Fish & Shellfish Immunology 30 (2011) 148–153

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Cloning and characterization of allograft inflammatory factor-1 (AIF-1) from manila clam *Venerupis philippinarum*

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ARTICLE INFO

Article history:
Received 23 August 2010
Received in revised form
30 September 2010
Accepted 30 September 2010
Available online 8 October 2010

Keywords:
Allograft inflammatory factor-1
Venerupis philippinarum
Immune response
Bacteria challenge

ABSTRACT

Allograft inflammatory factor-1 (AIF-1) is a 17 kDa interferon-γ-inducible Ca^{2+}-binding EF-hand protein that plays a significant role not only in different host responses to inflammatory stimuli, but in the whole host immune defense reaction. In the present study, the full-length cDNA of AIF-1 was identified from manila clam *Venerupis philippinarum* (denoted as VpAIF-1) by cDNA library and RACE approaches. The cDNA of VpAIF-1 consisted of a 5-terminal untranslated region (UTR) of 153 bp, a 3′UTR of 219 bp with a poly (A) tail, and an open reading frame (ORF) of 516 bp encoding a polypeptide of 171 amino acids with the putative molecular mass of 17 kDa. The deduced amino acid of VpAIF-1 shared two EF hand Ca^{2+}-binding motifs like other AIF-1s. Phylogenetic analysis further indicated that VpAIF-1 had higher evolutional conservation to invertebrate than vertebrate counterparts and should be a new member of the AIF-1 protein family. Spatial expression analysis indicated that mRNA transcript of VpAIF-1 was found to be most abundantly expressed in the tissues of haemocytes, gills and hepatopancreas, weakly expressed in the tissues of mantle, muscle, and foot. After challenged by *Vibrio anguillarum*, the mRNA level of VpAIF-1 in overall haemocytes population was recorded by quantitative real-time RT-PCR. VpAIF-1 mRNA was down-regulated in the first 12 h post-infection. Then, the expression level increased to the peak at 72 h and recovered to the 48 h-level at 96 h. All these results indicated that VpAIF-1 was involved in the immune response against microbe infection and might be contributed to the clearance of bacterial pathogens.

1. Introduction

Manila clam, *Venerupis philippinarum*, widely distributed in the coasts of China, is a kind of burrowing bivalve in Veneridae. Clam culture could be dated back to several centuries in China [1]. However, with the development of intensive culture and environmental deterioration, various diseases caused by bacteria, protozoa occurred in cultured *V. philippinarum* populations, resulted in enormous losses to the clam aquaculture [2]. Therefore, better understanding of the immune defense mechanisms of *V. philippinarum* might be contributed to the development of management strategies for disease control and long-term sustainability of clam farming.

Like other invertebrates, the absence of acquired immunity in clam makes them exclusively rely on the innate immune system to protect them against continuous threats from pathogens, especially in marine environments abundant in bacterial population [3]. The innate immune systems could provide the host immediate defense against infection in a non-specific manner [4]. Among the numerous different immune responses in clam, inflammation is one of the first responses of the immune system to infection, which is initiated upon pathogenic infection (bacterial, viral or parasitic), tissue injury, exogenous agents and so on [5–7]. As one of the key genes associated with inflammatory response, allograft inflammatory factor-1 (AIF-1), a 17 kDa interferon (IFN)-γ-inducible Ca^{2+}-binding EF-hand protein has attracted much attention especially in vertebrates [8,9].

AIF-1 was originally cloned from active macrophages in human (U49392) and rat (U17919) atherosclerotic allogenic heart grafts undergoing chronic transplant rejection [10]. It was demonstrated that AIF-1 was a modulator of the immune response during macrophage activation [9,10]. Later, Miyata et al. (2001) showed that AIF-1 transcripts were up-regulated in red seabream leukocytes upon LPS stimulation [11], implying that the AIF-1 in red seabream might have a similar function in activated leukocytes as AIF-1s did in mammals. In invertebrate, Michael et al. (1999)

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doi:10.1016/j.fsi.2010.09.021

reported that the mRNA expression of sponge AIF-1 was induced in cytokine-mediated allogenic responses during wound repair [12]. In addition, abalone AIF-1 has been proved that it could response against the pathogenic challenge and tissue injury [13]. Take these results together, we should conclude that AIF-1 plays a significant role not only in different host responses to inflammatory stimuli, but in the whole host immune defense reaction [14,15].

However, the studies regarding on molecular features and function of AIF-1 in mollusk were rare investigations except for disk abalone [13]. The main objectives of the present study were to clone the full-length cDNA of VpAIF-1 from *V. philippinarum*, and to investigate the tissue-specific expression and temporal expression profile of VpAIF-1 transcript after challenged by bacterial pathogen in order to better understand the roles of VpAIF-1 in the innate immune response of clam.

2. Materials and methods

2.1. Clams and bacteria challenge

The clams *Venerupis philippinarum* (average weight 9 ± 2 g) were collected from a farm in Qingdao, China, and maintained in six flat-bottomed rectangular tanks containing 50 L aerated fresh seawater, each containing 50 clams. The temperature was held at 20–22 °C and the salinity was kept at 30‰ for 10 days before processing.

Vibrio anguillarum was inoculated into 2216E liquid medium at 28 °C with shaking at 220 rpm. Centrifuged the above overnight culture at 12,000 rpm for 10 min and resuspended with seawater. For challenge experiment, one tank served as control, the other five tanks were immersed with high density of *V. anguillarum* with final concentration of 10^7 CFU ml^{-1}. The infected clams were randomly sampled at 6, 12, 24, 48, 72 and 96 h, respectively. The haemocytes from the control and infected groups was collected using a syringe and centrifuged at 2000 × g, 4 °C for 10 min to harvest the haemocytes. There were five replicates for each time point. The haemocyte pellets were used for RNA extraction using TRIzol reagent (Invitrogen).

2.2. Cloning the full-length cDNA of VpAIF-1 gene

A cDNA library was constructed from the haemocytes of manila clam challenged with *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 3226 successful sequencing reactions. BLAST analysis of the EST sequences revealed that an EST of 304 bp was highly similar to the known AIF-1 sequences. This sequence was then selected for further cloning of full-length cDNA of AIF-1 gene from *V. philippinarum*.

Four specific primers, sense primers P1 and P2, and reverse primers P3 and P4 (Table 1) were designed based on the known sequence to clone the full-length cDNA of AIF-1 from *V. philippinarum* (designated VpAIF-1). The nested PCR strategy was applied to clone the 3′ end of VpAIF-1 using sense primer P1, P2 and reverse primer T7, while sense primer oligo(dG)-adaptor and reverse primer P3, P4 were used to get the 5′ end of VpAIF-1. All the PCR reactions were performed in an ABI Veriti™ in a 25 μl reaction volume containing 2.5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ (2.5 mmol/l), 2 μl of dNTP (0.2 mmol/l), 1 μl of each primer (0.4 μmol l^{-1}), 15.8 μl of PCR-grade water, 0.2 ml of Taq polymerase (5 U μl^{-1}) (MBI) and 1 ml of cDNA template. The PCR temperature profile was 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The PCR products were cloned into the pMD18-T simple vector (TaKaRa) and sequenced bi-directionally with primers M13-47 and RV-M (Table 1). The sequencing results were verified and subjected to cluster analysis.

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Sequence information
P1	CTGGGACAAGCAAAGACA	3′-RACE primer
P2	CACCGGGTCAATCTGTTA	3′-RACE primer
T7	GTAATACGACTCACTATAGGGC	3′-RACE primer
P3	ACGCTTCTAAATGGTCTGTGA	5′-RACE primer
P4	CTCTTCCGGGTTCGCTAT	5′-RACE primer
Oligo(dG)-adaptor	GGCACGCGTCGACTAGTACG10	5′-RACE primer
P5	ATGGCAAATGAAGGAAATG	RT-PCR VpAIF-1 primer
P6	CAGATTGACCCGGTGTTT	RT-PCR VpAIF-1 primer
P7	CGCTTCTTCATCCTCCCTTGA	RT-PCR actin primer
P8	GGCGGTAATTTCCTTCTGCA	RT-PCR actin primer
P9	AAGATTATAGCGAACCCG	Real time VpAIF-1 primer
P10	TCTTTGCTTGTCCCAGTT	Real time VpAIF-1 primer
P11	CTCCCTTGAGAAGAGCTACGA	Real time actin primer
P12	GATACCAGCAGATTCCATACCC	Real time actin primer
M13-47	CGCCAGGGTTTTCCAGTCACGAC	Sequencing primer
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing primer

2.3. Sequence analysis of VpAIF-1

The VpAIF-1 cDNA sequence was analyzed by the BLAST algorithm at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used for multiple alignment of VpAIF-1, and the Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [16] and NLS prediction programs was employed for signal peptide prediction and nuclear localization signal. The motif sequences search was performed using InterPro Scan software (http://www.ebi.ac.uk/InterProScan/).

A phylogenetic tree was constructed according to amino acid sequences of the selected AIF-1 genes using the neighbor-joining method in program Mega 3.1 (http://www.megasoftware.net/). The bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

2.4. Tissue-specific expression of VpAIF-1 mRNA

The mRNA expression of VpAIF-1 in different tissues of healthy clams was measured by semi-quantitative RT-PCR. Total RNA was isolated from haemocytes, mantles, gills, hepatopancreas, muscles and foot. The cDNA first-strand synthesis was carried out based on Promega M-MLV RT Usage information (Promega) using total RNA treated with DNase I (Promega) as template. Single-strand cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μl containing 50 pmol of OligodT primer, 50 mmol/l Tris–HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 50 mmol/l DTT, 0.75 U of RNasin, 0.2 mmol/l of dNTP, and 200 U of MMLV reverse transcriptase (Promega). Reactions were incubated at 37 °C for 1 h, terminated by heating the mixture at 95 °C for 5 min, and subsequently stored at −80 °C. cDNA mix was diluted to 1:50 for subsequent experiment. There were four replicates for each tissue.

Two VpAIF-1 gene-specific primers P5 and P6 (Table 1) were used to amplify a product of 377 bp. A set of actin primers, P7 and P8 (Table 1), was used to amplify a product of 268 bp served as internal control. The PCR products were separated in 2% agarose gel and stained with ethidium bromide. Electrophoretic images and the optical densities of amplified bands were analyzed using the

software of Band Leader (version 3.00). All data were given in terms of relative mRNA expression as means ± S.E.

2.5. Temporal expression profile of VpAIF-1 transcript post Vibrio challenge

Haemocytes was selected to analyze temporal expression profile of VpAIF-1 challenged by *Vibrio* pathogen. Two VpAIF-1 gene-specific primers P9 and P10 (Table 1) were used to amplify a product of 205 bp from cDNA, and the PCR product was sequenced to verify the specificity of RT-PCR. Two actin primers, P11 and P12 (Table 1) were used to amplify a 121 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding clam samples.

Real-time PCR amplification was carried out in an Applied Biosystem 7500 fast real-time PCR system. Dissociation curve analysis of amplification product was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed using ABI 7500 SDS software V2.01 (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of VpAIF-1 [17]. All data were given in terms of relative mRNA expressed as mean ± S.E. ($N = 4$). The data were then subjected to analysis using *t*-test. Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.01$.

3. Results

3.1. Cloning and sequence analysis of VpAIF-1 gene

One EST from the cDNA library of manila clam *Venerupis philippinarum* was homologous to the previously known AIF-1 genes. Based on this EST, a 435 bp and a 373 bp DNA fragments were amplified by 3′-RACE and 5′-RACE technique, respectively. An 888 bp nucleotide sequence representing the complete cDNA sequence of VpAIF-1 was obtained by overlapping two fragments with this EST. The sequence encoding the *V. philippinarum* allograft inflammatory factor-1 (VpAIF-1) was deposited in GenBank under accession no. ACU83234. The full-length cDNA sequence and the deduced amino acid sequence were shown in Fig. 1.

The full-length cDNA of VpAIF-1 consisted of a 5′-terminal untranslated region (5′UTR) of 153 bp, a 3′UTR of 219 bp with a poly (A) tail, and an open reading frame (ORF) of 516 bp. The ORF encoded a polypeptide of 171 amino acids with a predicted molecular weight of 17 kDa. Two EF hand Ca^{2+}-binding motifs were found in amino acid sequence ranging from 82 aa to 94 aa and 69 aa to 104 aa by PROSITE program. Additionally, several numbers of biological active sites including casein kinase II (46 aa–49 aa), protein kinase C (51 aa–53 aa) phosphorylation and N-myristoylation (102 aa–107 aa) were also detected from the deduced amino acid of VpAIF-1. Interestingly, signal peptide and nuclear localization signal (NLS) were absent from the deduced amino acid of VpAIF-1.

3.2. Homologous and phylogenetic analysis of VpAIF-1

The protein sequences of AIF-1s were aligned using the ClustalW program (Fig. 2). The deduced amino acid sequence of VpAIF-1 shared high homology with other reported AIF-1s, such as 64% similarity to Amphioxus (XP_002597874), 60% similarity to Jellyfish (ABA42882), 59% similarity to Abalone (ACJ65689), 55% similarity to Sponge (CAC38780), 50% similarity to Sea anemone (XP_001635454), 49% similarity to Horse (XP_001490297), 48% similarity to Human (CAI18311), 47% similarity to Pig (NP_001123422) and Seabream (BAA36938), 46% similarity to Rat (NP_058892), and 41% similarity to Zebrafish (NP_942571) (Table 2).

```
   1 CCTAGTTGTTTTGAGGTTATTTTTGTGAACAAAATAAAAACTATGGGTGAACAAGGGCAA

  61 GATACGAATGCAATGGACACTGGAGAAAATGGGCATAATGGTGATAAAAATGTTGATAAT
   1                                  M  A  N  E  G  N  D  L  V
 121 AGTGTAGAAATTACTACATATGAAGAAGAAAAGATGGCAAATGAAGGAAATGATTTGGTT
  10 K  K  E  N  I  P  E  N  E  E  E  I  E  P  A  R  V  F  N  L
 181 AAGAAAGAAAACATTCCGGAAAACGAAGAGGAAATTGAGCCTGCACGTGTATTTAATCTC
  30 P  D  K  A  P  K  N  Y  K  I  I  A  N  P  E  E  E  A  L  D
 241 CCAGATAAAGCCCCCAAAAATTACAAGATTATAGCGAACCCGGAAGAGGAAGCTTTGGAT
  50 K  I  N  K  E  I  L  D  D  P  D  Y  K  E  V  D  D  L  T  D
 301 AAAATAAATAAGGAGATTCTTGATGATCCAGACTATAAAGAGGTTGATGATCTCACAGAC
  70 H  L  E  A  F  K  K  K  F  L  E  F  D  K  D  A  N  G  N  I
 361 CATTTAGAAGCGTTTAAAAAAAAGTTTCTCGAGTTTGATAAAGATGCAAATGGAAATATT
  90 D  M  F  G  L  S  R  M  M  E  K  L  G  Q  A  K  T  H  L  E
 421 GATATGTTTGGTTTATCTCGAATGATGGAGAAACTGGGACAAGCAAAGACACACCTGGAG
 110 M  K  K  M  I  R  E  I  D  T  T  N  T  G  S  I  C  Y  R  D
 481 ATGAAAAAAATGATTCGAGAAATTGATACTACAAACACCGGGTCAATCTGTTACAGAGAC
 130 F  I  T  M  M  I  G  P  K  T  S  V  L  K  L  I  L  L  F  E
 541 TTTATAACTATGATGATTGGTCCAAAAACGTCAGTTCTAAAACTAATTTTGTTGTTCGAA
 150 E  K  M  K  E  K  E  K  P  T  G  V  A  P  K  R  D  L  A  S
 601 GAGAAGATGAAAGAGAAGGAAAAGCCGACAGGGGTCGCCCCGAAAAGGGATTTAGCGAGT
 170 L  P
 661 TTGCCTTAAGTAAGTCCTTGCTTATGTGAAAAATGGACAGGGACCAAAATGTGCAATTTT
 721 GATGAAAGGCCGATCATGACCTACATATACCATTGTTGTACATAACTTCTACATGCTTAT
 781 AAAACAAATTATCAAATGACGTTGCTAAATTTGCATTGTATAACTAGAATGTGTTTCATA
 841 TAATACAAACGTGTTAAACTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. The full-length cDNA sequence of VpAIF-1 and its deduced amino acid sequence. The nucleotide and deduced amino acid sequence of the open reading frame and flanking region were numbered on the left. The start and stop codon were bold. The predicted EF hand Ca^{2+}-binding domain 1 was shaded. The predicted EF hand Ca^{2+}-binding domain 2 was underlined. The predicted N-glycosylation site was double underlined.

To evaluate the molecular evolutionary relationships of VpAIF-1 against other AIF-1s, a phylogenetic tree was constructed based on the protein sequences by the neighbor-joining method (Fig. 3). According to the phylogenetic tree, the AIF-1 members were mainly clustered into two groups according to their invertebrate and vertebrate origin. VpAIF-1 was firstly clustered with AIF-1 from abalone, and then formed a sister group with those from invertebrates and further grouped with those from vertebrates. The relationships displayed in the phylogenic tree were in good agreement with traditional taxonomy.

3.3. Tissues distribution of VpAIF-1

Semi-quantitative RT-PCR was employed to quantify the expression of VpAIF-1 mRNA in different tissues of manila clam *Venerupis philippinarum*. In healthy clams, the VpAIF-1 transcript was found to be constitutively expressed in a wide range of tissues with different expression levels. The expression of VpAIF-1 transcript was predominantly detectable in tissues of haemocytes, mantle, gill and hepatopancreas, and to a lesser degree in the tissues of muscle and foot (Fig. 4).

3.4. VpAIF-1 expression pattern in response to bacteria challenge

Temporal effect of bacteria challenge on the transcriptional activities of VpAIF-1 gene was investigated over a 96 h period (Fig. 5). After the bacterial challenge, the VpAIF-1 mRNA transcript decreased in the first 12 h (only 1/3 of the control group at 12 h), then increased gradually and reached to 6.7-fold compared to the control group at 72 h. At 96 h, the expression drop to 3.5-fold. An

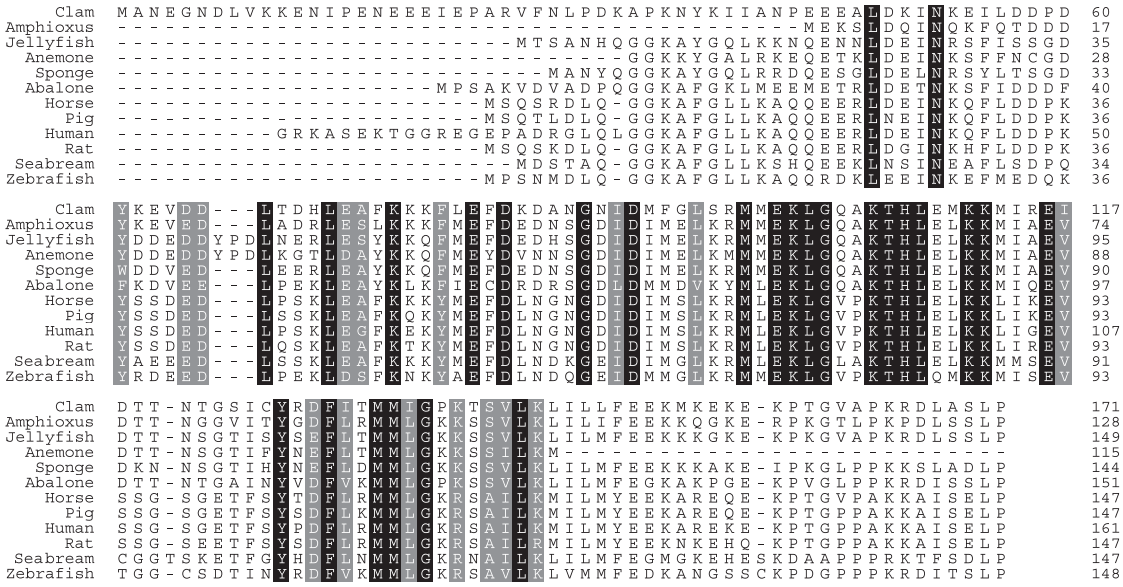

Fig. 2. Multiple sequence alignment of the VpAIF-1 with other registered counterparts from Amphioxus (XP_002597874), Jellyfish (ABA42882), Abalone (ACJ65689), Sponge (CAC38780), Sea anemone (XP_001635454), Horse (XP_001490297), Pig (NP_001123422), Rat (NP_058892), Seabream (BAA36938), Human (CAI18311), Zebrafish (NP_942571). The black shaded regions represent identical amino acids among the different species, while the gray shaded regions represent conservative replacements.

unpaired, two tailed *t*-test with control and challenged groups showed statistically significant difference in VpAIF-1 gene expression at 48 h, 96 h ($P < 0.05$), and 72 h ($P < 0.01$) post-infection. However, no significant difference was observed in other time points of the challenge group.

4. Discussion

The identification and characterization of genes involved in immune responses are now considered to be essential for the elucidation of immune defense mechanisms and for disease control. Accumulating evidence indicated that allograft inflammatory factor-1 (AIF-1) could: 1) be associated with several kinds of inflammatory response-related disease [9]; 2) regulate the immune response during macrophage activation and inflammatory stimuli [10,14]; 3) be involved in the whole immune defense reaction [15]. Recently, much more attention has been paid to AIF-1 in invertebrates, such as sponge, abalone and so on [12,13].

In this paper, the complete cDNA sequence of AIF-1 gene from manila clam *Venerupis philippinarum* was reported. The full-length cDNA of VpAIF-1 was of 888 bp, including an ORF of 516 bp encoding a polypeptide of 171 amino acids with the estimated molecular mass of 17 kDa. In the AIF family, AIFs were classified into four different subfamilies based on the amino acid length [13,18]. The identified clam AIF-1 has 171 aa and more similar to the average size of AIF-1 family as described by Deininger et al. (2002). Some conserved sequences and characteristic motifs were also found in the deduced amino acid sequence of VpAIF-1, such as AIF-

Table 2
Sequences used for multiple alignment and phylogenetic analysis.

Species	Common name	Taxonomy	Accession	Similarity %
Venerupis philippinarum	Manila clam	Invertebrate: Mollusca	ACU83234	100
Branchiostoma floridae	Amphioxus	Cephalo-chordata	XP_002597874.1	64
uncultured cnidarian	Jellyfish	Invertebrate: Cnidaria	ABA42882	60
Haliotis discus discus	Abalone	Invertebrate: Mollusca	ACJ65689	59
Suberites domuncula	Sponge	Invertebrate: Porifera	CAC38780	55
Nematostella vectensis	Sea anemone	Invertebrate: Cnidaria	XP_001635454	50
Equus caballus	Horse	Vertebrate: Mammalia	XP_001490297	49
Sus scrofa	Pig	Vertebrate: Mammalia	NP_001123422	47
Rattus norvegicus	Rat	Vertebrate: Mammalia	NP_058892.1	46
Pagrus major	Seabream	Vertebrate: Actinopterygii	BAA36938	47
Homo sapiens	Human	Vertebrate: Mammalia	CAI18311	48
Danio rerio	Zebrafish	Vertebrate: Teleostei	NP_942571	41

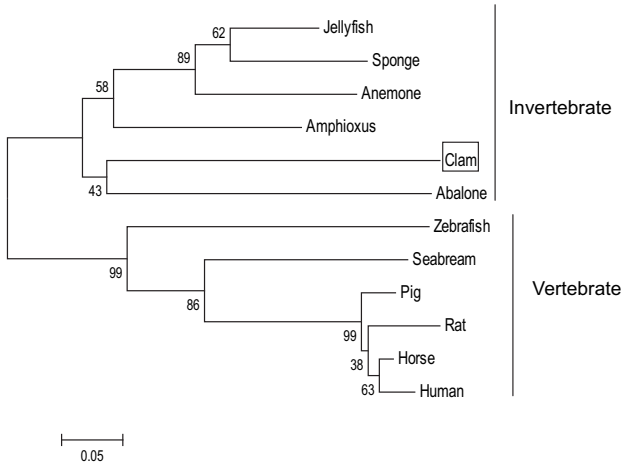

Fig. 3. A phylogenetic tree constructed with the neighbor-joining method. The common names and the GenBank accession numbers were the same as those in Fig. 2. Numbers at each branch indicate the percentage of times a node was supported in 1000 bootstrap pseudoreplication by neighbor joining.

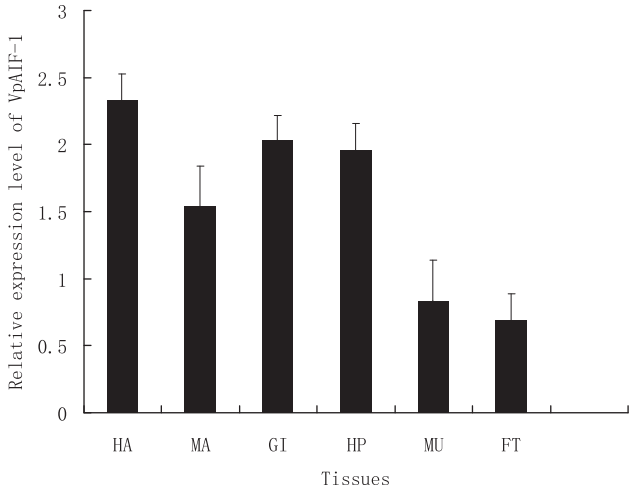

Fig. 4. Tissue distribution of VpAIF-1 transcripts measured by semi-quantitative RT-PCR. The value of the mRNA expression was achieved by comparing the density of VpAIF-1 band with that of the β-actin. Each symbol and vertical bar represented the mean ± S.E. ($N = 4$). HE: haemocyte; MA: mantle; GI: gill; HP: hepatopancreas; MU: muscle; FT: foot.

1 family signatures, EF hand Ca^{2+} binding, casein kinase II, protein kinase C-phosphorylation and N-myristoylation [18]. Consistent with abalone AIF-1, the signal peptide sequence and NLS signal were not identified in VpAIF-1, although found in invertebrate sponge AIF-1 [12,13]. Furthermore, phylogenetic analysis revealed that AIF-1 was a highly conserved gene among different Phyla of invertebrates and vertebrates, suggesting that this protein family might have a primarily similar functional role.

In previous studies, AIF-1 had been described that it was expressed in muscle, liver, spleen and thymus in rat [19] and human [20], and was expressed in haemocyte, gill, mantle, muscle, digestive tract and hepatopancreas in disk abalone [13]. In the present study, we observed that ubiquitously expressed VpAIF-1 transcripts in a variety of unstimulated clam tissues, suggesting it could participate in various processes in manila clam *V. philippinarum*. The VpAIF-1 transcript was higher expressed in the tissues of haemocytes, gill and hepatopancreas. Since the gill was constantly flushed with water that contained pathogens, the high expression level of VpAIF-1 mRNA in gills implied that VpAIF-1 could has a significant contribution in the prevention of microbial infection. Hepatopancreas was one of important immune centers and detoxification organs in most species [21], the enrichment of VpAIF-1 mRNA in hepatopancreas indicated VpAIF-1 might be involved in immunity defense. Moreover, VpAIF-1 was the highest expressed in haemocytes, which further supported the possible immune functions for this protein, since clam haemocytes were thought to play extremely important roles in immunity defense not only by direct sequestration and killing of foreign invaders, but also by synthesis and exocytosis of bioactive molecules [22–24].

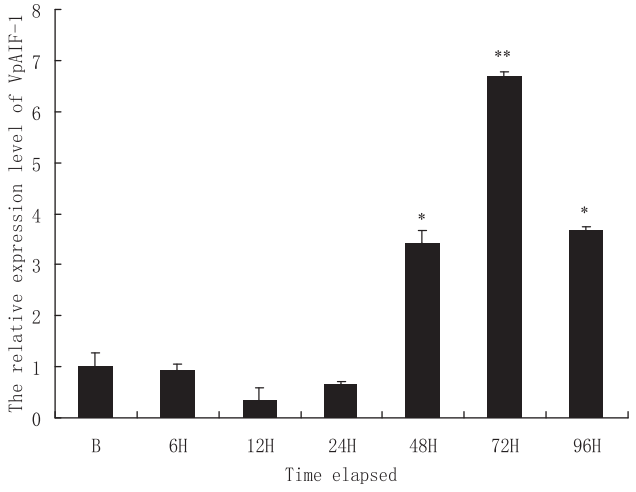

Fig. 5. Temporal expression of VpAIF-1 transcript in haemocytes after *Vibrio anguillarum* infection measured by quantitative real-time PCR at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. VpAIF-1 mRNA expression was normalized to the control group, and β-actin gene was used as internal control to calibrate the cDNA template for all the samples. Each bar represents the mean value from four determinations with standard error. Significant differences across control were indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

Haemocytes were demonstrated to play an important role in limiting infection in invertebrates [25–27], and it was always selected as candidate tissue for investigating the fluctuation of immune-related genes. Generally, bivalve haemocytes can be classified at least in two cell types: granulocytes and hyalinocytes. However, the classification schedules are so varied that three, four or even more morphologically different populations have been proposed for various bivalve species. In the present study, total haemocyte was used as our target tissue to examine the expression profiles of VpAIF-1 after bacteria challenge. During the early phase of *Vibrio* infection, VpAIF-1 transcript was decreased significantly in circulating haemocytes was probably be explained that there are massive granular haemocyte infiltrated at the site of infection and the neutralized localized infection resulted in lowering the number of circulating immune cells temporarily [28]. The similar phenomenon in AIF-1 expression had been described in rat [10] and red seabream [11]. After this phase, the number of VpAIF-producing haemocytes was recruited to supplement the infiltration haemocytes and protect other location from potential hazard, leading the expression level of VpAIF-1 to up-regulate and reach the peak at 72 h post-infection. Similar increased trends were also observed in other species AIF-1s. Miyata et al. (2001) have shown up-regulated AIF-1 transcripts in seabream leukocytes upon LPS stimulation from 3 to 24 h [11]. In abalone, elevated mRNA expression levels of AIF-1 were observed both in circulating haemocytes (3–24 h) and gills (3–48 h) after bacterial challenge [13]. These results collectively suggested that AIF-1 were involved in the immune response in these species.

In conclusion, a novel AIF-1 (VpAIF-1) gene was cloned from *V. philippinarum*, and it was constitutively expressed in the tissues of haemocyte, mantle, gill, hepatopancreas, muscle and foot with the highest level in haemocyte. The increased expression in haemocytes challenged with *V. anguillarum* indicated that VpAIF-1 was perhaps involved in the response to the bacterial challenge. More information about the functions of VpAIF-1 was required to better understand the immune defense mechanisms of clam *V. philippinarum*.

Acknowledgements

This work was financially supported by National Natural Science Foundation (No. 40776075), and Scientific Program of Zhejiang Province, China (No. 2006C13089), and K.C. Wong Magna Fund of Ningbo University and Sponsored by Start Research and Fund projects Fund at Ningbo University.

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