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Characterization of NLRP3-like gene from *Apostichopus japonicus* provides new evidence on inflammation response in invertebrates

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ABSTRACT

Inflammatory/defensive response after pathogen invasion is considered a local defense reaction in vertebrates. Inflammation response in *Apostichopus japonicus* was hardly determined due to scarce information available for nucleotide binding domain-like receptor family, pyrin domain-containing (NLRP) family. In the present study, invertebrate NLRP homologue was identified from *A. japonicus* (designated as *AjNLRP3-like*) by rapid amplification of cDNA ends. Full-length cDNA of *AjNLRP3-like* measured 2970 bp with 2265 bp open reading frame encoding a 754-amino acid (aa) residue protein. Structural analysis revealed that AjNLRP3-like processed characteristic domains of pyrin (32−102aa) and NACHT (183−339aa). Multiple sequence alignment and phylogenetic analysis supported that AjNLRP3-like belongs to a new member of NLRP3 protein subfamily. Spatial expression analysis revealed that *AjNLRP3-like* was ubiquitously expressed in all examined tissues with larger magnitude in coelomocytes. Both *Vibrio splendidus* challenge *in vivo* and lipopolysaccharide stimulation *in vitro* significantly upregulated mRNA expression of *AjNLRP3-like* when compared with the control group. NLRP3-mediated inflammation response depended on release of lysosomal cathepsin B (CTSB) and subsequent activation of high-mobility group box (HMGB) in vertebrates. We investigated expression profiles of *AjNLRP3-like* and *AjHMGB* after *AjCTSB* knock-down and discovered that *AjNLRP3-like* was depressed by 0.66-fold and 0.47-fold, whereas *AjHMGB* was depressed by 0.70-fold and 0.50-fold at 24 and 48 h in *AjCTSB*-silenced group, respectively. Similarly, down-regulation of *AjHMGB* was also observed after *AjNLRP3-like* knock-down. This study therefore suggests that *A. japonicus* feature similar inflammatory events as those in vertebrates, and activation of *AjNLRP3-like* depends on *AjCTSB* expression and release of *AjHMGB*.

1. Introduction

Inflammation refers to initial responses of hosts to external challenges through changes in local circulation (i.e., hyperemia and increased vessel permeability) and recruitment of immune cells (i.e., granulocytes, lymphocytes, and macrophages) to infected sites and eventually elimination of aggressors and promotion of tissue repair [1]. In this process, pro-inflammatory cytokines are produced and then released into extracellular milieu. Pro-inflammatory cytokines exert proinflammatory effects and activate inflammatory response by binding to extracellular domains of ubiquitous inflammatory cytokines receptor. Cleavage the precursor of pro-inflammatory cytokines is tightly controlled by inflammasomes, a family of proteins described for the first time in 2002 [2]. Inflammasomes were initially identified for their role in marginal or benign pathologies, such as periodic fevers and gout, but are now considered key factors in almost all inflammatory diseases [3–6]. Cytoplasmic receptors of the nucleotide binding domain-like receptor (NLR) family represent one of the key components of inflammasomes, of which NLRP3 was well documented and determined to play vital roles in initiating inflammatory process [7]. NLRP3 is activated by different common pathways, such as lysosomal dysfunction and consequent cathepsin B release, and effects of NADPH oxidase and potassium channel activation-associated K efflux [8]. Among these pathways, pivotal role of cathepsin B in activation of NLRP3 inflammasome was established by a large number of studies [9,10]. When phagocytic pathogens attack lysosomal membrane, which is then destabilized, cathepsin

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B is released and induces NLRP3-inflammasome activation through an unknown mechanism [11]. Subsequently, NLRP3 recruits apoptosis-associated speck-like protein and caspase-1 (also known as interleukin (IL)-1-converting enzyme) and assembles into large cytoplasmic complexes that are responsible for innate immune response to pathogens and/or danger signals. This response eventually leads to maturation and secretion of potent proinflammatory cytokines, such as precursors of IL-1β and IL-18 and damage-associated molecular patterns (DAMPs) of high-mobility group box (HMGB) family members of 1 or 3 [12–15]. However, few studies investigated signaling of HMGBs to combat pathogen infection in echinoderms.

Though invertebrates display various inflammation, immunity, and stress characteristics in extant species, similar inflammation events occur in invertebrates and mammals [16]. In invertebrates, early descriptions of inflammation were obtained through histological examinations of wound repair and demonstration of early activation of infiltrating phase; inflammatory responses were also reported in invertebrates, such as *Octopus vulgaris* [17], *Ruditapes decussatus* [18,19], *Crassostrea gigas* [20], and *Asterias rubens* [21]. Molecular patterns involved in immune-mediated and inflammatory responses in invertebrates were increasingly investigated over the last few years, and excellent reviews on the topic are available [22,23]. However, no definitive conclusion was made on inflammation indicator or mechanism of inflammation in invertebrates. *A. japonicus,* as a deuterostomia to vertebrates in evolution, played a special role in evolution of immunology. Understanding inflammatory response-related elements can clarify invertebrate inflammatory reaction and is a critical step for disease prevention and treatment of species aquaculture. In the current study, we cloned full-length cDNA of NLRP3 from *A. japonicus* (*AjNLRP3-like*) and investigated its spatial and time-course expression patterns to interpret its possible role in response to *Vibrio splendidus* and lipopolysaccharide (LPS) challenge. We confirmed regulatory relationship among AjNLRP3-like, AjCTSB, and AjHMGB in inflammatory responses by RNA interference. Our study provides new evidence for understanding inflammatory responses in invertebrates.

2. Materials and methods

2.1. Animals and challenge experiment

Sea cucumber *A. japonicus* (weight: 132 ± 17 g) were collected from Dalian Pacific Aquaculture Company and acclimatized in indoor aquaculture system with salinity of 30 and at 16 ± 1 °C [24]. For time-course expression analysis of AjNLRP3-like, one tank served as control, and another five tanks contained fresh *V. splendidus* at a final concentration of 10^7 CFU mL^{-1}. Coelomic fluid of five individuals from control and challenged groups were collected at 0, 6, 24, 48, 72, and 96 h post-inoculation. Coelomic fluids were collected and then centrifuged at 800 × *g* for 5 min at 4 °C to collect coelomocytes. For spatial expression analysis, coelomocytes and other four tissues, which include those of muscle, tentacle, respiratory trees, and intestine, were collected from control individuals using sterilized scissors and tweezers. Five biological replicates were obtained from experimental and control groups, and all samples were stored at −80 °C before RNA extraction and cDNA synthesis.

2.2. Rapid application cDNA ends of AjNLRP3-like

Partial sequence of NLRP3-like was collected from our transcriptome data [25], which was further validated by PCR. Full-length cDNA of *AjNLRP3-like* was obtained by rapid amplification of cDNA ends (RACE) with 3′, 5′-Full RACE kit (TaKaRa) following manufacturer's instructions. Gene-specific primers (Table 1) were designed based on the candidate NLRP3-like fragment. Desired polymerase chain reaction (PCR) products were cloned into pMD18-T simple vector (TaKaRa), and three positive clones were produced for each product sequenced at Sangon Biotechnology (Shanghai). We obtained the full-length cDNA of AjNLRP3-like gene by overlapping the EST and the fragment from RACE. The completed sequences including the opening reading frame was further amplified and sequenced to ensure its accuracy.

2.3. Sequence analysis

Sequence homology was analyzed using BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid (aa) sequence was assessed using Expert Protein Analysis System (http://www.expasy.org/). Domains in AjNLRP3-like aa sequence were detected using Simple Modular Architecture Research Tool (SMART) program (http://www.smart. emblheidelbergde/). Three-dimensional (3D) protein structures were predicted by Protein Homology/analog Y Recognition Engine V 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/login.html). A neighbor-joining (NJ) tree was constructed

Table 1
Primers used for cloning and quantitative real-time PCR.

Primer name	Primer sequence (5′-3′)	Used for
AjNLRP 3-1	TCCACCGCTGCTTACGGTTGA	3′ RACE
AjNLRP 3-2	CCAGGCGCGATATGCTGGGT	
AjNLRP 5-1	GTTGTAGAATGACAGCAACCCTTT	5′ RACE
AjNLRP 5-2	CTTCTCTCCACCCGATCATAGTCC	
AjNLRP qF	CTTCATCTTCCACCGCTGCTT	Real-time PCR
AjNLRP qR	GGCTTTTCCTTTATCTTGTTTCGTC	
AjCTSB qF	GAGGCCACGCCATTCGTATTCTC	Real-time PCR
AjCTSB qR	GATTCCAACTTCGTCCTTCCCAC	
AjHMGB qF	CCCTCCAGCCCTACAGACTTTA	Real-time PCR
AjHMGB qR	TGATCGCCCTCCTTCACGT	
Ajβ-Actin qF	CCATTCAACCCTAAAGCCAACA	Real-time PCR
Ajβ-Actin qR	ACACACCGTCTCCTGAGTCCAT	
AjCTSB specific siRNA	Sense: GCUAUGUCCGAUCGUUAUUTT Anti-sense: AAUAACGAUCGGACAUAGCTT	RNA interference
AjNLRP3-like specific siRNA	Sense: GGAUAUUACAGAGUGCGAATT Anti-sense: UUCGCACUCUGUAAUAUCCTT	RNA interference
Negative control siRNA	Sense: UUCUCCGAACGUGUCACGUTT Anti-sense: ACGUGACACGUUCGGAGAATT	RNA interference