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Molecular insights of the first gastropod TLR counterpart from disk abalone (*Haliotis discus discus*), revealing its transcriptional modulation under pathogenic stress

Don Anushka Sandaruwan Elvitigala [a], H.K.A. Premachandra [a], Ilson Whang [a,*], Bo-Hye Nam [b], Jehee Lee [a,c,*]

[a] Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea
[b] Biotechnology Research Division, National Fisheries Research and Development Institute (NFRDI), 408-1 Sirang-ri, Gijang-eup, Gijang-gun, Busan 619-705, Republic of Korea
[c] Marine and Environmental Institute, Jeju National University, Jeju Special Self-Governing Province 690-814, Republic of Korea

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ABSTRACT

Toll-like receptors (TLRs) are well-characterized pattern recognition receptors of innate immunity, known to induce immune responses against the pathogens by interacting with evolutionarily conserved pathogen-associated molecular patterns (PAMPs). In this study, a novel TLR homolog from disk abalone (*Haliotis discus discus*) was identified and characterized at molecular level. The open reading frame (ORF) of *AbTLR* is 3804 bp in length and encodes a 1268 amino acid peptide with a calculated molecular mass of 143.5 kDa. The deduced protein shows typical TLR domain architecture, with leucine rich repeats (LRR) and the toll-interleukin receptor (TIR) domain. Phylogenetic analysis revealed a close evolutionary relationship for AbTLR to its invertebrate counterparts, with close clustering to the molluscan homologs. Quantitative real-time PCR detected ubiquitous transcription of *AbTLR* in healthy tissues, but with highest levels in hemocytes. Differential transcriptional modulation of *AbTLR* was observed in abalone hemocytes and gills upon immune challenge, whereby *Vibrio parahaemolyticus* and purified lipopolysaccharide (LPS) enhanced the transcript level prominently. In addition, the viral hemorrhagic septicemia virus induced *AbTLR* transcription in hemocytes and gills, representing the first evidence of viral-induced immune response in mollusks to date. Collectively, our findings support a putative role for AbTLR in abalone antiviral and antibacterial defense.

1. Introduction

Invertebrates depend exclusively on innate immunity to combat infectious non self-agents, such as pathogenic microorganisms, since they do not possess an adaptive immune system. Recognition of invading pathogens to mount the innate immune response is generally mediated by the host-expressed pattern recognition receptors (PRRs), which interact with evolutionarily conserved, ubiquitously expressed molecular factors that decorate pathogenic microorganisms and are known as pathogen-associated molecular patterns (PAMPs) [1,2]. The most extensively studied PRRs are the Toll-like receptors (TLRs), and many of their PAMP ligands are well-characterized, including the lipopolysaccharide (LPS) and peptidoglycan cell wall components of bacteria and the flagellin protein, as well as free nucleic acid strands from lysed bacteria and viruses [3,4]. Moreover, these collective studies have revealed that the critical regulatory role of TLRs in innate immunity has been maintained throughout evolution, from cnidarians to mammals [5]. Indeed, recent studies demonstrating the compatibility of invertebrate and vertebrate innate immune systems have implicated the TLR-mediated immune signaling pathways as a key commonality [6,7].

The various TLR family members control different immune signaling pathways by activating a wide array of down-stream signaling molecules, such as myeloid differentiation factor 88

* Corresponding authors. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju, 690 756, Republic of Korea. Tel.: +82 64 754 3472; fax: +82 64 756 3493.
E-mail addresses: ilsonwhang@hanmail.net (I. Whang), jehee@jejunu.ac.kr, jeheedaum@hanmail.net (J. Lee).

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(MyD88), interleukin-1 (IL-1) receptor-associated kinase (IRAK), TNF receptor-associated factors (TRAFs), and nuclear factor kappa B (NF-κB) [8]. These various TLR signaling pathways culminate in the stimulation of particular genes' expression and immune-modulatory factors' activity, such as the cytokines. However, for all known TLRs the signal originates from a cellular membrane, either the plasma membrane (TLR1, 2, 4, 5, 6, and 10) or an intracellular endosome membrane (TLR 3, 7, 8, and 9) [9]. The common transmembrane localization is reflected in the characteristic protein structure of TLRs, which includes an ectodomain that protrudes outwards and is composed of ≤26 leucine rich repeat (LRR) motifs, a transmembrane domain, and an endodomain that extend inward and contains the signature Toll/IL-1 receptor (TIR) domain [10]. The TIR domain not only mediates localization of the TLR molecule to a particular membrane region but also initiates the down-stream signaling cascade [11,12].

Even though several invertebrate TLRs have been identified to date, only a few represent the molluscan species, including *Crassostrea gigas* [13] and *Chlamys farreri* (Akazara scallop) [14]. Yet, understanding the innate immune responses of marine invertebrate species is rapidly becoming important for the global food industry. Marine invertebrates, especially mollusks and arthropods, which are used as comestibles has reached to the levels of consumption, comparable to fish in many of the world's nations, with the most prominent use being in the East and Southeastern regions of Asia, including countries like China, Korea, and Japan. As a result, abalone has emerged as an economically important delicacy of the commercial invertebrate aquaculture industry. However, these marine gastropods are sensitive to a wide range of environmental conditions, some of which cause grievous impact on survival and growth. Pathogenic infections, in particular, have emerged as prominent threats to abalone integrity as a human food source [15–17]. The fact that the natural innate immune mechanisms functioning in abalones can tolerate some of these pathological invasions, to a certain extent, suggests that these mechanisms may represent targets of disease management strategies to help sustainable commercial farming efforts.

In this study, a novel TLR homolog (AbTLR) from disk abalone (*Haliotis discus discus*) was characterized at the molecular level. Moreover, transcriptional modulation of *AbTLR* was analyzed under pathological conditions, stimulated by live bacteria and virus, as well as purified LPS, to determine its potential role in immune defense of disk abalone.

2. Materials and methods

2.1. Identification of the partial cDNA sequences of AbTLR

A disk abalone sequence database was established based upon sequencing data (Roche 454 genome sequencer FLX systems (GS-FLX™); DNA Link, Republic of Korea) of a cDNA library generated from mRNA isolated from whole tissues of disk abalone [18]. Searching of this database with the Basic Local Alignment Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST) led to the identification of a TLR partial length cDNA sequences (isotig18424, isotig19106 and GSPZ16V01BZ56Q).

2.2. Identification of AbTLR complete coding sequence

A random shear bacterial artificial chromosome (BAC) library of abalone genomic DNA was custom constructed (Lucigen, USA) and screened for the full-length *AbTLR* gene by using the previously described pooling and super pooling strategies followed by polymerase chain reaction (PCR) [19]. The target gene sequence-specific primer pair, AbTLR-F and AbTLR-R (Table 1), was designed based on the partial cDNA sequence of *AbTLR* (Section 2.1). The PCR amplification was carried out in a total volume of 20 μL containing 0.5 U of Ex*Taq* polymerase (TaKaRa, Japan), 2 μL of 10× Ex*Taq* buffer, 1.6 μL of 2.5 mM dNTPs, 75 ng of template, and 10 pmol of each primer. The thermal cycling reaction included an initial 3 min incubation at 94 °C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The PCR products were analyzed on a 1.5% agarose gel and the band representing the amplicon containing the *AbTLR* gene was identified by expected size. Subsequently, detected BAC clone was sequenced (GS-FLX™) to obtain the genomic DNA sequence of the *AbTLR* gene. The complete putative coding sequence of *AbTLR* was then anticipated by analyzing the obtained genomic DNA sequence using the BLASTx algorithm and aligning with the existed partial cDNA sequences using DNAssist 2.2 (version 3.0) software. Coding sequence was further confirmed by cloning the respective fragment into pGEM-T vector (Promega – USA), after PCR amplification, using disk abalone multi tissue cDNA. Finally, the nucleotide sequence of *AbTLR* was deposited in GenBank under the accession number JX827423.

Table 1
Primers used in this study.

Name	Purpose	Sequence (5′ → 3′)
AbTLR-F	BAC library screening and qRT PCR of AbTLR	ACAGCTTCTTGGACGACGAATGGT
AbTLR-R	BAC library screening and qRT PCR of AbTLR	TCGTGCATGACGATGACGATGAGT
Ab-Rp-F	qRT-PCR of abalone ribosomal protein L5 gene	TCACCAACAAGGACATCATTTGTC
Ab-Rp-R	qRT-PCR of abalone ribosomal protein L5 gene	CAGGAGGAGTCCAGTGCAGTATG

2.3. In-silico analysis of AbTLR sequences

The nucleotide and predicted amino acid (aa) sequences of *AbTLR* were compared with those of various TLRs from different species that were retrieved by the BLAST search program. Pairwise sequence alignment and multiple sequence alignment were performed using the ClustalW2 program [20]. The phylogenetic relationship of these homologs was assessed by the Molecular Evolutionary Genetics Analysis (MEGA) software (version 5) using the neighbor-joining method with bootstrap values taken from 1000 replicates [21]. The characteristic TLR protein signatures were predicted by the ExPASy-Prosite server (http://prosite.expasy.org) and the SMART online server (http://smart.embl-heidelberg.de). The ExPASy protParam tool (http://web.expasy.org/protparam) was used to predict some physicochemical properties of the AbTLR protein. Furthermore, in order to prefigure and compare the three dimensional arrangement of the TLR primary protein structure, attributing to its committed role in physiology, degree of folding throughout the whole sequence area of AbTLR as well as its two homologues molluscan counterparts from *C. farreri* and *C. gigas* were predicted using FoldIndex© online bioinformatic tool [22]. Domain architectures of all three TLRs were identified using SMART online server.

2.4. Experimental animals and tissue collection

Healthy disk abalones (*H. discus discus*), with an average weight of 50 g and size of 8 cm, were purchased from the Youngsoo Abalone Farm of Jeju Island (Republic of Korea). Upon arrival, the live abalones were acclimated to the laboratory environment (seawater tanks with continuous filtering and aeration; salinity: