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Short communication

Identification and expression analysis of toll-like receptor genes (TLR8 and TLR9) in mucosal tissues of turbot (*Scophthalmus maximus* L.) following bacterial challenge

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ABSTRACT

Mucosal immune system is one of the most important components in the innate immunity and constitutes the front line of host defense against infection, especially for teleost, which are living in the pathogen-rich aquatic environment. The pathogen recognition receptors (PRRs), which can recognize the conserved pathogen-associated molecular patterns (PAMPs) of bacteria, are considered as one of the most important component for pathogen recognition and immune signaling pathways activation in mucosal immunity. In this regard, we sought to identify TLR8 and TLR9 in turbot (*Scophthalmus maximus*), as well as their mucosal expression patterns following different bacterial infection in mucosal tissues for the first time. The full-length TLR8 transcript consists of an open reading frame (ORF) of 3108 bp encoding the putative peptide of 1035 amino acids. While the TLR9 was 6730 bp long, containing a 3168 bp ORF that encodes 1055 amino acids. The phylogenetic analysis revealed both TLR8 and TLR9 showed the closest relationship to large yellow croaker. Moreover, both TLR8 and TLR9 could be detected in all examined healthy turbot tissues, with the lowest expression level in liver and a relatively moderate expression pattern in healthy mucosal tissues. Distinct expression patterns of TLR8 and TLR9 were comparatively observed in the mucosal tissues (intestine, gill and skin) following *Vibrio anguillarum* and *Streptococcus iniae* infection, suggesting their different roles for mucosal immunity. Further functional studies are needed to better characterize TLR8 and TLR9 and their family members, to better understand the ligand specificity and to identify their roles in different mucosal tissues in protecting fish from the pathogenically hostile environment.

1. Introduction

The innate immune system is the primary defense mechanism in host protection against invading microbial pathogens. Unlike higher vertebrates, such as birds and mammals, having adaptive immunity, lower vertebrates like fish rely primarily upon innate immune system, which confers faster but less specific immune response than that in adaptive immunity [1,2]. Mucosal immune system is one of the most important components in the innate immunity and constitutes the front line of host defense against infection [3]. As living in the pathogen-rich aquatic environment, fish mucosal surfaces are exposed to external pathogens or microbes directly, and are constantly colonized by a broad spectrum of pathogens [4]. Therefore, in order to prevent the pathogen attachment and invasion, the first step is to sense, screen and recognize the pathogen in mucosal surfaces. In this regard, germ-line-encoded pathogen recognition receptors (PRRs), which are distributed on cell surface, in intracellular compartments, or secreted into the blood stream and tissue, can recognize the conserved pathogen-associated molecular patterns (PAMPs) of the bacteria, and thus are considered as one of the most important components for pathogen recognition and immune signaling pathway activation in mucosal immunity [5]. However, our

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knowledge of the PRRs and their associated activities in mucosal immunity against pathogen infection in teleost is still very limited.

According to different properties of the PRRs, three major groups of PRRs have been reported so far, including Toll-like receptors (TLRs), NOD-like receptors and retinoic acid inducible gene I (RIG-I)-like receptors. Among them, the TLRs are the earliest characterized PRRs, playing vital roles in innate immunity from worm *Caenorhabditis elegans* to mammals. TLRs are transmembrane pattern recognition receptors, capable of recognizing PAMPs and regulating the subsequent immune response in mammals as well as in teleost fishes [5–7]. Structural and functional analysis of TLRs in vertebrates and comparison of TLR family members between teleost fishes, showed that specific divergence and function of TLR genes between fish and mammals as well as among fish species [8–10]. In general, TLRs are characterized by an ectodomain containing leucine-rich repeats (LRRs) that facilitate PAMP recognition, a transmembrane domain, and an intracellular Toll/IL-1 receptor (TIR) domain for downstream signaling transduction [1,2,6]. Based on their primary sequences, the recognition of specific pathogens and immune cell subtypes, two major TLR subfamilies are classified in human and mouse. Ten known functional TLRs have been identified in humans and 13 in mice; TLRs 1–9 are conserved in both species; three TLRs, namely, TLR11, 12, and 13, have been lost from human genome [8,11,12]. The first group includes TLR1, 2, 4, 5, 6 and 10, which are cell surface TLRs that are primarily expressed in the plasma membrane of immune cells, and they could recognize a variety of unique microbial membrane components like lipids, lipoproteins and proteins. The second group of TLRs, including 3, 7, 8 and 9, are endosomal/intracellular TLRs which are expressed on intracellular vesicular membranes and are commonly involved in recognition of nucleic acids [13]. To be specific, TLR9 has been shown to respond to unmethylated CpG DNA from viral or bacterial DNA, TLR3 to double-stranded RNA, and TLR7 and TLR8 have been demonstrated to be activated by synthetic antiviral imidazoquinoline compounds and are involved in recognizing single-stranded RNA. Consequently, PAMP recognition patterns and downstream signaling pathways differed as follows. Activation of endosomal TLRs leads to the production of nuclear factor (NF)-κb-mediated cytokines and type I interferon (IFN). Specifically, TLR8 and TLR9 recruit adaptor protein, MyD88, to signal the production of pro-inflammatory cytokines via the activation of NF-κb and/or type I interferon through the activation of IRF7 pathway. In case of the cell surface TLRs, they recruit MyD88 and TIRAP (TIR domain-containing adaptor protein) adaptor proteins to signal the NF-κb pathway to produce NF-κb-mediated cytokines [13].

Up to date, at least 14–20 different types of TLR have been identified in dozens of fishes, but orthologs of mammalian TLR6 and TLR10 have not been identified in fish [8,14]. Genome-wide characterizations of fish TLR genes and comparative genomics have assisted identification of more TLRs in fish. For example, in zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*), genome-wide searches have revealed high sequence conservation of fish TLRs to human TLRs, as well as distinct non-mammalian and even teleost unique TLRs. TLR8 and 9 have conserved domains as mammalian counterparts, and two different evolutionary history of gene duplication events have resulted in different paralogous, such as TLR8 in rainbow trout (*Oncorhynchus mykiss*) (i.e. TLR8a1 and a2) and in zebrafish (drTLR8a and b). Molecular phylogenetic analyses of fish TLRs predicted the TLR8 and 9 in the same clade among other clades [8]. Although the ligands recognition of TLR 8 and 9 in mammals have been well documented [15–17], yet this is not true in fishes. To date, many experiments have been performed in grass crap (*Ctenopharyngodon idella*) [18], Japanese flounder (*Paralichthys olivaceus*) [19], Atlantic salmon (*Salmo salar*) [20], large yellow croaker (*Larimichthys crocea*) [21], rainbow trout [22], zebrafish [23] for study TLR8, and for TLR9 associated studies in common carp (*Cyprinus carpio*) [24], cobia (*Rachycentron canadum*) [25], and zebrafish [26], but the ligand-binding properties of both TLRs have, so far, not been well evaluated. In teleost, although the number of reported TLR ligands progressively increases, continuous studies should be performed to identify the ligands specificity, and 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, turbot immune-related genes and their expression patterns following infection, including stomatin-like protein 2 [27], chemokine [28,29], MyD88 [30], PGRP2 [31], TLR2 [32], and G-type lysozyme [33], have been characterized. With high-throughput sequencing analysis of turbot in response to viral stimulations [34] and to bacterial infection [35], a wide varieties of immune pathways, such as toll-like receptor signaling pathway, the signatures of mucus barrier modification, pathogen entry, and host immune responses were revealed. Those efforts might provide new insights for drug intervention to manipulate immune responses, and also be used to assist future selection of disease resistant broodstock. But the characterization of mucosal immune cellular actors and their associate immune activities in mucosal tissues are still lacking. The successful clearance of the infected pathogen always starts with the early recognition of the invading pathogen, TLR8 and TLR9 might be some of the most important players in various intracellular compartments of mucosal tissues for host protection. Towards to this end, here, we sought to identify the TLR8 and TLR9 in turbot, including gene identification, comparative domain organization, phylogenetic, basal tissue distribution, and expression patterns following different bacterial infection in mucosal tissues for the first time. Our results will probably broaden the knowledge of interactions between host and pathogen, which will eventually help in the development of novel intervention strategies for improvement and enhancement of farming turbot.

2. Materials and methods

2.1. Sequence identification and analysis

The full length sequence of turbot TLR8 and TLR9 were captured from our transcriptome database [35] utilizing BLAST program with a cutoff E-value of 1e-10, using TLR8 and TLR9 sequences from other species as queries sequences. The potential open reading frame (ORF) of the retrieved sequences was identified by NCBI ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder). And the predicted ORF sequence was then verified in NCBI non-redundant protein sequence database by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/) was used to identify the conserved domains and signal peptides. Further analysis, such as the theoretical pI, molecular mass and N-glycosylation sites were carried out in ExPASy server (http://web.expasy.org/protparam/). The percentages of similarity and identity of TLR8 and TLR9 amino acids were calculated by MatGAT program [36].

2.2. Phylogenetic analysis

To further verify the sequence identification and determine the evolution relationships of TLR8 and TLR9, the amino acids sequences of turbot TLR8 and TLR9 as well as other species, including human (*Homo sapiens*), mouse (*Mus musculus*), western clawed frog (*Xenopus tropicalis*), zebrafish (*D. rerio*), fugu (*Takifugu rubripes*), large yellow croaker (*L. crocea*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*S. salar*) were selected to construct the phylogenetic tree. The selected amino acids sequences were first aligned in ClustalW2 program [37]. And a neighbor-joining phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis (MEGA 6) package [38]. The topological stability of the neighbor-joining tree was evaluated by 10,000 bootstrapping