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Short communication

A preliminary attempt to explore the potential functions of a tetraspanin gene (*MmTSPAN*) in the innate immunity of hard clam *Meretrix meretrix*: Sequence features and expression profiles

Yan Wang[a,c], Mengqiang Wang[a,d], Baojie Wang[a], Mei Liu[a], Keyong Jiang[a], Xuguang Hou[e], Lei Wang[a,b,f,*]

[a] CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China
[b] Laboratory for Marine Biology and Biotechnology, National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[c] University of Chinese Academy of Sciences, Beijing, 100049, China
[d] Research Platform for Marine Molecular Biotechnology, National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[e] Marine College, Shandong University at Weihai, Weihai, 264209, China
[f] CAS Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, 266400, China

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ABSTRACT

Tetraspanins belong to the transmembrane 4 superfamily (TM4SF), and play crucial roles in immune responses. In the present study, a novel tetraspanin gene (designated *MmTSPAN*) was cloned and characterized from the hard clam *Meretrix meretrix*. The complete cDNA sequence of *MmTSPAN* contained an open reading frame (ORF) of 816 bp, which encoded a protein of 271 amino acids. *Mm*TSPAN exhibited highly similarity with previously identified tetraspanins from other species. It contained four transmembrane domains (12–35 aa, 69–92 aa, 99–123 aa and 238–261 aa), characteristic CCG motif and four conservative cysteine residues. The mRNA transcripts of *MmTSPAN* were ubiquitously detectable in all the tested tissues, with the highest expression level in hepatopancreas. Temporal transcriptional levels in the hepatopancreas revealed significant up-regulation of *MmTSPAN* by *Vibrio splendidus* stimulation, with a 3.14-fold increase at 6 h compared to the control, and reaching 32.98-fold at 24 h. These results provide useful information for further study of the function of tetraspanin in the innate immune system of *M. meretrix*, and may offer a new therapeutic target for diseases of *M. meretrix*.

1. Introduction

Tetraspanins (also named as TSPAN or TM4SF proteins) are a large family of evolutionarily conserved four-transmembrane-domain proteins and widely found in multicellular eukaryotes [1]. The tetraspanin protein family has been discovered at least 32 members in mammals [2], 37 members in *Drosophila* [3], and also in fungi (excluding yeast), insects and fish [4–7]. Typical tetraspanin contains highly hydrophobic transmembrane domains (TM1-TM4) that delimit two extracellular loops (EL) and an intracellular loop (IL) [8]. Tetraspanin associate with several other tetraspanins and relate to some proteins form a huge of tetraspanin web [1]. Tetraspanin interacts with various types of proteins, such as integrin, immune receptor, signal molecule and so on, thus it participates in signal transduction, cell growth, migration, adhesion and plays an important role in the immune responses [9–12].

At least twenty tetraspanins can be expressed on the leukocytes surface [13], and the role of tetraspanin is inseparable in the immune response process, due to the immune function of leukocyte. The regulation of tetraspanin to immunological functions of the organism may be owing to its combination of complex and diverse proteins. Increasing evidences suggest that tetraspanin plays an important role in the interaction of cells with bacteria, viruses, fungi and parasites, so it has been studied as target protein for diseases caused by these pathogens in medicine [14,15].

The hard clam *Meretrix meretrix*, is an economically important species of marine bivalves, widely distributed along the coastal areas of South and Southeast Asia [16]. However, for the past few years, the tremendous economic losses in clam culture caused by bacteria and viruses is a serious problem that restricts the culture industry [17,18]. Especially, *Vibrio* has been reported to be the main pathogenic bacteria causing large mortalities of *M. meretrix* [19]. Research on tetraspanin of *M. meretrix* may be more effective and sustainable, compared with

* Corresponding author. CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China.
E-mail address: wanglei@qdio.ac.cn (L. Wang).

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Table 1
Nucleotide sequences of primers used in this study.

Primers	Sequence (5′-3′)	Brief information
TSPAN-F1	TTGGGTTTGACTGTTGTGGAGTAAATG	Gene specific primer for RACE
TSPAN-F2	GGGGCAGTAGAGGCTCGGACATCA	Gene specific primer for RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	Universal primers for RACE
3′CDS	AAGCAGTGGTATCAACGCAGAGTAC(T)30V N	Oligo (dT) for cDNA synthesizing
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer for sequencing
TSPAN-qPCR-F	TTACCTCAAGCAACGAAGTATCTG	Gene specific primer for real-time PCR
TSPAN-qPCR-R	TATGATGTCCGAGCCTCTACTG	Gene specific primer for real-time PCR
β-actin-qPCR-F	TTGTCTGGTGGTTCAACTATG	Internal control for real-time PCR
β-actin-qPCR-R	TCCACATCTGCTGGAAGGTG	Internal control for real-time PCR

traditional treatment. Many tetraspanins with unique functions have been found in invertebrates, but studies on its innate immune system are still rare. Therefore, the role of tetraspanin in the innate immune system of *M. meretrix* is worth our in-depth discussion and its application in immunopathology is promising.

In our present study, a novel tetraspanin gene has been cloned from *M. meretrix* (designated *MmTSPAN)*. The expression characteristics of *MmTSPAN* were analyzed in different tissues and its temporal mRNA expression pattern was detected after *Vibrio* stimulation. The results would provide useful information for further study of the function of tetraspanin in the innate immune system of *M. meretrix*, and may offer a new therapeutic target for diseases of *M. meretrix*.

2. Materials and methods

2.1. Animals and samples collection

Healthy adult hard clams were obtained from a local farm in Qingdao, China and kept in our laboratory for one week. To determine the tissue distribution of *MmTSPAN*, tissues of adductor muscle, foot, gill, hepatopancreas and mantle were sampled from five untreated clams, immediately frozen in liquid nitrogen and then stored at −80 °C until use. Hemocytes were collected from the adductor muscle of five untreated clams using a sterile syringe, and then centrifugation at 800*g* for 10 min at 4 °C, stored at −80 °C at once until use.

2.2. Vibrio stimulation experiment

Vibrio splendidus strain JZ6 was used for the immune challenge test. Approximately 300 clams were employed for *Vibrio* stimulation assay, and the clams were randomly divided into two groups. The challenge group was immersed with live *V. splendidus* at a final concentration of 1.0×10^8 CFU per 1 mL. After stimulation, samples were taken from each group at 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 96 h, with 5 replications at each time point, and each replication was a mixture of 3 individuals. The untreated clams were sampled at the same time point in the same way. The hepatopancreas from each sample was collected and stored at −80 °C for RNA extraction. Changes in *MmTSPAN* mRNA expression in response to *V. splendidus* challenge were then tested using quantitative real-time PCR (qPCR) technique.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from six tissues of *M. meretrix* using RNAiso Plus (9108, TaKaRa, China) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. The RNA quality and quantity were checked using a NanoDrop 2000 (Thermo scientific, USA). The cDNA synthesis was carried out by M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (M3683, Promega, USA) in accordance with the manufacturer's instructions, and then stored at −20 °C for subsequent cloning and expression analysis.

2.4. Cloning and sequencing of MmTSPAN

All of the *M. meretrix* EST sequences in NCBI database were annotation using Blastx, revealed that a sequence (GenBank Accession No. GR211299) homologous to tetraspanin and was used for cloning [20]. Based on the 748bp mRNA linear EST, gene specific primers (TSPAN-F1/2) were designed to clone the 3′-end of cDNA of *MmTSPAN* by the rapid amplification of cDNA ends (RACE) technique. The PCR products were cloned into the pMD-18T Cloning Vector (6011, TaKaRa, China), and then transformed into the competent cells of *Escherichia coli* strain *Trans*1-T1 (CD501, TransGen Biotech, China). The potentially positive recombinant clones were identified by colony PCR with M13 primers (M13-47 and RV-M), and three positive clones were picked for sequencing. The primers are listed in Table 1.

2.5. Bioinformatics analysis of MmTSPAN

The nucleotide and protein sequence similarities were searched via BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Multiple sequence alignment was generated using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The protein motif features were predicted by Simple Modular Architecture Research Tool (SMART, http://smart.emblheidelberg.de/). The transmembrane domain of protein was performed using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The structure of protein was predicted by the SWISS-MODEL Server and Repository (http://swissmodel.expasy.org/). The cartoon diagram was manufactured by Protter (http://wlab.ethz.ch/protter/). The phylogenetic tree was constructed based on the amino sequences alignment by the neighbor-joining (NJ) method with MEGA 7.0 software, bootstrap trials were replicated 1000 times.

2.6. Quantification analysis of MmTSPAN mRNA expression

The mRNA transcriptional levels of *MmTSPAN* in different tissues and its temporal expression pattern in hepatopancreas of clams stimulated with *V. splendidus* were analyzed by qPCR technique. All qPCR reactions were performed with TB Green Premix Ex Taq (Tli RNaseH Plus), ROX plus (RR42LR, TaKaRa, China) using about 100 ng cDNA template and 0.2 μM of each primer (Table 1), in ABI 7300 Real-Time PCR system (ThermoFisher, USA). The reaction conditions were denaturation for 10 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, and 31 s at 60 °C. The mRNA expression levels of *MmTSPAN* were normalized to that of β-actin gene for each sample.

Fig. 1. Nucleotide and deduced amino acid sequence of *Mm*TSPAN. CCG motif and conserved cysteine residues were boxed. The predicted transmembrane domain was in shade. The polyadenylation consensus signal was indicated in underline. The asterisk indicated the stop codon. The amino acid sequence of *Mm*TSPAN has been submitted to GenBank with the accession number KU301775.

2.7. Statistical analysis

The results of qPCR analyses were based on the melting curve analysis of the PCR products and the comparative C_T method ($2^{-\Delta\Delta Ct}$) was used to analyze the expression level of *MmTSPAN* mRNA. Final results are expressed as means ± standard deviation (SD). The statistical analysis was performed by one-way analysis of variance (one-way ANOVA) using SPSS Statistic 19.0 software to detect significant intergroup differences. The *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Cloning and characterization of MmTSPAN cDNA

The full-length cDNA of *MmTSPAN* was obtained by 3′-RACE according to the assembling of EST from a *M. meretrix* cDNA library and submitted to GenBank (Accession No. KU301775). The gene contained an 816 bp open reading frame (ORF), a 164 bp 5′-untranslated region (UTR) and 700 bp 3′-UTR, which included a putative polyadenylation consensus signal (AATAAA) and a poly A tail, all told 1680 bp long (Fig. 1). The ORF encoded a polypeptide of 271 amino acids with calculated molecular weight of 29.3 kDa and theoretical pI of 4.94. Hydrophobicity plot analysis of amino acid sequence identified four putative hydrophobic transmembrane domains (12–35 aa, 69–92 aa, 99–123 aa and 238–261 aa, respectively) using TMHMM. Like other tetraspanins, *Mm*TSPAN also has characteristic CCG motif and four conservative cysteines (Fig. 1).

The protein homologous modeling was performed to generate the three-dimensional (3D) structure of the *Mm*TSPAN based on its amino acid sequence. The template protein was human tetraspanin CD81 (PDB code: 5tcx) [21]. The tertiary structure of *Mm*TSPAN revealed six α helices and two 3_{10} helices (Fig. 2A). Corresponding to the predicted proteoforms of *Mm*TSPAN (Fig. 2B), four long α helices (TM 1-TM 4) are transmembrane α helices. Small extracellular loops (SEL) between TM 1 and TM 2, as well as large extracellular loops (LEL) connected TM 3 and TM 4 were outside the cell. The intracellular loop (IL) jointed TM 2 and TM 3, the C- terminal domain and N- terminal domain were located in cytoplasm.

3.2. Multiple alignment and phylogeny relationship of MmTSPAN

The deduced amino acid sequence of *Mm*TSPAN exhibited high similarity with previously identified tetraspanins, such as 44% with *Mizuhopecten yessoensis* CD63 (XP_021349411) and 43% with *Crassostrea virginica* terraspanin-9 (XP_022320724). Alignment of the protein sequence of *Mm*TSPAN was performed to determine its identity, compared with those of previously identified tetraspanins (Fig. 3). Comparison of tetraspanins revealed that they had conserved amino acid residues, and most of them were focused on transmembrane domains. The non-conserved amino acid sequences were mainly located in LEL, which also coexisted characteristic CCG motif and conservative cysteines. The construction of NJ phylogenetic tree from multiple tetraspanins was separated into four branches, *Mm*TSPAN was clustered with its homologue as one of branches, mollusc, other branches were echinoderm, arthropod and vertebrate (fish, avian, amphibian and mammalian) (Fig. 4).

3.3. The tissue distribution of MmTSPAN mRNA

MmTSPAN mRNA transcripts were quantified with qPCR in six different tissues with β-actin as internal control. The *MmTSPAN* mRNA

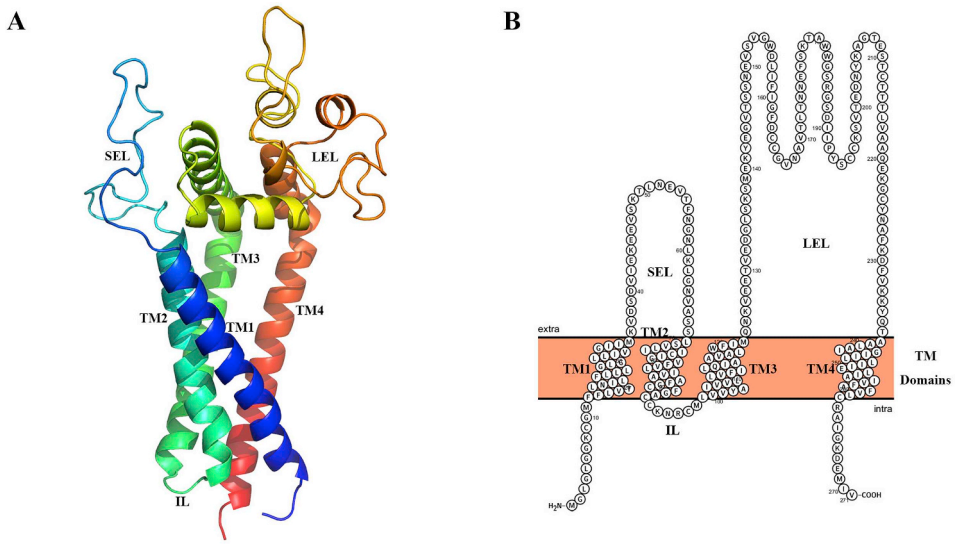

Fig. 2. (A) The three-dimensional (3D) structure of *Mm*TSPAN modeled by SWISS-MODEL. (B) The visualization proteoforms of *Mm*TSPAN made by Protter. The four transmembrane (TM 1–4) domains flank the small and large extracellular loops, as well as intracellular loop (SEL, LEL and IL, respectively).

transcripts could be detected in all the tested tissues, and the relative expression level was from high to low in hepatopancreas > gill > mantle > adductor muscle > foot > hemocytes. There were low expression levels in both the hemocytes and foot. The highest mRNA transcripts level was in hepatopancreas, which was 87.83-fold ($p < 0.05$) of that in hemocytes, then gill, mantle and adductor muscle, which were 26.67-fold, 20.01-fold and 12.25-fold ($p < 0.05$) of that in hemocytes, respectively (Fig. 5).

3.4. The temporal mRNA expression profile of MmTSPAN post Vibrio stimulation

The temporal mRNA expression level of *MmTSPAN* in hepatopancreas after *Vibrio* stimulation was detected via qPCR. The mRNA expression level of *MmTSPAN* in hepatopancreas was significantly up-regulated at 6 h after the *Vibrio* stimulation (3.14-fold compared with the control group, $p < 0.05$) and continuously increased at 12 h (4.61-

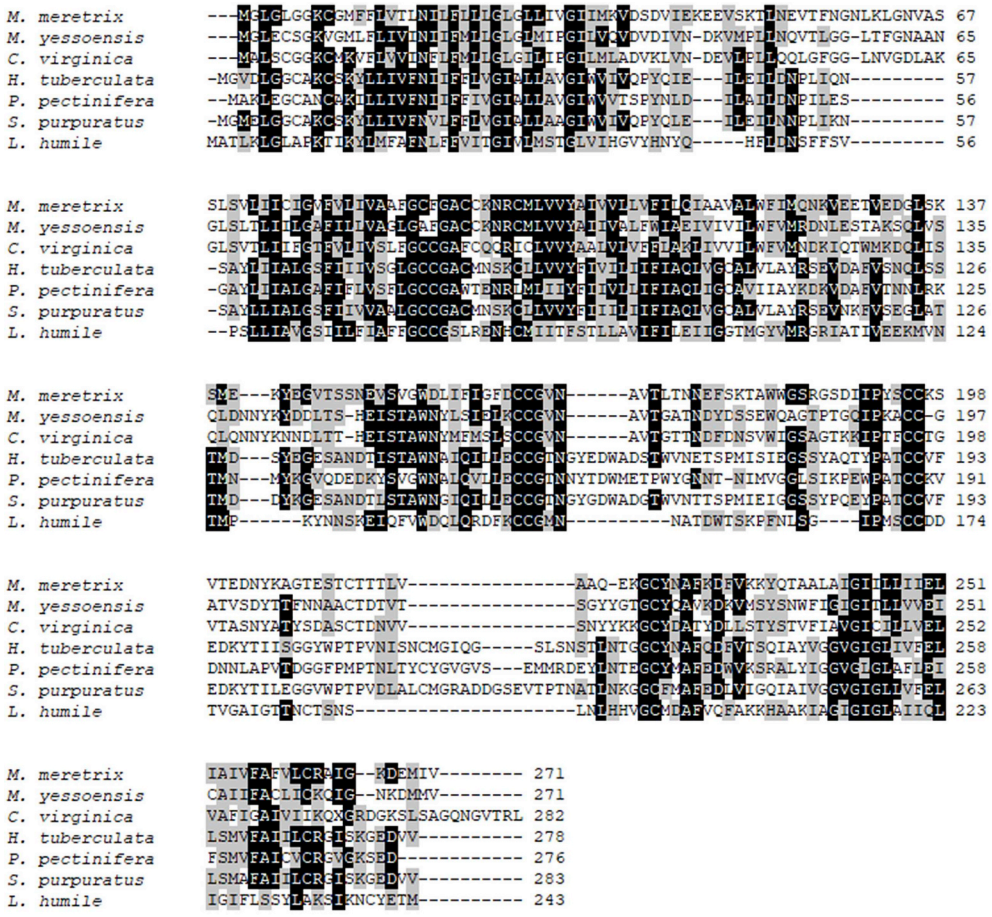

Fig. 3. Multiple alignments of *Mm*TSPAN with previous known tetraspanins. The same amino acid residues were shaded in black and the similar amino acids were shaded in grey. Gaps were indicated by dashes to improve the alignment. The sequences and their accession numbers are as follows: *Meretrix meretrix* (ANG56320), *Mizuhopecten yessoensis* (XP_021349411), *Crassostrea virginica* (XP_022320724), *Heliocidaris tuberculate* (ABE27955), *Patiria pectinifera* (BAP00631), *Strongylocentrotus purpuratus* (NP_001118229), *Linepithema humile* (XP_012229466).

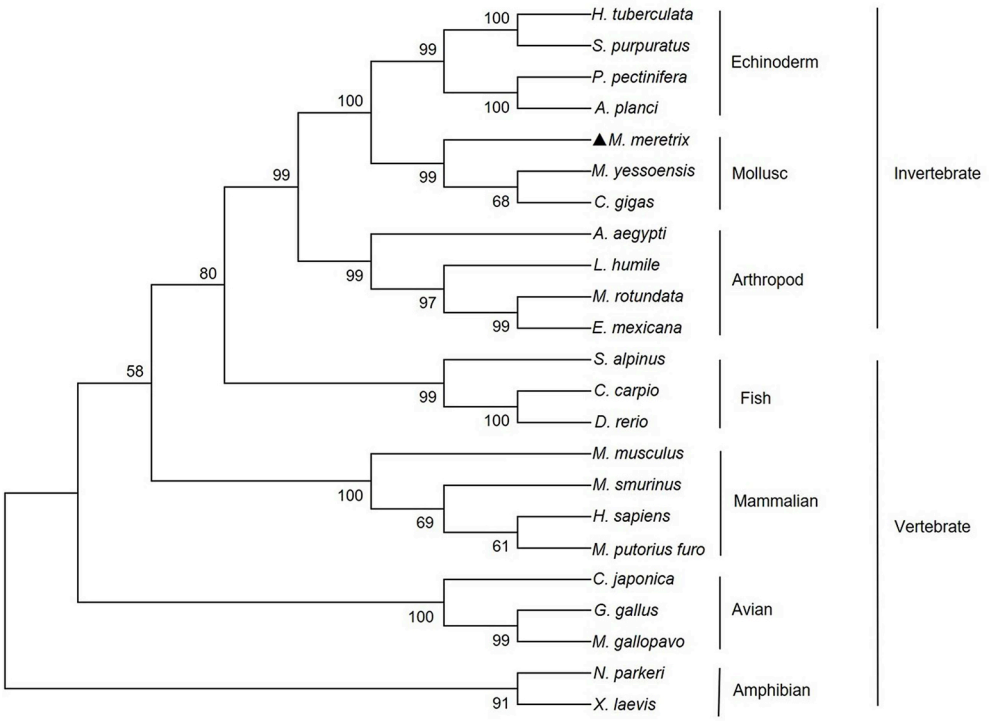

Fig. 4. Neighbor-Joining (NJ) phylogenic tree of *Mm*TSPAN constructed based on the protein sequences of tetraspanins from different organisms. To derive confidence value for the phylogeny analysis, bootstrap trials were 1000 replicates. The black triangle indicated the tetraspanin protein of *M. meretrix*. The numbers at the forks indicated the bootstrap value. Species and their protein accession numbers are as follows: *Meretrix meretrix* (ANG56320), *Mizuhopecten yessoensis* (XP_021349411), *Crassostrea gigas* (XP_011441833), *Heliocidaris tuberculate* (ABE27955), *Strongylocentrotus purpuratus* (NP_001-118229), *Patiria pectinifera* (BAP00631), *Acanthaster planci* (XP_022083554), *Aedes aegypti* (XP_021702248), *Linepithema humile* (XP_012229466), *Megachile rotundata* (XP_003704490), *Eufriesea mexicana* (XP_017757986), *Salvelinus alpinus* (XP_023847336), *Cyprinus carpio* (XP_01-8935977), *Danio rerio* (NP_955837), *Coturnix japonica* (XP_015742409), *Gallus gallus* (XP_015128066), *Meleagris gallopavo* (XP_010725346), *Xenopus. laevis* (NP_001082546), *Nanorana parkeri* (XP_0-18423488), *Mus musculus* (NP_031679), *Microcebus murinus* (XP_012608451), *Mustela putorius furo* (XP_004773100), *Homo sapiens* (NP_001771).

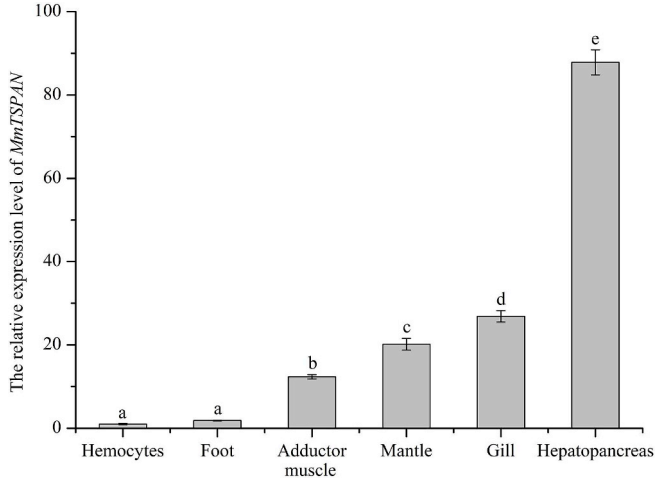

Fig. 5. Relative expression of *MmTSPAN* in different tissues. The β-actin gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$), while bars with same characters were not significantly different.

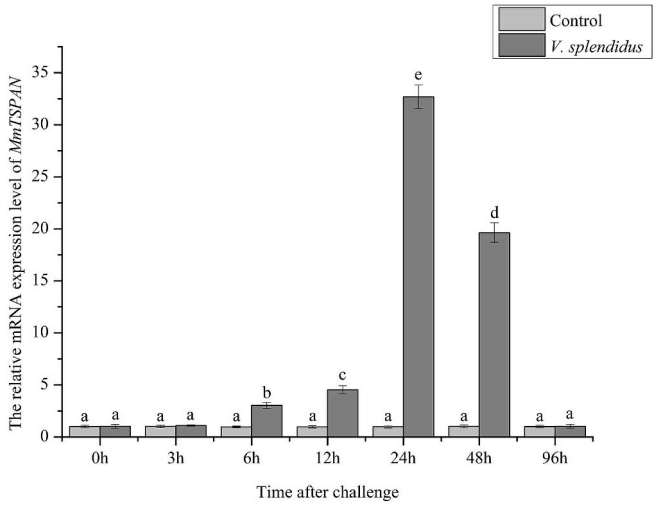

Fig. 6. Relative expression of *MmTSPAN* in hepatopancreas at 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 96 h after challenged with *V. splendidus*. The β-actin gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$), while bars with same characters were not significantly different.

fold, $p < 0.05$), then reached the peak at 24 h (32.98-fold, $p < 0.05$), kept at a high level at 48 h (19.23-fold, $p < 0.05$) and finally decreased to the origin level at 96 h ($p > 0.05$). While in the control group, no significant change of *MmTSPAN* mRNA expression was observed during the whole experiment (Fig. 6).

4. Discussion

Tetraspanins belong to a big family, TM4SF, and are abundantly and widely distributed in many cell types [1]. As a dock for signal transduction on the cell membrane, tetraspanin assembles membrane proteins and intracellular signaling proteins, obtains specific signals from the complex and varied external environment, and respond [8]. At present, the research hotspots of the tetraspanin family of higher vertebrate mammals mainly focus on the recognition of extracellular receptors, intracellular signaling pathways and the relationship with other cytokines during the immune response [22–24]. Tetraspanin is considered as a possible target of some pathogeny [25], thus as a target of drug action, tetraspanin provides a new way to prevent and treat relevant diseases. Many tetraspanins have been found in invertebrates,

such as Sm-TSP-2 in *Schistosoma mansoni* [26], *Lv*TSPAN8 in Pacific white shrimp *Litopenaeus vannamei* [27] and *SD*TM4SF in Demosponge *Suberites domuncula* [28], but studies on its innate immune system are relatively scarce.

In the present study, the complete cDNA sequence of *MmTSPAN* was cloned from the hard clam *M. meretrix*, which was 1680 bp and encoded a protein of 271 amino acids. As the same as previously reported tetraspanins, *Mm*TSPAN contains four TM domains, one SEL, a LEL and an IL. As shown in Fig. 2, LEL, which was located outside the cell membrane, was required for efficient interaction with certain associated proteins and the formation of the tetraspanin web [29]. The characteristic CCG motif and other four conserved cysteines were present in the LEL. Typically, 4–6 conservative cysteine residues are existing in the LEL of tetraspanin superfamily proteins [30]. These conserved cysteine residues formed intramolecular disulfide bonds crucial for the typical conformational integrity of LEL [31]. The amino acid sequence of *Mm*TSPAN shared over 30% similarities with other identified tetraspanins. The variable subdomain was mainly located in LEL, may be related to functional protein–protein interactions. Phylogenetic analysis of the various tetraspanins sequences clustered *Mm*TSPAN with its homologues from the Japanese scallop *M. yessoensis* and the Pacific oyster *C. gigas*. Taken together, *Mm*TSPAN was a novel member of tetraspanin family, and it could have the same functions to those from vertebrates and other invertebrates.

The previously reported tetraspanins, such as Human tetraspanin-3 and mouse OAP-1, were demonstrated to express in every examined tissues, suggesting that tetraspanin may participate in different biological processes [32,33]. Consistent with these reports, tissue expression analysis of the *MmTSPAN* gene revealed expression in each test tissues, indicating that *Mm*TSPAN was extensive tissue distribution. The highest mRNA transcript level of *MmTSPAN* was observed in hepatopancreas, which was 87.83-fold of that in hemocytes, followed by gill (26.67-fold). Compared with vertebrates, shellfish do not have independent immune organs. The hepatopancreas is the main organs of immunity and detoxification [34], and the mucosal system (including gill) is the first line of defense against invading pathogens in lower animals [35]. The high mRNA expression levels of *MmTSPAN* in hepatopancreas and gill, indicated that *Mm*TSPAN could be involved in the innate immune system of clam.

To further investigate the role of *MmTSPAN* in the immune response, its temporal expression profiles in hepatopancreas post *Vibrio* stimulation were detected by qPCR technique. When clams were stimulated with *V. splendidus*, the mRNA expression of *MmTSPAN* significantly increased and reached a maximum 24 h after challenge. In previous reports, *AbTSPAN33* from the disk abalone *Haliotis discus discus* could be induced after bacterial infection [36]. Temporal mRNA expression of CD63 in Chinese shrimp *Fenneropenaeus chinensis* documented that CD63 transcripts increased in hepatopancreas, hemocytes and lymphoid organ after challenge with white spot syndrome virus (WSSV) [37]. Flat oyster *Ostrea edulis* hemocytes *in vitro* infection with *Bonamia ostreae* induced an upregulated expression of tetraspanin ESTs [38]. Our research, together with those of previous studies, further verified that *Mm*TSPAN participated in the immune response as a part of response against the pathogen.

In conclusion, the full-length cDNAs of *MmTSPAN* were cloned and characterized from the hard clam, and the spatial and temporal expression profiles of its mRNA transcripts were detected. *Mm*TSPAN was found to be extensive tissue distribution, and could be induced after *V. splendidus* stimulation, as well as may participate in the immune response. The results obtained from this study would provide useful information of the potential role of *Mm*TSPAN in the innate immune system of *M. meretrix*, and might offer a new therapeutic target for diseases of *M. meretrix*.

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References

[1] M.E. Hemler, Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain, Annu. Rev. Cell Dev. Biol. 19 (2003) 397–422.
[2] S. Kohl, I. Giddings, D. Besch, E. Apfelstedt-Sylla, E. Zrenner, B. Wissinger, The role of the peripherin/RDS gene in retinal dystrophies, Acta Anat. 162 (2–3) (1998) 75–84.
[3] L.G. Fradkin, J.T. Kamphorst, A. DiAntonio, C.S. Goodman, J.N. Noordermeer, Genomewide analysis of the Drosophila tetraspanins reveals a subset with similar function in the formation of the embryonic synapse, P Natl Acad Sci USA 99 (21) (2002) 13663–13668.
[4] M. Gourgues, P.H. Clergeot, C. Veneault, J. Cots, S. Sibuet, A. Brunet-Simon, C. Levis, T. Langin, M.H. Lebrun, A new class of tetraspanins in fungi, Biochem. Biophys. Res. Commun. 297 (5) (2002) 1197–1204.
[5] E. Todres, J.B. Nardi, H.M. Robertson, The tetraspanin superfamily in insects, Insect Mol. Biol. 9 (6) (2000) 581–590.
[6] M.Z. Trikic, P. Monk, H. Roehl, L.J. Partridge, Regulation of zebrafish hatching by tetraspanin cd63, PLoS One 6 (5) (2011).
[7] J. Cao, X.N. Tan, Comparative analysis of the tetraspanin gene family in six teleost fishes, Fish Shellfish Immunol. 82 (2018) 432–441.
[8] C. Boucheix, E. Rubinstein, Tetraspanins, Cell Mol Life Sci 58 (9) (2001) 1189–1205.
[9] S. Levy, T. Shoham, The tetraspanin web modulates immune-signalling complexes, Nat. Rev. Immunol. 5 (2) (2005) 136–148.
[10] Y. Murayama, Y. Shinomura, K. Oritani, J.I. Miyagawa, H. Yoshida, M. Nishida, F. Katsube, M. Shiraga, T. Miyazaki, T. Nakamoto, S. Tsutsui, S. Tamura, S. Higashiyama, I. Shimomura, N. Hayashi, The tetraspanin CD9 modulates epidermal growth factor receptor signaling in cancer cells, J. Cell. Physiol. 216 (1) (2008) 135–143.
[11] M. Yamada, Y. Sumida, A. Fujibayashi, K. Fukaguchi, N. Sanzen, R. Nishiuchi, K. Sekiguchi, The tetraspanin CD151 regulates cell morphology and intracellular signaling on laminin-511, FEBS J. 275 (13) (2008) 3335–3351.
[12] C.K. Miranti, E. Park, Regulation of E-cadherin and cell-cell adhesion by the metastasis suppressor tetraspanin KAI1/CD82, Clin. Exp. Metastasis 28 (2) (2011) 176–177.
[13] J.M. Tarrant, L. Robb, A.B. van Spriel, M.D. Wright, Tetraspanins: molecular organisers of the leukocyte surface, Trends Immunol. 24 (11) (2003) 610–617.
[14] M. Yanez-Mo, O. Barreiro, M. Gordon-Alonso, M. Sala-Valdes, F. Sanchez-Madrid, Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes, Trends Cell Biol. 19 (9) (2009) 434–446.
[15] C.M. Guess, V. Quaranta, Defining the role of laminin-332 in carcinoma, Matrix Biol. 28 (8) (2009) 445–455.
[16] B.J. Tang, B.Z. Liu, G.D. Wang, T. Zhang, J.H. Xiang, Effects of various algal diets and starvation on larval growth and survival of *Meretrix meretrix*, Aquaculture 254 (1–4) (2006) 526–533.
[17] B. Allam, C. Paillard, A. Howard, M. Le Pennec, Isolation of the pathogen *Vibrio tapetis* and defense parameters in brown ring diseased Manila clams *Ruditapes philippinarum* cultivated in England, Dis. Aquat. Org. 41 (2) (2000) 105–113.
[18] M. Waechter, F. Le Roux, J.L. Nicolas, E. Marissal, F. Berthe, Characterisation of Crassostrea gigas spat pathogenic bacteria, Cr Biol 325 (3) (2002) 231–238.
[19] X. Yue, B.Z. Liu, L. Sun, Isolation and characterization of a virulent *Vibrio sp* bacterium from clams (*Meretrix meretrix*) with mass mortality, J. Invertebr. Pathol. 106 (2) (2011) 242–249.
[20] H.J. Li, W.D. Liu, X.G. Gao, D. Zhu, J. Wang, Y.F. Li, C.B. He, Identification of host-defense genes and development of microsatellite markers from ESTs of hard clam *Meretrix meretrix*, Mol. Biol. Rep. 38 (2) (2011) 769–775.
[21] B. Zimmerman, B. Kelly, B.J. McMillan, T.C.M. Seegar, R.O. Dror, A.C. Kruse, S.C. Blacklow, Crystal structure of a full-length human tetraspanin reveals a cholesterol-binding pocket, Cell 167 (4) (2016) 1041–1051 e11.
[22] P. Banse, R. Moeller, J. Bruening, L. Lasswitz, S. Kahl, A.G. Khan, J. Marcotrigiano, T. Pietschmann, G. Gerold, CD81 receptor regions outside the large extracellular loop determine hepatitis C virus entry into hepatoma cells, Viruses-Basel 10 (4) (2018).
[23] S.N. Hurwitz, M.R. Cheerathodi, D. Nkosi, S.B. York, D.G. Meckes, Tetraspanin CD63 bridges autophagic and endosomal processes to regulate exosomal secretion and intracellular signaling of epstein-barr virus LMP1, J. Virol. 92 (5) (2018).
[24] W.M. Peng, C.F. Yu, W. Kolanus, A. Mazzocca, T. Bieber, S. Kraft, N. Novak, Tetraspanins CD9 and CD81 are molecular partners of trimeric Fc epsilon RI on human antigen-presenting cells, Allergy 66 (5) (2011) 605–611.
[25] F. Martin, D.M. Roth, D.A. Jans, C.W. Pouton, L.J. Partridge, P.N. Monk, G.W. Moseley, Tetraspanins in viral infections: a fundamental role in viral biology?

J. Virol. 79 (17) (2005) 10839–10851.
[26] P.M. Cupit, M.L. Steinauer, B.W. Tonnessen, L.E. Agola, J.M. Kinuthia, I.N. Mwangi, M.W. Mutuku, G.M. Mkoji, E.S. Loker, C. Cunningham, Polymorphism associated with the Schistosoma mansoni tetraspanin-2 gene, Int. J. Parasitol. 41 (12) (2011) 1249–1252.
[27] J.X. Peng, P.Y. Wei, X.L. Chen, D.G. Zeng, X.H. Chen, Identification of cold responsive genes in Pacific white shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridization, Gene 575 (2) (2016) 667–674.
[28] T. Adell, V. Gamulin, S. Perovic-Ottstadt, M. Wiens, M. Korzhev, I.M. Muller, W.E.G. Muller, Evolution of metazoan cell junction proteins: the scaffold protein MAGI and the transmembrane receptor tetraspanin in the demosponge Suberites domuncula, J. Mol. Evol. 59 (1) (2004) 41–50.
[29] T.A. Hopf, L.J. Colwell, R. Sheridan, B. Rost, C. Sander, D.S. Marks, Three-dimensional structures of membrane proteins from genomic sequencing, Cell 149 (7) (2012) 1607–1621.
[30] M. Seigneuret, A. Delaguillaumie, C. Lagaudriere-Gesbert, H. Conjeaud, Structure of the tetraspanin main extracellular domain - a partially conserved fold with a structurally variable domain insertion, J. Biol. Chem. 276 (43) (2001) 40055–40064.
[31] C.S. Stipp, Functional domains in tetraspanin proteins (vol 28, pg 106, 2003), Trends Biochem. Sci. 28 (3) (2003) 124-124.
[32] S.C. Todd, V.S. Doctor, S. Levy, Sequences and expression of six new members of the tetraspanin/TM4SF family, Bba-Gene Struct Expr 1399 (1) (1998) 101–104.
[33] S.K. Tiwari-Woodruff, A.G. Buznikov, T.Q. Vu, P.E. Micevych, K. Chen, H.I. Kornblum, J.M. Bronstein, OSP/claudin-11 forms a complex with a novel member of the tetraspanin super family and beta 1 integrin and regulates proliferation and migration of oligodendrocytes, J. Cell Biol. 153 (2) (2001) 295–305.
[34] L. Zhang, L.L. Wang, J.M. Zhao, L.M. Qiu, L.S. Song, C.H. Dong, F.M. Li, The responsive expression of heat shock protein 22 gene in zhikong scallop *Chlamys farreri* against a bacterial challenge, Aquacult. Res. 41 (2) (2010) 257–266.
[35] A.E. Ellis, Innate host defense mechanisms of fish against viruses and bacteria, Dev. Comp. Immunol. 25 (8–9) (2001) 827–839.
[36] T.T. Priyathilaka, S.D.N.K. Bathige, H.M.L.P.B. Herath, S. Lee, J. Lee, Molecular identification of disk abalone (*Haliotis discus discus*) tetraspanin 33 and CD63: insights into potent players in the disk abalone host defense system, Fish Shellfish Immunol. 69 (2017) 173–184.
[37] B. Wang, F. Li, J. Xiang, L. Gui, Z. Luo, H. Yan, Three tetraspanins from Chinese shrimp, *Fenneropenaeus chinensis*, may play important roles in WSSV infection, J. Fish. Dis. 33 (1) (2010) 15–29.
[38] B. Morga, I. Arzul, N. Faury, A. Segarra, B. Chollet, T. Renault, Molecular responses of *Ostrea edulis* haemocytes to an in vitro infection with *Bonamia ostreae*, Dev. Comp. Immunol. 35 (3) (2011) 323–333.