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RESEARCH REPORT

Molecular characterization of a defender against apoptotic cell death 1 gene (*Cf*DAD1) from the mollusk *Chlamys farreri*

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Abstract

The defender against apoptotic cell death 1 (DAD1) was a negative regulator to inhibit the apoptosis process. In the present study, a DAD1 gene (designated as *Cf*DAD1) was identified in Zhikong scallop *Chlamys farreri*. Its full-length cDNA sequence of *Cf*DAD1 contained a 342 bp open reading frame (ORF), which encoded a mature protein of 113 amino acids. A DAD domain was revealed from the deduced protein sequence of *Cf*DAD1. The mRNA transcripts of *Cf*DAD1 could be detectable in all the investigated tissues, including hemocytes, muscle, mantle, gill, hepatopancreas and gonad, among which the maximum expression level were found in hemocytes. Being infected with *Vibrio splendidus*, *Staphylococcus aureus* or *Yarrowia lipolytica*, the mRNA expression levels of *Cf*DAD1 in hemocytes were all significantly up-regulated. Moreover, the apoptosis level of *Cf*DAD1-suppressed group was significant higher than those of control groups. All these results indicated that *Cf*DAD1 was efficient negative regulator of apoptosis and involved in the innate immune responses of scallop.

Key Words: Apoptosis; *Chlamys farreri*; Innate immunity

Introduction

Apoptosis, also termed as programmed cell death (PCD), was one of the most fundamental processes essential for normal growth and regular development in multicellular organisms (Nagata, 2018). The main characteristic morphological features of apoptosis included cellular shrinkage, chromatin condensation, chromosomal DNA fragmentation, global mRNA decay, membrane blebbing and nuclear condensation (Banfalvi, 2017). Apoptosis was considered as a pivotal component of various biological processes, such as chemical induced cell death, embryonic development, hormone dependent atrophy, normal cell turnover, proper functioning of the immune defense system, and so on (Lockshin, 2016). Several factors required for execution or regulation of apoptosis were rather conserved in evolution, including caspases (Galluzzi *et al*., 2016), inhibitor of apoptosis proteins (IAPs) (Kocab and Duckett, 2016), and also defender against apoptotic cell death 1 gene (DAD1) (Zhang *et al*., 2016). However, the activation of apoptotic pathways and the interaction between the key genes differ between the main taxonomic groups of invertebrates, and it is important to investigate the diversity of the apoptotic pathways found in the animal kingdom (Estévez-Calvar *et al*., 2013).

DAD1, a regulatory protein to inhibit the apoptosis process, was first described and characterized as a negative regulator of mammalian cell death that may act downstream of the B-cell lymphoma 2 (BCL2) protein (Nakashima *et al*., 1993). The function of DAD1 has been well investigated in multicellular model organisms, for examples, mutants in mouse DAD1 exhibited aberrant morphology, developmental delay and increased apoptosis during embryogenesis (Nishii *et al*., 1999; Hong *et al*., 2000), while over expression of DAD1 inhibited developmental apoptosis during

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embryogenesis in *Caenorhabditis elegans* (Zhang *et al*., 2016). These research achievements indicated that DAD1 would play irreplaceable roles in regulating apoptosis and cell viability. Compared with the extensive identification and investigation of DAD1 in vertebrates, although DAD1 has already been identified and primary characterized in the bay scallop *Argopecten irradians* (Zhu *et al*., 2008), black tiger shrimp *Penaeus monodon* (Molthathong *et al*., 2008) and kuruma shrimp *Marsupenaeus japonicas* (Zheng *et al*., 2016), the information on DAD1 in marine invertebrate is still rare and fragmentary.

Zhikong scallop *Chlamys farreri* (Mollusca; Bivalvia; Pteriomorphia) is an important and representative species of mollusk, not only for its economic and ecological importance, but also for its increasing value in the invertebrate innate immunity investigation (Matozzo, 2016; Tascedda and Ottaviani, 2016; Gerdol, 2017). The research on DAD1 in *C. farreri* would enhance the understanding of its potential functions in apoptosis process and invertebrate innate immunity. However, no information on DAD1 was available in Zhikong scallop yet. To bridge this gap, a novel DAD1 gene (designated as *Cf*DAD1) has been identified and investigated in *C. farreri*, and the main objectives of the present study were (1) to characterize the molecular features of *Cf*DAD1 (2) to validate the spatial and temporal expression pattern of its mRNA transcripts, and (3) to confirm its function via double strand RNA (dsRNA) mediated RNA interference (RNAi).

Material and Method

Scallops, in vivo *experimental infection and samples collection*

Adult scallops (average 50 mm in shell length) were purchased from a local farm in Qingdao, China, and maintained in aerated seawater at about 20 °C. Approximately two hundred scallops were used for *in vivo* experimental infection assay according to our previous report (Wang *et al*., 2017). Animals were infected by bath exposure using different bacteria and yeast. Being acclimated for two weeks, fifty scallops were transferred to tanks containing live bacteria *Vibrio splendidus* strain JZ6 at a final concentration of 1.0×10^8 colony forming units (CFUs) per 1 mL, defined as Gram-negative bacterial infection group. Another fifty scallops were transferred and kept in the tanks containing live bacteria *Staphylococcus aureus* strain 33025 (SanYao, China) at a final concentration of 1.0×10^8 CFUs mL^{-1}, defined as Gram-positive bacterial infection group. The third fifty scallops were maintained in the fungi-containing tanks with live yeast *Yarrowia lipolytica* strain N11b at a final concentration of 1.0×10^8 CFUs mL^{-1}, defined as fungi infection group. And the last fifty scallops were treated as the control group. Five scallops from every group were randomly sampled at 0, 3, 6, 12, 24 and 48 h post stimulation, respectively. The hemolymphs were collected and centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes for RNA isolation. Hemocytes, muscle, mantle, gill, hepatopancreas and gonad from five untreated scallops (one animal was employed as one repeat for one tissue sample) were collected to determine the spatial expression pattern of *Cf*DAD1 mRNA transcripts.

RNA isolation and cDNA synthesis

Raw RNA was isolated using RNAiso Plus (9108, Takara, Japan). The SuperScript IV Reverse Transcriptase (18090010, Thermo Fisher Scientific, USA) was employed to synthesize the first-strand cDNA synthesis using RQ1 DNase I (M6101, Promega, USA) treated raw RNA as template and adaptor primer-oligo (dT) as primer (Table 1). The reaction were carried out at 55 °C for 1 h, terminated by heating at 80 °C for 5 min, then a homopolymeric tail was added with dCTP (4028, Takara, Japan) and terminal deoxynucleotidyl transferase (TdT, 2230, Takara, Japan) and then stored at -80 °C till use.

Cloning the complete cDNA sequence of Cf*DAD1*

In our previous works (Wang *et al*., 2018b), a transcript sequence homologues to previous identified DAD1s was generated via assembling and screening public available transcriptomic data and expression sequence tags (ESTs) in *C. farreri.* And this transcript sequence was selected for further cloning the complete cDNA sequence of *Cf*DAD1. Four gene-specific primers, *Cf*DAD1-RACE-R1/2 and *Cf*DAD1-RACE-F1/2, were designed based on this sequence for 5` and 3` rapid amplification of cDNA ends (RACE) technique, respectively (Table 1). All PCR amplification was carried out in a MJ Mini Personal Thermal Cycler (Bio-Rad, USA), and PCR products were gel-purified using Monarch DNA Gel Extraction Kit (T1020S, NEB, USA), ligated into the pMD18-T simple vector (D103A, Takara, Japan), and then transformed into the competent cells *Escherichia coli* strain DH5α (CB101, Tiangen, China). The positive recombinants were identified via anti-ampicillin selection and verified by PCR using M13-47 and RV-M as primers (Table 1). Five of the positive clones were sequenced using a PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

Bioinformatical analysis of Cf*DAD1 cDNA and deduced amino acid sequences*

The blast+ 2.7.1 was employed to perform search for protein sequence similarity. The deduced protein sequences of *Cf*DAD1 were analyzed by Lasergene suite 7.1.0.44 using the EditSeq module. The presence and location of signal peptide and the function domains were predicted with SignalP 4.1 and Simple Modular Architecture Research Tool (SMART) 7.0, respectively. Multiple sequence alignments were generated using Clustal Omega 1.2.4 combined with Sequence Manipulation Suite 2.0. A Neighbor-Joining (NJ) phylogenic tree was generated using MEGA-X 10.0.1. Bootstrap trials were replicated 1000 times to derive confidence value for the phylogeny analysis.

Quantitative real-time PCR analysis of Cf*DAD1 mRNA expression profiles*

The spatial and temporal mRNA expression profiles of *Cf*DAD1 in hemocytes of scallops infected by various microbes were detected by quantitative real-time PCR (qRT-PCR). All qRT-PCR reactions

Table 1 Oligonucleotide primers used in the experiments

Primers	Sequence (5`-3`)	Brief information
adaptor primer	GGCCACGCGTCGACTAGTAC	Anchor primer for 3' RACE
adaptor primer-oligo (dG)	GGCCACGCGTCGACTAGTACG_{10}HN	Anchor primer for 5' RACE
adaptor primer-oligo (dT)	GGCCACGCGTCGACTAGTACT_{17}VN	Olido (dT) for cDNA synthesizing
*Cf*EF-1α-qRT-F*	ATCCTTCCTCCATCTCGTCCT	Internal control for real-time PCR
*Cf*EF-1α-qRT-R*	GGCACAGTTCCAATACCTCCA	Internal control for real-time PCR
*Cf*DAD1-CDS-F	ATGCCTGATAGTTTATTTTCCGTGGTG	Gene specific primer for CDS
*Cf*DAD1-CDS-R	CTATCCAATGAAGTTGATAACAACTAA	Gene specific primer for CDS
*Cf*DAD1-dsRNA-Basic-F	TTATATTCTGTCATTCACTTC	Gene specific primer
*Cf*DAD1-dsRNA-Basic-R	TGTAAAATAATATGAGCAAAAATAAAG	Gene specific primer
*Cf*DAD1-dsRNA-T7-F	GGATCCTAATACGACTCACTATAGGGATCCTTATATTCTGTCATTCACTTC	Gene specific primer with T7 promoter
*Cf*DAD1-dsRNA-T7-R	GGATCCTAATACGACTCACTATAGGGATCCTGTAAAATAATATGAGCAAAAATAAAG	Gene specific primer with T7 promoter
*Cf*DAD1-qRT-F*	CGGCTACAAGTGAATCCACAG	Gene specific primer for real-time PCR
*Cf*DAD1-qRT-R*	TCCTTCCCATCACCATTTCCA	Gene specific primer for real-time PCR
*Cf*DAD1-RACE-F1	CATTGGATAGTATGTTATGGAAATGGT	Gene specific primer for RACE
*Cf*DAD1-RACE-F2	ATTCTTCAACAGTACAGATGTGTATAT	Gene specific primer for RACE
*Cf*DAD1-RACE-R1	GGATGACGCCTGTCATCAGGATGTAGA	Gene specific primer for RACE
*Cf*DAD1-RACE-R2	TTTTTGCAAAATAAAATCAACGTGGCA	Gene specific primer for RACE
EGFP-dsRNA-Basic-F	CGACGTAAACGGCCACAAGT	GFP primer
EGFP-dsRNA-Basic-R	CTTGTACAGCTCGTCCATGC	GFP primer
EGFP-dsRNA-T7-F	GGATCCTAATACGACTCACTATAGGGATCCGACGTAAACGGCCACAAGT	GFP primer incorporated with T7 promoter
EGFP-dsRNA-T7-R	GGATCCTAATACGACTCACTATAGGGATCCTTGTACAGCTCGTCCATGC	GFP primer incorporated with T7 promoter
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing primer
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing primer

* The efficiency of *Cf*EF-1α-qRT-F/R and *Cf*DAD1-qRT-F/R were 98% and 101%, respectively.

were carried out with the SYBR premix ExTaq (RR420, Takara, Japan) in a LineGene K FQD-48A (A4) Fluorescence Quantitative PCR Detection System (Bioer, China) using 100 ng cDNA as template. All the primer information for qRT-PCR was listed in Table 1. The efficiency of qRT-PCR primers were checked using serial two-fold dilutions of cDNA (Pfaffl, 2001), and the efficiency of *Cf*EF-1α-qRT-F/R and *Cf*DAD1-qRT-F/R were almost the same (98% and 101%, respectively). The mRNA expression of *Cf*DAD1 was normalized to that of elongation factor 1 α (EF-1α) for each sample. The relative mRNA expression levels of *Cf*DAD1 were calculated using comparative C_T method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) as mean ± S.D. (n = 5). The data were tested with one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and the *p* values less than 0.05 were considered statistically significant.

Knock-down of Cf*DAD1 gene* in vivo *via dsRNA mediated RNA interference and apoptotic assay*

T7 promoter adapted primers *Cf*DAD1-dsRNA-T7-F/R and EGFP-dsRNA-T7-F/R (Table 1) were used to amplify cDNA fragments from *Cf*DAD1 and enhanced green fluorescent protein (EGFP), and the PCR products were gel-purified and used as templates to synthesize dsRNA. The dsRNA products were synthesized via *in vitro* transcription according to the our previously reports (Wang *et al*., 2011; Wang *et al*., 2016a,b; Wang *et al*., 2018a), and its concentration was quantified using Nanodrop Lite (Thermo Fisher Scientific, USA) by the absorbance at 260 nm and adjusted to a final concentration of 1 mg mL^{-1}. One hundred micrograms of *Cf*DAD1 dsRNA was injected into the adductor of each scallop, and the control groups received an injection of 100 μg EGFP dsRNA or PBS, while the untreated scallops were employed as blank group. Hemocytes from five randomly sampled scallops of each treatment were collected every 12 h (0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h post dsRNA injection) and used for total RNA isolation and cDNA synthesis. The effect of dsRNA-mediate RNAi for *Cf*DAD1 in scallops was verified via qRT-PCR, and 70% inhibition of mRNA abundance after dsRNA injection was a threshold for an effective RNAi assay (Krueger *et al*., 2007). Five individuals were randomly sampled at 0, 48, 72 and 120 h post dsRNA injection, and the hemolymphs were collected and centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes for apoptotic assay. The apoptosis level of scallops was assayed via caspase-3 colorimetric assay kit (KGA204, KeyGEN BioTECH, China). All data were given in terms of OD_{450} as means ± S.D (n=5). The data were tested with one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (S-N-K) using IBM SPSS Statics 23.0.0.0, and the *p* values less than 0.05 were considered statistically significant.

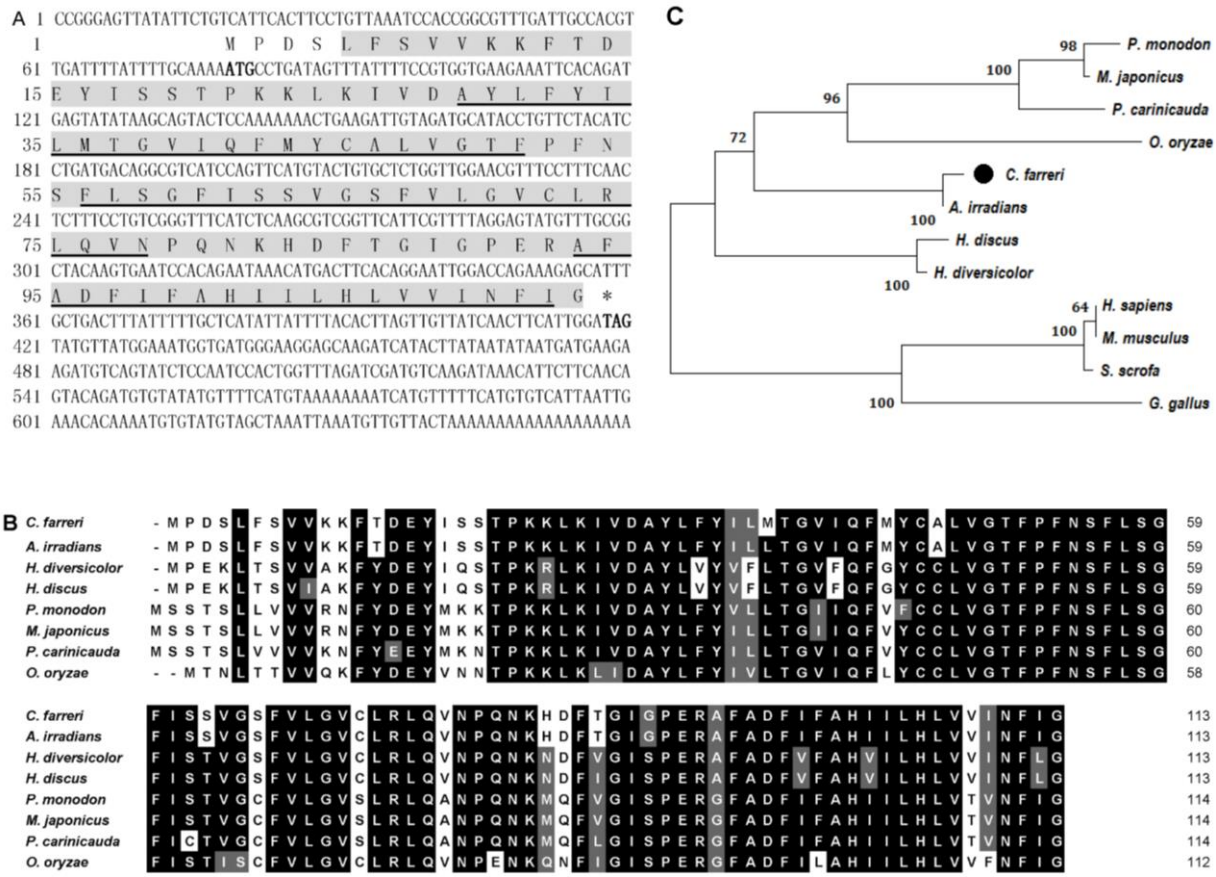

Fig. 1 Sequence features, multiple alignments and phylogenetic analysis of *Cf*DAD1. A: Nucleotide and deduced protein sequences of *Cf*DAD1. The nucleotides and amino acids were numbered along the left margin. The function domain was in shade. The transmembrane segments were underlined. The stop codon was indicated by asterisks. B: Multiple alignments of *Cf*DAD1 with previous known ones. The black shadow region indicated positions of same amino acid residues shared by all sequences. Similar amino acid residues were shaded in grey. Gaps were indicated by dashes to improve the alignment. C: Consensus phylogenetic tree based on the amino acid sequences of different DAD1s. Neighbor-Joining method was selected to infer the evolutionary history. The numbers at the forks stood for the bootstrap value (%). The sequences and their accession numbers are as follows: *Argopecten irradians*, AAX56947; *Gallus gallus*, AAC60276; *Haliotis discus*, ANF99571; *Haliotis diversicolor*, AGA92566; *Homo sapiens*, AAB58540; *Marsupenaeus japonicas*, AON76443; *Mus musculus*, CAA73779; *Orseolia oryzae*, ALH18096; *Palaemon carinicauda*, AGJ03553; *Penaeus monodon*, ABU54835; *Sus scrofa*, BAA13115

Results

The molecular feathers and phylogeny relationship of CfDAD1

The complete cDNA sequence of *Cf*DAD1 was obtained via 5` and 3` RACE, and then submitted to GenBank with the accession number KU361830. It comprised 660 bp, containing a 78 bp 5` untranslated regions (UTR), a 240 bp 3` UTR with a poly A tail and a 342 bp open reading frame (ORF). This ORF encoded a mature protein of 113 amino acid residues with a theoretical molecular mass at about 12.66 kDa and a calculated isoelectric point (pI) of 7.99. No signal peptide could be predicted in the deduced protein sequence. The deduced amino acid sequence of *Cf*DAD1 contained a DAD domain (from L^5 to G^{113}). Additionally, three transmembrane segments (from A^{30} to F^{51}, from F^{56} to N^{78}, and from A^{93} to I^{112}) were also revealed (Figure 1A). An alignment of the protein sequence of *Cf*DAD1 with those previously identified ones was shown in Figure 1B. The deduced amino acid sequence of *Cf*DAD1 exhibited high similarity with previously identified ones, such as 99% identity with that of *A. irradians* (AAX56947), 94% with *Mizuhopecten yessoensis* (OWF34780), and 81% with *Haliotis diversicolor* (AGA92566). A NJ phylogenetic tree based on protein sequences from multiple DAD1s was positioned separately into two main branches, sequences form invertebrates and vertebrates were separated. Within the invertebrates, *Cf*DAD1 was closer to similar sequences from another mollusc such as bay scallop *A. irradians* and far from those from crustaceans (Figure 1C).

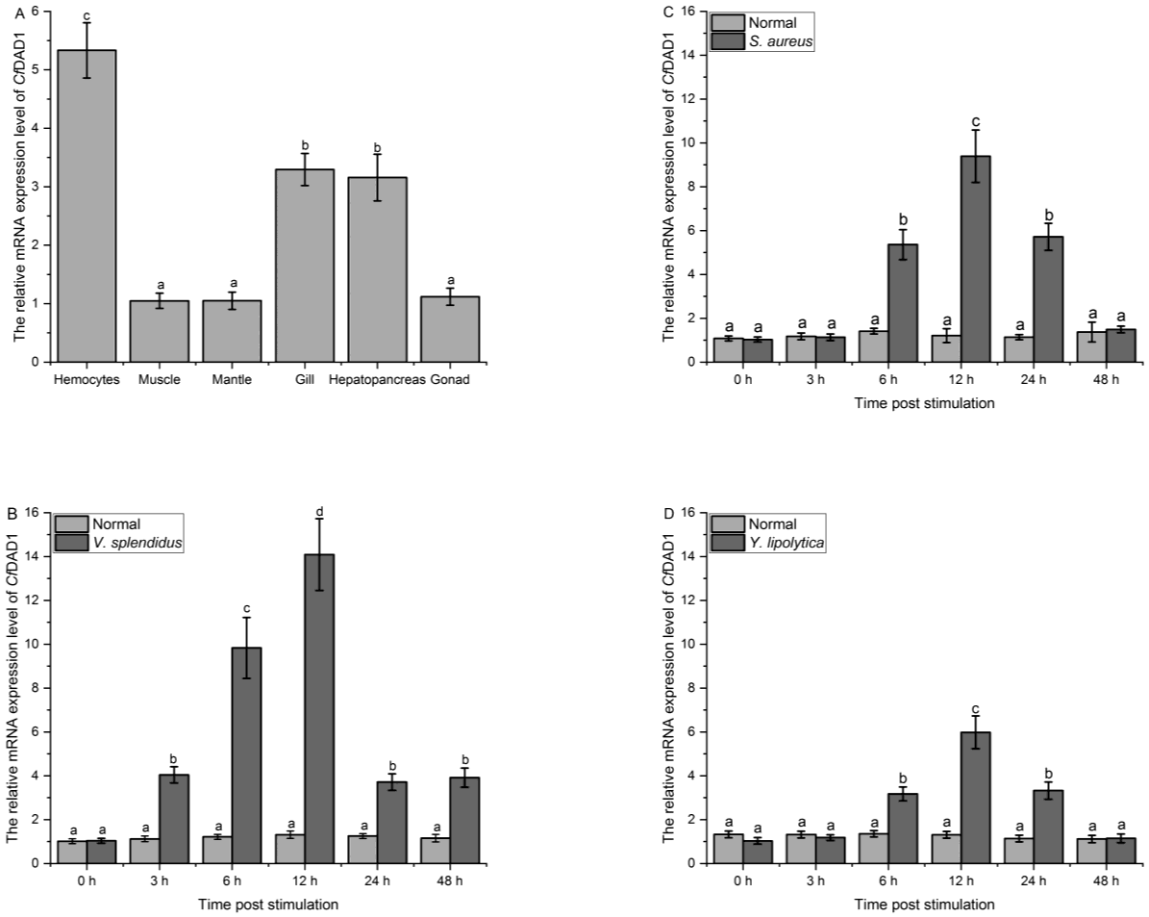

Fig. 2 Spatial and temporal mRNA expression patterns of *Cf*DAD1. A: Spatial mRNA expression pattern of *Cf*DAD1. The mRNA expression level in hemocytes, mantle, gill, hepatopancreas and gonad of five adult scallops was normalized to that of muscle. B-D: Temporal mRNA expression patterns of *Cf*DAD1 in hemocytes at 0, 3, 6, 12, 24 and 48 h post microbe infection (B: *V. splendidus*, C: *S. aureus*, D: *Y. lipolytica*). Vertical bars represented mean ± S.D. (n = 5), and bars with different characters were statically significant ($p < 0.05$)

The spatial and temporal expression profile of Cf*DAD1 mRNA transcripts*

The qRT-PCR was employed to detect the spatial and temporal mRNA expression profiles of *Cf*DAD1 with EF-1α as internal control. The *Cf*DAD1 mRNA was detectable in all the tested tissues. The maximum level was observed in hemocytes, which was 5.33-fold ($p < 0.05$) of that in muscle, followed by gill and hepatopancreas, which were 3.29-fold and 3.16-fold of that in muscle ($p < 0.05$), respectively. And the mRNA expression levels in mantle and gonads were 1.05-fold and 1.12-fold of that in muscle ($p > 0.05$), respectively (Figure 2A). The mRNA expression levels of *Cf*DAD1 were all up-regulated post different *in vivo* experimental infection. The mRNA expression level of *Cf*DAD1 significantly up-regulated at 3 h post *V. splendidus* infection (4.04-fold compared with the origin level, $p < 0.05$), and reached to the peak level at 12 h (14.08-fold, $p < 0.05$, Figure 2B). In the *S. aureus* infection group, its mRNA expression level was significantly up-regulated at 6 h post infection (5.36-fold, $p < 0.05$), reached the peak level at 12 h post infection (9.38-fold, $p < 0.05$), maintained at a high level at 24 h (5.71-fold, $p < 0.05$) and then decreased to the origin level at 48 h (Figure 2C). Being infected by *Y. lipolytica*, its mRNA transcripts were significantly induced at 6 h post infection (3.17-fold, $p < 0.05$) and reached the maximum level at 12 h post infection (5.98-fold, $p < 0.05$), maintained at a high level at 24 h (3.32-fold, $p < 0.05$) and then decreased to the origin level at 48 h (Figure 2D).

The apoptotic levels in Cf*DAD1-suppressed scallops*

In the present study, the effect of dsRNA-mediate RNAi was verified via qRT-PCR, and the mRNA expression level of *Cf*DAD began to decrease at 24 h post dsRNA injection (0.66-fold, $p < 0.05$) and kept at a rather low level (less than 0.3-fold of the normal expression level) during 36 to 84 h post RNAi (Figure 3A). After dsRNA injection, the apoptosis level of *Cf*DAD1-suppressed scallops lagged behind the expression change of CfDAD1 and was obviously higher than those of other groups at 48, 72 and 120 h post injection (Figure 3B).

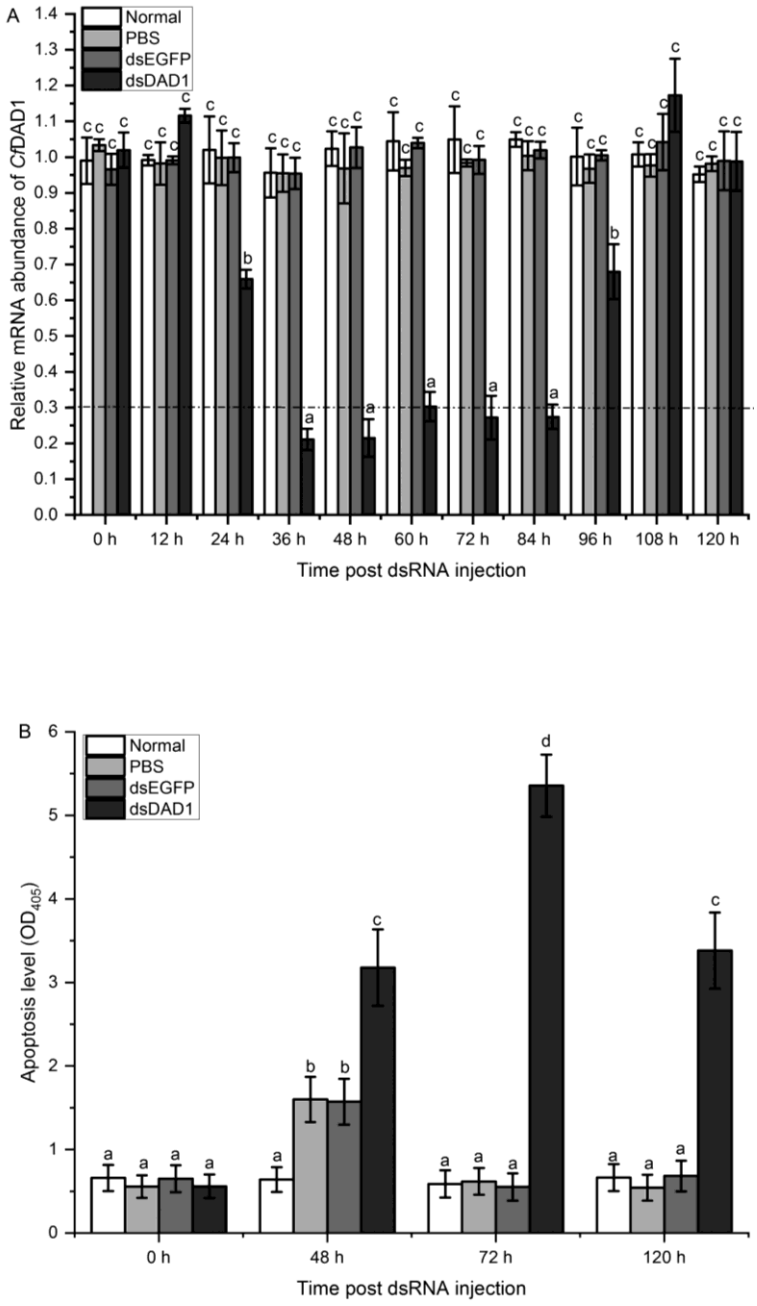

Fig. 3 RNAi of *Cf*DAD1 and apoptosis level. A: The relative abundance of *Cf*DAD1 mRNA during RNAi. Each values was shown as mean ± S.D. (n = 5), and bars with different characters indicated statically significant ($p < 0.05$). B: Gene silencing of *Cf*DAD1 increased apoptosis level. Each values was presented as OD_{405} and shown as mean ± S.D. (n = 5), and bars with different characters indicated statically significant ($p < 0.05$)

Discussion

DAD1 was a negative regulatory protein to inhibit the apoptosis process (Kelleher and Gilmore, 1997; Hong *et al*., 2000; Lockshin, 2016; Nagata, 2018). In the present study, the complete cDNA sequence of *Cf*DAD1 was identified and characterized in *C. farreri*. Its sequence feathers, high similarity and close phylogenetic relationship to previously identified ones collectively suggested that it was a novel member of invertebrate DAD1 family and may have similar functions.

DAD1 it has been observed to be ubiquitously expressed in various tissues in transcriptional level in marine invertebrates (Molthathong *et al*., 2008; Zhu *et al*., 2008; Zheng *et al*., 2016), and in the present study, the *Cf*DAD1 mRNA could be also detected in all the tested tissues. The highest mRNA expression level was found in hemocytes, followed with gill and hepatopancreas, and the variable spatial distribution of *Cf*DAD1 mRNA transcripts was speculated to be related with tissue function. The low apoptotic activity in hemocytes compared to other tissues has been reported in other bivalves

such as the *Mytilus galloprovincialis* (Romero *et al*., 2011), which was consist with our observation, for the function of *Cf*DAD1, the higher gene expression the lower apoptotic activity. Moreover, the hepatopancreas was believed to be the central immune related organ in crustaceans and mollusks (Chai *et al*., 2010), while gill was regarded as the first defense line against invading microbes in fish and invertebrates (Ellis, 2001; Wang *et al*., 2016c). The high mRNA expression level of *Cf*DAD1 in these three tissue indicated that DAD1 might be involved in the innate immune responses of scallop. It has been reported that DAD1 could respond to various stimuli in transcriptional level. For examples, *Mj*DAD1 showed both dose-dependent and time-dependent responses to nitrite stress in *M. japonicas* (Zheng *et al*., 2016), while the mRNA expression levels of *Ai*DAD1 gene of hemolymph were significantly high after injury in *A. irradians* (Zhu *et al*., 2008). In the present study, *Cf*DAD1 mRNA transcripts in hemocytes could be significantly induced by the stimulation of three typical microbes. One potential speculation would be that the increase of *Cf*DAD1 could reduce the apoptotic activity in hemocytes, for the low apoptotic level could favor the phagocytic activity of hemocytes to engulf invading pathogens as more as possible before its dead (Canesi *et al*., 2002). So, the rapid responses of *Cf*DAD1 to various invading pathogens indicated that apoptosis and its regulators could play pivotal role in the innate immune response of scallops.

The response of DAD1s to inhibit apoptosis has been well studied in an array of model organisms. For examples, mutants of DAD1 could increase cell apoptosis during embryogenesis in mouse (Hong *et al*., 2000), while the over expression of DAD1 would inhibit developmental apoptosis in *C. elegans* (Sugimoto *et al*., 1995). For neither gene mutation technique nor gene overexpression method was available in marine mollusk yet, dsRNA-mediated RNAi technique was applied to inhibit the expression of *Cf*DAD1 to investigate its potential effects. In our previous reports, this dsRNA-mediated RNAi technique has been successfully applied to inhibit the expression of several target genes in scallop. In the present study, this protocol was employed as a powerful tool to analyse more in detail the apoptosis. After dsRNA injection, the apoptosis level of *Cf*DAD1-suppressed scallops was obviously higher than those of other groups, suggesting the negative regulating roles of *Cf*DAD1 during apoptosis in scallop.

In conclusion, the complete cDNA sequence of a novel DAD1 gene was identified and characterized in *C. farreri*. Its mRNA expression levels significantly increased post microbe infection. The *Cf*DAD1-suppressed scallops exhibited higher apoptosis level. All these results indicated that *Cf*DAD1 was efficient negative regulator of apoptosis and involved in the innate immunity.

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Reference

Banfalvi G. Methods to detect apoptotic cell death. Apoptosis 22: 306-323, 2017.

Canesi L, Gallo G, Gavioli M, Pruzzo C. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Techniq. 57: 469-476, 2002.

Chai YM, Yu SS, Zhao XF, Zhu Q, Wang JX. Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to white spot syndrome virus. Fish Shellfish Immun. 29: 480-486, 2010.

Ellis A. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immun. 25: 827-839, 2001.

Estévez-Calvar N, Romero A, Figueras A, Novoa B. Genes of the Mitochondrial Apoptotic Pathway in *Mytilus galloprovincialis*. PLoS ONE 8: e61502, 2013.

Galluzzi L, López-Soto A, Kumar S, Kroemer G. Caspases connect cell-death signaling to organismal homeostasis. Immunity 44: 221-231, 2016.

Gerdol M. Immune-related genes in gastropods and bivalves: A comparative overview. ISJ-Invert. Surviv. J. 14: 95-111, 2017.

Hong N, Flannery M, Hsieh S, Cado D, Pedersen R, Winoto A. Mice lacking Dad1, the defender against apoptotic death-1, express abnormal N-linked glycoproteins and undergo increased embryonic apoptosis. Dev. Biol. 220: 76-84, 2000.

Kelleher DJ, Gilmore R. DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. P. Natl. Acad. Sci. USA. 94: 4994-4999, 1997.

Kocab AJ, Duckett CS. Inhibitor of apoptosis proteins as intracellular signaling intermediates. FEBS J. 283: 221-231, 2016.

Krueger U, Bergauer T, Kaufmann B, Wolter I, Pilk S, Heider-Fabian M, *et al*. Insights into effective RNAi gained from large-scale siRNA validation screening. Oligonucleotides 17: 237-250, 2007.

Lockshin R. Programmed cell death 50 (and beyond). Cell Death Differ. 23:10, 2016.

Matozzo V. Aspects of eco-immunology in molluscs. ISJ-Invert. Surviv. J. 13: 116-121, 2016.

Molthathong S, Senapin S, Klinbunga S, Puanglarp N, Rojtinnakorn J, Flegel TW. Down-regulation

of defender against apoptotic death (DAD1) after yellow head virus (YHV) challenge in black tiger shrimp *Penaeus monodon*. Fish Shellfish Immun. 24: 173-179, 2008.

Nagata S. Apoptosis and clearance of apoptotic cells. Annu. Rev. Immunol. 36: 489-517, 2018.

Nakashima T, Sekiguchi T, Kuraoka A, Fukushima K, Shibata Y, Komiyama S, *et al*. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell. Biol. 13: 6367-6374, 1993.

Nishii K, Tsuzuki T, Kumai M, Takeda N, Koga H, Aizawa S, *et al*. Abnormalities of developmental cell death in Dad1-deficient mice. Genes Cells 4: 243-252, 1999.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: e45, 2001.

Romero A, Estévez-Calvar N, Dios S, Figueras A, Novoa B. New Insights into the Apoptotic Process in Mollusks: Characterization of Caspase Genes in *Mytilus galloprovincialis*. PLoS ONE 6: e17003, 2011.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. 3: 1101-1108, 2008.

Sugimoto A, Hozak RR, Nakashima T, Nishimoto T, Rothman J. dad-1, an endogenous programmed cell death suppressor in *Caenorhabditis elegans* and vertebrates. EMBO J. 14: 4434-4441, 1995.

Tascedda F, Ottaviani E. Biologically active peptides in molluscs. ISJ-Invert. Surv. J. 13: 186-190, 2016.

Wang MQ, Wang BJ, Jiang KY, Liu M, Shi XW, Wang L. A mitochondrial manganese superoxide dismutase involved in innate immunity is essential for the survival of *Chlamys farreri*. Fish Shellfish Immun. 72: 282-290, 2018a.

Wang MQ, Wang LL, Guo Y, Yi QL, Song LS. An LRR-only protein representing a new type of pattern recognition receptor in *Chlamys farreri*. Dev. Comp. Immun. 54: 145-155, 2016a.

Wang MQ, Wang LL, Jia ZH, Wang XD, Yi QL, Lv Z, *et al*. The versatile functions of LRR-only proteins in mollusk *Chlamys farreri*. Dev. Comp. Immun. 77: 188-199, 2017.

Wang MQ, Wang LL, Jia ZH, Yi QL, Song LS. The various components implied the diversified Toll-like receptor (TLR) signaling pathway in mollusk *Chlamys farreri*. Fish Shellfish Immun. 74: 205-212, 2018b.

Wang MQ, Wang LL, Xin LS, Wang XD, Wang L, Xu JC, *et al*. Two novel LRR-only proteins in *Chlamys farreri*: Similar in structure, yet different in expression profile and pattern recognition. Dev. Comp. Immun. 59: 99-109, 2016b.

Wang MQ, Yang JL, Zhou Z, Qiu LM, Wang LL, Zhang H, *et al*. A primitive Toll-like receptor signaling pathway in mollusk Zhikong scallop *Chlamys farreri*. Dev. Comp. Immun. 35: 511-520, 2011.

Wang XQ, Wang LL, Wang MQ, Zhang H, Song LS. cDNA cloning, characterization and mRNA expression of cryptocyanin from the Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853. Crustaceana 89: 273-290, 2016c.

Zhang Y, Cui C, Lai ZC. The defender against apoptotic cell death 1 gene is required for tissue growth and efficient N-glycosylation in *Drosophila melanogaster*. Dev. Biol. 420: 186-195, 2016.

Zheng JB, Mao Y, Su YQ, Wang J. Effects of nitrite stress on mRNA expression of antioxidant enzymes, immune-related genes and apoptosis-related proteins in *Marsupenaeus japonicus*. Fish Shellfish Immu. 58: 239-252, 2016.

Zhu L, Song LS, Zhang H, Zhao JM, Li CH, Xu W. Molecular cloning and responsive expression to injury stimulus of a defender against cell death 1 (DAD1) gene from bay scallops *Argopecten irradians*. Mol. Biol. Rep. 35: 125-132, 2008.