Fish and Shellfish Immunology 93 (2019) 542–550

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

The first cloned echinoderm tumor necrosis factor receptor from *Holothuria leucospilota:* Molecular characterization and functional analysis

Haipeng Li[a,1], Ting Chen[b,d,1], Hongyan Sun[c], Xiaofen Wu[b,d], Xiao Jiang[b,d], Chunhua Ren[b,d,*]

[a] Guangzhou University, School of Environmental Science and Engineering, Guangzhou, 510006, PR China
[b] CAS Key Laboratory of Tropical Marine Bio-resources and Ecology (LMB); Guangdong Provincial Key Laboratory of Applied Marine Biology (LAMB), South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, 510301, PR China
[c] College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, PR China
[d] Institution of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences, ISEE, CAS, PR China

ARTICLE INFO

Keywords:
Tumor necrosis factor receptor
Holothuria leucospilota
Apoptosis
Nuclear factor-κB
Activator protein-1
Proinflammatory cytokines
Innate immune

ABSTRACT

In this study, an echinoderm tumor necrosis factor receptor named *HLTNFR-16* was first cloned from the tropical sea cucumber *Holothuria leucospilota*. The full-length cDNA of *HLTNFR-16* is 3675 bp in size, containing a 415 bp 5′-untranslated region (UTR), a 2024 bp 3′-UTR and a 1236 bp open reading frame (ORF) encoding a protein of 411 amino acids with a deduced molecular weight of 45.63 kDa. The HLTNFR-16 protein contains a signal peptide, four TNFR domains (the last three were identified as extracellular cysteine-rich domains), a transmembrane region and a death domain. Phylogenetic analysis showed that HLTNFR-16 was clustered into a clade with TNFR-16s in other species, indicating that this echinoderm TNFR may be a new member of the TNFR-16 subfamily. The results of TUNEL assay showed that the over expression of HLTNFR-16 could induce apoptosis in HEK293T cells. When HLTNFR-16 was silenced by siRNA, the apoptosis of sea cucumber coelomocytes induced by inactivated *Vibrio harveyi* was suppressed significantly, indicating that HLTNFR-16 is important for apoptosis induction. Additionally, luciferase reporter assay exhibited that the over-expressed HLTNFR-16 in HEK293T cells could activate the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Moreover, the secretion of proinflammatory cytokines interleukin (IL)-1β, IL-6 and IL-18 in HEK293T cells was increased by the over-expression of HLTNFR-16. This study provides evidences for the potential roles of sea cucumber TNFR in the innate immunity.

1. Introduction

The tumor necrosis factor receptor (TNFR) family is a cytokine receptor superfamily [1] and the extracellular cysteine-rich domain (CRD) for binding to the tumor necrosis factors (TNFs) is the hallmark of this superfamily [2]. As the transducers of the TNF-induced signaling, TNFRs mediate a wide spectrum of physiological and pathological events, such as apoptosis, inflammation, proliferation, cell survival and differentiation [3–5]. According to the presence/absence of the death domain (DD) within the cytoplasmic region, TNFRs are divided into two categories: the TNFRs without DD, and the TNFRs with an intracellular DD which are also known as death receptors [5–7].

In mammals, it is well certified about the roles of death receptors (such as TNFR-1) in downstream signaling, particularly in the apoptosis and the activation of the transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [8–12]. Taking TNFR-1 as an example, when activated by its ligand TNF-α, TNFR-1 recruits an adaptor protein TNFR-1-associated death domain protein (TRADD) through its DD [10]. As a platform adapter protein, TRADD can further recruit several signaling molecules, including Fas-associated death domain protein (FADD), TNFR-associated factor-2 (TRAF2) and receptor-interacting protein (RIP), to transmit the extracellular signals that mediated by the activated receptor [13–16]. The interaction between TRADD and FADD is necessary for the initiation of extrinsic apoptosis [17]. After TRADD recruits FADD, caspase-8 is activated and the apoptotic caspase is subsequently initiated [9,11,12]. On the other hand, TRAF2 and RIP indirectly bind to TNFR-1 via TRADD, leading to the activation of the transcription factors NF-κB and AP-1 [5]. In this case, TRAF2 and RIP can activate the NF-κB inducing kinase (NIK) by phosphorylation, followed by the activation of the inhibitor of κB (IκB)

* Corresponding author. Institution of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences, ISEE, CAS, PR China.
E-mail addresses: lihaipeng08@163.com (H. Li), chan1010@scsio.ac.cn (T. Chen), hongyanlucky@scau.edu.cn (H. Sun), wuxiaofen17@mails.ucas.ac.cn (X. Wu), jiangxiao@scsio.ac.cn (X. Jiang), rosemary166@sina.com (C. Ren).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2019.08.008
Received 23 April 2019; Received in revised form 2 August 2019; Accepted 5 August 2019
Available online 05 August 2019
1050-4648/

kinase complex (IκK) and the degradation of IκB proteins, and final lead to the activation of NF-κB [18–21]. In addition, TRAF2 can also activate the c-Jun NH_2-terminal kinase (JNK) pathways by interacting with the mitogen-activated protein kinase (MAPK), resulting in the activation of AP-1 [22–24]. The activation of NF-κB and AP-1 leads to the expression of immuno-inflammatory-related cytokines [25–27]. Moreover, the genes that control cell proliferation, differentiation and survival may be transcriptionally regulated by the activation of NF-κB and/or AP-1 [6]. Thus, it is generally considered that the death receptors play important roles in the apoptosis, immunity, inflammation, proliferation, and cell survival and differentiation by the induction of apoptosis, and the activation of NF-κB and JNK signaling [3–5].

To date, 29 members of TNFR superfamily have been identified in human [28], and the molecular mechanisms of TNFR-mediated signaling are well documented in mammals [1,5,6,28–35]. Since the first invertebrate TNFR (named as Wengen) was identified from *Drosophila* [36], more and more studies on invertebrate TNFRs have been conducted. These studies on invertebrate TNFRs mainly focused on the response of TNFRs under immune challenge. In insects, the *Drosophila melanogaster* TNFR-like protein Wengen has been demonstrated to participate immune defense against gram-negative bacterial and fungal invasion [36]. In crustaceans, the up-regulated *LvTNFRSF* transcripts in the *Litopenaeus vannamei* intestine was observed under challenges of *Vibrio alginolyticus* or *Staphylococcus aureus* [37]. In mollusks, the *CfTNFR* mRNAs in the gonad, gill, mantle and hepatopancreas of *Chlamys farreri* were significantly up-regulated after *Listonella anguillarum* challenge [38], and the expression of 4 TNFR isoforms in the *Crassostrea hongkongensis* hemocytes were also increased after challenged with pathogenic infection or air stress [39]. The intracellular signaling pathways of invertebrate TNFRs have been further studied in oyster *C. hongkongensis*, in which the transcription factor NF-κB could be activated by the 4 TNFRs [39]. In echinoderm, the highest group of invertebrates, there is still no cloned TNFR cDNA but only several TNFR homologs were predicted from the genome data of *Acanthaster planci* (Genbank: XR_002598173.1, XM_022244638.1, XM_022234782.1, XM_022234671.1, XM_022231173.1, XM_022249475.1 and XM_022227257.1), *Strongylocentrotus purpuratus* (Genbank: XM_795305.4, XM_011678353.1 and XM_011 665629.1) and *Apostichopus japonicas* (PIK52601.1). In the current study, we first cloned an echinoderm *TNFR* (named as *HLTNFR-16*) from the tropical sea cucumber *Holothuria leucospilota*, a start-up aquaculture species in Southern China [40]. The structural and phylogenetic characteristics of HLTNFR-16 were investigated. In addition, the involvements of HLTNFR-16 in apoptosis and the intracellular signaling pathways (including NF-κB and JNK pathways) were further examined.

2. Materials and methods

2.1. Animals

Healthy and sexually immature adult *H. leucospilota* with weights of 190–210 g were obtained from the Daya bay in Guangdong province, China, and maintained in an aquarium with filtrated and aerated seawater (35‰ salinity) at 32 °C for a week before experiments.

2.2. Molecular cloning of HLTNFR-16 full-length cDNA

Total RNA was extracted from the intestine of sea cucumber *H. leucospilota* using TRIzol reagent (Invitrogen, USA) and reverse transcribed into the first strand cDNA by using the SuperScript™ III First-Strand Synthesis System (Invitrogen). A 993-bp expression sequence tag (EST) for TNFR-16 homolog (Suppl. 1) obtained from a cDNA library of *H. leucospilota* previously constructed in our lab was verified with the gene-specific primers HLTNFR-F and HLTNFR-R (Table 1). The full-length cDNA sequence of *HLTNFR-16* was obtained by the 3′- and 5′-rapid amplification of cDNA ends (RACE) with the gene-specific primers (3′ RACE1/3′ RACE2 and 5′ RACE1/5′ RACE2, Table 1). The amplification conditions for 3′- and 5′-RACE were as follows: 94 °C for 60s, 30 cycles of 98 °C for 10 s, 55 °C for 15 s and 68 °C for 60 s. The obtained amplicons were sub-cloned into the pMD™-20 vector (TaKaRa, Japan), and three positive clones for each amplicon were sequenced.

Table 1
Primers and siRNA sequences used in this study.

Name	Sequence (5′-3′)
For sequence verification	
HLTNFR-F	CGAAGAGTGTCGGCCATGTA
HLTNFR-R	AATCCTCTCTCCTAATGTCT
For cDNA cloning	
3′ RACE1	ATTGGCCTCCTCATCTATGTCCTA
3′ RACE2	CCACAACTACTTACACAGGTCCCA
5′ RACE1	CGCTGAACACACTGTATCGCTGT
5′ RACE2	TGTCGCCCTGGTGATGTCATCTG
For protein expression	
PHLTNFR-F	GGAATTCATGAGCGGAATTTTGTCCAT
PHLTNFR-R	CCGCTCGAGTCATCGTGACTTATCATTCC
For qPCR	
QHLTNFR-F	TGTGAGGAGTGCGTGCCT
QHLTNFR-R	CAAATCGGACCCTGAAGTA
For RNAi	
siRNA-1 sense	CCAUGUAUGGAGUGCAAUUTT
siRNA-1 antisense	AAUUGCACUCCAUACAUGGTT
siRNA-2 sense	GCGACAAUGUGUAAGCCUUTT
siRNA-2 antisense	AAGGCUUACACAUUGUCGCTT
siRNA-3 sense	CCUCAUCUAUGUCCUAUUUTT
siRNA-3 antisense	AAAUAGGACAUAGAUGAGGTT
EGFP siRNA sense	GCAUCAAGGUGAACUUCAATT
EGFP siRNA antisense	UUGAAGUUCACCUUGAUGCTT

2.3. Bioinformatics analysis

Structural domains of HLTNFR-16 were predicted using the SMART (http://smart.embl-heidelberg.de/) and ScanProsite (https://prosite.expasy.org/) programs. Alignment of TNFR-16 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 the amino acid difference (*p*-distance) with the Neighbor-joining method (pairwise deletion) with 1000 bootstrap replicates using MEGA 6.0.

2.4. Construction of the recombinant plasmid

The coding region of *HLTNFR-16* was amplified by PCR using the gene-specific primers PHLTNFR-F and PHLTNFR-R (Table 1) with a restriction enzyme site for *Eco*RI or *Xho*I at their 5′-ends, respectively. The amplified DNA fragment and pcDNA3/myc plasmid (Invitrogen) were digested with *Eco*RI and *Xho*I (TaKaRa) and ligated with T4 DNA ligase (TaKaRa) at 16 °C for 2 h. Then, the inserted DNA was transformed into XL1-blue competent cells. The pcDNA3/myc/HLTNFR-16 recombinant plasmids (rHLTNFR-16) were confirmed by double-enzyme digestion and purified using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany).

2.5. Cell culture and transfection

HEK293T cells were inoculated into a 24-well tissue culture plate with the density of 2.0×10^5 cells/well and cultured at 37 °C with 5% CO_2 for 24 h. Then, 1 μg pcDNA3/myc/HLTNFR-16 plasmid was transfected into HEK293T cells by 1 μL lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. As a control, the pcDNA3/myc blank plasmid was transfected into HEK293T cells using the same method.

2.6. Western blot

After transfection for 4 h, HEK293T cells that untransfected (blank group), transfected with pcDNA3/myc (control group) or pcDNA3/myc/HLTNFR-16 (experimental group) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 for 24 h. Then, the cells in the above three groups were detected by Western blot as described previously [41]. In this case, the rabbit anti-myc antibody (Saier Biotech Inc, China) at 1:500 dilution and rabbit anti-GAPDH antibody (Saier Biotech Inc) at 1:400 dilution were used as the primary antibodies. The horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Abcam, UK) at 1:1000 dilution was used as the secondary antibody.

2.7. Detection of apoptosis induced by over-expressed HLTNFR-16

After transfection for 4 h, HEK293T cells in the blank, control and experimental groups were respectively cultured in 10 mL DMEM (supplemented with 10% FBS) at 37 °C with 5% CO_2 for 48 h. Then, the apoptotic cells were detected by using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay and observed under fluorescence microscope as described previously [42,43]. To count the apoptosis rate, six cellular fields randomly allocated were respectively selected in the blank, control and experimental groups, and the FITC-stained apoptotic cells (green) and the DAPI-stained total cells (blue) in the selected fields were counted. The apoptotic rate (AR) was calculated as follows: AR = number of apoptotic cells/number of total cells × 100%.

2.8. RNA interference (RNAi)-mediated silencing of HLTNFR-16

Three small interfering RNAs (siRNAs), siRNA-1, siRNA-2, and siRNA-3 (Table 1), which were specific to *HLTNFR-16* gene were designed using the siDirect version 2.0 online program (http://sidirect2.rnai.jp/) and synthesized by Sangon Biotech, Shanghai (China). In this case, a non-targeting siRNA (EGFP siRNA, Table 1) which is specific to enhanced green fluorescent protein (EGFP) was used as the negative control. Before the RNAi experiment, 150 μg of siRNA for each group was dissolved in 300 μl of 150 mM RNase free saline solution (RFSS).

Fifteen healthy *H. leucospilota* (weighing about 50 g) were randomly divided into five groups and each group contains three individuals. In the blank control group, 100 μl of 150 mM RFSS was injected into each sea cucumber. In the negative control group, 100 μl of EGFP siRNA solution which contains 50 μg EGFP siRNA was injected into each sea cucumber. In the three experimental groups, each cucumber was injected with 100 μl of siRNA-1, siRNA-2 or siRNA-3 solution, respectively.

2.9. Detection of apoptosis in sea cucumber coelomocytes after RNAi

After RNAi for 24 h, all the sea cucumbers were dissected on ice and the coelomocytes which have been determined as the effector cells of immune system in echinoderms [45] were isolated as described previously [46]. To assess the interference efficiency of each siRNA, a small amount of the coelomocytes from each sea cucumber were retained for the real-time PCR analysis. The extraction and inverse transcription of the total RNA from the sea cucumber coelomocytes were carried out as described previously [46]. The expression levels of *HLTNFR-16* after RNAi were measured by quantitative real-time PCR using the specific primers QHLTNFR-F and QHLTNFR-R (Table 1). The internal control *HLβ-actin* and the amplification conditions of real-time PCR were identical to previous report [42]. The rest coelomocytes were collected from each sea cucumber and cultured in Leiboviz's L-15 cell culture medium as described previously [46]. On the following day, the coelomocytes from the blank control group (RFSS injected), negative control group (EGFP siRNA injected) and experimental group (HLTNFR-16 siRNA injected) with the highest interference efficiency were respectively challenged by the heat inactivated *Vibrio harveyi*. The inactivated bacteria were prepared in Leiboviz's L-15 cell culture medium to a final concentration of 10^7 cells/ml and gently overlaid onto coelomocytes after removal of old culture medium. After 24 h, the apoptosis of the *H. leucospilota* coelomocytes (including those treated with inactivated bacteria and untreated) from the above three groups were measured by TUNEL assay.

2.10. Luciferase reporter assay

The luciferase reporter gene assay was performed as described before [44]. Briefly, HEK293T cells cultured in a 24-well plate were incubated with the mixture containing 600 ng of pcDNA3/myc/HLTNFR-16, 400 ng of NF-κB-luc (or AP-1-luc) reporter plasmid, 8 ng pRL-TK reference plasmid (Promega, USA) and 1 μL lipofectamine 2000 (Invitrogen) for 4 h. In this case, pcDNA3/myc was used as the negative control. After transfection for 48 h, luciferase reporter activity was detected by using a Dual-Luciferase Reporter Assay System (Promega). Each experiment was performed in triplicates.

2.11. ELISA assay

To explore whether HLTNFR-16 could induce the secretion of proinflammatory cytokines, 3 μg pcDNA3/myc/HLTNFR-16 plasmid (experimental group) or pcDNA3/myc blank plasmid (control group) were transfected respectively into HEK293T cells by using 3 μL lipofectamine 2000. The supernatants from the transfected cells were collected at 24 h and 48 h post-transfection and the concentrations of interleukin (IL)-1β, IL-6 and IL-18 were determined by using human IL-1β, IL-6 and IL-18 ELISA Kits (Elabscience Biotechnolog Co. Ltd, China) according to the manufacturer's instructions [44]. The data obtained in this experiment were performed in triplicates.

2.12. Data transformation and statistical analysis

For luciferase reporter assay and ELISA assay, data were expressed as mean ± standard error (SE) and analyzed using Student's *t*-test followed by Fisher least significant difference (LSD) test with SPSS (IBM Software, USA).

3. Results

3.1. Molecular cloning and sequence analysis of HLTNFR-16

By using RT-PCR coupled with 3'-/5'-RACE, the full-length cDNA of *HLTNFR-16* was obtained and deposited in GenBank under the accession number MK797985. The *HLTNFR-16* cDNA is 3675 bp in size, containing a 415 bp 5′-untranslated region (UTR), a 2024 bp 3′-UTR and an 1236 bp open reading frame (ORF) encoding a protein of 411 amino acids (Fig. 1A) with a deduced molecular weight of 45.63 kDa and a predicted isolectric point of 7.61. A typical polyadenylation signal (AATAAA) is located at 15-bp upstream of the poly-A tail. Based on the SMART program, a signal peptide (residues 1–33) at the N-terminus, four TNFR domains (residues 44–76, 79–118, 120–156, 159–199), a transmembrane region (residues 225–247) and a DD (residues 311–407) at the C-terminal were predicted in the amino acid sequence of HLTNFR-16 (Fig. 1B). The last three TNFR domains were identified as CRDs (Fig. 1B) by using ScanProsite program. In each CRD, six conserved cysteine residues and a conserved motif (CXXCXXC) were found.

3.2. Phylogenetic and homology analysis

To analyze the evolutionary relationship of TNFR homologs among different species, a phylogenetic tree was constructed. As shown in

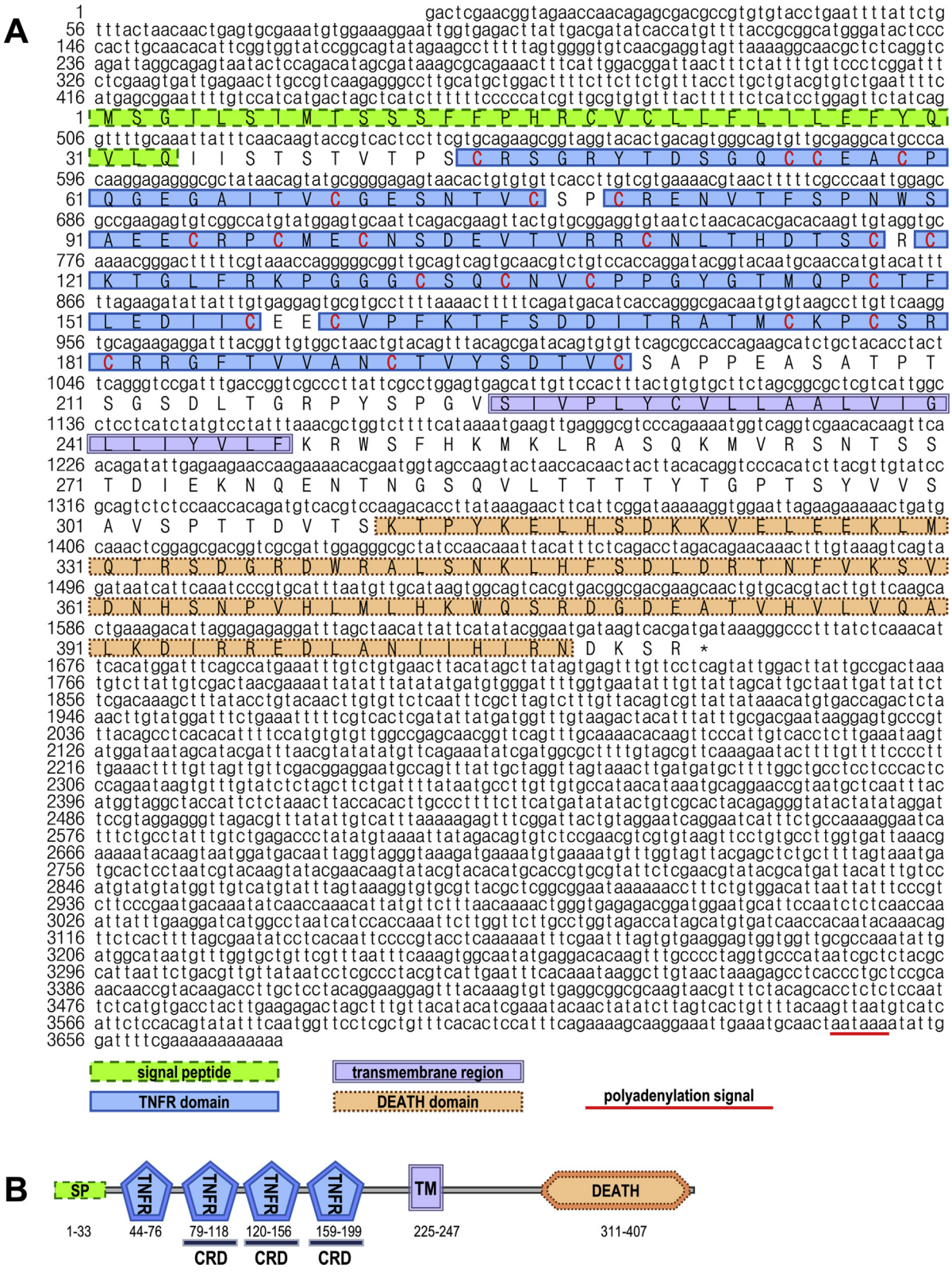

Fig. 1. Sequences and structural domains of HLTNFR-16. A: Nucleotide and deduced amino acid sequences of *HLTNFR-16* cDNA. The typical polyadenylation signal in 3′-UTR is underlined and the stop codon is marked by an asterisk. In the amino acid sequence, the signal peptide, TNFR domains, transmembrane region and death domain are marked in different symbols. B: Structural domains of HLTNFR-16 predicted by using the SMART and ScanProsite programs. The numbers represent the amino acid residues of HLTNFR-16. The signal peptide, TNFR domains, transmembrane region, death domain and cysteine-rich domains are boxed.

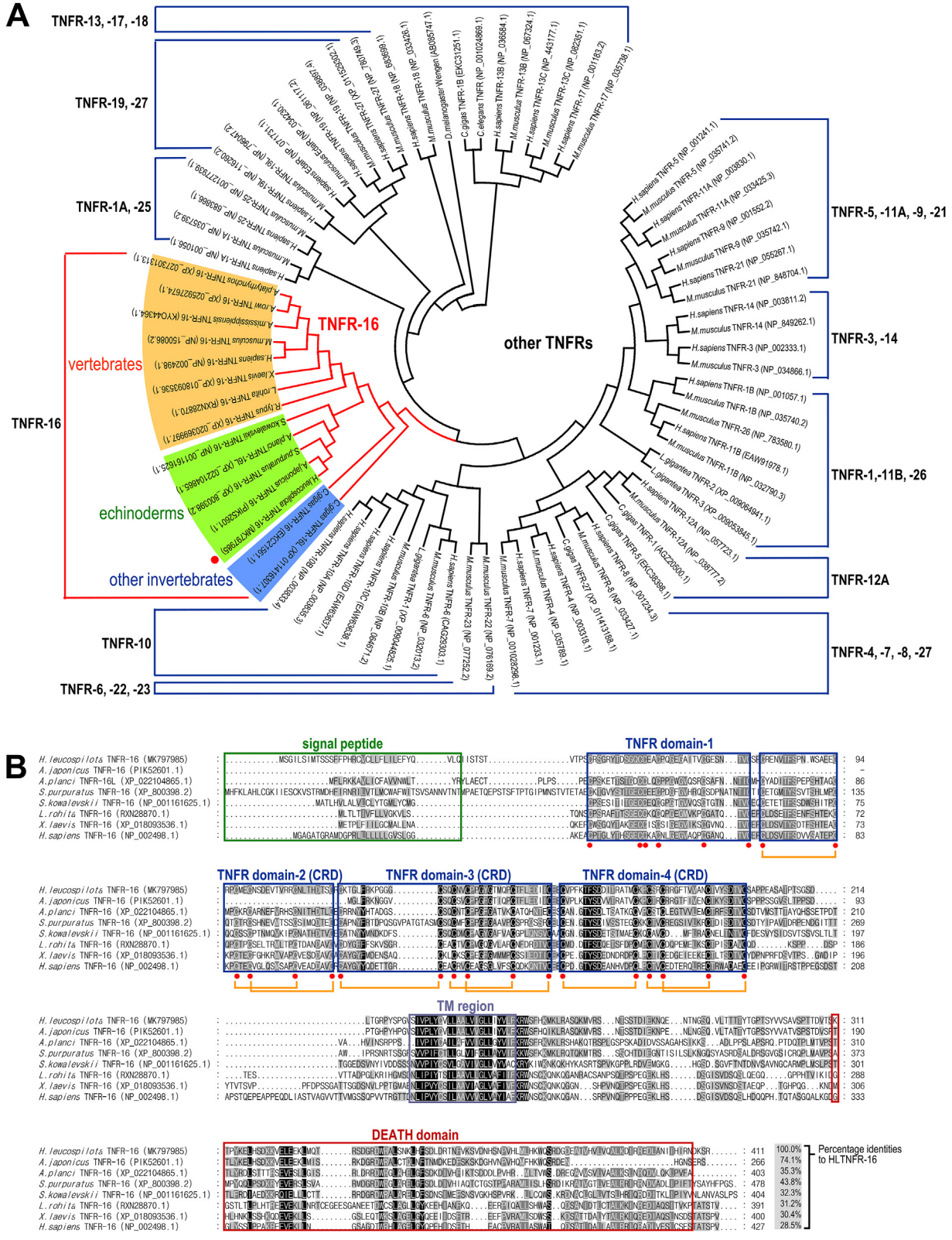

Fig. 2. Phylogenetic analysis and amino acid sequence alignment. A: Phylogenetic analysis of TNFR among various species using Neighbor-Joining method with bootstrap value of 1000. B: Amino acid sequence alignment of TNFR-16s among eight deuterostome species. The conserved amino acid residues are boxed in dark gray and similar amino acid residues are labelled in light gray. The TNFR domains, transmembrane region and death domain are boxed, and the conserved cysteines and the predicted disulfide bridges within the cysteine-rich domains are indicated.

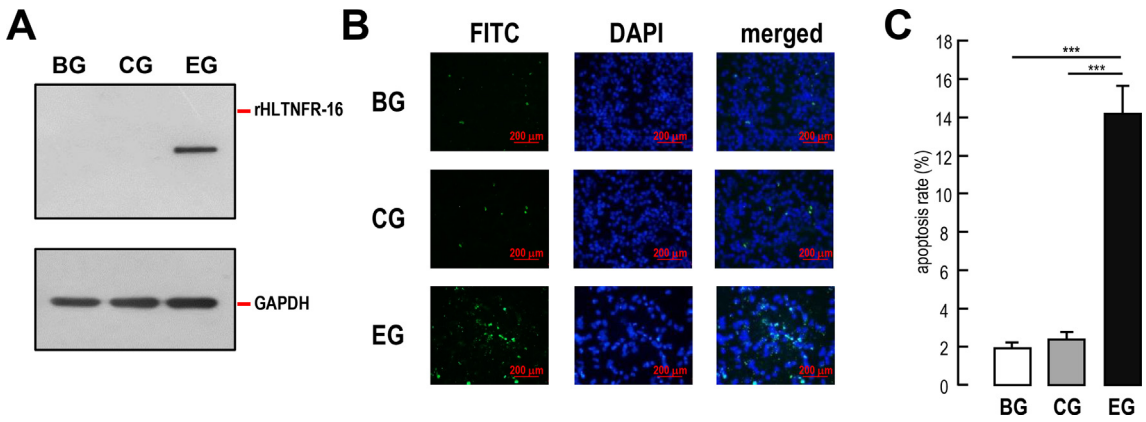

Fig. 3. Western blot analysis and apoptosis detection induced by over-expressed HLTNFR-16. A: Western blot analysis of HLTNFR-16 transfection. Parallel blotting of GAPDH was used as an internal control. B: Detection of apoptosis by TUNEL assay. The "FITC" represents the FITC-stained broken DNA fragments (marker for apoptosis). The "DAPI" represents the DAPI-stained cell nuclei. The "merged" represents the combination of cell nuclei and broken DNA fragments. C: Comparison of apoptosis rates of HEK293T cells in different groups. BG: blank group (HEK293T cells untransfected); CG: control group (HEK293T cells transfected with pcDNA3/myc); EG: experimental group (HEK293T cells transfected with pcDNA3/myc/HLTNFR-16). The values are expressed as mean ± standard error (n = 3). Significant difference was shown as $^{***}P < 0.001$.

Fig. 2A, our newly cloned HLTNFR-16 first forms a cluster with *A. japonicas* TNFR-16, and further groups into a branch with other members of TNFR-16 subfamily. In the branch of TNFR-16, the TNFR-16s from vertebrates, echinoderms and other invertebrates group into three independent clades, respectively, based on the evolutionary relationships of their original species.

Amino acid alignments showed that the TNFR-16s in different species shared considerable consensus sequences from echinoderms to mammals (Fig. 2B). The signal peptide, TNFR domains, CRDs, transmembrane region and DD could be identified from all the primary sequences of TNFR-16s we used for comparison. Moreover, the HLTNFR-16 shared relative high sequence identity with *A. japonicus* TNFR-16 (74.1%), but low sequence identities (28.5–43.8%) with the TNFR-16s from other animal species (Fig. 2B).

3.3. Effects of over-expressed HLTNFR-16 on apoptosis

To assess the effects of HLTNFR-16 on apoptosis, pcDNA3/myc/HLTNFR-16 was transfected into HEK293T cells. As shown in Fig. 3A, over-expression of rHLTNFR-16 protein was detected in the transfected HEK293T cells. By TUNEL assay, a large number of fragmented DNAs were observed in the cells with over-expressed HLTNFR-16, whereas only a few were observed in the control group. The results indicated that over-expressed HLTNFR-16 could significantly induce apoptosis (Fig. 3B) in HEK293T cells and the percentage of apoptotic cells were 1.82%, 2.33% and 14.21% in the blank group, control group and experimental group, respectively (Fig. 3C).

3.4. RNAi-mediated silencing of HLTNFR-16

To verify the effects of HLTNFR-16 on apoptosis, the RNAi-mediated silencing of HLTNFR-16 was carried out. The results showed that siRNA-2 had the highest RNAi efficiency (Fig. 4A). After injection of siRNA-2 for 24 h, the expression of the *HLTNFR*-16 mRNA in the sea cucumber coelomocytes was dropped to 11.19% of the blank control. Thus, the coelomocytes from the sea cucumber injected with siRNA-2 were used in the subsequent experiment for apoptosis detection.

3.5. Effects of HLTNFR-16 silencing on the apoptosis

When the sea cucumber coelomocytes were not challenged with inactivated *V. harveyi*, the apoptosis rates in the three groups including the blank control group, negative control group and siRNA-2 injection group were not significantly different (Fig. 4B and C). The apoptosis rate in the blank control group, negative control group and siRNA-2 injection group was 3.29%, 3.79% and 2.58% (Fig. 4B and C), respectively. When the sea cucumber coelomocytes were challenged with inactivated *V. harveyi* for 24 h, a large number of fragmented DNAs were observed in the blank and negative control groups, but not the siRNA-2 injection group (Fig. 4B). The percentages of apoptosis were 10.29%, 9.30% and 3.50% in the blank control group, negative control group and siRNA-2 injection group, respectively (Fig. 4C). Treatment of inactivated *V. harveyi* could not induce a significant increase of apoptosis in coelomocytes when HLTNFR-16 was silenced by siRNA-2 (Fig. 4B and C), indicating that HLTNFR-16 is important for apoptosis induction.

3.6. Activation of NF-κB and JNK signal pathways mediated by HLTNFR-16

To assess the roles of HLTNFR-16 in NF-κB and JNK signaling pathways, luciferase reporter gene assay was performed. Compared to the control group that transfected with blank pcDNA3 vector, the expression of HLTNFR-16 could significantly increase the activities of NF-κB ($P < 0.001$) and AP-1 ($P < 0.001$) luciferase reporters in HEK293T cells, and the luciferase activity of NF-κB and AP-1 in transfected HEK293T cells were 2.11- and 2.84-fold up-regulated compared to the respective control groups (Fig. 5A).

3.7. Induction of proinflammatory cytokines by HLTNFR-16

To investigate the effects of HLTNFR-16 on secretion of proinflammatory cytokines, pcDNA3/myc/HLTNFR-16 and pcDNA3/myc were transfected into HEK293T cells, respectively. As shown in Fig. 5B, HLTNFR-16 could induce the secretion of IL-1β, IL-6 and IL-18. After 24 h and 48 h post-transfection, the concentrations of IL-1β were 2.54-fold ($P < 0.001$) and 2.00-fold ($P < 0.001$) higher than the control groups, the concentrations of IL-6 were 3.50-fold ($P < 0.001$) and 3.01-fold ($P < 0.001$) higher than the control groups, and the concentration of IL-18 were 3.54-fold ($P < 0.001$) and 2.67-fold ($P < 0.001$) higher than the control groups, respectively.

4. Discussion

Compared to higher vertebrates, studies on the TNFR superfamily members in invertebrates are still limited. The sequence identification and functional characterization of TNFR in echinoderm, the highest group of invertebrates, would hopefully provide new insights into the

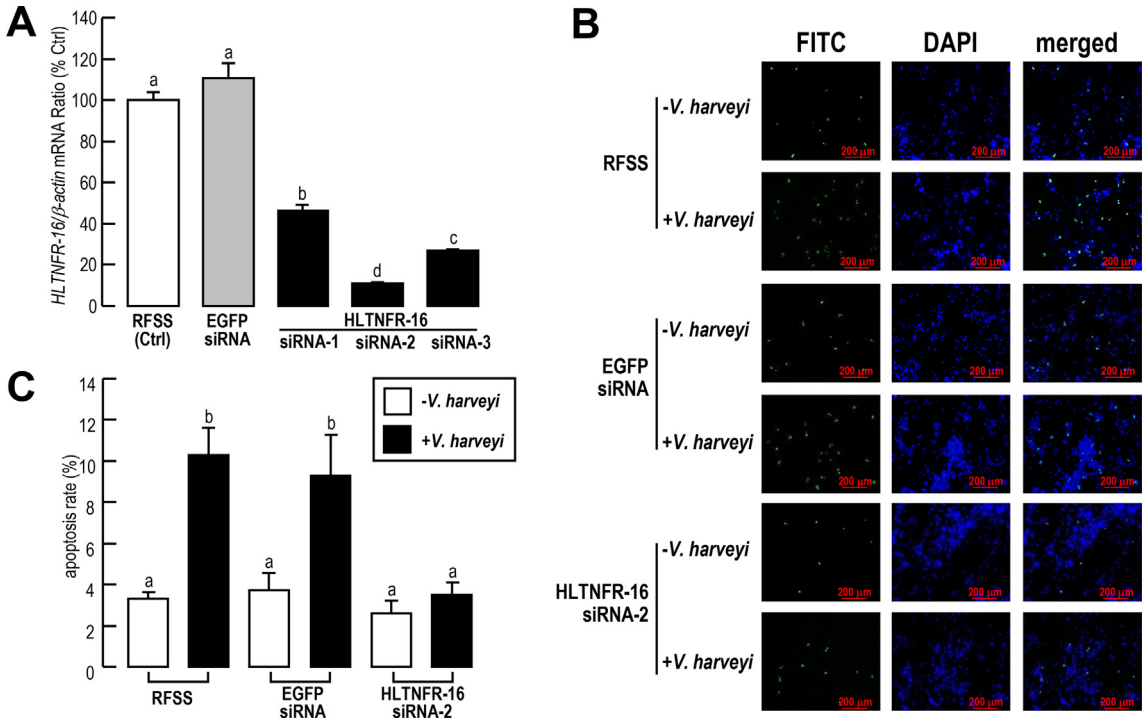

Fig. 4. Effects of HLTNFR-16 silencing on apoptosis. A: Expression levels of *HLTNFR*-16 mRNA in sea cucumber coelomocytes after injection of RFSS, EGFP siRNA and HLTNFR-16 siRNA-1, siRNA-2 and siRNA-3 for 24 h. B: Detection of apoptosis of coelomocytes from the sea cucumbers injected with RFSS, EGFP siRNA and HLTNFR-16 siRNA-2 by TUNEL assay. C: Comparison of apoptosis rates of sea cucumber coelomocytes in different groups. The values are expressed as mean ± standard error (n = 3). The same letter represents a similar expression level ($p > 0.05$), and the different letter represents significant difference in expression levels between two groups ($p < 0.05$).

evolutionary origin of TNFRs as well as their functional roles in vertebrates. In the current study, an echinoderm TNFR was first identified from the tropical sea cucumber *H. leucospilota*. In this case, the 3 conserved CRDs (the hallmark of TNFR family) and a DD are presented in the extracellular and intracellular portions of HLTNFR-16, respectively. The CRDs have deep evolutionary origins, which have been reported within several primitive groups, such as cnidarians, choanoflagellates, sponges and ctenophores [47]. Despite low sequence similarity, all members from the TNFR family possess at least one CRD consisting of 6 highly conserved cysteines that forming three disulfide bridges, which is necessary for binding of the ligands [48–50].

Phylogenetic analysis showed that HLTNFR-16 formed a clade with TNFR-16s from other species, indicating that our newly cloned HLTNFR-16 may be a member of the TNFR-16 subfamily. Like TNFR-16 and other death receptors (such as TNFR1, DR3, DR4 and DR5) in human [6], HLTNFR-16 protein contains a DD at its C-terminus. The DD within the death receptors in human is required for the induction of apoptosis [3,48]. Previous studies have showed that the overexpression of human TNFR-l, DR3, DR4 or DR5 could lead to apoptosis [1,28,30]. Our current study showed that overexpression of HLTNFR-16 could also induce apoptosis in HEK293T cells. When HLTNFR-16 was silenced by siRNA, the apoptosis of sea cucumber coelomocytes induced by inactivated *V. harveyi* was suppressed significantly, indicating that HLTNFR-16 is important for apoptosis induction. To our knowledge,

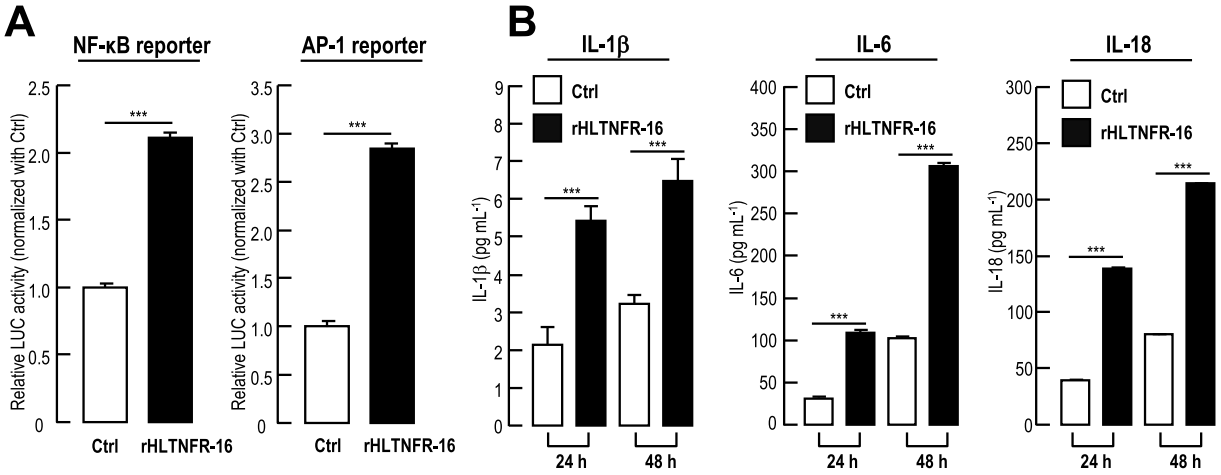

Fig. 5. Luciferase reporter gene and ELISA assays. A: Effects of over-expressed HLTNFR-16 on the activation of NF-κB and AP-1. B: Induction of proinflammatory cytokines (IL-1β, IL-6 and IL-18) secretion by over-expressed HLTNFR-16. The data for experiments are expressed as mean ± standard error (n = 3). Significant difference was shown as ***$P < 0.001$.

this is the first report on the roles of invertebrate TNFR in apoptosis. In addition, overexpression of other sea cucumber intracellular proteins with DD, such as myeloid differentiation factor 88 (MyD88) [44] and fas-associated death domain (FADD) [51], have been reported to induce apoptosis, providing evidences for the roles of DD contained protein in mediation of apoptosis in echinoderms.

Invertebrates lack acquired immunity, and innate immunity is the primary defense line of invertebrates against invading pathogens [25]. In the innate immune system, NF-κB and JNK signaling are two important pathways which could induce the expression of inflammatory cytokines [52–55]. Previous studies showed that the death receptor TNFR-1 in human could activate both NF-κB and JNK signal pathways [5] and the other death receptors in human such as DR3, DR4 and DR5 have also been demonstrated to activate NF-κB signal pathway [1,30]. In human, the activation of NF-κB and JNK signal pathways by death receptors is caused by a ligand-receptor complex with three additional mediators, including TRADD, TRAF2 and RIP [5]. In invertebrates, the TNFRs from arthropods (*D. melanogaster* and *L. vannamei*) and molluscs (*C. farreri* and *C. hongkongensis*) have been speculated to be involved in the immune defense against invasions of pathogens [36–39]. However, the studies on the activation of signal pathways by TNFRs have only been reported in the oyster *C. hongkongensis* and the results showed that TNFRs from this species could active NF-κB signal pathway [39]. In order to make clear the potential function of HLTNFR-16 in the NF-κB and JNK signal pathways, luciferase reporter gene assay was performed. Our present results showed that HLTNFR-16 could activate NF-κB signaling pathway. In mammals, IL-1β and IL-6 are important inflammatory cytokines and the expression of these cytokines is mainly controlled by the NF-κB signaling pathway [54,55]. Our study further showed that the overexpressed HLTNFR-16 could induce both IL-1β and IL-6 in *in vitro* experiments, which further confirmed that HLTNFR-16 could activate NF-κB signaling pathway to induce the secretion of downstream proinflammatory cytokines. In addition, our results showed that HLTNFR-16 could significantly active the AP-1 luciferase reporter, thereby providing the first evidence for the activation of AP-1 by an invertebrate TNFR. Previous studies revealed the involvement of AP-1 in regulating the expression of proinflammatory cytokine IL-18, and the critical role of JNK signaling pathway in mediating the production of IL-18 [56,57]. Our current study showed that IL-18 could be induced by overexpressed HLTNFR-16, which further supported that HLTNFR-16 could activate JNK signaling pathway.

In conclusion, an echinoderm TNFR which belongs to TNFR-16 subfamily was cloned from the tropical sea cucumber *H. leucospilota* and functionally characterized in this study. The functional domains of the deduced HLTNFR-16 protein were identified. Similar to human death receptors, the overexpressed HLTNFR-16 could activate NF-κB and JNK signaling pathways and induce the secretion of downstream proinflammatory cytokines, indicating that the sea cucumber TNFR plays an important role in the innate immune responses. Moreover, we further confirmed that HLTNFR-16 is important for apoptosis induction. As a whole, our study may give new insights on the concrete roles of echinoderm TNFR in induction of apoptosis, and in activation of NF-κB and JNK signal pathways.

Acknowledgments

This study was supported by the National Key R & D 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), the Science and Technology Research Programs of Guangdong Province (2016A020210136, 2017B030314052, 2018A030313857), the Scientific Research Projects of City-affiliated Universities in Guangzhou (1201421216) and the Institution of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences (No. ISEE2018PY03).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.008.

References

[1] A.M. Chinnaiyan, K. O'Rourke, G.L. Yu, R.H. Lyons, M. Garg, D.R. Duan, et al., Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95, Science 274 (1996) 990–992.
[2] G.D. Wiens, G.W. Glenney, Origin and evolution of TNF and TNF receptor superfamilies, Dev. Comp. Immunol. 35 (2011) 1324–1335.
[3] L.A. Tartaglia, D.V. Goeddel, Two TNF receptors, Immunol. Today 13 (1992) 151–153.
[4] R. Beyaert, W. Fiers, Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we do understand and what we do not, FEBS Lett. 340 (1994) 9–16.
[5] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (1998) 1305–1308.
[6] L.A. Gravestein, J. Borst, Tumor necrosis factor receptor family members in the immune system, Semin. Immunol. 10 (1998) 423–434.
[7] S. Nagata, Apoptosis by death factor, Cell 88 (1997) 355–365.
[8] B.G. Darnay, B.B. Aggarwal, Early events in TNF signaling: a story of associations and dissociations, J. Leukoc. Biol. 61 (1997) 559–566.
[9] J. Han, C.Q. Zhong, D.W. Zhang, Programmed necrosis: backup to and competitor with apoptosis in the immune system, Nat. Immunol. 12 (2011) 1143–1149.
[10] L. Cabal-Hierro, P.S. Lazo, Signal transduction by tumor necrosis factor receptors, Cell. Signal. 24 (2012) 1297–1305.
[11] M. Pasparakis, P. Vandenabeele, Necroptosis and its role in inflammation, Nature 517 (2015) 311–320.
[12] O. Micheau, J. Tschopp, Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes, Cell 114 (2003) 181–190.
[13] M. Rothe, M.G. Pan, W.J. Henzel, T.M. Ayres, D.V. Goeddel, The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins, Cell 83 (1995) 1243–1252.
[14] H. Hsu, H.B. Shu, M.G. Pan, D.V. Goeddel, TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways, Cell 84 (1996) 299–308.
[15] H. Hsu, J. Huang, H.B. Shu, V. Baichwal, D.V. Goeddel, TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex, Immunity 4 (1996) 387–396.
[16] A.M. Chinnaiyan, C.G. Tepper, M.F. Seldin, K. ORourke, F.C. Kischkel, S. Hellbardt, et al., FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis, J. Biol. Chem. 271 (1996) 4961–4965.
[17] H.L. Hsu, J. Xiong, D.V. Goeddel, The TNF Receptor 1-associated protein TRADD signals cell-death and NF-Kappa-B activation, Cell 81 (1995) 495–504.
[18] C.H. Regnier, H.Y. Song, X. Gao, D.V. Goeddel, Z. Cao, M. Rothe, Identification and characterization of an IkappaB kinase, Cell 90 (1997) 373–383.
[19] J.D. Woronicz, X. Gao, Z. Cao, M. Rothe, D.V. Goeddel, IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK, Science 278 (1997) 866–869.
[20] E. Zandi, D.M. Rothwarf, M. Delhase, M. Hayakawa, M. Karin, The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation, Cell 91 (1997) 243–252.
[21] F. Mercurio, H. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J. Li, et al., IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation, Science 278 (1997) 860–866.
[22] T.W. Mak, W.C. Yeh, Signaling for survival and apoptosis in the immune system, Arthritis Res. 4 (Suppl 3) (2002) S243–S252.
[23] G. Natoli, A. Costanzo, A. Ianni, D.J. Templeton, J.R. Woodgett, C. Balsano, et al., Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway, Science 275 (1997) 200–203.
[24] H.Y. Song, C.H. Regnier, C.J. Kirschning, D.V. Goeddel, M. Rothe, Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2, Proc. Natl. Acad. Sci. U. S. A 94 (1997) 9792–9796.
[25] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.
[26] G. Hajishengallis, M. Wang, S. Liang, Induction of distinct TLR2-mediated proinflammatory and proadhesive signaling pathways in response to *Porphyromonas gingivalis* fimbriae, J. Immunol. 182 (2009) 6690–6696.
[27] J.C. Kagan, R. Medzhitov, Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling, Cell 125 (2006) 943–955.
[28] N.P. Malek, J. Pluempe, S. Kubicka, M.P. Manns, C. Trautwein, Molecular mechanisms of TNF receptor-mediated signaling, Recent Results Cancer Res. 147 (1998) 97–106.
[29] C. Akgul, S.W. Edwards, Regulation of neutrophil apoptosis via death receptors, Cell. Mol. Life Sci. 60 (2003) 2402–2408.
[30] P.M. Chaudhary, M. Eby, A. Jasmin, A. Bookwalter, J. Murray, L. Hood, Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway, Immunity 7 (1997) 821–830.
[31] S. Gupta, Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review), Int. J. Oncol. 22 (2003) 15–20.
[32] K. Kuwano, N. Hara, Signal transduction pathways of apoptosis and inflammation

induced by the tumor necrosis factor receptor family, Am. J. Respir. Cell Mol. Biol. 22 (2000) 147–149.
[33] R.M. Locksley, N. Killeen, M.J. Lenardo, The TNF and TNF receptor superfamilies: integrating mammalian biology, Cell 104 (2001) 487–501.
[34] L.M. Sedger, M.F. McDermott, TNF and TNF-receptors: from mediators of cell death and inflammation to therapeutic giants - past, present and future, Cytokine Growth Factor Rev. 25 (2014) 453–472.
[35] T. Hehlgans, K. Pfeffer, The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games, Immunology 115 (2005) 1–20.
[36] H. Kanda, T. Igaki, H. Kanuka, T. Yagi, M. Miura, Wengen, a member of the Drosophila tumor necrosis factor receptor superfamily, is required for Eiger signaling, J. Biol. Chem. 277 (2002) 28372–28375.
[37] P.H. Wang, D.H. Wan, L.R. Pang, Z.H. Gu, W. Qiu, S.P. Weng, et al., Molecular cloning, characterization and expression analysis of the tumor necrosis factor (TNF) superfamily gene, TNF receptor superfamily gene and lipopolysaccharide-induced TNF-alpha factor (LITAF) gene from *Litopenaeus vannamei*, Dev. Comp. Immunol. 36 (2012) 39–50.
[38] L. Li, L. Qiu, L. Song, X. Song, J. Zhao, L. Wang, et al., First molluscan TNFR homologue in Zhikong scallop: molecular characterization and expression analysis, Fish Shellfish Immunol. 27 (2009) 625–632.
[39] Z.M. Xiang, S. Xiao, F.X. Wang, Y.P. Qin, J. Wu, H.T. Ma, et al., Cloning, characterization and comparative analysis of four death receptorTNFRs from the oyster *Crassostrea hongkongensis*, Fish Shellfish Immunol. 59 (2016) 288–297.
[40] W. Huang, D. Huo, Z.H. Yu, C.H. Ren, X. Jiang, P. Luo, et al., Spawning, larval development and juvenile growth of the tropical sea cucumber *Holothuria leucospilota*, Aquaculture 488 (2018) 22–29.
[41] C.H. Ren, T. Chen, H.Y. Sun, X. Jiang, C.Q. Hu, J. Qian, et al., The first echinoderm poly-U-binding factor 60 kDa (PUF60) from sea cucumber (*Stichopus monotuberculatus*): molecular characterization, inducible expression and involvement of apoptosis, Fish Shellfish Immunol. 47 (2015) 196–204.
[42] A. Yan, C. Ren, T. Chen, D. Huo, X. Jiang, H. Sun, et al., A novel caspase-6 from sea cucumber *Holothuria leucospilota*: molecular characterization, expression analysis and apoptosis detection, Fish Shellfish Immunol. 80 (2018) 232–240.
[43] A. Yan, C. Ren, T. Chen, X. Jiang, H. Sun, D. Huo, et al., The first tropical sea cucumber caspase-8 from *Holothuria leucospilota*: molecular characterization, involvement of apoptosis and inducible expression by immune challenge, Fish Shellfish Immunol. 72 (2018) 124–131.
[44] L. Zhao, X. Jiang, T. Chen, H. Sun, C. Ren, Molecular characterization and functional analysis of MyD88 from the tropical sea cucumber, *Holothuria leucospilota*, Fish Shellfish Immunol. 83 (2018) 1–7.
[45] F. Ramirez-Gomez, F. Aponte-Rivera, L. Mendez-Castaner, J.E. Garcia-Arraras, Changes in holothurian coelomocyte populations following immune stimulation with different molecular patterns, Fish Shellfish Immunol. 29 (2010) 175–185.
[46] C. Ren, T. Chen, X. Jiang, Y. Wang, C. Hu, Identification and functional characterization of a novel ferritin subunit from the tropical sea cucumber, *Stichopus monotuberculatus*, Fish Shellfish Immunol. 38 (2014) 265–274.
[47] S.D. Quistad, N. Traylor-Knowles, Precambrian origins of the TNFR superfamily, Cell Death Dis. 2 (2016) 16058.
[48] C.A. Smith, T. Farrah, R.G. Goodwin, The TNF receptor superfamily of cellular and viral-proteins - activation, costimulation, and death, Cell 76 (1994) 959–962.
[49] D.W. Banner, A. Darcy, W. Janes, R. Gentz, H.J. Schoenfeld, C. Broger, et al., Crystal-structure of the soluble Human 55 KD TNF receptor-Human TNF-beta complex - implications for TNF receptor activation, Cell 73 (1993) 431–445.
[50] N. Itoh, S. Nagata, A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen, J. Biol. Chem. 268 (1993) 10932–10937.
[51] L. Zhao, C. Ren, T. Chen, H. Sun, X. Wu, X. Jiang, et al., The first cloned sea cucumber FADD from *Holothuria leucospilota*: molecular characterization, inducible expression and involvement of apoptosis, Fish Shellfish Immunol. 89 (2019) 548–554.
[52] S. Janssens, R. Beyaert, A universal role for MyD88 in TLR/IL-1R-mediated signaling, Trends Biochem. Sci. 27 (2002) 474–482.
[53] R. Medzhitov, P. Preston-Hurlburt, E. Kopp, A. Stadlen, C.Q. Chen, S. Ghosh, et al., MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways, Mol. Cell 2 (1998) 253–258.
[54] H. Kumar, T. Kawai, S. Akira, Toll-like receptors and innate immunity, Biochem. Biophys. Res. Commun. 388 (2009) 621–625.
[55] K.B. Narayanan, H.H. Park, Toll/interleukin-1 receptor (TIR) domain-mediated cellular signaling pathways, Apoptosis 20 (2) (2015) 196–209.
[56] Y. Wang, C. Li, X. Wang, J. Zhang, Z. Chang, Heat shock response inhibits IL-18 expression through the JNK pathway in murine peritoneal macrophages, Biochem. Biophys. Res. Commun. 296 (2002) 742–748.
[57] Y.M. Kim, J.Y. Im, S.H. Han, H.S. Kang, I. Choi, IFN-gamma up-regulates IL-18 gene expression via IFN consensus sequence-binding protein and activator protein-1 elements in macrophages, J. Immunol. 165 (2000) 3198–3205.