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RESEARCH PAPER

Transforming Growth Factor β1 Gene Play a Novel Role in Innate Immune Response in *Pelteobagrus fulvidraco*

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Abstract

Transforming growth factor-beta 1 (TGF-β1) as a member of TGF-β superfamily plays crucial roles in regulation of immune responses. Yellow catfish, *Pelteobagrus fulvidraco*, is one of the most important freshwater aquaculture species in China, but little is known about its genes related to immune response. The present study cloned cDNA encoding TGF-β1 from *P. fulvidraco*. The results showed the full-length cDNA of TGF-β1 is 2,816 bp and encodes 461 amino acids. The homology of *P. fulvidraco* TGF-β1 depicted 83% and 81% similarity to the TGF-β1 in *Stegastes partitus* and *Pundamilia nyererei* respectively. The phylogenetic tree construction revealed that *P. fulvidraco* is closely related to *Astyanax mexicanus*. Analysis of mRNA expression of TGF-β1 in different tissues (gonads, brain, liver, kidney, spleen, intestine, blood, gills, muscle and heart) revealed that TGF-β1 is predominantly expressed in liver and brain, followed by gill and spleen. TGF-β1 gene in gill and spleen up-regulated for 48h after *Edwardsiella ictaluri* and *Flavobacterium columnare* injection, then the expression showed a significant decrease ($p<0.05$). These results indicated that TGF-β1 contributes to the inherent immune reaction of *P. fulvidraco*.

Keywords: *Pelteobagrus fulvidraco*, TGF-β1, Inherent immune reaction.

Introduction

The identification and characterization of fish cytokine and immune-regulatory genes in recent years have allowed numerous research on the diseases which are related to the genes. Thus, a number of studies on the expression of immune-regulatory genes in fish infected with various bacterial, viral as well as parasitic pathogens, and their involvement in specific disease processes have been performed (Border & Noble, 1994; Li *et al*., 2015; Wu & Hill, 2009). Most of the immune-related cells are regulated by transforming growth factor-β (TGF-β) directly or indirectly, such as in the emergence of diseases, participation stress signal transduction and cell response (Gorelik & Flavell, 2000; Li *et al*., 2015).

Recent research focused on TGF–β family in aquatic animals includes four isomers, TGF-β1, TGF-β2, TGF-β3 and TGF-β6. Similar to mammals, TGF-β1 in fish can also function as an immunosuppressive mediator. For example, goldfish TGF-β1 inhibits nitric oxide production in TNF-γ-activated macrophages (Dabiri *et al*., 2008). The full-length cDNA sequence of TGF-β1 in many fish species have been cloned (Kohli *et al*., 2003; Haddad *et al*., 2008; Hardie *et al*., 1998; Mu & Hong, 2008; Tafalla *et al*., 2003; Yin & Jimmy, 2000). Blocking TGF-β1 in immune cells can cause chronic inflammation associated with diseases such as fibrosis (Bridle *et al*., 2006). Expression of TGF-β1 increased significantly in rainbow trout *Oncorhynchus mykiss* after infection of amoebic gill disease and *Aeromonas salmonicida* (Border & Noble, 1994; Tafalla, Coll, & Secombes, 2005); and TGF-β1 expression in Atlantic salmon immune organs also increased when infected with salmon louse (Skugor, Glover, Nilsen, & Krasnov, 2008). TGF-β1 has function to inhibit trout macrophage respiratory burst activity as well as leukocyte-derived macrophage-activating factor in head-kidney (Jang, Hardie, & Secombes, 1994; Jang, Hardie, & Secombes, 1995). However, the precise regulatory roles of TGF-β1 under the bacterial infection remain unknown in yellow catfish.

The host resistance against bacterial or viral infection is a way to detect immune responses, and the broadly used tests nowadays are based on the

measurement of the inhibitory influence on survival or mortality after infection with virulent strains of bacteria (Kotha, Frazier, Welply, Segarini, & Grotendorst, 1997; Shibanuma, Mashimo, Kuroki, & Nose, 1994). In recent decades, polymerase chain reaction (PCR) has become an important method for the rapid, sensitive and specific detection of immune response after different bacterial infection. Bacteria *Edwardsiella ictaluri* and *Flavobacterium columnare* are two common pathogens of cultured fish. Enteric septicemia of catfish caused by *E. ictaluri* infection is responsible for approximately 50% of economic losses to catfish farmers in the United States (Shoemaker, Klesius, Evans, & Arias, 2009). Albeit yellow catfish is the most susceptible species to *E. ictaluri* infection, bacterium *F. columnare* is the causative agent of bacterial cold water disease. Previous studies have demonstrated that when fish were infected with *F. columnare* the pathogen could be detected from mucus, skin as well as jaws by electron microscopy (Martinez, Casado, & Enriquez, 2004).

Yellow catfish restricted to freshwater habitats has become a favorite food fish due to its excellent meat quality; and has become an important freshwater aquaculture species due to its high commercial value (Sun, Zhong, Huang, & Yang, 2014). However, many diseases have become the restricted factor for catfish farming development. Therefore, the aims of this study were to: (1) clone the TGF-β1 cDNA sequence from immune organs; (2) compare its deduced amino acid sequence with other known TGF-β1; (3) analyze TGF-β1 mRNA expression from different adult organs; (4) compare the expression changes after infection. Our results provided the basic information to identify the function of TGF-β1 and to research the molecular mechanism of immune responses in *P. fulvidraco*.

Materials and Methods

Fish

Two-year-old *P. fulvidraco* (Female: 14.5±2.4 cm length, 43.6±1.8 g fresh mass; Male: 23.5±2.5 cm length, 100.6±2.8 g fresh mass) were obtained from the Chinese Academy of Fishery Sciences *Pelteobagrus* sp. Breeding Engineering Center, China. They were acclimated in a 500 L circular fibreglass tank for 2 weeks. During acclimation *P. fulvidraco* were fed minced trash fish at 3% of body weight twice daily (8:00 and 16:00), feces and uneaten feed were collected using a siphon tube about an hour after feeding. Then left them unfed for 24 hours before the experiment to reduce stress during fish translocation. Fish were killed on ice using anaesthesia (MS 222, Sigma Inc.). During acclimation and the experiment fish were subjected to natural photoperiod (12 h light:12 h dark), dissolved oxygen in seawater (salinity 31‰) was maintained above 6.0 mg L^{-1}, temperature at 25±0.5°C, and total ammonia level was less than 0.05 mg L^{-1}.

TGF-β1 Cloning and Sequence Analysis

Total RNA from various yellow catfish organs was extracted with Trizol reagent (Invitrogen, USA) following the manufacturer's instruction. RNA concentration was spectrophotometrically determined by measuring the absorbance at 260 nm, and the integrity of 28S and 18S rRNA was checked by electrophoresis. Total RNA was then reverse-transcribed into cDNA using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's instructions. cDNA was then used as the template to amplify. PCR primers of TGF-β1 were designed according to TGF-β1 of *Ietalurus Punetaus* in Genbank (KT852370) (Table 1).

The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, and primer extension at 72°C for 10 min. Each of the 25 μL reaction volume contained 5 ng of cDNA, 12.5 μL 2X reaction buffer, 0.2 mM dNTP, 10 pM of each primer and 0.5 U of Taq DNA polymerase (TaKaRa, Japan). Then the gene was subcloned into the vector pMD-18T (TaKaRa). Positive clones containing inserts of an

Table 1 The primer used for cloning and tissue expression of TGF-β1 gene in the experiment

Primer	Sequence (5'-3′)	°C T_m	bp size
TGF-F1	ACCGCTTATCCGAACTTCTC	56	1289
TGF-R1	GGATACCGGTTGCTGCAAAG	56	
TGF-F2	TCATTTTCTACCGCTTATCCGA	58.5	300
TGF-R2	TGAGCAGCAGTGGGTTTAGG	58.5	
TGF-F3	GTGAATGTGAACCGGGGAAC	56	1243
TGF-R3	GCCACGCTACACTGTTATATTGT	56	
TGF-F4	TGCATACACACCTCAGCAGA	59	687
TGF-R4	AAGTAATCCGGCTCGCAGTA	59	
TGF-F5	TGCATACACACCTCAGCAGA	56	178
TGF-R5	GCAGCAAGTGGTCAAGTTCA	56	
Actin-F	CCACCTTCAACTCCATCA	60	223
Actin-R	TACCACCAGACAGAACAG	60	

expected size were sequenced using M13 primers and sequenced by BGI (Shanghai, China).

Nucleotide and deduced amino acid sequences of TGF-β1 cDNA were analyzed using the software Vector NTI Advance 11 (Life Technologies, Grand Island, NY, USA). TGF-β1 sequences from different organisms were obtained from NCBI. Full-length cDNA sequences of *P. fulvidraco* was assembled using DNAMAN 6.0 software (version 5.0, Lynnon Biosoft, Quebec, Canada). The comparison of cDNA sequences of TGF-β1 to their genomic sequences is accomplished by analyzing the exonic sequences structure. BLAST software (www.ncbi.nlm.nih.gov) was used to obtain the TGF-β1 exonic structures. A Multiple sequence alignment was created by the ClustalW program (www.ebi.ac.uk/clustalw/).

Phylogenetic Analysis

ClustalW was used to create multiple sequence alignments, while MEGA version 4.1. TGF-β1 sequence was applied to assess the similarity amongst the aligned sequences (www.ebi.ac.uk/clustalw/). A phylogenetic tree based upon the deduced amino acid sequences was constructed using the neighbor-joining (NJ) algorithm. Sequences of TGF-β1 in *Poecilia latipinna*, *Poecilia formosa*, *Xiphophorus maculatus*, *Fundulus heteroclius*, *Stegastes partitus*, *Pundamilia nyererei*, *Esox lucius*, *Salmo salar*, *Tiphophrus fulvidraco*, *Astyanax mexicanus*, *Sinocyclocheilus rhinocerous* and *Sinocyclocheilus grahami* retrieved from databases were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). Amino acid sequences with 1,000 bootstrapping replications were finished with the Molecular Evolutionary Genetics Analysis (MEGA4.0) package.

Tissue Expression of TGF-β1 Gene

Gonads, brain, liver, kidney, spleen, intestine, blood, gills, muscle and heart were separated aseptically. These tissues were then used for total RNA extraction with the RNAprep Tissue Kit (Omega Bio-tek, Doraville, GA, USA). 1 μg of total RNA was used for cDNA synthesis with the Superscript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) and stoRTF/stoRTR primers (Table 1).

Bacterial Challenge

Healthy male (100.6±2.8 g body weight, 23.5±2.5 cm total length) and female (43.6±1.8 g body weight, 14.5±2.4 cm total length) yellow catfish (2-year-old) were used for bacterial challenge. During bacterial challenge fish were kept at 26ºC in a flow-through system, and sterile water was used for the challenge. Fish applied in *E. ictaluri* challenge were divided into a control group (phosphate buffered saline, 100 μL PBS injected) and an *E. ictaluri* challenged group (injection). To inoculate bacteria for the challenge, a single colony of *E. ictaluri* was isolated and cultured in BHI broth at 27ºC overnight. The bacterial culture was diluted with PBS (pH 7.4), then 1×10^5 CFU of bacteria in 100 μL PBS were injected intraperitoneally into the channel *P. fulvidraco*.

In *F. columnare* challenge fish were also divided in each of two groups: control group (without bacteria), and bacterial challenge group that the fish were immersed with a dose of 3 ml of 107 CFUml-1 of *F. columnare* for 1 h. In an attempt to inoculate bacteria for challenge, a single colony of *F. columnare* had been isolated and cultured in BHI both at 27°C overnight. The spleen and gills from 24 individuals (three pools of 8 fish each) at 4 h, 48 h, 3 d and 7 d after *F. columnare* treatment were separated for RNA extraction. Corresponding uninfected control samples were taken at each time interval.

Statistical Analysis

Significant differences in gene expression levels between diseased and normally appearing fish organs were determined using the non-parametric Mann-Whitney U-test.

Results

Phylogenetic Analysis of the TGF- β1 Gene

A phylogenetic tree was constructed based on the amino acid sequences of selected TGF-β1 using the neighbor-joining (N-J) tree algorithm (Figure 1 and 2). Data were analyzed using Poisson's correction, and gaps were removed by complete deletion. *P. nyererei* and *S. partitus* TGF-β1 belonged to Perciformes; *P. latipinna*, *X. maculatus* and *P. formosa* TGF-β1 belonged to Cyprinodontiformes; *F. heteroclius* and *P. fulvidraco* TGF-β1 amino acids were on different branches. The homology similarity of TGF-β1 was 83% and 81% compared with *S. partitus* and *P. nyererei* respectively. The TGF-β1 gene shared high homology with three species: *A. mexicanus* (87%), *E. lucius* (82%) and *S. salar* (81%). These results indicated that the structure of TGF-β1 is highly conserved in both lower and higher teleosts. This speculation is consistent with the fundamental role in normal cell physiology.

Tissue Expression of TGF-β1 Gene

The expression of both male and female *P. fulvidraco* TGF-β1 in various tissues was examined by quantitative real-time PCR. TGF-β1 shows a high expression in gonads, brain, spleen, blood, gills, muscle and heart, and a relatively lower expression in

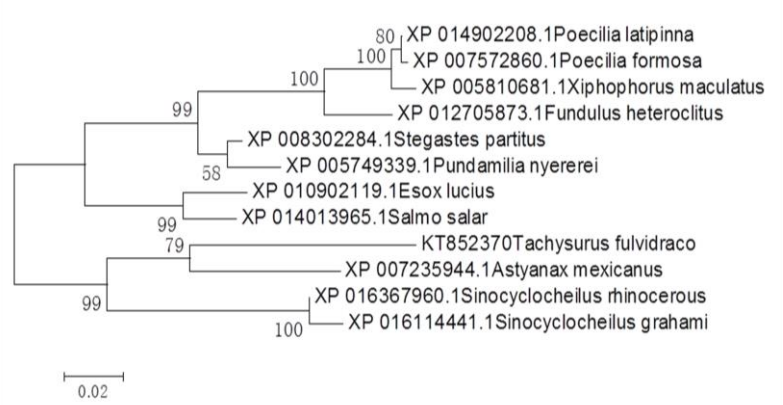

Figure 1 Alignment of amino acid sequence of TGF-β1 gene in *P. fulvidraco* and other 5 species fish. The identical, highly conserved, and less conserved amino acid resides were indicated by "*"、" :"、".".

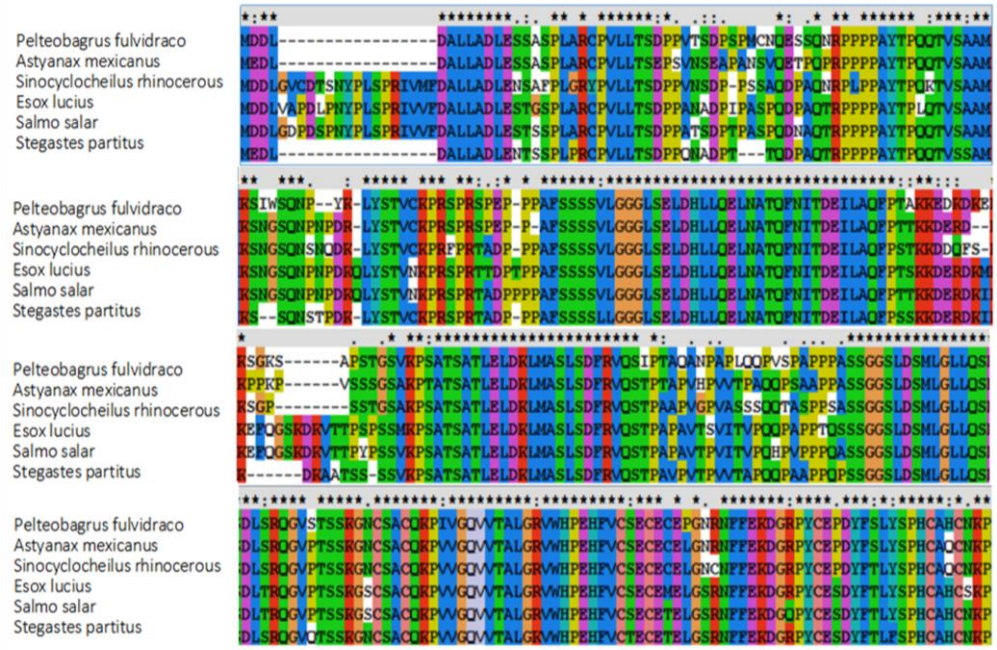

Figure 2 Phylogenetic tree of TGF-β1 amino acid sequences in *P. fulvidraco* from different species by maximum likelihood (NJ) analyses. The numbers on the branches are bootstrap values for NJ.

intestine and kidney and no expression was detected in liver (Figure 3). TGF-β1 expression in most tissues in male was clearly lower than in female except for gill and spleen.

Expression of TGF-β1 After Bacterial Challenge

To investigate whether TGF-β1 was involved in responses to disease infection with the catfish pathogen, qRT-PCR analysis was performed in infected spleen and gill and the time-dependent variations of TGF-β1 expression were shown in Figure 4.

As for the *E. ictaluri* infection group (Figure 4A), TGF-β1 in gills of both male and female appeared more sensitive than in spleen. In gills TGF-β1 expression up-regulated clearly in 4 h after *E. ictaluri* infection (3.38-fold), followed by a strong promotion (9.26-fold) within 48 h. Similar situation was also revealed in spleen where TGF-β1 expression exhibited an obvious increase in the first 48 h with an induction of 6.09-fold ($P<0.05$). Thereafter, TGF-β1 expression in all experimental organs decreased and only a slight variation was exhibited between the 3rd and 7th day ($P>0.05$).

As for the *F. columnare* infection group (Figure 4B), TGF-β1 in spleen of both male and female appeared more sensitive infection than in gills. TGF-β1 in spleen demonstrated a stronger response to *F. columnare* infection that the gene expression

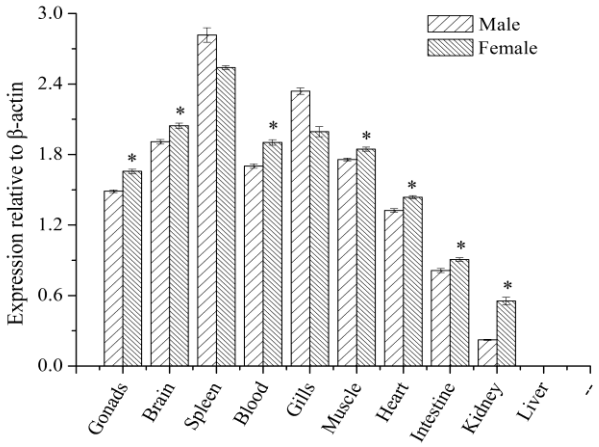

Figure 3 Tissue expression of TGF-β1 mRNA as determined by real time quantitative RT-PCR in 10 healthy tissues from male and female *P. fulvidraco* (heart, gill, muscle, kidney, brain, blood, intestate, gonad, liver and spleen). The bars indicate mean expression of 3 tested pools (8 fish each) ± SE. Specific expression of TGF-β1 relative to β-actin was analysed using densitometry.

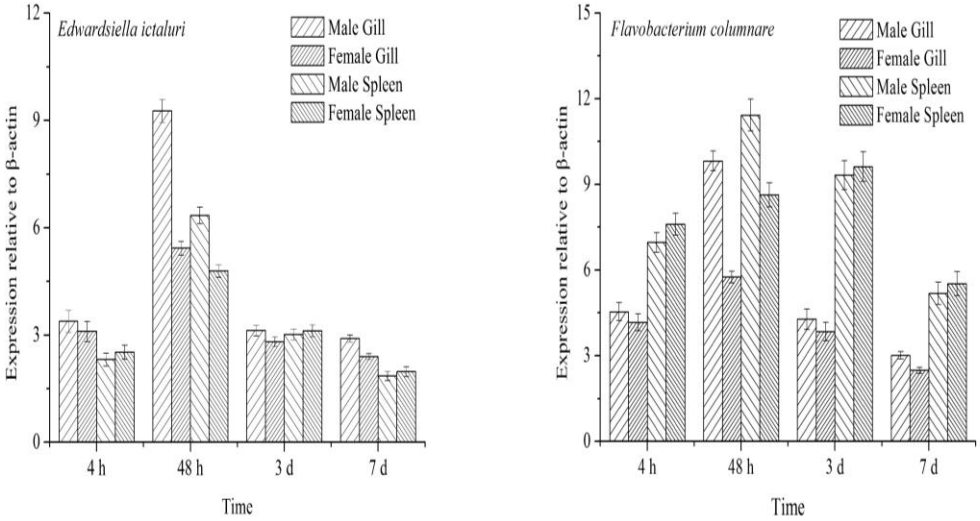

Figure 4 Gene expression of the two TGF-β1 genes as determined by real time quantitative RT-PCR at 4 h, 48 h, 3 d and 7 d after *Edwardsiella ictaluri* (A) or *Flavobacterium columnare* (B) infection in the spleen and gill tissue of both male and female *P. fulvidraco*. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Specific expression of TGF-β1 relative to β-actin was analysed using densitometry.

significantly up-regulated within 48 h after infection (11.45-fold) then decreased. Comparable phenomenon was also revealed in gills.

Discussions

Our present study has successfully cloned a TGF-β1 gene from *P. fulvidraco*. It can be seen that the TGF-β1 gene encodes a protein possessing high homology to other fishes, which implies the TGF-β1 genes are highly conserved. Meanwhile, TGF-β1 gene might have similar functions compared with other fishes. The expression level in our study differed from tissues, little or no expression was found in most of the digestive organs except for spleen; on contrary, high expression was found in other parts such as brain, gills and spleen. This result is similar to a previous research on trout that constitutive expression of TGF-β1 was found in brain, gills, spleen, head kidney and peripheral blood leucocytes except in liver (Li *et al*., 2015).

TGF-β1 is also the prototype for a superfamily of secreted proteins that control fish growth and development. In teleosts, some genes in TGF-β1 super family may control sex associated with growth (Li *et al*., 2015). Brain, muscle and other organs have

been identified greatly contributing to fish growth. In zebrafish, TGF-β1 may inhibit oocyte maturation (Kotha *et al*., 1997). It has been reported that male fish grows significantly faster than female in many species such as tilapia (Mair, Abucay, Beardmore, & Skibinski, 1995), common carp (Kocour, Linhart, & Gela, 2003) and yellow catfish (Wang, Mao, Chen, Liu, & Gui, 2009). Our results revealed that the TGF-β1 expression in male was lower than in female among most tissues except spleen and gills. Spleen as an important immune organ in teleosts generates a great amount of TGF-β that plays a key role in fish skeletal muscle development (Yang & Zhou, 2008; Funkenstein Olekh, & Jakowlew, 2010). High level of TGF-β1 expression also has been found in the gill of sea bream (*Sparus aurata*) (Tafalla *et al*., 2003).

According to our present research, TGF-β1 expression in gills of all experimental animals in *E. ictaluri* infection group was induced much stronger than in spleen, and the expression level in both gills and spleen experienced a clear increase within the first 48h after injection, then went down. The TGF-β1 expression in spleen of all experimental animals in *F. columnare* infection group was higher than in gills, while the expression tendency was similar to *E. ictaluri* infection group during the experimental seven days. This result illustrated a role for TGF-β1 in antibacterial defense that *E. ictaluri* and *F. columnare* induced TGF-β1 gene expression at early infection times post-infection; and with time went by the titer decreased. Such the situation was also seen in rainbow trout *O. mykiss* infected with viral haemorrhagic septicemia virus (Tafalla *et al*., 2005). Hence, TGF-β1 gene in yellow catfish can be considered a candidate to explore mechanism of growth rate.

The reason that induced TGF-β1 expression in terms of *E. ictaluri* was contrary to the expression considering *F. columnare* might be due to tissue specificity that gills and spleen are more sensitive to *F. columnare* than to *E. ictaluri*. In addition, TGF-β1 expression in males was significantly higher than in females at the first 2 days then the expression declined with insignificant difference in all fish, this might because the males in our study were more than twice heavier than the females that they would generate greater response than females.

TGF-β1 is expressed in all immune cells and its isoform are usually regulated precisely in response to stresses and diseases (Letterio & Roberts, 1998). A series of research have demonstrated that TGF-β1 was involved in innate immunity. After *Aeromonas salmonicida* infection, TGF-β1 expression in rainbow trout increased significantly (Border & Noble, 1994; Tafalla *et al*., 2005). After homes seabass infection, TGF-β1 expression in spleen was obviously increased (Scapigliati *et al*., 2010). Genes encoding cd8-a and tgf-b genes are over-expressed in spleen of trout after vaccination against *Y. ruckeri* (Raida & Buchmann, 2008). In our study, the immunity-involved gene TGF-β1 in the gill and spleen of diseased farmed *P. fulvidraco* were repressed compared to normally appearing species. The suggestion that gill and spleen are two main tissues for immune response is similar to a recent report on trout *O. mykiss* (Orieux, Douet, Le Hénaff, & Bourdineaud, 2013). As a well-known immuno-regulatory cytokine, TGF-β1 has a remarkable function in maintaining the immunological balance and re-establishing immune homeostasis by possibly down-regulating expression of other cytokines and cytokine-induced effect (Johnson, Harms, Levine, & Law, 2006). It is known that TGF-β1 at early stages of infection can facilitate CD8+T responses such as differentiation (Shibanuma *et al*., 1994) and IL-2 secretion (Swaing, Huston, Tonkonogy, & Weinberg, 1991). We therefore hypothesized the up-regulation of TGF-β1 was induced by inflammatory mediator increment such as IL-1b or might be aimed to restrict the inflammatory process (Lilleeng *et al*., 2009).

Conclusions

TGF-β1 in yellow catfish might have a function to resist to pathogens, and to become a key factor to regulate the growth between male and female. These findings illustrate the involvement and importance of the immune-regulatory gene TGF-β1. At a molecular level, future studies using *in situ* hybridization are highly required to identify the cellular origin of mRNA transcripts, whilst at a protein level further studies are necessary to examine the protein expression resulting from immune-regulatory genes. The characterization at a molecular level of TGF-β1 in teleosts constitutes a powerful tool to develop methods in order to study its function in immune system.

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