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Short communication

Identification and expression analysis of TLR2 in mucosal tissues of turbot (*Scophthalmus maximus* L.) following bacterial challenge

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ABSTRACT

The pathogen recognition receptors (PRRs), which can recognize the conserved pathogen-associated molecular patterns (PAMPs) of the bacteria, play key roles in the mucosal surfaces for pathogen recognition and activation of immune signaling pathways. However, our understanding of the PRRs and their activities in mucosal surfaces in the critical early time points during pathogen infection is still limited. Towards to this end, here, we sought to identify the Toll-like receptor 2 (TLR2) in turbot as well as its expression profiles in mucosal barriers following bacterial infection in the early time points. The full-length TLR2 transcript consists of open reading frame (ORF) of 2451 bp encoding the putative peptide of 816 amino acids. The phylogenetic analysis revealed the turbot TLR2 showed the closest relationship to *Paralichthys olivaceus*. The TLR2 mRNA expression could be detected in all examined tissues, with the most abundant expression level in liver, and the lowest expression level in skin. In addition, TLR2 showed different expression patterns following *Vibrio anguillarum* and *Streptococcus iniae* infection, but was up-regulated following both challenge, especially post *S. iniae* challenge. Characterization of TLR2 will probably contribute to understanding of a number of infectious diseases and broaden the knowledge of interactions between host and pathogen, which will eventually help in the development of novel intervention strategies for farming turbot.

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1. Introduction

In teleost species, the innate immune system is the primary host protection mechanism against pathogen infection, but it is the mucosal immune system that constitutes the first line of host defense. The mucosal immune system is more critical for aquatic animals, which are living in the pathogen rich aquatic environment. Their mucosal surfaces are exposed to external pathogen directly, while also serve as dynamic interfaces that simultaneously mediate a diverse array of critical physiological processes [1]. In constantly contact with a broad spectrum of pathogens, the mucosal surfaces are colonized by a wide range of commensals, opportunistic and primary pathogens all the time [2]. In this situation, the main function of mucosal immune system is to sense, screen and recognize the attached pathogen, and then to active the immune system to clear the infected pathogen. In this regard, the pathogen recognition receptors (PRRs), which can recognize the conserved pathogen-associated molecular patterns (PAMPs) of the bacteria, should play key roles in the mucosal surfaces for pathogen recognition and activation of immune signaling pathways. However, our understanding of the PRRs and their activities in mucosal surfaces in the critical early time points during pathogen infection is still limited.

According to different properties of the PRRs, they were divided into three major groups, i.e., Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs). Among them, the TLRs were the earliest

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characterized and also the most extensively studied PRRs in teleost species [3]. Members of the Toll family play key roles in innate antibacterial and antifungal immunity from worm *Caenorhabditis elegans* to mammals, and confer the host ability to detect a diverse range of PAMPs to initiate a well-coordinated immune response to limit or eradicate invading microbes [4,5]. Toll-like receptor 2 (TLR2) is a member of the vertebrate protein family of TLRs that are very important components of innate immunity. Typically, the type I receptor molecular of TLR2 is characterized by the extracellular domains containing 18 to 20 leucine rich repeats (LRR), a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain for signaling transducing [5]. TLR2 was proven to be directly involved in recognition of specific PAMPs and activated pro-inflammatory cytokines and type-I interferons through conserved singling pathways, including MyD88-depedent, MyD88-independent, and TRIF (TIR-domain-containing adapter-inducing interferon-β)-dependent pathways [6]. The mechanisms of the pathways were detailed somewhere [7]. In detail, TLR2 is well known as a receptor recognizing conserved components of Gram-positive bacteria such as lipoteichoic acid, peptidoglycans, lipoproteins, yeast zymosan, glycosylphosphatidylinositols from protozoan parasites, LPS of Gram-negative bacterium *Porphyromonas gingivalis*, LPS of zoonotic pathogen *Leptospira interrogans*, and Gram-negative bacterium *Edwardsiella ictaluri* and others [8–13]. Complex formation with itself constituting a homodimer [14] or with other molecules as heterodimer, such as TLR1 or TLR6 [15] involved in pattern recognition of various PAMPs. Thus, TLR2 may be a sensor and inductor of specific defense processes, such as cellular necrosis initially spurred by microbial compounds. More recently in teleost fish, the structures and expression patterns of TLR have been characterized in fugu (*Takifugu rubripes*) [16], zebrafish (*Danio rerio*) [17], Japanese flounder (*Paralichthys olivaceus*) [18], channel catfish (*Ictalurus punctatus*) [13], common carp (*Cyprinus carpio*) [19], orange-spotted grouper (*Epinephelus coioides*) [20]. Collectively, the TRL2 plays vital roles for fish innate immune responses against infection, but its activities in mucosal immune responses were always overlooked. Up to date, many studies have explored their critical roles against bacterial infection in fish species, and found that their activities were dramatically changed during infection. In teleost, the number of reported TLR ligands progressively increases, and characterizations of structure and expression pattern after infection have seen TLR specificity and bacterium-dependent, thus, attempts to characterize TLR specificity and function are still necessary.

Turbot (*Scophthalmus maximus*), one of the most important maricultured species in China, suffers from widespread disease outbreaks due to a number of pathogens, including

Vibrio anguillarum, *Streptococcus iniae* and *Edwardsiella tarda*, resulting in dramatic economic losses. Recently in turbot, many innate immune actors and their associated activities following infection have been characterized, including Stomatin-like protein 2 [21], chemokines [22,23], MyD88 [24] and lysozyme [25]. With the first high-throughput sequencing analysis of turbot in response to viral stimulations, a wide varieties of immune pathways were identified, such as complement pathway, toll-like receptor signaling pathway, B cell receptor signaling pathway, T cell receptor signaling pathway and cytokines [26]. But the characterization of mucosal immune cellular actors and their associated immune activities related to mucosal resistance is still lacking. The successful clearance of the infected pathogen always starts with the early recognition of the invading pathogen, TLR2 could be one of the most important players on the mucosal surfaces for host protection. Towards to this end, here, we sought to identify the TLR2 in turbot as well as its expression profiles in mucosal barriers following bacterial infection in the early time points which are critical for establishment of infection. Therefore, we reported the identification, phylogenetic, basal tissue distribution, and mucosal expression patterns following different bacterial infection for the TLR2 in turbot for the first time. Characterization of TLR2 will probably contribute to the understanding of a number of infectious diseases and broaden the knowledge of interactions between host and pathogen, which will eventually help in the development of novel intervention strategies for farming turbot.

2. Materials and methods

2.1. Sequence identification and analysis

The turbot TLR2 sequence was captured from our transcriptome database (Li, unpublished data) by BLAST program using the TLR2 protein sequences from other species as queries with a cutoff E-value of 1e-10. The NCBI ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to identify the potential open reading frame (ORF) of the retrieved sequences. The predicted ORF sequence was further verified by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI non-redundant protein sequence database. The conserved domains and signal peptides were identified by the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/). And the ExPASy server (http://expasy.org/tools/) was utilized to analyze the theoretical pI, molecular massand N-glycosylation sites [27].

2.2. Phylogenetic analysis

To identify the evolution relationships of TLR2 and also to further verify the sequence identification, the amino acids sequences of TLR2 from turbot as well as the other species were selected to construct the phylogenetic tree, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), western clawed frog (*Xenopus tropicalis*), green sea turtle (*Chelonia mydas*), olive flounder (*Paralichthys olivaceus*), Nile tilapia (*Oreochromis niloticus*), zebrafish (*D. rerio*), medaka (*Oryzias latipes*), and stickleback (*Gasterosteus aculeatus*). The ClustalW2 program was used to conduct the multiple sequence alignments of TLR2 amino acids sequences [28]. Phylogenetic and molecular evolutionary analyses were performed in Molecular Evolutionary Genetics Analysis (MEGA 6) package using the neighbor-joining method [29]. The phylogenetic tree was analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 10,000 bootstrapping replications.

2.3. Bacteria challenge and sample collection

Samples of turbot fingerlings (average weight of 15.6 ± 0.23 g) were obtained from the Turbot hatchery in Haiyang, Shandong Province, China. Prior to experimentation, fish were acclimated in the laboratory in a flow-through system (27 ± 0.5 °C) for at least one week for overall fish health evaluation. Each aquarium was supplied with constant aeration, continuous water flow at approximately 7 L/min and a light and dark period of 12 h: 12 h was maintained. The fish received no feed during the challenge experiment. Only healthy turbot fingerlings determined by the external appearance and level of activity were used for this study.

Two bacteria, the Gram-negative bacteria *V. anguillarum* and the Gram-positive bacteria *S. iniae* were selected to conduct the bath challenge to characterize the immune roles of TLR2 gene in the host mucosal defense against bacterial infection. The bacterial isolate of *V. anguillarum* and *S. iniae* were provided by the disease lab in Qingdao Agricultural University. After a pre-challenge, the bacteria

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