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RESEARCH REPORT

Molecular characterization and expression of *AjNLRP3* in the antibacterial host defense of the sea cucumber (*Apostichopus japonicus*)

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Abstract

The NOD-like receptor proteins (NLRP) is an important genes primarily involved in innate immunity. In this study, we identified a novel *NLRP* gene designated as *AjNLRP3* (GenBank accession number MF663701) in *Apostichopus japonicus* using transcriptome sequencing and the rapid amplification of cDNA ends approach. The full-length of *AjNLRP3* is 3642 bp and a putative open reading frame of 2424 bp encodes a polypeptide with 807 amino acid residues. The predicted molecular mass of the protein sequence predicted for *AjNLRP3* is 93.03 kDa and its theoretical pI is 6.14. AjNLRP3 contains two low complexity regions, two internal repeat regions, and a NACHT domain. Spatial distribution expression analysis detected *AjNLRP3* in all of the tissues tested, with greater expression levels in the body wall, moderate expression in the respiratory tree, and weaker levels in the intestine, tube feet, celomocytes, and longitudinal muscle. The expression levels of *AjNLRP3* increased by 2.60-fold in the celomocytes and decreased by 0.87-fold in the respiratory tree of diseased sea cucumbers compared with those in healthy sea cucumbers. Time-course expression analysis under bacterial challenge in celomocytes showed that the expression of *AjNLRP3* was significantly decreased by 0.23-fold at 8 h and by 0.20-fold at 24 h. In conclusion, this study showed that the AjNLRP3 protein found in the sea cucumber has a similar structure and biological function to that in other organisms, where it appears to be involved with the innate immune responses against bacterial infection.

Key Words: AjNLRP3; *Apostichopus japonica*; cloning and expression; antibacterial experiment

Introduction

Animal immune mechanisms include innate immunity and acquired immunity, where both mechanisms protect the body from invasive bacteria, fungi, viruses, and other pathogens. Previously, it was considered that complex molecular immune responses only occur in higher vertebrates, but recent studies have shown that Echinodermata also possess many immune gene families and various immune responses. In general, immune cells move to chemical irritants along a concentration gradient. Thus, when the source of an invasive pathogen is located in the body, the damaged cells release certain chemical substances to attract large inflammatory cells and induce the inflammatory response. Subsequently, large numbers of inflammatory cells infiltrate the damaged tissue. Studies have shown that this type of response also occurs in the coelom cells of members of the Echinodermata. Indeed, *Pisaster ochraceus* possesses an analogue of interleukin-1, which is found in higher animals (Meng *et al*., 2009), and this analogue participates in phagocytosis. The sea cucumber, *Apostichopus japonicus*, can also respond to pathogens via cell-mediated immunity and humoral immunity, although the mechanism of action is not clear. In recent years, some genes related to immunity in *A. japonicus* have been identified using expressed sequence tag (EST) libraries, gene chips, proteomics, and cloning, including agglutinin, antimicrobial peptide, muramidase, immune-related enzymes, clotting protein, pattern recognition receptors (PRRs), Toll-like receptors, complement C3, and other serum factors (Sun *et al*., 2013; Ji *et al*., 2014; Yang *et al*., 2016). Most of the mechanisms related to immune molecules have not been elucidated in *A. japonicus*.

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Inflammation refers to the initial response of hosts to external challenges via changes in the local circulation (*i.e.,* hyperemia and increased vessel permeability) and the recruitment of immune cells (*i.e.,* granulocytes, lymphocytes, and macrophages) to infected sites, and the eventual elimination of aggressors and the promotion of tissue repair (Cone, 2001). Proinflammatory cytokines are produced in this process and released into the extracellular milieu. Proinflammatory cytokines have proinflammatory effects where they activate the inflammatory response by binding to the extracellular domains of ubiquitous inflammatory cytokine receptors. The cleavage of proinflammatory cytokine precursors is strictly controlled by inflammasomes, which comprise a family of proteins that were first described in 2002 (Martinon *et al*., 2002). Inflammasomes were initially identified based on their roles in marginal or benign pathologies, such as periodic fevers and gout, but they are now considered key factors in most inflammatory diseases (Hawiger, 2001; Brigati *et al*., 2002; Lin *et al*., 2014; Dutartre, 2016). Cytoplasmic receptors of the nucleotide binding domain-like receptor (NLR) family are key components of inflammasomes, where NLRP3 plays vital roles in initiating the inflammatory process (Davis *et al*., 2011).

A. japonicus (Selenka) belongs to Echinodermata, Holothuroidea, Aspidochirotida, Stichopodidae, *Apostichopus*, and it is one of the most economically important aquatic animals in China (Chang, 2004). In recent years, outbreaks of diseases such as skin ulcer syndrome (Wang *et al*., 2006), stomach atrophy (Deng *et al*., 2008), red body disease (Hao *et al*., 2013), and bad side disease (Zhang *et al*., 2010) have severely affected the development of sea cucumber aquaculture.

Sea cucumbers are lower marine animals and like other invertebrates, they can only rely on the innate immune system to resist infection by pathogenic bacteria. Therefore, by studying the characteristic nonspecific immune signal transduction pathways in the sea cucumber, we can determine the immune functions of key genes and their characteristic responses in important cell signaling pathways, thereby clarifying the molecular mechanisms of nonspecific immune responses to facilitate disease prevention, molecular therapy, and the development of new drugs for sea cucumbers.

Materials and Methods

Preparation of animals and collection of samples

Healthy sea cucumbers (body weight 68.00 ± 4.59 g) were obtained from Dalian Heshengfeng Marine Product Farm and maintained at 16 °C - 17 °C in our laboratory for 1 week (The sea cucumbers did not feed and change water from the 1st to 3rd day, and start feeding and changing water every two days from the 4th to 7th day). To examine the spatial expression of *AjNLRP3*, six tissues comprising the intestine, respiratory tree, tube feet, coelomocytes, body wall, and longitudinal muscle were dissected carefully from five healthy animals. The *Vibrio splendidus* D4501 was initially isolated from a skin ulceration dieased *A. japonicus* in our laboratory. The *V. splendidus* was grown in 2216E liquid medium at 28 ℃ with shaking at 200 rpm for 12 h and the cultures was centrifuged at 5,000 rpm for 10 min to harvest the bacteria. The experiment employed a control group and a bacterial challenge group. Each group was divided into seven replicates containing five individuals. The sea cucumbers in the bacterial challenge group were immersed in a suspension of *V. splendidus* D4501 at a density of 10^7 CFU/mL, whereas the other group was not treated with *V. splendidus*. The celomic fluids were collected at 0, 4, 8, 12, 24, 48, and 72 h post-immersion. In order to harvest coelomocytes, the coelomic fluid was centrifuged immediately after collection at 1,000 rpm and 4 °C for 5 min. All of the tissues were snap frozen immediately in liquid nitrogen and stored at -80 °C.

Total RNA extraction, cDNA synthesis, and cloning of the full-length AjNLRP3

Total RNA was extracted from all of the tissues collected from *A. japonicus* using Trizol (Ambion) according to the manufacturer's protocol. The quality and quantity of the isolated RNA were measured

Table 1 The primers used in this research

Primer	Sequence (5'→3')	Purpose
AjN3 5-1	TATCAGGGTATTCGTCAAATCCATCC	5'- RACE PCR
AjN3 5-2	CTCTCAGTTGTCTTAGCCGCAGGTA	5'- RACE PCR
AjN3 3-1	CCTGACCATGGCCTTCAAGGAGTG	3'- RACE PCR
AjN3 3-2	GAATGTCTGCTGCCTCTTTCGCC	3'- RACE PCR
UMP-1	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	3'&5' RACE
UMP-2	CTAATACGACTCACTATAGGGC	3'&5' RACE
M13F	TGTAAAACGACGGCCAGT	colony PCR
M13R	CAGGAAACAGCTATGACC	colony PCR
AjNLRP3-F	CTGAGAAAACGACTTGGGGATG	qRT-PCR
AjNLRP3-R	GATAATCAGCAATGTAGTGGGCA	qRT-PCR
Cytb F	TGAGCCGCAACAGTAATC	qRT-PCR of *Cytb*
Cytb R	AAGGGAAAAGGAAGTGAAAG	qRT-PCR of *Cytb*

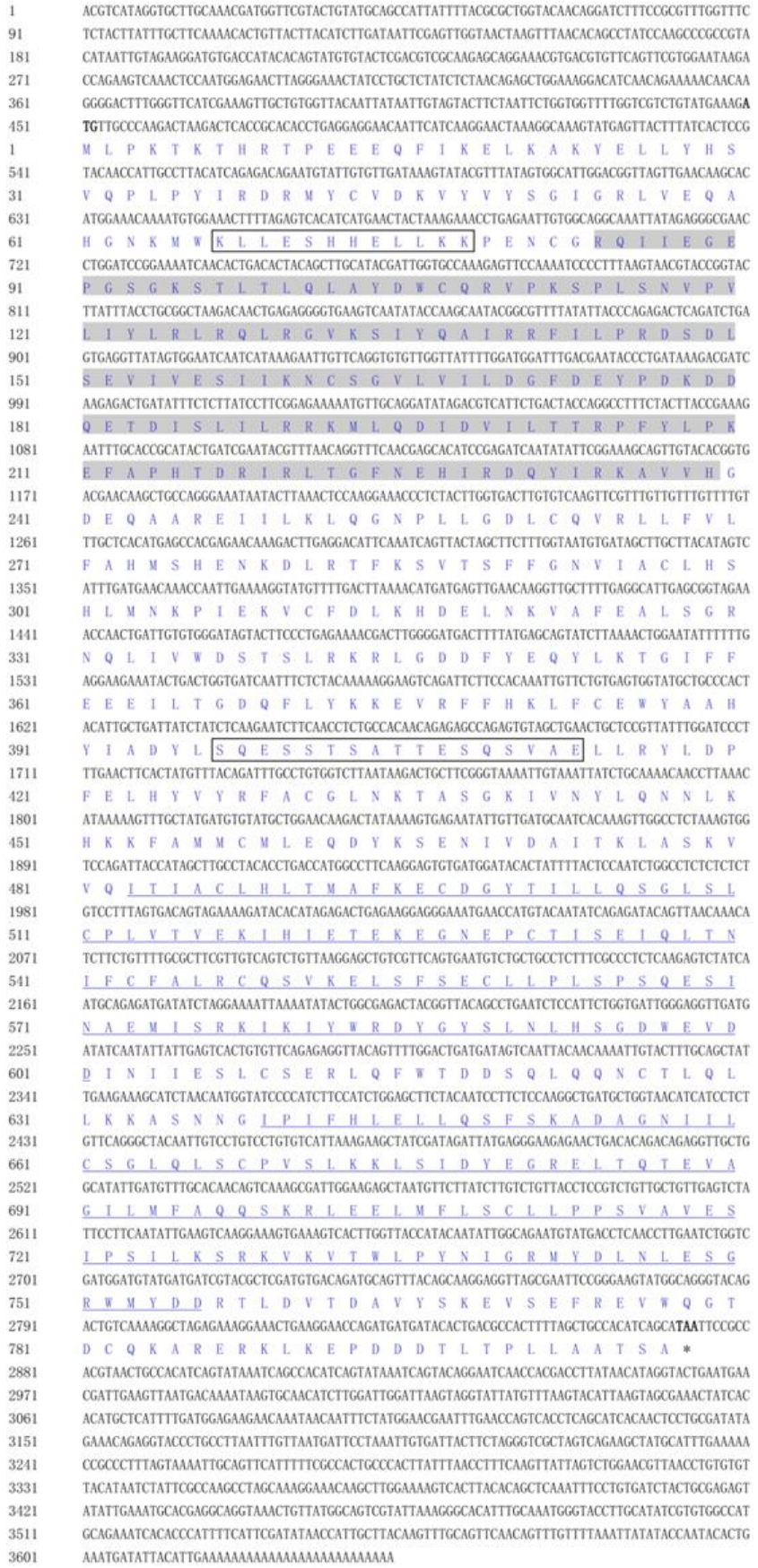

Fig. 1 Nucleotide and deduced amino acid sequences of *AjNLRP3* in sea cucumber. The termination codon is marked with an asterisk. The low complexity regions are boxed. The putative NACHT domain is highlighted in gray. The internal repeat regions are underlined.

with 1.0 % agarose gel electrophoresis and using a spectrophotometer (NanoPhotometer, Munich, Germany). First strand cDNA was synthesized in a 20-μL reaction mixture comprising 1000 ng total RNA, 4 μL of 5× PrimerScript buffer, 1 μL Oligo dT Primers (50 mM), 1 μL random 6-mers (100 mM), 1 μL PrimerScript RT Enzyme Mix I (PrimerScript™ RT reagent Kit, TaKaRa, Japan), and RNase-free double-distilled H_2O (ddH_2O) was added to make up the remaining volume. The reaction mixture was incubated at 37 °C for 15 min, and then at 85 °C for 5 s to denature the reverse transcriptase. All of the cDNA samples were stored at -20 °C until use. The partial cDNA sequence of *AjNLRP3* was obtained from our transcriptome assembly data (unpublished data). Based on the partial sequence, nested PCR with rapid amplification of cDNA ends (RACE) was performed to obtain the full length of *AjNLRP3* using universal primers and the specific primers *NLRP3*-5′RACE1/2 (for 5′RACE) and *NLRP3*-3′RACE1/2 (for 3′RACE). The cDNA template for RACE-PCR was prepared using a SMART RACE cDNA Amplification Kit (TaKaRa). The PCR protocol was as follows: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Nested PCR was conducted using the first PCR products as templates and *NLRP3*-5′RACE or *NLRP3*-3′RACE with NUP as primers. The PCR protocol was the same as that used in the first round of PCR. The specific products were purified with a Gel Extraction Kit (QIAgen), before cloning into the T1 vector (TRAN, China) and transforming into Trans1-T1 phage-resistant chemically competent cells (TRAN). The positive recombinants were identified by colony PCR using M13 primers (TRAN). Three independent clones were sequenced to confirm the sequence. The primer sequences are shown in Table 1.

Bioinformatics analysis

The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to analyze the nucleotide sequences and to search for protein sequences in other species via the NCBI website (http://www.ncbi.nlm.nih.gov/). Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Potential N-glycosylation sites were identified using the NetNGlyc 1.0 prediction server. Multiple sequence alignments were generated by ClustalW2 (http://www.ebi.ac.uk/tools/clustalw2). The features of protein motifs were predicted with the Simple Modular Architecture Research Tool (SMART; http://smart.emblheidelberg.de/). A neighbor-joining phylogenic tree was constructed based on the deduced amino acid sequences using the MEGA 7.0 program. Bootstrap sampling was repeated 1,000 times.

Expression analysis of AjNLRP3

Six types of tissues comprising the intestine, respiratory tree, tube feet, celomocytes, body wall, and longitudinal muscle were obtained from the healthy sea cucumbers for RNA extraction. Total RNA extraction and real-time reverse transcription PCR (RT-PCR) analysis were performed as described previously. RT-PCR was conducted in a 20-μL reaction mixture comprising 2 μL of the original cDNA diluted at 1:10, 10 μL of 2× SYBR Green Master mix (SYBR PrimeScript™ RT-PCR kit II, TaKaRa), 0.4 μL of ROX Reference Dye II, 0.8 μL (10 mM) of each primer (Table 1), and 6 μL ddH_2O. The quantitative RT-PCR (qRT-PCR) parameters were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 32 s. The specific amplification of the PCR products was confirmed by melting curve analysis. The relative *AjNLRP3* mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. The expression quantities of each sample were analyzed by using SPSS Statistics 19.0 software. The cytochrome b (*Cytb*) gene was used as the reference gene.

Bacterial challenge experiment

In the bacterial challenge experiment, 70 healthy sea cucumbers were split between the treatment and control groups, where the treatment group was soaked in 1×10^7 CFU/mL *V. splendidus* and the control group did not receive the bacterial treatment. The two groups were kept in water at the same temperature. After soaking for 0, 4, 8, 12, 24, 48, and 72 h, the body cavity fluid was collected with five biological replicates in each group. The fluid samples were centrifuged at 1,000 rpm for 5 min, and the body cavity cells were collected and frozen quickly in liquid nitrogen. All of the samples were stored at -80 °C for later use.

Results

Sequence analysis of AjNLRP3

The full-length *AjNLRP3* (GenBank accession number MF663701) gene comprises 3642 bp encoding 807 amino acids, where leucine is the most abundant and tryptophan is the least abundant. The predicted molecular mass of the protein deduced from *AjNLRP3* ($C_{4178}H_{6596}N_{1104}O_{1220}S_{38}$) is 93.03 kDa and the theoretical pI of 6.14. The amino acid sequence contains two low complexity regions (amino acids 67 - 78 and 397 - 413) and two internal repeat sequences (amino acids 483 - 601 and 639 - 756), where amino acids 84 - 239 comprise a NACHT domain. The amino acid sequence contains no tailing signal, transmembrane structure, or signal peptide sequence (Fig. 1).

Analysis of the amino acid composition of AjNLRP3

We analyzed the amino acid hydrophilicity/hydrophobicity of the full-length *AjNLRP3* cDNA using ProtScale online. The fatty acids coefficient for AjNLRP3 is 97.24, the maximum hydrophilic coefficient is 2.52, the minimum hydrophilic coefficient is -3.089, and the total average hydrophilic coefficient is -0.239, which indicates that the protein is hydrophilic (Fig. 2).

Homology and evolutionary analysis based on the amino acid sequence of AjNLRP3

We used ORF Finder to identify the open reading frame of the *AjNLRP3* gene in the sea cucumber and then compared it with other *NLRP3*

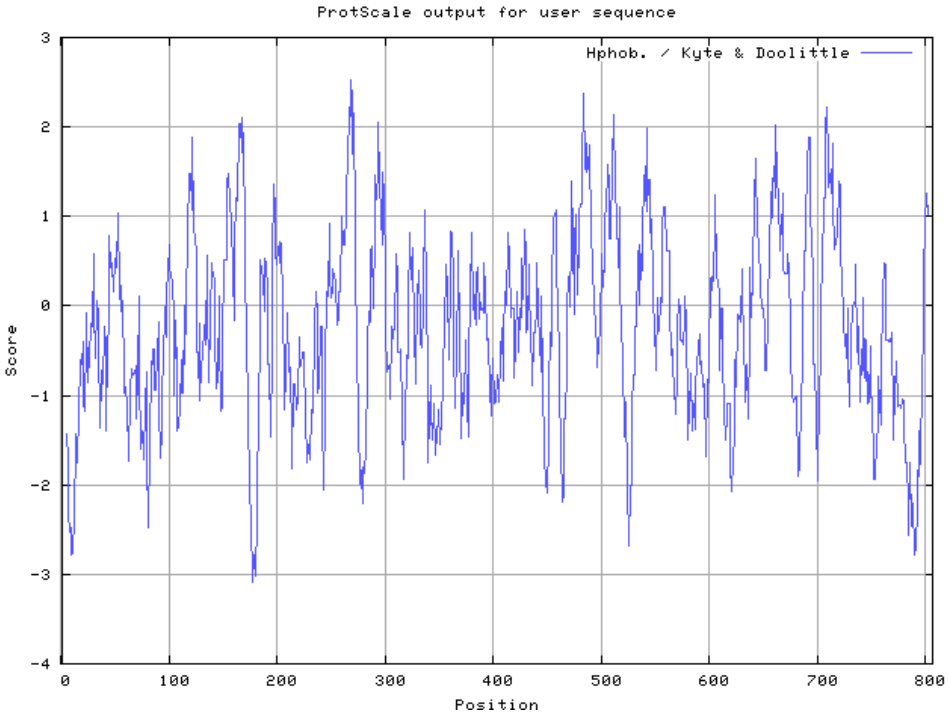

Fig. 2 The hydrophilicity/hydrophobicity results of *AjNLRP3*. Horizontal indicated the position of amino acid, vertical coordinates indicated the score.

genes using SmartBlast. The results showed that the amino acid sequence predicted for AjNLRP3 shared 24 % similarity compared with the amino acid sequence of NLRP3 in *Homo sapiens*, while the shared similarity with that in *Mus musculus* was 25 % and that in *Danio rerio* was 23 %. In order to determine the phylogenetic relationships between AjNLRP3 and other NLRP3s, we constructed a phylogenetic tree based on the deduced amino acid sequences of AjNLRP3 and different members of the NLRP3 superfamily using the neighbor-joining method with 1,000 replicates, and the results are shown in Figure 3. The phylogenetic tree showed that the sequence in *A. japonicus* clustered with those in the echinoderm subgroup and it had a close evolutionary relationship with the sequence in *Strongylocentrotus purpuratus.* The sequences in *A. japonicus* and *S. purpuratus* had high genetic distances from those in vertebrates.

Tissue distribution of AjNLRP3

The spatial expression pattern of *AjNLRP3* mRNA was investigated using qRT-PCR with the *Cytb* gene as the internal control and the results are shown in Figure 4. The results show relative expression in arbitrary units normalized to longitudinal muscle expression. We found that the *AjNLRP3* gene was expressed in all of the tissues tested, with a significantly higher expression level in the body wall compared with the other tissues ($p < 0.01$), whereas the lowest expression level was found in the longitudinal muscle. There were no significant differences in the expression levels in the respiratory tree, intestine, tube feet and body cavity cells.

Expression of the AjNLRP3 *gene in diseased sea cucumbers*

We examined the expression of the *AjNLRP3* gene in the intestine, respiratory tree, and coelomocytes in healthy sea cucumbers and those with skin ulcer syndrome. The results show relative expression in arbitrary units normalized to healthy sea cucumber expression. The expression level of the *AjNLRP3* gene was increased significantly by 2.6-fold in celomocytes in the infected sea cucumbers compared with those in the control group. In the respiratory tree, the expression level was reduced to 0.87-fold and it differed significantly from that in the control group. There was no significant difference in the intestine tissues (Fig. 5).

Expression of AjNLRP3 *in celomocytes after bacterial challenge*

The effects of challenge with *V. splendidus* on the transcriptional expression levels of the *AjNLRP3* gene were compared in celomocytes by RT-PCR (Fig. 6). The results show relative expression in arbitrary units normalized to healthy sea cucumber expression. The results showed that the expression level of the *AjNLRP3* gene did not differ significantly from that in the control group at 4 h, but the expression level decreased significantly by 0.23-fold at 8 h. After 12 h, the difference between the two groups was not significant. At 24 h, the expression level of the gene was decreased significantly by 0.20-fold compared with the control group, but it then increased to the level in the control.

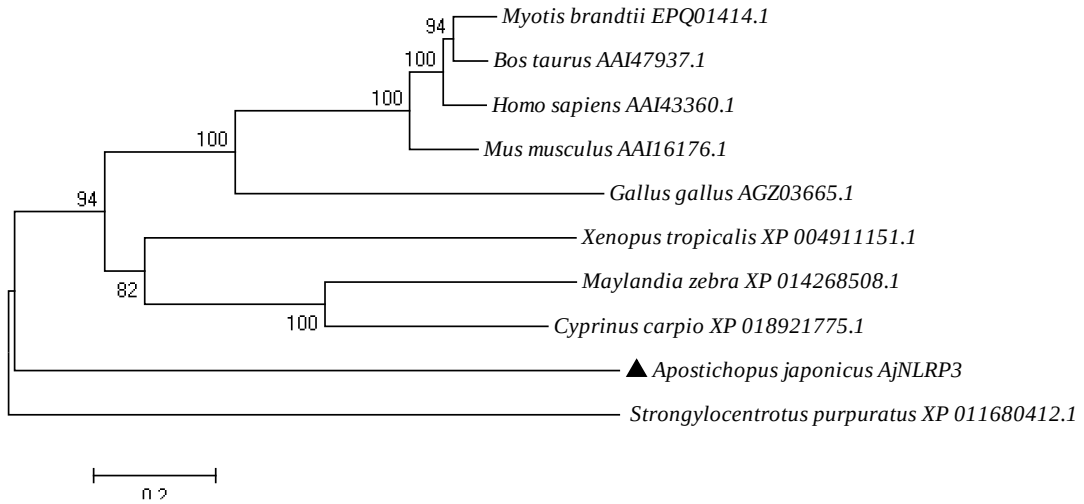

Fig. 3 Consensus neighbor-joining tree based on the amino acid sequence of*AjNLRP3*from other species. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches Evolutionary analyses were conducted in MEGA7.0.

Discussion

In this study, we characterized and analyzed the expression of a cDNA for the *AjNLRP3* gene in *A. japonicus*. A 3642-bp nucleotide sequence representing the complete cDNA sequence of *AjNLRP3* was obtained by overlapping an EST from the *A. japonicus* cDNA library and 5′/3′RACE products. Domain and motif analysis showed that the *AjNLRP3* gene contains two low complexity regions, two internal repeat regions, and a NACHT structural domain. NACHT is a common domain structure in the members of the NLR family, where it participates in the innate immune recognition process, as well as apoptosis and the major histocompatibility complex transcriptional activation process (Orlowski *et al*., 2016). BLASTp analysis showed that the deduced amino acid sequence of AjNLRP3 shares 24 % identity with NLRP3 from *Homo sapiens* (AAC37547.1), 25 % with that from *Mus musculus* (AAA37375.1) and 23 % with that from *Danio rerio* (AAQ97764.1). Multiple comparisons showed that *AjNLRP3* and other NLRP3s share low homology. In addition, the relationships in the phylogenetic tree showed that *A. japonicus* has a closer evolution relationship with *S. purpuratus.* The cDNA sequence did not contain the same protein structural domain and repeated leucine domain that are found in all vertebrate NLRP3s. Our analysis of NLRP3 genes in vertebrates using the NCBI database showed that some vertebrate NLRP3 genes also lack the same protein structural domain and leucine repeated domain, thereby suggesting effects due to gene duplication during their evolution. Our results demonstrated that *AjNLRP3* is a novel member of the NLR family.

To better understand the role of AjNLRP3 in innate immunity in the sea cucumber, we analyzed the tissue distribution of AjNLRP3 by real-time PCR. AjNLRP3 was distributed in all of the tissues that we tested, which indicates that it has important roles in physiological processes. NLRP3 regulates various processes in inflammation and thus it is distributed in a wide range of tissue (Tohno *et al*., 2011; Huang *et al*., 2014; Ye *et al*., 2015). The mRNA highest expression level of *AjNLRP3* was found in the body wall tissues. Seawater contains a large number of pathogenic microorganisms and the body wall of the sea cucumber is the first line of defense against infection by pathogens via direct contact with seawater, which may explain why the highest mRNA expression level of *AjNLRP3* was detected in the body wall. The relatively high mRNA expression levels of *AjNLRP3* in the respiratory tree, intestine, tube feet, and celomocytes indicate that it is involved in the immune response of *A. japonicus* (Motta *et al*., 2015). There were no significant differences in the expression levels among the different tissues, but the lowest expression level was found in the longitudinal muscle. The expression profiles indicate that AjNLRP3 is involved in important physiological functions.

PRRs play vital roles in the recognition of pathogenic microorganisms and the initiation of the natural immune response. The four known categories of PRRs comprise Toll-like receptors and C-type lectin receptors in the cell membrane, and retinoic acid-inducible gene-I-like receptors and NOD-like receptors (NLRs) in the cytoplasm. Studies have shown that *NLRs* have important roles in the inflammatory response (Kate *et al.*, 2010; Tee *et al.*, 2013). *AjNLRP3* is a member of the NLR genes family. As reported previously, celomocytes are the main cellular components of the immune system as freely circulating cells in sea cucumbers. *V. splendidus* is regarded as a major pathogen that affects sea cucumbers in aquaculture (Wang *et al.*, 2007; Wang *et al.*, 2009; Zhao *et al.*, 2011). Thus, to

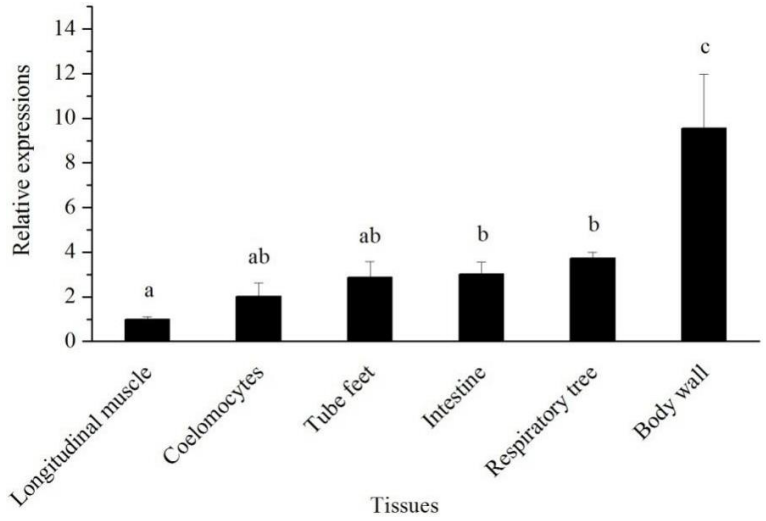

Fig. 4 Relative expression of *AjNLRP3* in different tissues. The results show relative expression in arbitrary units normalized to longitudinal muscle expression.Each vertical bar represents the mean ± SD (n = 3), *Cytb* used as a reference gene. Different Roman letters above the bars indicate significant differences in different tissues at $p < 0.05$.

better understand the role of *AjNLRP3* in the response of *A. japonicus* to bacterial challenge, we tested the effect of treatment with *V. splendidus* as a potential pathogen. We considered that the expression level of the *AjNLRP3* gene in the celomocytes after bacterial challenge might provide insights into the defensive mechanisms and regulatory processes in the sea cucumber after infection. We found that infection with *V. splendidus* caused changes in the expression level of the *AjNLRP3* gene, where the expression level of *AjNLRP3* decreased significantly from 8 to 24 h after the bacterial challenge, thereby indicating that the transcription of *AjNLRP3* was suppressed before tissue damage occurred. In the NOD signal transduction pathway, multiple tissue proteases act as upstream regulatory genes to promote the synthesis of the interleukin-1β precursor and the secretion of interleukin-1β is mediated by NLRP3 (Fujisawa *et al*., 2007; Hirota *et al*., 2011; Morishige *et al*., 2010). Similar regulatory effects may occur in the sea cucumber. The expression level of the *AjNLRP3* gene was decreased significantly in the respiratory tree tissues of diseased sea cucumbers compared with the control group. The expression level was also significantly higher in the body cavity cells of diseased sea cucumbers compared with the control group, thereby indicating that this gene is involved in the response to infection by pathogens.

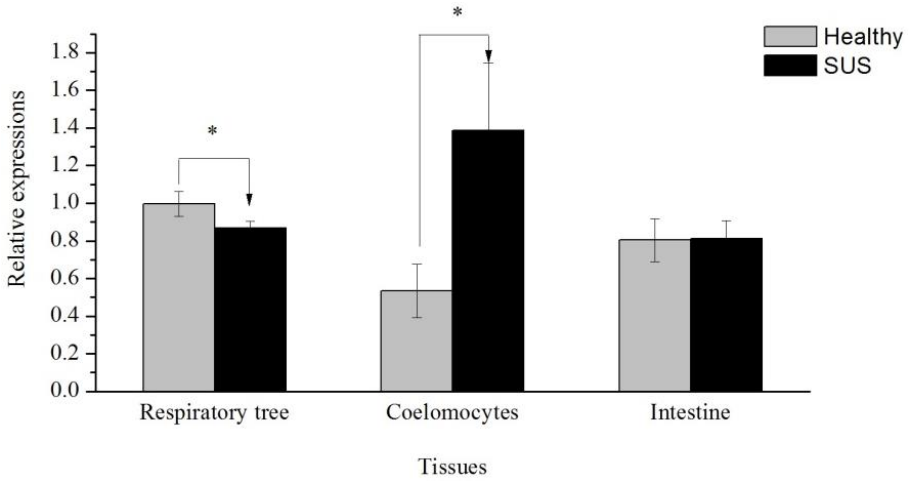

Fig. 5 Relative expression of *AjNLRP3* in coelomocytes after challenged with *V. splendidus*. The results show relative expression in arbitrary units normalized to healthy sea cucumber expression. Each vertical bar represents the mean ± SD (n = 3). The asterisks (*) and double asterisks (**) above the bars represent differences at $p < 0.05$ and significant differences at $p < 0.01$, respectively.

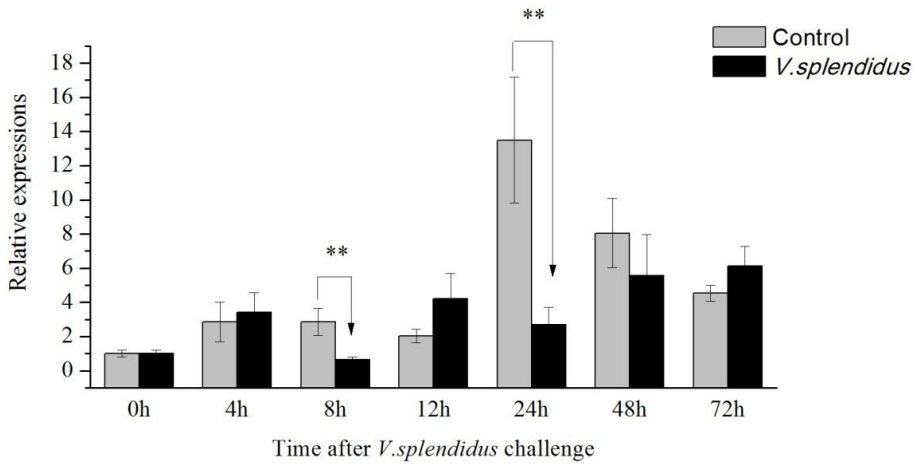

Fig. 6 Relative expression of *AjNLRP3* in celomocytes after challenged with *V. splendidus*. The results show relative expression in arbitrary units normalized to healthy sea cucumber expression.Each vertical bar represents the mean ± SD (n = 5).The asterisks (*) and double asterisks (**) above the bars represent differences at $p < 0.05$ and significant differences at $p < 0.01$, respectively.

Inflammatory response is an immune defense process caused by cell infection or cell damage and plays an important role in the process of wound repair and anti-infection. The cytoplasmic receptor of the nucleotide binding receptor (NLR) family is one of the key components of the inflammatory body, and in many animals and sea cucumber genome sequencing, there are NLRs, a large family of genes. Inflammatory small bodies induced by NLRP3 are the most important types of inflammatory complexes (Kanneganti *et al.*, 2007; Franchi *et al.*, 2009). Unlike other known inflammatory reactions, NLRP3 inflammation is induced by various types of activators, including pathogenic bacteria, portotoxins, environmental irritants and some internal molecules. The inflammatory response can be induced when the transcriptional level of NLRP3 exceeds the threshold. The high and low levels of the threshold are associated with the type of cell, such as the lower threshold of dendritic cells compared to phagocytes. As the inflammatory response worsens, the host cell can synthesize mir-223. The transcription products of NLRP3 were degraded by the 3 'non-coding region of NLRP3 (Bauernfeind *et al.*, 2012; Haneklaus *et al.*, 2012). *NLRP3* genes have been detected in *Homo sapiens* (Gross *et al.*, 2009; Hise *et al.*, 2009), *Mus* (Bauer *et al.*, 2010; Heneka *et al.*, 2013), *Vitulus* (Zimin *et al.*,2009), *Porcorum* (Tohno *et al.*, 2016), *Pullum* (Ye *et al.*, 2015), and other vertebrates, but few studies have investigated them in invertebrates, and thus the results obtained in the present study provide important new insights into the role of the *NLRP3* gene in invertebrates.

Acknowledgments

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