Fish and Shellfish Immunology 89 (2019) 548–554

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

The first cloned sea cucumber FADD from *Holothuria leucospilota*: Molecular characterization, inducible expression and involvement of apoptosis

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ARTICLE INFO

Keywords:
Fas-associated death domain
Holothuria leucospilota
Apoptosis
Innate immune

ABSTRACT

In this study, a sea cucumber Fas-associated death domain (FADD) named *HLFADD* was first cloned from *Holothuria leucospilota*. The full-length cDNA of *HLFADD* is 2137 bp in size, containing a 116-bp 5′-untranslated region (UTR), a 1334-bp 3′-UTR and a 687-bp open reading frame (ORF) encoding a protein of 228 amino acids with a deduced molecular weight of 26.42 kDa. HLFADD protein contains a conserved death effector domain at its N-terminal and a conserved death domain at its C-terminal, structurally similar to its counterparts in vertebrates. The over-expressed HLFADD protein could induce apoptosis in HEK293 cells, suggesting a possible death receptor-mediated apoptosis pathway in echinoderms adapted with FADD. Moreover, *HLFADD* mRNA is ubiquitously expressed in all examined tissues, with the highest transcript level in the coelomocytes, followed by intestine. *In vitro* experiments performed in the *H. leucospilota* coelomocytes, the expression of *HLFADD* mRNA was significantly up-regulated by lipopolysaccharides (LPS) or polyriboinosinic-polyribocytidylic acid [poly (I:C)] challenge, suggesting that HLFADD might play important roles in the innate immune defense of sea cucumber against the invasion of bacteria and viruses.

1. Introduction

Fas-associated death domain (FADD) protein is an essential adaptor protein that transmits the apoptotic signals mediated by the main death receptors (DRs) [1]. These death receptors include tumor necrosis factor receptor super-family member 6 (Fas), tumor necrosis factor receptor 1 (TNF-R1), DR3, TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, DR4) and TRAIL-R2 (DR5) [1–8].

As an essential biological process, apoptosis plays critical roles in cellular and tissue homeostasis, embryonic development and immunity [9–13]. In multicellular organisms, apoptosis mediates the immune defense against the invading parasites, viral and bacterial pathogens by eliminating the infected, damaged, instable and needless cells [10–13]. Apoptosis is considered to be triggered via two major pathways: the intrinsic pathway (also called the mitochondrial pathway) and the extrinsic pathway (also called the receptor-mediated pathway) [10]. In the extrinsic pathway, FADD is a pivotal adapter protein that couples the transmembrane receptors (Fas, TNF-R1, DR3, DR4 and DR5) to intracellular cysteine-dependent aspartate-directed proteases (caspases) [1–8], which initiate and execute the downstream apoptosis [14]. Once the death receptor (such as Fas) was activated by extracellular ligand (such as Fas ligand), it rapidly recruited FADD through the homophilic interaction between death domains (DDs) in the death receptor and FADD [15,16]. Then, FADD recruits procaspase-8 via its N-terminal death effector domain (DED), leading to the formation of the death-inducing signaling complex (DISC) and the initiation of the apoptotic caspase cascade [15–17]. Additionally, the intrinsic pathway is initiated in response to intracellular damage and characterized by the

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https://doi.org/10.1016/j.fsi.2019.04.030
Received 1 February 2019; Received in revised form 29 March 2019; Accepted 10 April 2019
Available online 13 April 2019
1050-4648/

formation of apoptosome [10,18]. In this pathway, the mitochondrial cytochrome c is released from the mitochondrial intermembrane space into the cytosol and forms an apoptosome with procaspase-9, resulting in the activation of the apoptotic caspase cascade [19].

In addition to the functional roles in apoptosis, FADD is also implicated in the innate immunity [20]. Previous studies have suggested that the requirement to FADD in innate immune against invading pathogens may be evolutionarily conserved from *Drosophila* to mammals [21,22].

In contrast to the detailed descriptions of the biological roles of FADD in mammals [1–8,20,23–26], little is known regarding the function of FADD in invertebrates, with an exception of *Drosophila* [21,27–33]. The FADD orthologs are widely found in invertebrates. In echinoderm, the highest group of invertebrates, several hypothetical *FADD* sequences were predicted from the genome data of *Strongylocentrotus purpuratus* (GenBank: XM_786882.3, XM_011665781.1 and XM_011665512.1), *Paracentrotus lividus* (GenBank: EF555572.1), *Acanthaster planci* (GenBank: XM_022241283.1, XM_022241282.1, XM_022241281.1 and XM_022231863.1) and *Apostichopus japonicas* (Genbank: PIK53982.1). However, the further confirmation of *FADD* gene in echinoderm by molecular cloning is still lacked. To clarify the molecular characteristics of FADD from echinoderm and its involvement in apoptosis and innate immunity, we firstly cloned the cDNA sequence of FADD from the tropical sea cucumber *Holothuria leucospilota*, a start-up aquaculture species in Southern China. The functional domains and 3-D structure of the *H. leucospilota* FADD (HLFADD) were analyzed and a phylogenetic tree was constructed. In addition, the mRNA expression profiles of *HLFADD* in various tissues, specifically in the primary coelomocytes after challenged *in vitro* by lipopolysaccharides (LPS) and polyriboinosinic polyribocytidylic acid [Poly (I:C)] were also investigated. Furthermore, HLFADD was transfected into HEK293 cells and apoptosis was examined.

2. Materials and methods

2.1. Animals and tissue collection

Eight healthy sea cucumbers (*H. leucospilota*) with weight of 190–210 g were obtained from the Daya bay in Guangdong province, China, and acclimated for a week in a seawater aquarium with filtered seawater (35‰ salinity) at 32 °C before experimentation. The sea cucumbers were dissected on ice and the tissues including intestine, respiratory tree, rete mirabile, transverse vessel, cuvierian tubules and body wall were collected and rapidly frozen in the liquid nitrogen and stored at −80 °C until RNA extraction. To get the coelomocytes, the coelomic fluids were centrifuged at 1000 × *g* for 10 min at 4 °C, then the sediments (coelomocytes) were stored in TRIzol reagent (Invitrogen, USA) at −80 °C.

2.2. Molecular cloning of HLFADD full-length cDNA

Total RNA was extracted from the intestine of sea cucumber *H. leucospilota* by using TRIzol reagent (TaKaRa, Japan) and reverse transcribed to the first-strand cDNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa). To clone the full-length cDNA sequence of *HLFADD*, the 5′- and 3′-rapid amplification of cDNA ends (RACE) were operated using 5′ Full Race Kit and 3′ Full Race Core Set Ver. 2.0 (TaKaRa), respectively. The specific primers (5′ RACE1/5′ RACE2 and 3′ RACE1/3′ RACE2, Table 1) for *HLFADD* used in the 5′- and 3′-RACE were designed based on a verified expression sequence tag (EST) of 1122 bp (Suppl. 1), which was obtained from a previously constructed cDNA library of *H. leucospilota* in our lab and shared high sequence similarity to the known FADDs in other species. The 5′- and 3′-RACE were conducted under the following conditions: 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 120 s. The obtained amplicons were sub-cloned into the pMD-18 vectors (TaKaRa), and three positive clones for each amplicon were sequenced.

Table 1
Primers used in this study.

Primers	Primer sequences (5′-3′)
For cDNA cloning	
3′ RACE1	GGTTGTGTTGACCAGAAACAGCGACAT
3′ RACE2	TGCTTGCTCTATGTCCACTTAGTGAGCTACAA
5′ RACE1	AAAAAGTCACATTATCTGGGCTAA
5′ RACE2	TGTGAAAACATCTCCTCTTCTGGT
For protein expression	
P*HLFADD*-F	GAATTCATGGCAAACTATTCAAATGAAATTACAA
P*HLFADD*-R	CTCGAGAGCGTAGTCTGGGACGTCGTATGGGTA
For qPCR	
Q*HLFADD*-F	TCAAGTGGTGATGGCACCTA
Q*HLFADD*-R	TCACCCTGTACCGTTCCTTC
Q*β-actin*-F	TCCACGTGCAGTGTTTCCA
Q*β-actin*-R	CGCTTGCTTTGTGCTTCATC

2.3. Bioinformatics analysis

Structural domains of HLFADD were predicted using the SMART program (http://smart.embl-heidelberg.de/). The three-dimensional (3-D) model of HLFADD was deduced with Swiss modeling software provided by the SWISS-MODEL server (https://swissmodel.expasy.org/). Alignment of FADD among various species was performed with ClustalX (http://wwwigbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and demonstrated by using the GeneDoc program. The phylogenetic tree was constructed based on amino acid difference (*p*-distance) with the Neighbor-joining method (pairwise deletion) with 1000 bootstrap replicates using MEGA 6.0.

2.4. Expression pattern of HLFADD mRNA in different tissues

The expressions of *HLFADD* mRNA in the coelomocytes, intestine, respiratory tree, rete mirabile, transverse vessel, cuvierian tubules and body wall of *H. leucospilota* were detected by real-time PCR. The total RNA was extracted as described in section 2.2 and reverse transcribed using PrimeScript™ RT reagent Kit (TaKaRa). Specific primers Q*HLFADD*-F and Q*HLFADD*-R (Table 1) were designed based on the obtained cDNA sequence. Real-time PCR was performed using a RotorGene RG-3000 real-time PCR System (Corbett research, Australia) in a 25 μl reaction volume containing 12.5 μl of 2 × SYBR *Premix Ex Taq* II, 1 μl of each primer (10 μM), 2 μl of cDNA (50 ng/μl) and 8.5 μl of dH_2O. The thermocycling conditions were 95 °C for 30 s, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 82 °C for 20 s. In this case, the housekeeping gene *HLβ-actin* was used as an internal control and the specific primers Q*β-actin*-F and Q*β-actin*-R (Table 1) were designed based on a 613 bp EST (Suppl. 2) from the previously constructed cDNA libraries of *H. leucospilota* in our lab.

2.5. Expression of HLFADD mRNA in sea cucumber primary coelomocytes after immune challenge

To explore the immune function of HLFADD in sea cucumber, coelomocytes, the effector cells of immune system in echinoderms [34], were isolated and used as the target cells in this study. Isolation and primary culture of *H. leucospilota* coelomocytes were performed as described previously [35,36]. After culture for one day, the *H. leucospilota* coelomocytes were challenged by LPS (10 μg/mL, from *Escherichia coli* O111:B4, Sigma, Germany) and poly (I:C) (10 μg/mL, Sigma), respectively, and collected at the time points of 0, 3, 6, 12, 24 and 48 h post-challenge. In this case, the untreated coelomocytes were used as a control. The temporal expression pattern of *HLFADD* after immune challenge was measured by real-time PCR. The procedures for real-time PCR were identical to section 2.4.

2.6. Construction of the recombinant plasmid pcDNA3/HA/HLFADD

The coding region of *HLFADD* was amplified by PCR using the gene-specific primers P*HLFADD*-F and P*HLFADD*-R (Table 1) with a restriction enzyme site for *Eco*R I or *Xho* I at their 5′-ends, respectively. The amplified DNA fragment and pcDNA3/HA plasmid (Invitrogen, USA) were digested with *Eco*R I and *Xhol* I (TaKaRa) and ligated with T4 DNA ligase (TaKaRa) at 16 °C for 4 h. Then, the recombinant product was transformed into XL1-blue competent cells. The transformant was cultured in lysogen broth (LB) medium with ampicillin (100 μg/mL) at 37 °C with shaking overnight. Soon afterwards, the pcDNA3/HA/HLFADD recombinant plasmid (rHLFADD) was purified using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany) and confirmed by double-enzyme (*Eco*R I and *Xhol* I) digestion and sequencing.

2.7. Transfection and western blot analysis

HEK293 cells under logarithmic phase were inoculated into a 24-well tissue culture plate with 2.0×10^5 cells per well and cultured at 37 °C with 5% CO_2 for 24 h. Then, 1.5 μg pcDNA3/HA/HLFADD plasmid was transfected into the HEK293 cells by using 1.5 μl lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. As a control, the pcDNA3/HA blank plasmid was transfected into HEK293 cells by using the same method.

After transfection of 4 h, the HEK293 cells that were transfected with pcDNA3/HA/HLFADD (experimental group) or pcDNA3/HA (control group) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% FBS at 37 °C with 5% CO_2 for 48 h. Then, the cells in the above two groups were lysed and the supernatants were put into western-blot assays as described previously [37]. In this case, the rabbit anti-HA antibody (Sigma, Germany) and rabbit anti-GAPDH antibody (Saier Biotech Inc, China) were used as the primary antibodies. The horseradish peroxidase conjugated goat anti-rabbit IgG (Abcam, UK) was used as the secondary antibody.

2.8. Detection of apoptosis

After transfection for 4 h, the untransfected HEK293 cells (blank group) and the HEK293 cells that were transfected with pcDNA3/HA (control group) or pcDNA3/HA/HLFADD (experimental group) were cultured in 10 mL DMEM containing 10% FBS at 37 °C with 5% CO_2 for 48 h. Then, the apoptotic cells were observed by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay as described previously [37].

2.9. Data transformation and statistical analysis

For *HLFADD* mRNA expression, data expressed as mean ± standard deviation (SD) were analyzed using Student's *t*-test or ANOVA followed by Fisher least significant difference (LSD) test with SPSS (IBM Software, USA). Differences were considered as significant at $P < 0.05$.

3. Results

3.1. Molecular cloning and sequence analysis of HLFADD

By using RT-PCR coupled with 3'-/5′-RACE, a full-length cDNA of FADD was obtained from *H. leucospilota* and deposited in GenBank under the accession no. MK455781. The *HLFADD* cDNA is 2137 bp in size, containing a 116 bp 5′-untranslated region (UTR), a 1334 bp 3′-UTR and a 687 bp open reading frame (ORF) encoding a protein of 228 amino acids (Fig. 1A) with a deduced molecular weight of 26.42 kDa and a predicted isolectric point of 7.32. An atypical polyadenylation signal (AATTAA) is located at 19-bp upstream of the poly-A tail. Based on the SMART program, a death effector domain (DED, residues 6–91) at the N-terminal and a death domain (DD, residues 130–224) at the C-terminal were predicted in the amino acid sequence of HLFADD (Fig. 1A and B). In addition, the identities of the N-terminal DEDs within HLFADD and *H. leucospilota* caspase-8 (HLcaspase-8) [38], and the C-terminal DDs within HLFADD and *H. leucospilota* TNF-R (cloned in our lab recently) were identified, which are 22.4% and 33.0%, respectively.

3.2. Phylogenetic and homology analysis

Result of phylogenetic analysis by neighbor-joining method revealed that FADDs from multiple animal species were classified into two branches: one was the protostomia FADDs and the other was the deuterostomia FADDs. In the deuterostomes, FADDs were further separated into three clades, namely the vertebrate FADDs, tunicate FADDs and echinoderm FADDs. Our newly identified HLFADD was found in the echinoderm clade, which shared the shortest evolutional distant with the *A. japonicus* FADD (Fig. 2A).

The 3-D modeling was performed for the vertebrate FADDs from human and medaka (*Oryzias latipes*), and the invertebrate FADDs from *H. leucospilota* and fruit fly (*Drosophila melanogaster*). In this case, the 3-D structures of the invertebrate and vertebrate FADDs were highly comparable, with conserved characteristics of the N-terminal DED and the C-terminal DD (Fig. 2B). Within the DED and DD, the α-helices were similarly organized among all the FADDs we analyzed (Fig. 2B).

Multiple alignment of the FADD amino acid sequences from various species indicated that the FADDs shared considerable consensus sequences from echinoderms to mammals (Fig. 2C). The DED and DD could be identified from all the primary sequences of FADDs we used for comparison. Moreover, the HLFADD shared relative high sequence identity with *A. japonicus* FADD (42.5%), but very low sequence identities (19.7–32.4%) with the FADDs from other animal species (Fig. 2C).

3.3. Expression pattern of HLFADD in different tissues

To examine the distribution of *HLFADD*, the *HLFADD* mRNA expression in multiple tissues of *H. leucospilota* including the coelomocytes, intestine, respiratory tree, rete mirabile, transverse vessel, cuvierian tubules and body wall was analyzed by the quantitative real-time PCR. As shown in Fig. 3A, *HLFADD* mRNA was ubiquitously expressed in all the examined tissues and the strongest expression was found in the coelomocytes, followed by the intestine, body wall, transverse vessel, cuvierian tubules, rete mirabile and respiratory tree. Compared to the respiratory tree, 17.40-, 17.26-, 3.74-, 2.19-, 1.74- and 1.53-fold expression levels were presented in the coelomocytes, intestine, body wall, transverse vessel, cuvierian tubules and rete mirabile of *H. leucospilota*, respectively.

3.4. Temporal expression pattern of HLFADD after immune challenge

The temporal expression of the *HLFADD* mRNA in primary culture coelomocytes challenged with LPS or poly (I:C) was determined by real-time PCR. Responding to LPS treatment, the expression of *HLFADD* was first up-regulated at 6 h post-challenge ($P < 0.05$) and reached a peak of 5.51-fold at 24 h post-challenge. Then, the expression of the HLFADD mRNA dropped and reached to 2.53-fold of the basal level at 48 h post-challenge. After challenge with poly (I:C), the expression of HLFADD was also first up-regulated at 6 h post-challenge ($P < 0.05$) and reached a peak of 4.48-fold at 48 h post-challenge (Fig. 3B).

3.5. Effects of HLFADD over-expression on cell apoptosis

In order to investigate the involvement of HLFADD in the procedures of apoptosis, pcDNA3/HA/HLFADD was transfected into HEK293 cells. As shown in Fig. 4A, expression of recombinant HLFADD (rHLFADD) protein was detected in the transfected HEK293 cells (Fig. 4A). By TUNEL assay, the apoptosis rate was detected. As shown in

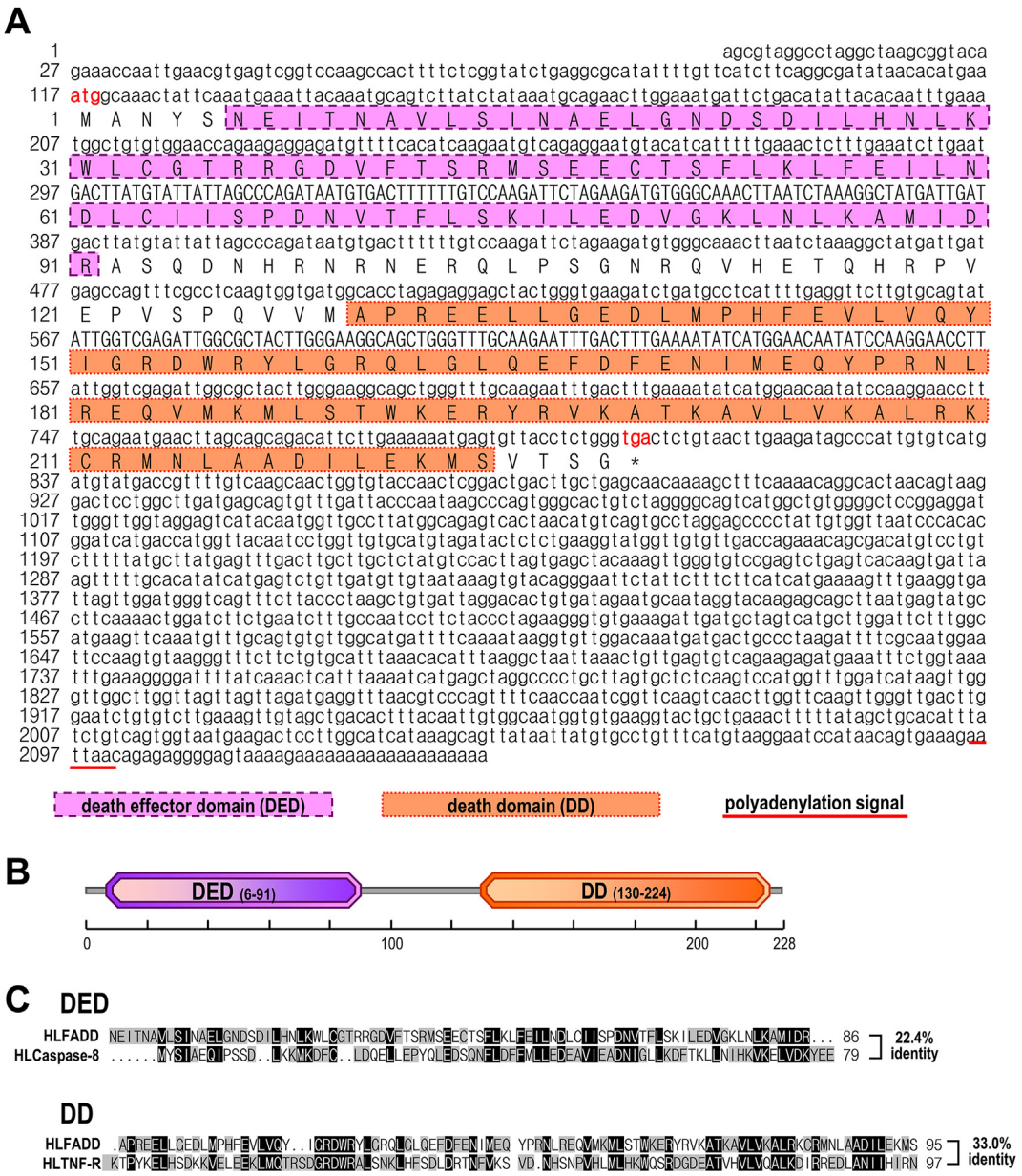

Fig. 1. A: Nucleotide and deduced amino acid sequences of *HLFADD* cDNA. The atypical polyadenylation signal in 3′-UTR is underlined and the initiation codon as well as stop codon are bolded. In the amino acid sequence, the characteristic death effector domain (DED) and death domain (DD) are marked in different symbols. B: Structural domain of HLFADD predicted by using the SMART program. The numbers represent the amino acid residues of HLFADD. The functional domains DED and DD are boxed. C: Alignment of the DEDs between HLFADD and HLcaspase-8, and the DDs between HLFADD and HLTNF-R.

Fig. 4B, the over-expression of exogenous HLFADD could significantly induce apoptosis in HEK293 cells with DNA fragmentation in cell nuclei (Fig. 4B) and the percentage of apoptosis was 2.42%, 2.46% and 22.72% in the blank, control and experimental groups, respectively (Fig. 4C).

4. Discussion

In this report, a FADD named as HLFADD was first cloned from the tropical sea cucumber *H. leucospilota*. The results of the conserved functional domains prediction (Fig. 1B) and phylogenetic analysis (Fig. 2A) indicated that our newly cloned HLFADD is a new member of the FADD family. Although HLFADD protein shared low identity with its counterpart in other animal species (Fig. 2C), the amino acid sequences in the DEDs and DDs within the FADD proteins are relatively conserved among various species (Fig. 2C). The conservation of DED and DD between echinoderm and other animal species was further supported by the high similarity of their 3-D structures (Fig. 2B).

In mammals, FADD is a pivotal adaptor protein involved in death receptor-mediated apoptosis. The presence of DED and DD in FADD is essential for the functions of FADD. When the cell death signal (such as Fas ligand) was specifically recognized by the corresponding death receptor (such as Fas), the death receptor was activated and the adaptor protein FADD was recruited through the homophilic interaction between DDs in the death receptor and FADD [15,16]. Then, the DED of FADD interacts with the DED in the procaspase-8, resulted in the formation of DISC and the activation of caspase-8, eventually leading to cell death (apoptosis) [38]. In the current study, the *H. leucospilota* FADD (HLFADD) contains an N-terminal DED and a C-terminal DD, structurally similar to its mammalian counterparts. In addition, the identity of the N-terminal DEDs between HLFADD and the caspase-8 from *H. leucospilota* (HLcaspase-8) [38] is 22.4%, and the identity

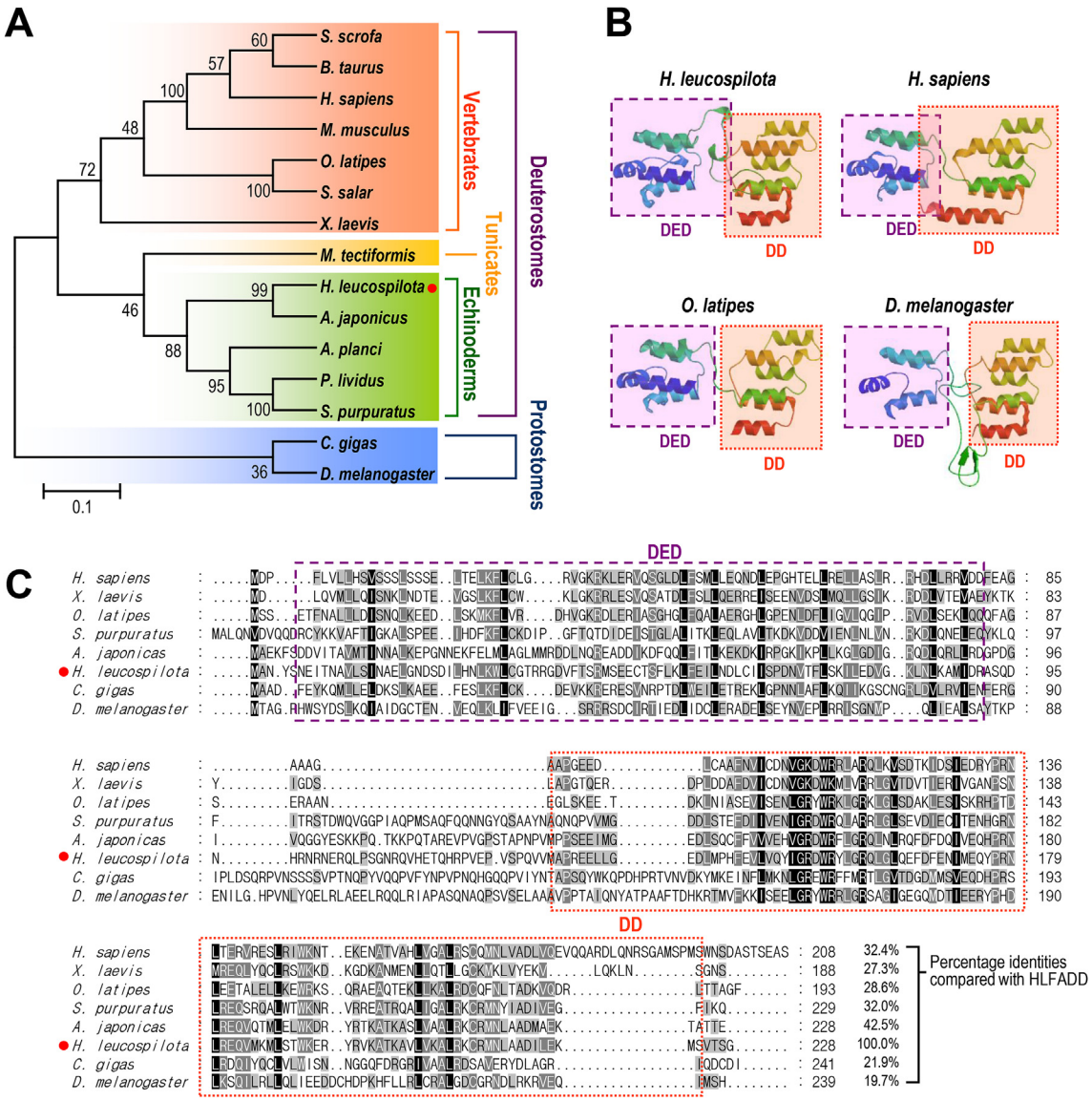

Fig. 2. A: Phylogenetic analysis of FADDs among various species using Neighbor-Joining method with bootstrap value of 1000. B: Comparison of the three-dimensional (3-D) of the DED and DD domains in FADDs among four species. C: Amino acid sequence alignment of FADDs among eight deuterostome species. The functional domains DED and DD are boxed with purple broken line and orange dot line, respectively. The conserved amino acid residues are respectively shown in black, dark gray and light gray background based on their similarities. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

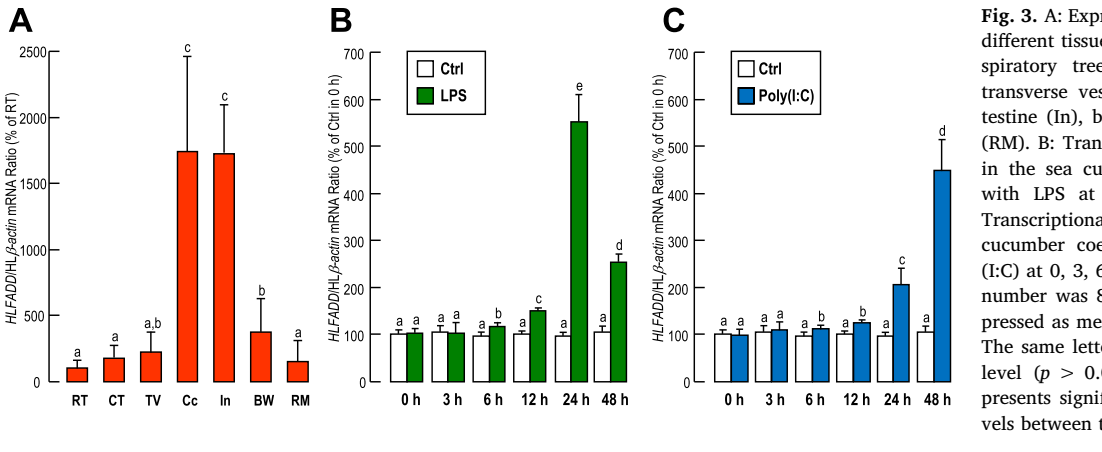

Fig. 3. A: Expression profile of *HFADD* mRNA in different tissues of *H. leucospilota*, including: respiratory tree (RT), cuvierian tubules (CT), transverse vessel (TV), coelomocytes (Cc), intestine (In), body wall (BW) and rete mirabile (RM). B: Transcriptional expression of *HLFADD* in the sea cucumber coelomocytes challenged with LPS at 0, 3, 6, 12, 24 and 48 h. C: Transcriptional expression of *HLFADD* in the sea cucumber coelomocytes challenged with poly (I:C) at 0, 3, 6, 12, 24 and 48 h. The individual number was 8 in this case. The values are expressed as mean ± standard deviation (n = 3). The same letter represents a similar expression level ($p > 0.05$), and the different letter represents significant difference of expression levels between two groups ($p < 0.05$).

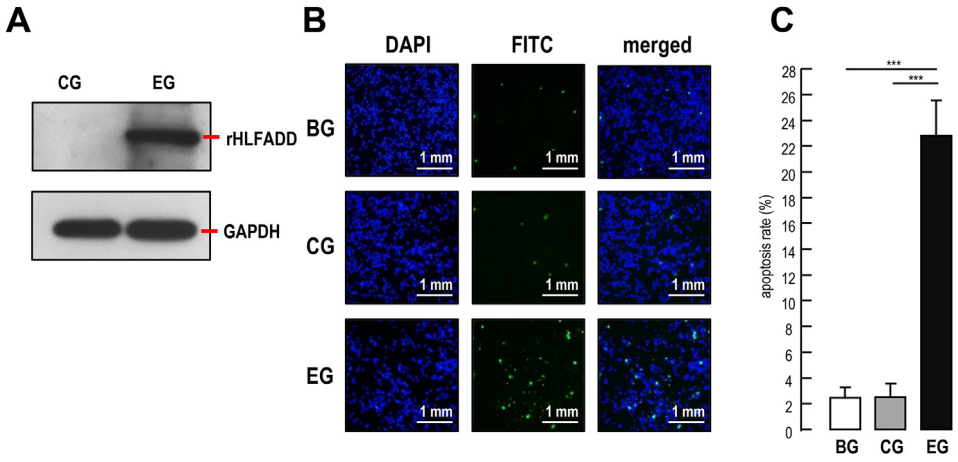

Fig. 4. A: Western blot analysis of HLFADD transfection. Parallel blotting of GAPDH was used as an internal control. B: Detection of apoptosis by TUNEL assay. The "DAPI" represents the DAPI-stained cell nuclei. The "FITC" represents the FITC-stained fractured DNA fragments (marker for apoptosis). The "merged" represents the combination of cell nuclei and fractured DNA fragments. C: Comparison of apoptosis rates of HEK293 cells in different groups. BG: blank group (HEK293 cells untransfected); CG: control group (HEK293 cells transfected with pcDNA3/HA); EG: experimental group (HEK293 cells transfected with pcDNA3/HA/HLFADD). The values are expressed as mean ± standard deviation (n = 3). Significant difference was shown as ***$P < 0.001$.

shared by the C-terminal DDs of HLFADD and a TNF-R from *H. leucospilota* is 33.0% (Fig. 1C).

Studies regarding the apoptosis of sea cucumber have been reported, including the regulatory effects of *β*-integrin, cytochrome c, microRNAs, interleukin 1 receptor-associated kinase 4 (IRAK4), poly-U-binding factor 60 kDa (PUF60), caspase-6, caspase-8 and myeloid differentiation factor 88 (MyD88) on apoptosis in *A. japonicas* and *H. leucospilota* [37–46]. However, the role of FADD in the apoptosis process of sea cucumber has not been illustrated. Previous studies have provided evidences for the induction of apoptosis by FADDs in human cell lines. For example, overexpression of human FADD could induce apoptosis in malignant glioma cells and rheumatoid synoviocytes [47,48]; overexpression of murine FADD could induce apoptosis in HEK293 cells [49]; overexpression of African clawed frog FADD could induce apoptosis in wild-type mouse embryonic fibroblasts (MEF) [50]; overexpression of Medaka fish FADD could induce apoptosis in HeLa cells [51]. In order to investigate the involvement of sea cucumber FADD in apoptosis, HLFADD plasmid was transfected into HEK293 cells. Our current study showed that the *H. leucospilota* FADD could induce apoptosis in the human cell line HEK293, similar to the cases of vertebrate FADDs listed above. Compared with HEK293 cells that were not transfected or transfected with empty vector, the apoptosis rate of HEK293 cells transfected with HLFADD was significantly increased (Fig. 4B and C), indicating that the over-expression of FADD from sea cucumber could induce apoptosis.

FADD, TNF-R and caspase-8, the core components necessary for the death receptor-mediated pathway, were all identified in *H. leucospilota* in our lab. Functional analysis indicated that HLFADD and HLcaspase-8 [38] could induce apoptosis in mammalian cell lines upon overexpression. All these results suggested that the receptor-mediated apoptosis pathway may exist in sea cucumber. It used to be believed that the mechanism of apoptosis induced by death receptors was established during the appearance of vertebrates [51]. However, our results give the evidence for the possible existence of the receptor-mediated apoptosis pathway in invertebrates. This is a new viewpoint that the mechanism of apoptosis via death receptors may exist not only in vertebrates but also in invertebrates.

Previous studies showed that FADD were ubiquitously expressed in all tissues either in vertebrate or invertebrate species [50,52,53]. In this study, the transcript expression of *HLFADD* was detected in all examined tissues and the highest mRNA level was found in the coelomocytes, the effector cells of the echinoderm immune system [34], followed by intestine, a gathering site for immune genes of echinoderms [54]. The high mRNA expression levels of *HLFADD* observed in the immune-related tissues implied that *HLFADD* might be an immune-related gene in this species. In other invertebrates, the high expression levels were also observed in the immune-related tissues. For example, the highest expression of FADD was found in the hemolymph of oyster *Crassostrea gigas* [52], as well as the fatbody and gut tissues of mosquitoes *Aedes aegypti* [55].

FADD has been reported to be involved in the innate immune response to bacterial infection in *Drosophila* [29,56] and its antiviral activity has also been clarified [53]. The transcriptional expression of *FADD* induced by various immune stimulants has been reported in both vertebrate and invertebrate species including *Drosophila*, *C. gigas*, *A. aegypti*, *Xenopus laevis* and *Epinephelus coioides* [50,52,53,55,56]. In this study, when the *H. leucospilota* coelomocytes were incubated with LPS (the cell wall component of Gram-negative bacteria) or poly (I:C) (the analogue of dsRNA virus), the expression of HLFADD transcripts was significantly up-regulated. The high mRNA expression levels in the immune-related tissues and the noteworthy up-regulation by challenge of LPS or poly (I:C) indicated that HLFADD from *H. leucospilota* is potentially involved in the immune defense of sea cucumber against the bacterial and viral infections.

In current study, our results showed that the expression of HLFADD was up-regulated by immune stimulants and over-expressed HLFADD could induce apoptosis. Our previous study showed that the apoptosis rate of sea cucumber coelomocytes was increased after challenge by LPS or poly (I:C) [38]. Thus, we speculate that the apoptosis induced by HLFADD is one of the innate immune mechanisms for sea cucumber to attack the invasive bacteria and viruses.

In conclusion, we first cloned a sea cucumber FADD from *H. leucospilota* in this study. The functional domains of the deduced HLFADD protein were identified, and the involvement of HLFADD in apoptosis was also investigated. Our results showed that over-expressed HLFADD could induce apoptosis and the apoptosis induced by HLFADD might be triggered via a death receptor-mediated pathway. Moreover, the up-regulation of *HLFADD* mRNA was observed in the coelomocytes after LPS or poly (I:C) challenge, suggesting that the HLFADD might play important roles in the innate immune defense of sea cucumber against the infections of bacteria and viruses.

Acknowledgments

This study was supported by the National Key Research and Development Program of China (2018YFD0901605), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020205), the Science & Technology Promoting Projects for Oceanic & Fishery in Guangdong Province (SDYY-2018-01) and the Guangdong Province Program (2017B030314052, 2018A030313857, 2015A030310120, A2015230).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.030.

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