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Molecular characterization and functional analysis of Japanese flounder (*Paralichthys olivaceus*) *thbs2* in response to lymphocystis disease virus

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ABSTRACT

In mammals, a matricellular protein, thrombospondin 2 (Thbs2) has been reported to play important roles in modulating cell-matrix interactions, vascular integrity and thrombosis formation. However, the role of gene, *thbs2* has not yet been studied in teleost. In the present study, this novel fish gene from Japanese flounder was cloned and its function in resistant to lymphocystis disease virus was elucidated. The Japanese flounder *thbs2* encoded a 1176-amino acid protein with 91% identity to medaka. Amino acid sequence indicated that Japanese flounder Thbs2 contained 10 typical conserved domains. The *thbs2* was expressed in all stages of embryo development, and in hatched larva stage, its expression was significantly higher than that in other stages ($P < 0.05$). The relative expression level of *thbs2* was significantly higher in the head kidney, liver, blood, gill, and heart of the lymphocystis disease virus resistant fish than in sensitive fish ($P < 0.05$); and in muscle, this difference was at highly significant ($P < 0.01$). Additionally, the distribution of Thbs2 in tissue was evaluated by immunohistochemical staining. Subcellular localization analysis showed that Thbs2 was distributed throughout the cytoplasm of the cells. Taken together, our results provide new basic data for *thbs2* function, especially its role in anti-lymphocystis disease virus immune response.

1. Introduction

Thrombospondin 2 (Thbs2) is a matricellular protein that belongs to thrombin-sensitive family, and plays important roles in modulating cell-matrix interactions, as well as in vascular integrity and thrombosis formation [1,2]. Thbs2 could mediate the interaction between cell-cell, cell-matrix, regulate cell apoptosis, cell proliferation and cell adhesion [3]. Thbs2 also plays a role in anti-angiogenesis by inhibiting cell migration in the process of microangiogenesis in most tumor tissues [4]. The expression rate of *thbs2* was significantly lower in gastric cancer tissues than that in normal gastric mucosa. Additionally, Thbs2 promotes the down-regulation of microRNA-376c by MAPK signaling pathway, which is a new target of research in prostate cancer [5]. At present, as a potent angiogenesis and tumor growth inhibitor, Thbs2 has been identified as prognostic marker in several cancers such as gastric, rectal, urothelial and bladder urothelial cancers and lung adenocarcinoma. However, the function of *thbs2* in immune response, especially in virus infection has not been reported yet.

Japanese flounder (*Paralichthys olivaceus*) is one of the important marine species in China. Their peculiar characteristics includes fast growth, succulent meat, high nutritional content and suitability for intensive industrial cultivation. In 1997, lymphocystis disease virus (LCDV) first appeared in the cultured Japanese flounder in China, and gradually became one of the most important diseases endangering the Japanese flounder culture industry. Several studies have been conducted to understand the nature of LCDV and the associated disease [6–12]. The genome sequence of virus isolated from Japanese flounder in China was determined, that consisted of 186250 base-pairs with 240

Abbreviations: LCDV, lymphocystis disease virus; ORFs, open reading frames; AF, after fertlization; CIK, *Ctenopharyngodon idellusere* kidney; qRT-PCR, Quantitative real time PCR; PFA, paraformaldehyde

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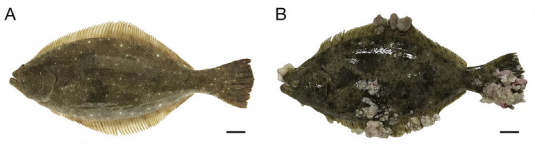

Fig. 1. Lymphocystis disease virus resistant fish and sensitive fish of Japanese flounder (*Paralichthys olivaceus*). (A) Lymphocystis disease virus resistant fish; (B) Lymphocystis disease virus sensitive fish. Bar depicts 4 cm.

potential open reading frames (ORFs) [13]. The initial infection is followed by inhibition of apoptosis, cell division before enlargement, hypertrophy due to cell fusion and encircling of hyaline capsule associated with the alteration of collagen fibers for lymphocystis cell formation [8]. In Japan, a microsatellite marker, *Poli9-8TUF* was selected as a candidate locus for maker-assisted breeding of LCDV-resistant Japanese flounder [10,11]. In a transcriptome analysis of head kidney of LCDV-resistant and -sensitive fish, we found that *thbs2* was highly expressed in LVDV-resistant fish (unpublished data). Thus, we speculate that, *thbs2* may play a role in resistance to LCDV after recovery from infection.

In the present paper, we cloned cDNA *thbs2* of Japanese flounder, expression levels of mRNA in different tissues of LCDV-resistant and -sensitive fish; as well as, various stages of embryo development were evaluated. We also studied immunohistochemistry and the subcellular localization. The results of our study provide new insights on function of *thbs2*, and mechanism of resistance to LCDV in Japanese flounder.

2. Material and methods

2.1. Ethics

All experiments were approved by and, performed in accordance of regulations and guidelines of the Animal Care and Use Committee, Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences (No. BCES2018-02).

2.2. Fish and cells

The Japanese flounder were cultured at Changli culture base of Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences. That culture base was a high incidence area of LCDV. The LCDV challenge culture began on October 12, 2015 and ended on June 3, 2016. During the culture, the temperature and salinity of water were maintained at 13–23 °C and 10–27‰. At the end of culture, three LCDV-resistant (Fig. 1A) and three LCDV-sensitive (Fig. 1B) fish were sampled with following criteria: if granular cysts were observed on the surface or gill of a fish by eyes, the fish would be identified as a LCDV-sensitive individual; if granular cysts were not observed on the surface or gill, the fish would be determined as a LCDV-resistant individual. The blood, gill, liver, head kidney, gut, gonad, muscle, heart and spleen of each fish were quickly sampled frozen in liquid nitrogen for 48 h, and stored at - 80 °C until use.

In order to collected different development stages samples of Japanese flounder embryo, there batches of normal fertilization were conducted using different female and male fish with 16.0 ± 0.5 °C sea water. The fertilized eggs (0 h after fertilization, 0 h AF), 4 cell (2 h AF), 32 cell (4 h AF), 128 cell (5 h AF), high blastocyst (6 h AF), low blastocyst (11 h AF), early gastrula (15 h AF), late gastrula (27 h AF), sarcomere (32 h AF), heartbeat (55 h AF) and hatched larva (62 h AF) were quickly sampled frozen in liquid nitrogen for 48 h, and stored at - 80 °C until use.

Ctenopharyngodon idellusere kidney cells (CIK) were grown in Medium 199 (Gibco, USA) supplemented with 10% fetal bovine serum (BI, China), 1% Antibiotic-Antimycotic (Gibco, USA), and cultivated at 28 °C without CO_2.

2.3. Cloning of thbs2 and bioinformatics

According to the genome sequences of Japanese flounder [14,15] and head kidney transcriptome, the full length cDNA of Japanese flounder *thbs2* was cloned using RACE method with primers as listed in Table 1. The homology analysis of *thbs2* was performed using BLAST program. ExPASy translate program (https://web.expasy.org/translate/) was used for amino acid sequence analysis; Molecular weight and theoretical isoelectric point were calculated by Compute pI/Mw software (http://web.expasy.org/protparam/). NCBI Conserved Domain program (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) was used for prediction of protein structure; TMHMM server v.2.0.0.0 was used for analysis of amino acid transmembrane region (http://www.cbs.dtu.dk/services/TMHMM-2.0/); Multiple sequences alignment was performed using ClustalX1.83 software and the data was edited using END script program [16]. Neighbor-Joining (NJ) phylogenetic tree (bootstrap 1000) was constructed using MEGA X software [17].

2.4. Quantitative real time PCR (qRT-PCR)

To understand the relative expression pattern of *thbs2* in different stages of embryo development, and in different tissues of LCDV-resistant and -sensitive Japanese flounder, total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. The qRT-PCR assay was performed using Power SYBR Green PCR Master Mix (ABI, USA) in an ABI 9700 PCR. Each assay was performed in triplicate with cycling condition: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The β-actin was used as reference gene, and the $2^{-\Delta\Delta Ct}$ method was used to analyze the expression levels [18].

2.5. Immunohistochemical staining

Gill, heart, head kidney and liver of LCDV-resistant and -sensitive fish were fixed with 4% paraformaldehyde (PFA) by incubating for

Table 1
Primers information for cloning and qRT-PCR of *thbs2* in Japanese flounder (*Paralichthys olivaceus*).

Primer	Sequence (5′-3′)	Application
thbs2-5′RACE-F1	TGCTGGCCACGAACACAAAGC	5′RACE
thbs2-5′RACE-F2	CTCACTGGGGGGATGTGGTCG	
thbs2-3′RACE-F1	GATTGGGCTACTGGCACTG	3′RACE
thbs2-3′RACE-F2	CAGTGATTGTCTGAGGAGGAA	
thbs2-RTF	CTCCCAAAGCAAACTTGACACA	qRT-PCR
thbs2-RTR	GGATCAAGGCACATGGGAAT	
thbs2-INF	TCTCGAGCTCAAGCTTATGATACTCAGGAGAAGTCTCTTCTTGCTGCTC	pEGFP-*thbs2*
thbs2-INR	GGCGACCGGTGGATCCCATCTTGTGAACACTGTCGGCAACTCCGGC	
β-actin-F	AGGGAAATCGTGCGTGACAT	*β-actin* qRT-PCR
β-actin-R	GCCCATCTCCTGCTCGAA	

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gttgcgcctggagtcctgcagactgtggatgtagtggaccaacgtggactttacgcgttttacgcacgaggttttctgagggagacaccacttttcatccatttcat
catcggttcaggtcactacagatctgaacttgaactttaaaaaaaataaaaagatattgtcggatttcagagtgattaaaaaagctgcattcgtcaggagagATGATA
                                                                                                        M  I
CTCAGGAGAAGTCTCTTCTTGCTGCTCTTATCATTTAACTTCCTCCATGCATTATCTGAAGATGGTGAGCAGGAGGACGAGACATCGTTCGACTTGTTTGAAATTAGC
 L  R  R  S  L  F  L  L  L  L  S  F  N  F  L  H  A  L  S  E  D  G  E  Q  E  D  E  T  S  F  D  L  F  E  I  S
CACATCTCACGTAAAACTCTGGGGGCCAAACAGTTCCGGGGTCAAAACTCAGACATCCCTGCCTACCGCTTCATCCGCTTCGACCACATCCCCCCAGTGAGCCCCCCC
 H  I  S  R  K  T  L  G  A  K  Q  F  R  G  Q  N  S  D  I  P  A  Y  R  F  I  R  F  D  H  I  P  P  V  S  P  P
ATATTAAAACAAATACTACAACAGATCCAAAACAACGAGGGCTTTGTGTTCGTGGCCAGCATACGGCAGGACCGTGCCTCGAGGGGCACCCTGATTGCTTTGGAGGGC
 I  L  K  Q  I  L  Q  Q  I  Q  N  N  E  G  F  V  F  V  A  S  I  R  Q  D  R  A  S  R  G  T  L  I  A  L  E  G
CCTGATGGCCGGCGCCAGTTTGAGATCGTGTCCAACGGACGAGCCAACACTCTGGACCTGGTGTACTGGGTGGATGGCTCACAGAATGTGGTATCGTTCGAGGACGTG
 P  D  G  R  R  Q  F  E  I  V  S  N  G  R  A  N  T  L  D  L  V  Y  W  V  D  G  S  Q  N  V  V  S  F  E  D  V
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 D  L  S  D  S  Q  W  K  N  I  T  L  H  V  H  G  E  N  A  N  L  F  V  G  C  S  P  I  D  S  F  I  L  D  E  P
TTCTACGAGCACCTGCGGGCTGAGGGAAGCCGCATGTATGTTGCAAAGGGATCCATCCGAGAGAACCACTTCAGGGGCCTTCTGCAGAATGTGCGTTTCATCTTTGAC
 F  Y  E  H  L  R  A  E  G  S  R  M  Y  V  A  K  G  S  I  R  E  N  H  F  R  G  L  L  Q  N  V  R  F  I  F  D
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 T  P  V  E  D  V  L  L  T  R  D  C  E  A  T  K  Q  D  D  A  N  I  V  S  E  S  T  E  I  V  D  V  S  P  S  I
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 T  T  N  I  I  G  Q  K  T  D  E  T  G  A  D  M  C  E  R  S  C  E  E  L  S  T  M  F  Q  E  L  K  G  L  R  V
GTCGTCAGTAACCTTATTGATGGCCTGCAAAAAGTGACAGAGGAGAACACACTCATGAAGGAGGCCCTTGGGAGGATTAAGAACCCTAAAGAGAAAAACATGTGCTGG
 V  V  S  N  L  I  D  G  L  Q  K  V  T  E  E  N  T  L  M  K  E  A  L  G  R  I  K  N  P  K  E  K  N  M  C  W
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 Q  D  G  R  L  F  D  D  K  E  D  W  V  V  D  S  C  T  K  C  T  C  Q  E  S  K  I  V  C  H  Q  I  T  C  P  P
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 V  A  C  A  T  P  S  F  V  D  G  E  C  C  P  V  C  L  P  K  Y  S  E  D  G  W  S  P  W  S  E  W  T  E  C  T
GTCACCTGCGGGACAGGAACTCAACAGAGGGGTCGGTCATGTGATGCAACCAGCAACCCTTGCACCGGACCCTCGATCCAGACCCGCAAGTGCAGCCTAGGCAAATGT
 V  T  C  G  T  G  T  Q  Q  R  G  R  S  C  D  A  T  S  N  P  C  T  G  P  S  I  Q  T  R  K  C  S  L  G  K  C
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 D  S  R  V  R  Q  D  G  G  W  S  L  W  S  P  W  S  S  C  S  V  T  C  G  E  G  Q  I  T  R  I  R  H  C  N  A
CCCGTGCCGCAGCTGGGGGGCAAAGACTGCGAAGGAAGTGGACGGGAGACTCAGCGCTGTACCACAGAGCCATGTCCCGTGGATGGTGGTTGGGGTCCTTGGTCTCCA
 P  V  P  Q  L  G  G  K  D  C  E  G  S  G  R  E  T  Q  R  C  T  T  E  P  C  P  V  D  G  G  W  G  P  W  S  P
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 W  A  T  C  S  A  T  C  G  G  G  V  R  S  R  T  R  E  C  N  S  P  H  P  Q  Y  G  G  K  K  C  I  G  E  A  D
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 G  P  C  P  A  G  F  R  G  N  G  T  H  C  E  D  I  N  E  C  D  M  V  P  D  V  C  F  K  V  S  G  S  P  R  C
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 E  P  E  N  P  C  K  E  K  T  H  N  C  H  R  F  A  E  C  I  Y  I  S  H  F  N  D  P  M  Y  K  C  E  C  R  T
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 G  L  F  V  F  S  Q  E  L  V  F  F  S  D  L  K  Y  E  C  R  D  N  *
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tctcttcatgtttttcatcctgaatgtgttttataacggagaacttgagttaacaatctaaataagccattttagtaagtgccaaaaaaaaaaaaaaaaaa
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Fig. 2. The nucleotide and amino acid sequences of Japanese flounder (*Paralichthys olivaceus*) *thbs2*. An asterisk represents the stop codon, and the lowercase for noncoding region in cDNA.

24 h at 4 °C, and then stored in methanol at −20 °C. The fixed tissues were sliced into 5 μm thickness layers using paraffin section. The slides were then rehydrated, incubated with 3% hydrogen peroxide in dark for 25 min, and blocked using 3% BSA in PBS for 30 min. Primary rabbit anti-human Thrombospondin 2 antibody (ab84469, Abcam, UK) (with consistency of 83.17% to residues 724–824 of Japanese flounder Thbs 2) was used at a concentration of 10 μg/mL. Slides were incubated with primary antibody at 4 °C, overnight. After that, and then washed thrice with PBS and incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (GB23303, Servicebio, China). The DAB kit (G1211, Servicebio, China) was used for immunohistochemical (IHC) staining. After IHC staining, the slides were counterstained with hematoxylin, mounted with neutral balsam, and observed under microscope.

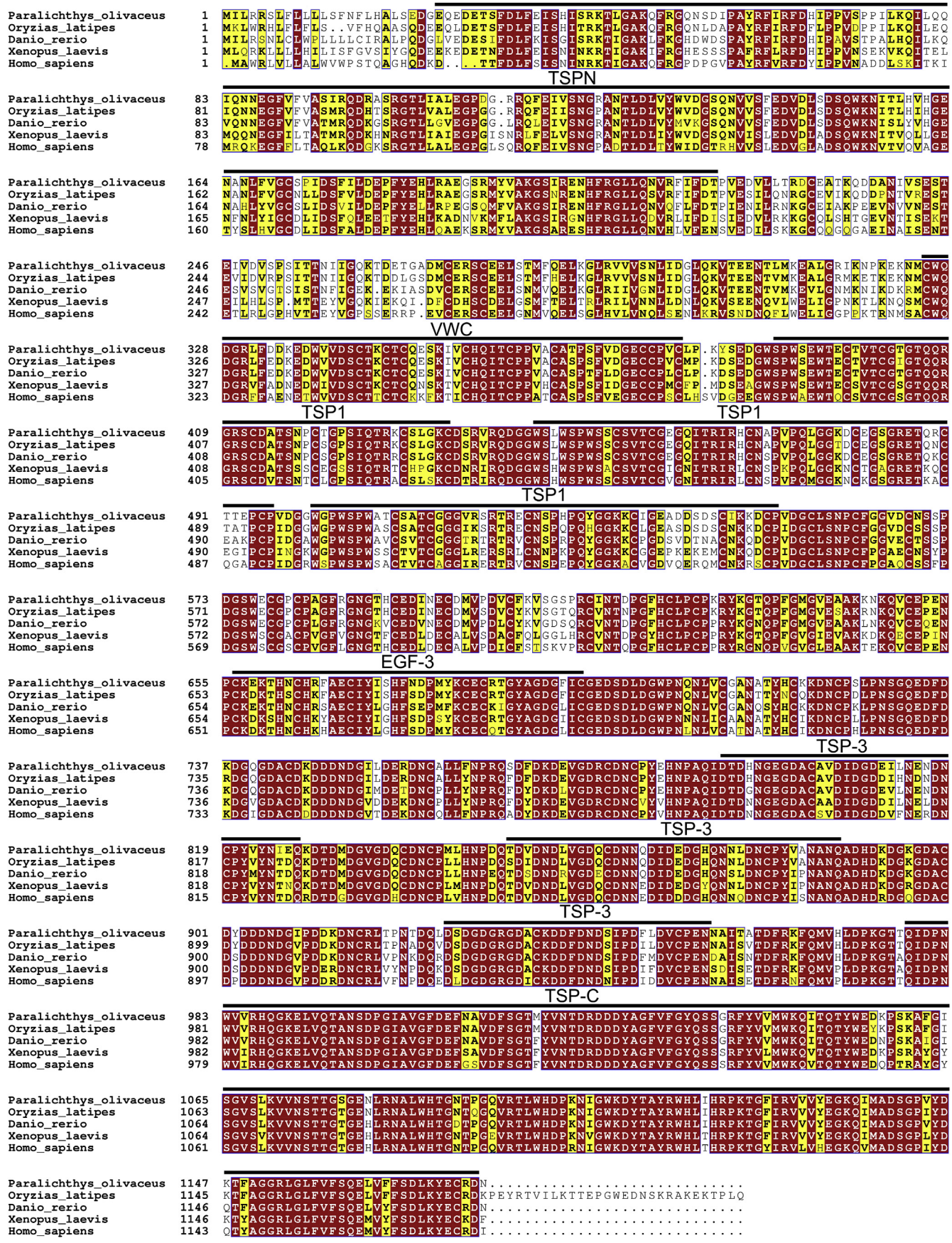

Fig. 3. Sequence alignment of Thbs2 homologs in different species. The conserved domains, including thrombospondin N-terminal -like domains, von Willebrand factor type C domain, thrombospondin type 1 repeats; EGF domain, thrombospondin type 3 repeat, and thrombospondin C-terminal region were indicated above the sequences.

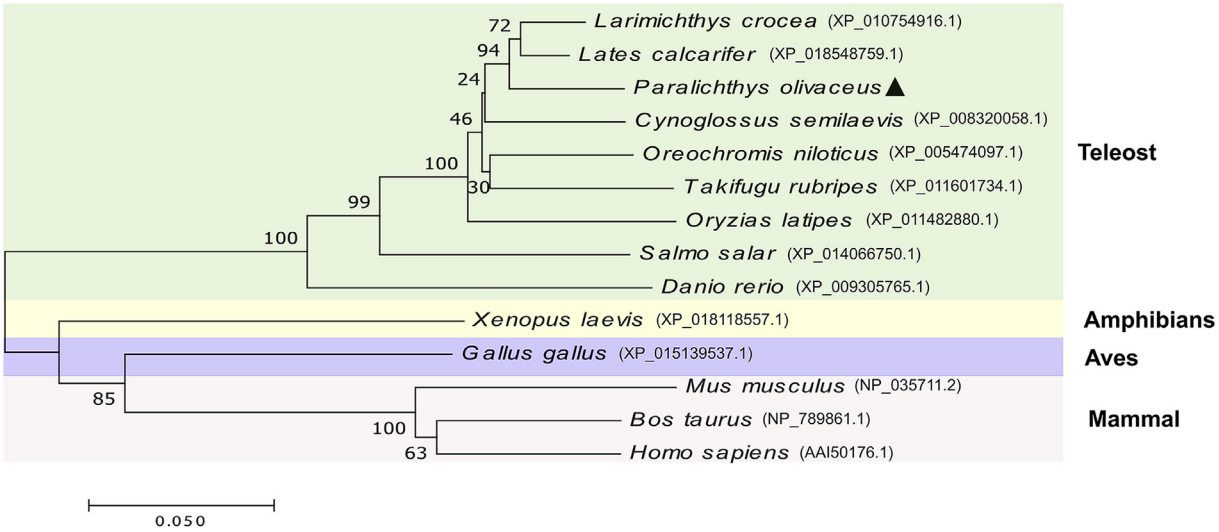

Fig. 4. Phylogenetic analysis of Thbs2 using neighbor-joining method based on the amino acid sequences of Thbs2 homologs from different species. Bootstrap values are shown at the nodes.

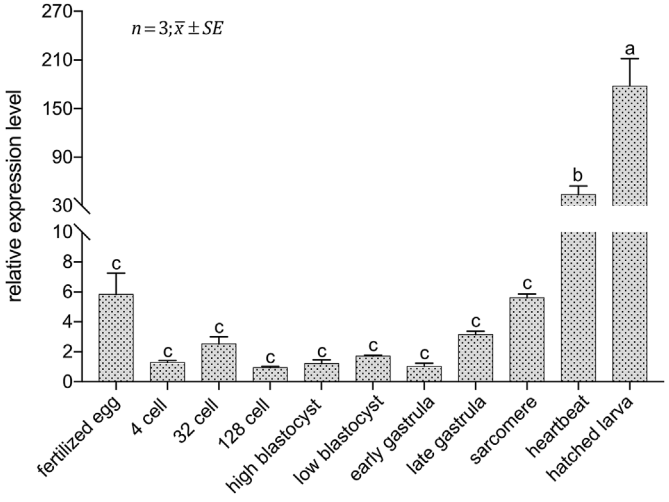

Fig. 5. The relative expression level of Japanese flounder (*Paralichthys olivaceus*) *thbs2* in different stages of embryo development.

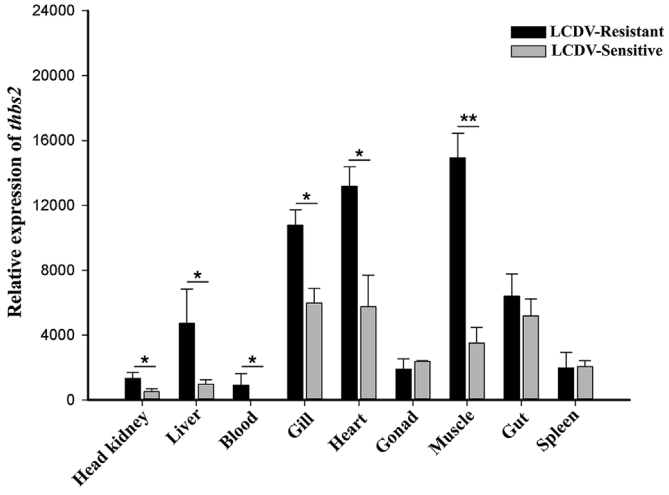

Fig. 6. The relative expression level of Japanese flounder (*Paralichthys olivaceus*) *thbs2* in different tissues of lymphocystis disease virus -resistant and -sensitive fish. * indicates significant difference ($P < 0.05$), **indicates significant difference ($P < 0.01$).

2.6. Plasmid construction

To uncover the molecular function of Thbs2, the CDS region of *thbs2* gene was amplified by RT-PCR using the primers as listed in Table 1 and purified with agarose gel DNA extraction kit (9762, Takara, Japan). The pEGFP-N1 vector was double enzyme digested by *Hin* III/*Bam* HI, and then purified (9762, Takara, Japan). Finally, the pEGFP-*thbs2* plasmid was constructed by mixing purified target fragment and pEGFP-N1 according to the protocol of In-Fusion HD Cloning Kit (639650, Clontech, USA). The constructed plasmid was subsequently confirmed by Sanger sequencing.

2.7. Cell transfection

Cell transfection was carried out using Lipo8000 transfection reagent (Beyotime, China) according to the manufacturer's instruction. CIK cells were pre-plated in 12-well plates one day prior to transfection and seeded to be 70–80% confluent. 1 μg plasmid and 0.8 μL Lipo8000 was mixed and diluted by 50 μL Opti-MEM (Gibco, USA). Then 400 μL of Opti-MEM and 50 μL diluted mixture were added to cells and incubated for 24 h. Next, the medium was replenished and cells were cultured for another 12 h.

2.8. Subcellular localization

After the used medium was removed, the transfected cells were washed with 1xPBS and fixed with 4% PFA for 10 min and washed twice with 1xPBS. Subsequently, the cell nucleus was dyed with DAPI for 10 min and washed 5 times with 1xPBS in dark. Then the cells were observed under fluorescence microscope.

3. Results

3.1. Sequencing characterization of thbs2

The Japanese flounder *thbs2* cDNA was 4313-bp in length, containing a 3528-bp ORF, 210-bp 5′ untranslated region (UTR) and 575-bp 3′UTR with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail (Fig. 2). The ORF encoded 1176-aa protein with predicated molecular weight of 130.77 KDa and theoretical isoelectric point of 4.57. The Japanese flounder Thbs2 protein shared 91% identity with medaka (*Oyzias latipes*), 85% with zebrafish (*Danio rerio*), 75% with African clawed frog (*Xenopus laevis*), and 73% with human (*homo*

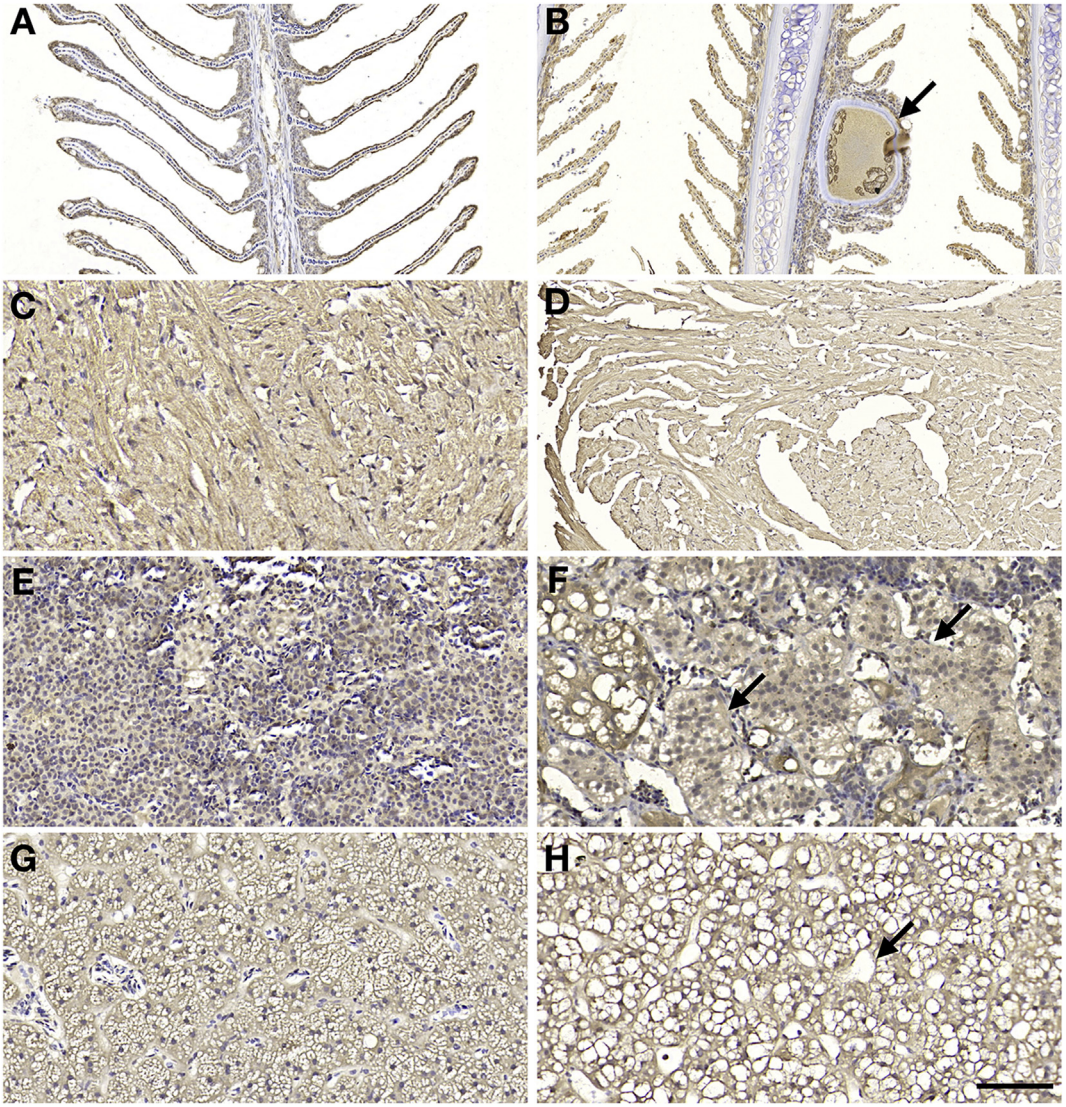

Fig. 7. Immunohistochemical staining of different tissues from Japanese flounder (*Paralichthys olivaceus*) lymphocystis disease virus -resistant (LCDV-R) and -sensitive (LCDV-S) fish. A: gill of LCDV-R fish; B: gill of LCDV-S fish, arrow indicates lymhocystis cell; C: heart of LCDV-R fish; D: heart of LCDV-S fish; E: head kidney of LCDV-R fish; F: head kidney of LCDV-s fish, arrows indicate necrotic epithelial cells of renal tubules; G: liver of LCDV-R fish; H: liver of LCDV-S fish, arrow indicates hepatocyte with vacuolation. Bar depicts 50 μm.

sapiens).

Amino acid alignment indicated that Japanese flounder Thbs2 protein contained 10 highly conserved domains, including thrombospondin N-terminal-like domains (TSPN, 25–219 aa), von Willebrand factor type C domain (VWC, 325–379 aa), thrombospondin type 1 repeats (TSP1; 389–434 aa, 444–496 aa and 501–553 aa); EGF domain (EGF-3, 656–695 aa), thrombospondin type 3 repeat (TSP-3; 793–827 aa, 851–888 aa and 926–955 aa), and thrombospondin C-terminal region (TSP-C, 978–1175 aa)(Fig. 3). No transmembrane region was detected in Japanese flounder Thbs2. Phylogenetic analysis indicated that Japanese flounder Thbs2 was closely associated with large yellow croaker (*Larimichthys crocea*) and Barramundi (*Lates calcarifer*). All the Thbs2 from teleost were clustered in a group, which was separated from amphibians, aves, and mammals (Fig. 4).

3.2. Expression pattern of thbs2 in embryo development

The expression pattern of Japanese flounder *thbs2* in different stages of embryo development was analyzed by qRT-PCR. The expression of *thbs2* were detected in all the analyzed stages. However, the relative expression levels of *thbs2* from newly fertilized egg to sarcomere stage were not significantly different ($P > 0.05$). After the sarcomere stage, this relative expression level increased sharply, with highest level observed in hatched larva stage, that was significantly different than all other stages ($P < 0.05$) (Fig. 5).

3.3. Tissue expression of thbs2 in LCDV-resistant and -sensitive fish

Fig. 6 shows the expression of *thbs2* mRNA in all analyzed tissue samples of LCDV-resistant and -sensitive fish. Among which, the relative expression level of *thbs2* in head kidney, liver, blood, gill and heart was significantly higher in LCDV-resistant fish than in sensitive fish ($P < 0.05$). In muscle, the difference of *thbs2* relative expression level was highly significant in LCDV resistant than sensitive fish ($P < 0.01$); however, in gonad, gut and spleen, this expression was not significant.

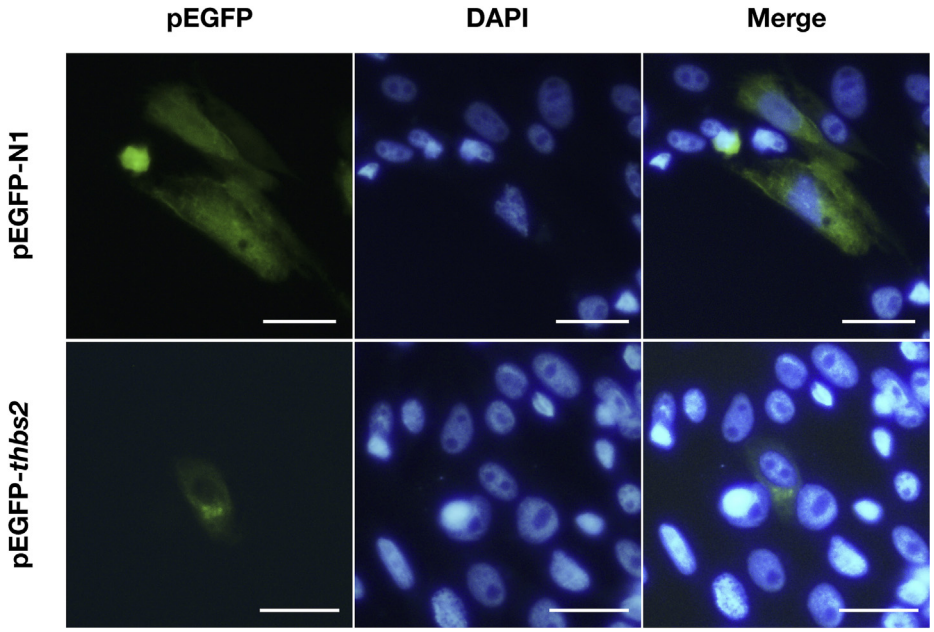

Fig. 8. Subcellular localization of THBS2 in *Ctenopharyngodon idellusere* kidney cells (CIK). The CIK cells were first transfected with pEGFP-N1 or pEGFP-*thbs2*, and then stained with DAPI. Cells were observed under fluorescence microscopy. Bar depicts 20 μm.

3.4. Immunohistochemical staining of Thbs2 in LCDV-resistant and sensitive tissues

We observed the morphological changes and assessed Thbs2 expression in gill, heart, head kidney, and liver from LCDV-resistant and -sensitive fish. The Thbs2 expression was detected in all evaluated tissues. The LCDV resistant group showed normal appearance of gill, heart, head kidney, and liver (Fig. 7A, C, E, G). However, the morphological changes were observed in appearances of LCDV sensitive group due to the virus infection. In the gill lamella of LCDV-sensitive fish, a lymphocystis cell with diameter around 50 μm was observed (Fig. 7B). The heart of LCDV-sensitive fish showed server pathological changes including shortening of myocardial fibers and enlargement of interstitial voids between myocardial fibers (Fig. 7D). Epithelial cells of renal tubules in the head kidney of LCDV-sensitive fish were swollen, vesicular, karyopyknotic, and necrotic (Fig. 7F). In the liver of LCDV-sensitive fish, the hepatocytes showed edema and vacuolation, however, no obvious enlarged cells were observed (Fig. 7H).

3.5. The subcellular localization of Thbs2

To determine the subcellular localization of Thbs2 *in vitro*, pEGFP-N1 and pEGFP-*thbs2* plasmids were transfected into CIK cells and the nucleus were counter stained with DAPI. As shown in Fig. 8, the green fluorescence was observed both in cytoplasm and nucleus of pEGFP-N1 transfected cells. Contrastingly, green fluorescence was detected only in cytoplasm of pEGFP-*thbs2* transfected cells. Thus, we proposed that Thbs2 was a cytoplasmic protein.

4. Discussion

Defects in expression of *thbs2* is known to cause abnormalities in collagen fibrillogenesis, fragility of skin, and laxity of tendons and ligaments in mouse [3]. Polymorphisms in Thbs2 were associated with lumbar disk herniation and lumbar spinal stenosis in the Japanese and Korean population, as well as progression of intervertebral disc degeneration in the Chinese Han population [19]. However, *thbs2* has not been studied in teleost.

In the present study, using RACE method, we cloned the full length of Japanese flounder thbs2 which encoded a 1176-aa protein with 91% identity with medaka. Like mammalian Thbs2, Japanese flounder Thbs2 consisted of 10 typical conserved domains. Thbs2 was expressed in all development stages of Japanese flounder embryo and was highly expressed in tissues such as liver, gill, heart, and muscle. Additionally, we found that Japanese flounder Thbs2 was localized in the cytoplasm of CIK cells.

The thrombospondin type 3 repeats, together with the EGF domain (thrombospondin type 2 repeats) and thrombospondin C-terminal region, constitutes the most conserved portion of thrombospondin [20]. The thrombospondin type 3 repeats are aspartate rich, and constitute the calcium binding domain of thrombospondin. Several active sequences including the cell adhesion RGD sequence, the binding sites for neutrophil elastase and cathepsin G [21], and attachment sites for neutrophils [22] and sickle red blood cells [23] were mapped in this domain. Another important domain in Thbs2 were thrombospondin type 1 repeats, which were reported could interact with matrix metalloproteinase 9 (MMP9), transforming growth factor (TGF) β and the membrane protein CD36 [24].

LCDV can spread horizontally between fishes through skin contact and external trauma, and the gill and skin are the primary targets of LCDV invasion [25,26]. Contrarily, LCDV can enter the digestive tract with food and then proliferate in tissue of stomach, muscles, and liver. Next, LCDV can enter the blood circulation by binding to receptors on the peripheral leukocyte and infect other tissues and organs [27–29]. In this study, the expression level of Thbs2 was significantly up-regulated in the gill and muscle of the LCDV-resistant fish, implying its role in immune regulation against LCDV infection. The up-regulation of Thbs2 expression in the liver may be because Thbs2 contains several structural domains that could resist to protease digestion, and bind macromolecular of liver heparin and fibrinogen [3]. When the fish is subjected to stress by external stimuli, the expression of *thbs2* may be activated via this macromolecular medicated signal transduction.

Thbs2-deficient mice exhibit connective tissue abnormalities, associated with collagen fiber formation disorders and abnormal enlargement, increased vascular density with hemorrhagic properties, skin fragility, and reduced tensile strength. Additionally in *in vitro* culture, the fibroblasts from the Thbs2-deficient mice skin were found to accumulate on the surface of plastic or glass culture dishes containing

bacteria [30,31]. Yang et al., proposed that the decreased expression of *thbs2* leads to the increased expression of matrix metalloproteinase 2, causing adhesion dysfunction of fibroblasts [32]. Therefore, Thbs2 is not only identified as an anti-angiogenic agent, but also as a powerful fibroblast inhibitor. This function of *thbs2* in fibroblasts may also play role in the immune response to LCDV infection in Japanese flounder.

The major target organ of LCDV invasion is the connective tissue under the skin. When LCDV continuously proliferates in cells of connective tissue, fibroblasts gradually expand and become rounded. When diameter of the cell grows greater than 20 μm, it implies the formation of lymphocystis cells [33]. The typical feature of lymphocystis cell is a hyaline capsule covered with collagen fibers [8,34,35] that elongates with the increase in time of infection [34]. Therefore, we propose that the Thbs2 plays an anti-LCDV role by inhibition of fibroblast, which is a critical for the formation of lymphocystis cells.

5. Conclusion

In the present study, the *thbs2* gene was cloned in teleost for the first time, and its function in response to LDCV was preliminary investigated. Based on results obtained, we propose that the Thbs2 plays an anti-LCDV role by inhibition of fibroblast, which is a critical for the formation of lymphocystis cells.

Declaration of interest

The authors have declared that no competing financial interests exist.

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