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Short communication

Involvement of tetraspanin 8 in the innate immune response of the giant prawn, *Macrobrachium rosenbergii*

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ABSTRACT

The tetraspanins, representing a conserved superfamily of four-span membrane proteins, are highly involved in viral and bacterial infections. Thus far, the function of the tetraspanins in crustaceans remains largely unknown. In this study, we report the cloning and expression analysis of a tetraspanin 8 from the giant freshwater prawn, *Macrobrachium rosenbergii* (named as *MrTspan8*). *MrTspan8* contains a 720-bp open reading frame encoding a 239-amino acids protein, which exhibits four transmembrane domains and two extracellular loops that are typical for tetraspanins. *MrTspan8* was found to be widely expressed in a variety of prawn tissues including heart, gill, muscle, gut, and hepatopancreas. Additionally, *MrTspan8* expression was significantly increased in the hepatopancreas and gill of the prawns challenged by the bacterial pathogen *Aeromonas hydrophila*. Moreover, we show that pre-incubation of the peptides from the large extracellular loop of MrTSPAN8 protein reduced the cell death caused by *A. hydrophila* infection in prawn tissue, suggesting that MrTSPAN8 could be a mediator for bacterial infection to prawn.

1. Introduction

Tetraspanins represent a widely distributed superfamily of glycoproteins which play critical roles in multiple physiological and pathological processes [1–8]. Tetraspanins are able to form lateral associations with themselves and multiple partner proteins in the membrane, giving rise to tetraspanin enriched microdomains (TEM) that are involved in diverse cellular activities such as motility, adhesion, fusion, and trafficking [3,9].

Members of the tetraspanin superfamily exhibit several defining features, including the intracellular N and C terminus, four transmembrane domains with conserved polar residues, one small extracellular loop (SEL) and one large extracellular loop (LEL) [9]. The LEL in tetraspanin has been suggested as the primary region of connection between cells and other proteins or pathogens [3]. Although few pathogens adhere to host cells by binding directly to tetraspanins [1,10], the majority of the bacterial adhesion requires an indirect interaction with tetraspanins through TEM-embedded receptors [1,9,11]. It has been reported that application of anti-tetraspanin antibodies or recombinant LEL of some tetraspanins can disrupt TEM on endothelial cells leading to impaired adherence of bacteria to the host cells [1,12]. Although the tetraspanins in human and mouse have been widely studied [4], the information of tetraspanins in crustaceans is relatively limited. For example, three tetraspanins from the Chinese shrimp, *Fenneropenaeus chinensis,* were found to be up-regulated in the live white spot syndrome virus (WSSV)-challenged shrimp tissues [13]. CD63, one member of the tetraspanin family, has been shown to play an important role in mediating entry of WSSV into the shrimp, *Litopenaeus vannamei* [14].

The giant freshwater prawn, *Macrobrachium rosenbergii*, represents one of the most economically important aquaculture species in many countries in Asia [15]. In recent years, the prawn aquaculture was threatened by bacterial and viral pathogens [15–24]. In this report, the full-length cDNA sequence of tetraspanin 8 from *M. rosenbergii* (*MrTspan8*) has been cloned. *MrTspan8* was widely expressed in various prawn tissues, and significantly increased in hepatopancreas and gill when challenged by bacterial pathogen *Aeromonas hydrophila*. Blocking experiments *in vivo* further suggest that MrTSPAN8 acts as a mediator for bacterial infection to prawns.

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2. Materials and methods

2.1. Animals

Adult prawns (*M. rosenbergii*,15–20 g) of both sexes were obtained from a local market and reared in glass aquariums with circulating freshwater at 25 °C and were fed twice a day with commercial bait as previously reported [25]. Prawns were acclimated for at least 2 weeks before experimentation. A variety of prawn tissues were dissected, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The experiments were performed in triplicate. For each replicate, each type of tissue was pooled from five shrimps. Our experiments performed on animals, animal care and all protocols were followed the ethical guidelines of Hangzhou Normal University for the care and use of laboratory animals as described in Ref. [26].

2.2. A. hydrophila challenge

The prawns were randomly divided into challenge and control groups. Each group was performed with five prawns and prepared in triplicate. Bacterial suspensions were prepared and the amount of the bacteria used for challenge was determined as described in Ref. [22]. For the challenge groups, the prawns were injected intramuscularly with 20 μl of 1×10^6 cfu ml^{-1} cultured *A. hydrophila.* For the control groups, the prawns were injected with 20 μl PBS. The prawns were sacrificed at the time of 0, 3, 9, 16 and 24 h of challenge. The hepatopancreas and gill were carefully dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. For blocking experiments, peptides, DLKCCGVNNYTDWSDYPYGQ (aa 138 to 157) and ADGCCKVMSEGCGNGILNKP (aa 163 to 182), were designed and synthesized based on the LEL region of MrTSPAN8 according to previous study [1]. In one group, each prawn was injected intramuscularly with 20 μl of 1×10^6 cfu ml^{-1} cultured *A. hydrophila* pre-incubated with the MrTSPAN8 peptide mixtures (20 μg/prawn). In another groups, each prawn was injected with 20 μl of 1×10^6 cfu ml^{-1} cultured *A. hydrophila* pre-incubated with bovine serum albumin (BSA, 20 μg/prawn). Prawns injected with PBS were used as negative controls. The prawns were sacrificed at 9 h post injection. The hepatopancreas was dissected and fixed by 4% paraformaldehyde for cell death analysis.

2.3. RNA extraction and cDNA synthesis

RNA was isolated using TRIzol reagent (Invitrogen), and then transcribed into first strand cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara).

2.4. Molecular cloning of MrTspan8

We have performed whole transcriptome sequencing analysis to discover genes whose expression are changed in prawn tissue in response to *A. hydrophila* [27]. One of the cDNA fragments that were upregulated in response to the bacterial challenge was predicted to encode tetraspanin 8. The gene-specific primers for amplifying the full-length prawn tetraspanin 8 were designed based on this sequence (Table 1). The full-length cDNA fragments of prawn tetraspanin *(MrTspan8)* was obtained by the RACE technology. Briefly, first strand cDNAs were synthesized from 2 μg of hepatopancreas RNA using GeneRacer™ Advanced RACE Kit (ThermoFisher) and used as templates to amplify the 5′- and 3′- region cDNA fragments of prawn tetraspanin 8. The full-length cDNA sequence was obtained by assembling the original cDNA fragment with its 5′- and 3′ cDNA ends, and submitted to GenBank under the accession number of MG755750.

2.5. Quantitative real-time PCR (RT-PCR)

Quantitative RT-PCR was performed using SsoFast EvaGreen Supermix with CFX384 real-time PCR Detection System (both from BioRad Laboratories) as previously described [26]. The quantitative RT-PCR primers for *MrTspan8* were Q8F and Q8LF (Table 1). *GAPDH* of *M. rosenbergii* was used as the reference gene. The quantitative RT-PCR primers for *GAPDH* are QMrGAPDHF and QMrGAPDHF (Table 1 [26,27]). The $2^{-\Delta\Delta Ct}$ method was used for calculating the relative fold change of gene expression in quantitative RT-PCR.

Table 1
Nucleotide sequences of primers used in PCR.

Primer	Direction	sequence
5RACE8L	R	CCCAAGAACCCGATAAGGAGGATGAGGAG
3RACE8L	F	CGATGCCATTAAAGACGACATCCAAGGAGT
Q8F	F	CCGGACTCCTCATCCTCCTTAT
Q8R	R	GACGTTTTCGGCTTGGTTTGTG
QMrGAPDHF	F	AACGACCCGTTCATCGCTCTA
QMrGAPDHR,	R	CGTCCTCAGCCTTCACCTCA
P8F	F	TGGTATTGGGGTTGCAGTTGTAGT
P8R	R	GCGTTGGCCGTAAGGGTAGTC

2.6. In situ hybridization and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

For *in situ* hybridization and cell apoptotic assay, tissues were dissected, fixed by 4% paraformaldehyde, dehydrated through gradient ethanol series, and processed for paraffin sections. *In situ* hybridization was performed on paraffin-embedded sections as described previously [26]. For synthesizing RNA probe for *MrTspan8,* a pair of primers, P8F/R, was designed on the basis of *MrTspan8* sequence to generate a 406-bp cDNA fragment. The fragment was then subcloned into pGEM®-T Easy Vector (Promega) and sequenced. The recombinant plasmid for *MrTspan8* was purified and linearized and then used as template for probe synthesis. DIG RNA labeling mix (Roche) was used as substrate. The anti-sense and sense Digoxin-labeled RNA probes were synthesized by T7 and SP6 RNA polymerase (Promega), respectively. Cell apoptosis was determined by TUNEL staining using the In Situ Cell Death Assay kit (Roche) as described previously [28].

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (one-way ANOVA) using SPSS. The results were shown as means ± SEM. The differences were considered to be significant for $p < 0.05$.

3. Results and discussion

The full-length *MrTspan8* cDNA is 1736 bp in length and contains 563-bp of 5′ untranslated regions (UTR), 720-bp of open reading frame (ORF), and 453-bp of 3′UTR (Fig. 1A). The putative amino acid sequence of MrTSPAN8 displayed 32–60% identity with known tetraspanin proteins of other animals, with the highest (60%) identity to the TSPAN8 from the Pacific white shrimp, *Litopenaeus vannamei*. The transmembrane helicase in MrTSPAN8 protein was predicted using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). As its counterpart in other species, MrTSPAN8 was predicted to contain four transmembrane domains as well as a small and a large extracellular loop (LEL, Fig. 1B). Two signature motifs, a Cys-Cys-Gly sequence and two further Cys residues, are highly conserved in the LEL of all tetraspanins [9]. They were also found in the LEL region of MrTSPAN8 (Fig. 1B). SmartBLAST results showed that the amino acid sequence of MrTSPAN8 exhibited 31–32% identify with Tetraspanins from well-studied reference species including *Homo sapiens*, *Mus musculus*, *Danio rerio*, and *Drosophila melanogaster* (Fig. S1A). Phylogenetic trees were constructed based on the amino acid sequence of MrTSPAN8 and those from different classes of organisms (Fig. S1B). The MrTSPAN8 was in

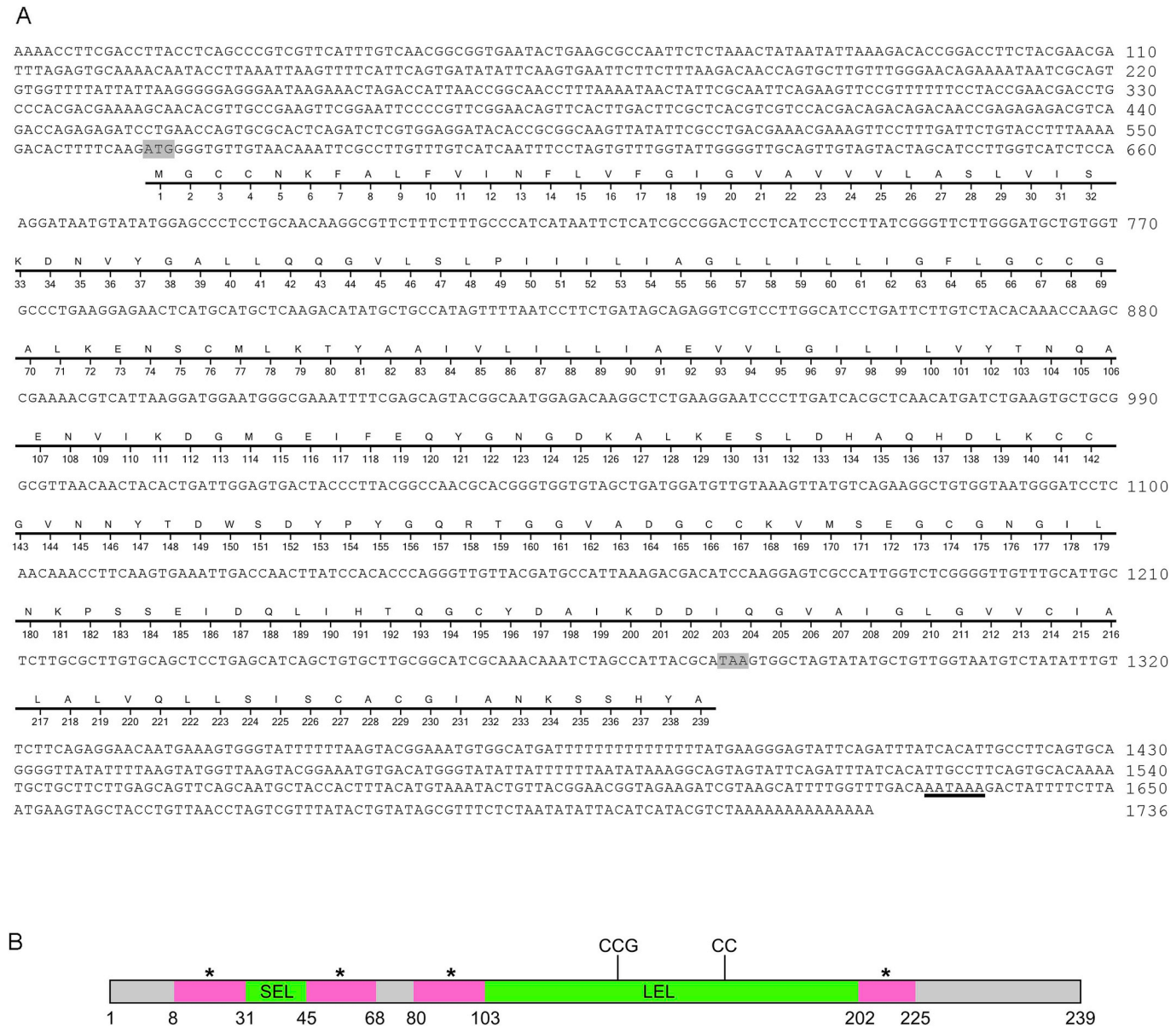

Fig. 1. The full-length cDNA and deduced amino acid sequence of *MrTspan8* gene (A) and a schematic representation showing the structure of MrTSPAN8 protein (B). The putative polyadenylation signals are underlined. The start and the stop codons are shaded. SEL, small extracellular loop; LEL, large extracellular loop; *, transmembrane region.

the same branch with the TSPAN8 of the Pacific white shrimp, *L. vannamei* (Fig. S1B).

To investigate the function of *MrTspan8* in the prawn, we performed quantitative RT-PCR to analyze the gene expression of *MrTspan8*. We found that *MrTspan8* was expressed in various prawn tissues including heart, gill, muscle, gut, and hepatopancreas (Fig. 2A). *In situ* hybridization analysis further showed that *MrTspan8* was expressed in heart, gill, muscle, gut epithelium, and tubule epithelial cells of the hepatopancreas (Fig. 2B–F), suggesting that *MrTspan8* is probably involved in physiological processes of multiple organs in prawn. To date, only a few tetraspanins have been studied in crustaceans. In consistence with our results, *CD63* from *L. vannamei* was also ubiquitously expressed [14]. In addition, *Tetraspanin-3* from Chinese shrimp, *F. chinensis,* was widely expressed in all examined tissues [13]. By contrast, *CD9* from *F. chinensis* was specifically in the hepatopancreas and *CD63* from *F. chinensis* was not detected in gut and muscle [13]. These results support previous results that most of the tetraspanins are ubiquitously expressed and may have similar functions, while some have restricted expression and specialized functions [4,5,29,30].

Next, we investigated whether the expression of *MrTspan8* in hepatopancreas and gill, two important organs involved in prawn immunity, was changed in response to *A. hydrophila* challenge. Quantitative RT-PCR results showed that the expression of *MrTspan8* in hepatopancreas was dramatically increased in response to *A. hydrophila* challenge, reached the peak at 3 h post injection, and then decreased gradually but still remained at a much higher level than the control in the next 21 h (Fig. 3A). *MrTspan8* expression in the gill was also significantly upregulated by *A. hydrophila* challenge, peaked at 3 h of challenge, and then decreased to normal level (Fig. 3B). Similar to *MrTspan8*, three tetraspanins from *F. chinensis* were significantly upregulated in hepatopancreas by WSSV challenge [13]. The *L. vannamei CD63* expression was up-regulated in gill in response to WSSV challenge; however its expression in hepatopancreas was down-regulated [14]. Given that tetraspanins have been shown to play a critical role in

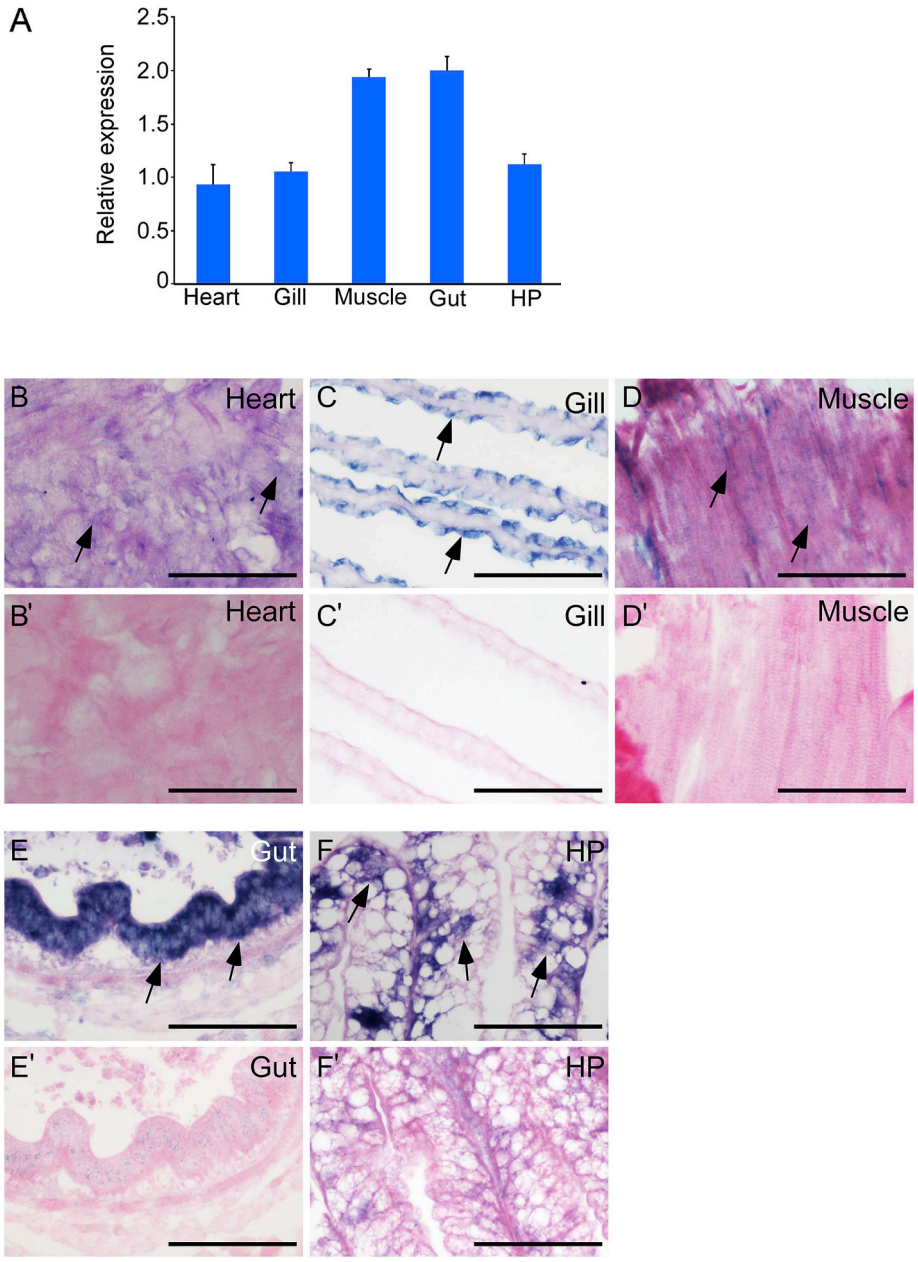

Fig. 2. Gene expression analysis of *MrTspan8* in various *M. rosenbergii* tissues. (A) The relative mRNA level of *MrTspan8* in a variety of prawn tissues was analyzed by quantitative RT-PCR. *GAPDH* was used as the reference gene. Results were shown as mean ± SEM. (B–F) *In situ* hybridization showing the expression of *MrTspan8* in heart, gill, muscle, gut, and hepatopancreas of *M. rosenbergii*. (B′-F′) *In situ* hybridization performed with sense Digoxin-labeled RNA probe of *MrTspan8* for negative control. Positive staining was indicated by arrows. HP, hepatopancreas. Scale bars, 100 μm.

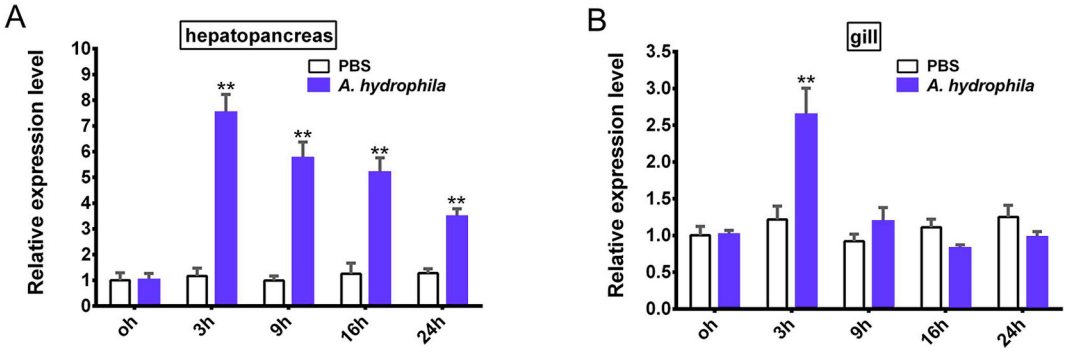

Fig. 3. Expression profiles analysis of *MrTspan8* in hepatopancreas (A) and gill (B) in response to *A. hydrophila* at various time points. Results were shown as mean ± SEM. $^{**}p < 0.01$.

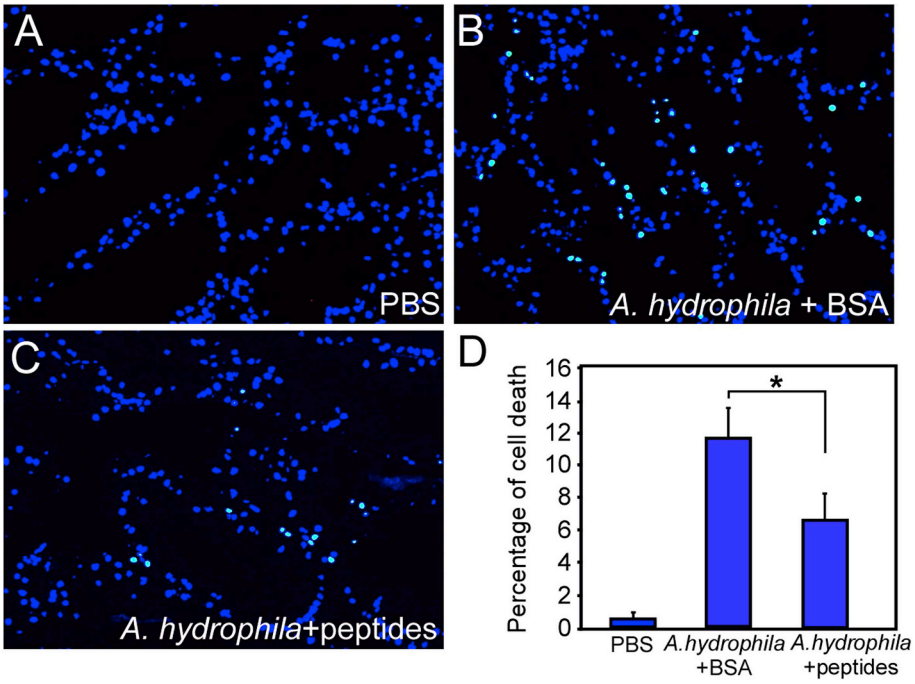

Fig. 4. Analysis of cell death induced by *A. hydrophila* pre-incubated with BSA and MrTSPAN8 peptides by TUNEL assay. (A–C) TUNEL staining showing cell apoptosis in hepatopancreas of the prawns injected with PBS, *A. hydrophila* plus BSA, and *A. hydrophila* plus MrTSPAN8 peptides, respectively. Nuclei were stained by DAPI (blue). Apoptotic cells were stained in green. (D) Statistic analysis showing an obvious decrease in cell apoptosis in the peptide pre-incubation group. Results were shown as mean ± SEM. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the adherence of pathogens to host cells, these data suggest that MrTSPAN8, like its other family members, may also be a host factor that facilitates pathogens entry into shrimp cells.

Anti-tetraspanin antibodies or short peptides of LEL have been shown to disrupt TEM on endothelial cells which result in impaired adherence of bacteria to the host cells [1,12]. For example, the recombinant LEL of CD9 could substantially decrease the adherence of multiple species of bacteria such as *Neisseria meningitidis* and *Salmonella enterica* to mammalian cells [12]. And synthetic peptides of the keratinocyte-expressed CD9, can inhibit bacterial adhesion to cultured keratinocytes and in a tissue engineered model of human skin infection [1]. In addition, blocking the large extracellular loop (LEL) domain of *FcTspan3* efficiently inhibits the infection of WSSV in *F. chinensis* [31]. However, RNAi of *FcTspan3* in the Chinese shrimp did not significantly decrease the viral copy numbers of WSSV [31], which may due to the compensation of functionally redundant tetraspanin family proteins [5]. To investigate the function of MrTSPAN8, we synthesized the short peptides of the LEL region of MrTSPAN8 to determine their anti-bacterial effects. Three groups of prawns were used. The control group of prawns, which were injected with PBS, showed little apoptotic cells in their hepatopancreas as revealed by TUNEL assay (Fig. 4A). In contrast, prawns injected with BSA pre-incubated-*A. hydrophila* showed about 12% of apoptotic cells in the hepatopancreas (Fig. 4B and D). Interestingly, the percentage of cell apoptosis was significantly decreased in prawns injected with MrTSPAN8 peptides pre-incubated-*A. hydrophila* (Fig. 4C and D), suggesting that MrTSPAN8 plays a key role in the bacterial invasion process to the prawn, *M. rosenbergii*. Our results, together with previous studies, also support that pre-incubation of LEL peptides to disrupt the function of TEM could efficiently inhibit bacterial invasion into host cells. However, further studies are required to elucidate in depth the function of prawn MrTSPAN8 in response to pathogenic infections.

In summary, we report the cloning of a novel tetraspanin gene encoding a tetraspanin 8 homolog of the prawn, *M. rosenbergii*. The fact that *MrTspan8* expression was modulated by bacterial infection suggests an important role for *MrTspan8* in the innate immune response of the prawn. Finally, the blocking experiments suggests that *MrTspan8* is possibly required for bacterial invasion into the prawn.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.055.

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