylic acid (poly I:C), polysaccharides, flagellin, double-stranded and single-stranded viral RNA and so on [3,4]. Toll-like receptors (TLRs), a family of conserved and germline-encoded PRRs, belong to type I transmembrane proteins that contain an extracellular leucine-rich repeats (LRRs) domain responsible for recognizing PAMPs, a transmembrane domain (TM) and an intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain involved in downstream signal transduction [5]. TIR domain is highly conserved not only between the different TLRs of one species but also between different animal species [6].

Among various TLRs, TLR4 was the first member of the TLR family to be identified and plays the central role in recognition of LPS, a major component of the outer membrane of Gram-negative bacteria. TLR4 binds to LPS and triggers a signaling cascade through MyD88-dependent and MyD88-independent pathway resulting in the expression of downstream pro-inflammatory cytokines [7]. Until now, TLR4 homologues have been identified, and their molecular characteristics and expression patterns have been analyzed in ictalurids [e.g., channel catfish (*Ictalurus punctatus*)],

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Table 1
Sequences of primers used in this study.

Primer	Sequence (5′→3′)	Comment
TLR4b-F	GTTCTTGTGGATRTTSGTCT	Gene cloning
TLR4b-R	ATKGRGTTRRARGACAAATC	
3′-Adaptor primer	GCTGTCAACGATACGCTACGTAACG GCATGACAGTG(T)$_{18}$	3′RACE
3′-Primer	GCTGTCAACGATACGCTACGTAACG	
3′-Nested primer	CGCTACGTAACGGCATGACAGTG	
TLR4b-3′-GSP	TTATCGGCACACAGAAACTAAGGCATT	
TLR4b-3′-NGSP	TGATGTTCTCCAAAAGTTGCCCA	
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	5′RACE
AUAP	GGCCACGCGTCGACTAGTAC	
TLR4b-5′-GSP	GGAGGGACACAGACTGGATG	
TLR4b-5′-NGSP	TGTAGCTTGGTTAGGTTATTCA	
TLR4b-gF1	GCTGGACAAGGACAGGAATG	Intron-1
TLR4b-gR1	GTGATAGGAAGACTGCTGGG	
TLR4b-gF2	TCCCAGCAGTCTTCCTATCA	Intron-2
TLR4b-gR2	TGTAAGGACGGGAGACGAAT	
TLR4b-qF	CCAACCAGAGCTGAAACGTC	Real-time PCR
TLR4b-qR	TGAAAGGTTTGTGGGCAACT	
TLR4a-qF	GATTCTCACTGGAAACCC	
TLR4a-qR	TTGAAGCGGTAAGGACAC	
β-actin-F	TCTACAACGAGCTGCGTGTTG	
β-actin-R	TCAATCCCAAAGCCAACAGG	

and cyprinids [e.g., zebrafish (*Danio rerio*), rare minnow (*Gobiocypris rarus*), common carp (*Cyprinus carpio*), mrigal (*Cirrhinus mrigala*), grass carp (*Ctenopharyngodon idella*) and naked carp (*Gymnocypris przewalskii*)] [8–15].

Owing to herbivorous habit, faster growth rate and delicate flesh quality, blunt snout bream (*Megalobrama amblycephala*), endemic to China and commonly known as Wuchang fish, has been accepted as a principal species in the Chinese freshwater fish polyculture system during the last few decades [16]. The latest data shows that its production has reached 0.73 million tons [17].

However, due to the intensive rearing, a incidence of bacterial diseases, especially *Aeromonas. hydrophila,* highly increases, which have led to catastrophic economic losses in *Megalobrama amblycephala* culture industry [18]. Obviously, the characterization of immune-related gene might significantly contribute to the elucidation of disease defense mechanisms in *M. amblycephala* and can shed new insights into its health management, diseases control and developing molecular markers related to disease resistance in aquaculture. TLR4 has been proved to be responsive to *Aeromonas hydrophila* challenge or LPS stimulation in some cyprinid species. Although a TLR4 homologue (*maTLR4a*) has been reported in *M. amblycephala* [19], in the present study, we identified and characterized a novel TLR4 homologue (*maTLR4b*). We also obtained the genomic sequence of *maTLR4b*, investigated its tissue distribution and expression modulation after LPS stimulation to better understand its role in innate immunity.

2. Materials and methods

2.1. Fish, LPS stimulation and RNA isolation

Blunt snout bream (weight: 405 ± 12.4 g) were obtained from a fish farm in Yingcaiyuan, Changsha, China. Fish were maintained with a flow-through water supply at room temperature. After acclimating for 7 days, the normal fish were used for the stimulation experiments. A group of fish received an intraperitoneal injection of LPS isolated from *Escherichia coli* (L2880, Sigma) at a dose of 0.1 mg/100 g fish, while a second group of fish injected with equal dose of sterilized phosphate buffered saline (PBS, pH 7.4) was served as a control group. Fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO, USA). Various tissues from three healthy individuals, including kidney, head kidney, muscle, liver, spleen, gill and heart, were collected. Similarly, the spleen from three individuals was collected at different time points (0, 2, 4, 6, 8, 12 and 24 h) after stimulation. Total RNA from above tissues was extracted using TRIzol reagent (Invitrogen, CA, USA), and quantified based on the absorbance at 260 nm. The integrity of RNA was checked in agarose gel electrophoresis. The tissues collected at 0 h were from non-stimulated fish.

2.2. Cloning and characterization of maTLR4b

The partial cDNA of *maTLR4b* was amplified by a pair of degenerate primers designed based on an alignment of TLR4 sequences of other teleost fish (Table 1). To obtain the full-length cDNA sequence of *maTLR4b*, 3′-Rapid Amplification of cDNA Ends (RACE) and 5′-RACE were performed using the GeneRacer™ (Invitrogen, CA, USA) and Rapid Amplification of cDNA Ends Kits (GibcoBRL, USA), respectively, according to the manufacturer's instructions. After comparison the cDNA sequence of *maTLR4b* with the genomic sequence of *Ctenopharyngodon idellus* TLR4.4, two pairs of primers were designed for amplifying the introns of *maTLR4b* (Table 1). All resulting PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced bi-directionally. All sequences were assembled to obtain the full-length cDNA sequence of *maTLR4b*. Protein domain structures were predicted using the Simple Modular Architecture Research Tool (SMART) [20] and SignalP 4.1 [21]. The multiple sequence alignments were performed with the Clustal W program [22]. The phylogenetic tree was constructed by Neighbor-Joining method in Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.06), and support for each node was bootstrapped with 1000 replicates [23].

2.3. Quantitative analysis of maTLR4b

To determine the *maTLR4b* expression level in different normal tissues and spleen after LPS stimulation, the quantitative real-time PCR (qRT-PCR) was performed using a gene-specific primer sets (Table 1). Furthermore, we compare expression levels between *maTLR4a* and *maTLR4b* after LPS stimulation. The β-actin was amplified as an internal control using the primer set of β-actin-qF and β-actin-qR (Table 1). The SYBR Green fluorescent qRT-PCR was implemented in a 20 μL reaction volume using an ABI 7500 Real-time PCR system (Applied Biosystems), containing 10 μL SYBR® Premix Ex Taq™ II (2×) (TaKaRa, Dalian, China), 0.4 μL ROX