A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Comparative Molecular Characterization of Tumor Necrosis Factor alpha (TNFα) and Lipopolysaccharide Induced TNFα Factor (LITAF) genes from mullet *Liza haematocheila*

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DEPARTMENT OF MARINE LIFE SCIENCES

SCHOOL OF MARINE BIOMEDICAL SCIENCES

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(Supervised by Professor Jehee Lee)

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The thesis has been examined and approved by

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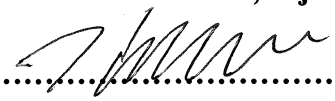

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Abstract

Mullets are commercially important fishes throughout the world. Even though, production from mullet farming is increasing in recent years, mass mortality associated with pathogenic infections causes heavy economic loss. For the success of Korean mullet aquaculture, comprehensive understanding of mullet immune system is essential for better health management. TNF alpha and LITAF genes are important immune genes studied from wide range of aquatic organism for better understanding. This study showed that TNF alpha and LITAF genes are expressed ubiquitously in various tissues of mullet and upregulated with pathogenic challenge. LITAF genes were showed to be participating in apoptosis. All three mullet LITAF genes induce capase-3 activity. These LITAFs also induce TNF alpha expression. These study showed that TNF alpha and LITAF are critical immune genes could be targeted for better disease control in mullet.

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1. Introduction

1.1 Global mullet fishery

Mullets (Family Mugilidae) are marine fishes which are globally distributed in coastal shallow water, including all temperate and tropical seas. There are some species spend part or whole life cycle in coastal lagoons, freshwater lakes and/or rivers. Mullets contribute substantially to the global fishery production.

Global mullet fishery production in 2015 by species

Fish Species	Capture production (tonnes)	Aquaculture production (tonnes)
Liza haematocheila	158, 964	N/A
Mugil cephalus	149, 631	15, 005
Mugil curema	6, 385	N/A
Liza aurata	2, 362	N/A
Mugil liza	2, 242	20
Liza ramada	1, 443	N/A
Chelon labrosus	713	N/A
Mugil soiuy	295	900
Chelon saliens	95	N/A

(Source: FAO Fishery and Aquaculture Statistics 2015)

Mullets have been traditionally cultivated in many countries for hundreds of years. It is reported that Mullets were cultivated in enclosures even in ancient Roman civilization. Even though,

traditional culture based mullet fishery has still been a significant contributor to fish protein in many part of the world, mullet farming has become popular in last few decades. While extensive and/or semi-intensive mullet farming has been practiced with stocking wild caught fries and fingerlings in countries such as Egypt, Israel, Italy, Korea, Hong Kong, Taiwan and Singapore, Commercial intensive aquaculture of mullet species have been promoted with aquaculture based seed production in Taiwan and Hawaii[1].

Mullet farming encounters challenges with disease outbreak partly because of infection from wild stock seeds or from wild mullets crowding in drainage channels of fish ponds. Meanwhile, pathogenic infections are one of the major constraints for commercial intensive aquaculture. Mullets are susceptible to a wide range of pathogens which includes, viruses, bacteria, fungi, protozoans and myxozoans. Several studies reported mortality associated with viral infection in mullet (iridoviral disease in *Mugil cephalus* and viral nervous necrosis disease in *Liza aurata* and *Chelon saliens)*. Several bacterial pathogens were reported to cause mortality to mullets. The Gram-negative bacterial pathogenic species included *Flavobacterium* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Vibrio* spp. The Gram-positive *Streptococcus* sp and L*actococcuss garvieae* were also notable pathogens infecting mullets. In addition, fungal pathogens such as *Ichthyophonus* sp, protozoans such as *Amyloodinium* sp, *Trichodina* sp and myxozoan species such as *Myxobolus* sp and *Kudoa* sp were other notable microparasite pathogens[2][3].

1.2 Korean mullet fishery and aquaculture

Mullet species *M. cephalus* and *L. haematocheila* have been successfully cultured in Korea. The previous studies identified infections of *Vibrio* spp., *L.garviae, Amyloodinium* sp and *Myxobolus* sp to mullets in Korea. While *Vibrio* and *L.garviae* were identified in cultured mullet *L.*

haematocheila, Amyloodinium sp and *Myxobolus* sp were isolated from wild *M. cephalus.* Out of these two bacterial pathogens recorded in *L. haematocheila, L.garviae* was recognized as a causative agent of mass mortality in several aquaculture farms[4][5].

An understanding of the immunology of the cultured species is important for better disease control. Growth in commercial aquaculture has demanded comprehensive understanding of immune system of the commercially important aquatic species. The understanding of the immune system of the target species are crucial for securing the optimum natural immune response of the fish through aquaculture conditions and the selection of fish stock and also for development and improvement of prophylactic measures such as vaccines and probiotics. Moreover, comparative immunology studies are important to understand the evolutionary aspects of structure and function of vertebrate immune system from fish to mammals. Hence, fish has become an excellent model organism for rervealing the evolution of adaptive immune system in vertebrates as well as crosstalk between innate and adaptive immune systems. Numerous immune related genes for both innate and adaptive immunity, involved in pathogen recognition, cytokine production, complement pathway, antimicrobial peptides, and certain cell membrane proteins, have been characterized from various fish species. *Oplegnathus fasciatus*[6], *Sebastes schlegelii*[7], and *Hippocampus abdominalis*[8] were among the commercially important aquatic species in Korea from which immune related genes were studied in last few years.

Even though, production from mullet farming is increasing in recent years, mass mortality associated with pathogenic infections causes heavy economic loss. For the success of Korean mullet aquaculture, comprehensive understanding of mullet immune system is essential for better health management.

1.3 Lipopolysaccharide induced tumor necrosis factor α factor

Lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF) α factor (LITAF) was first identified from human and characterized as a transcription factor which activates transcription of cytokines such as TNFα, interleukin-6, interleukin-1β in response to LPS. Mammalian LITAF was shown to be induced by LPS through a mechanism involving toll-like receptor 2 or 4 (TLR2 /4) and recruitment of the adaptor molecule MyD88. Upon stimulation, cytoplasmic LITAF and signal transducer and activator of transcription 6(B) (STAT6B) are phosphorylated by p38 alpha. Phosphorylated LITAF and STAT6B form a heterodimer which translocates into the nucleus and binds to the specific promoter to induce the expression of these cytokines[10].

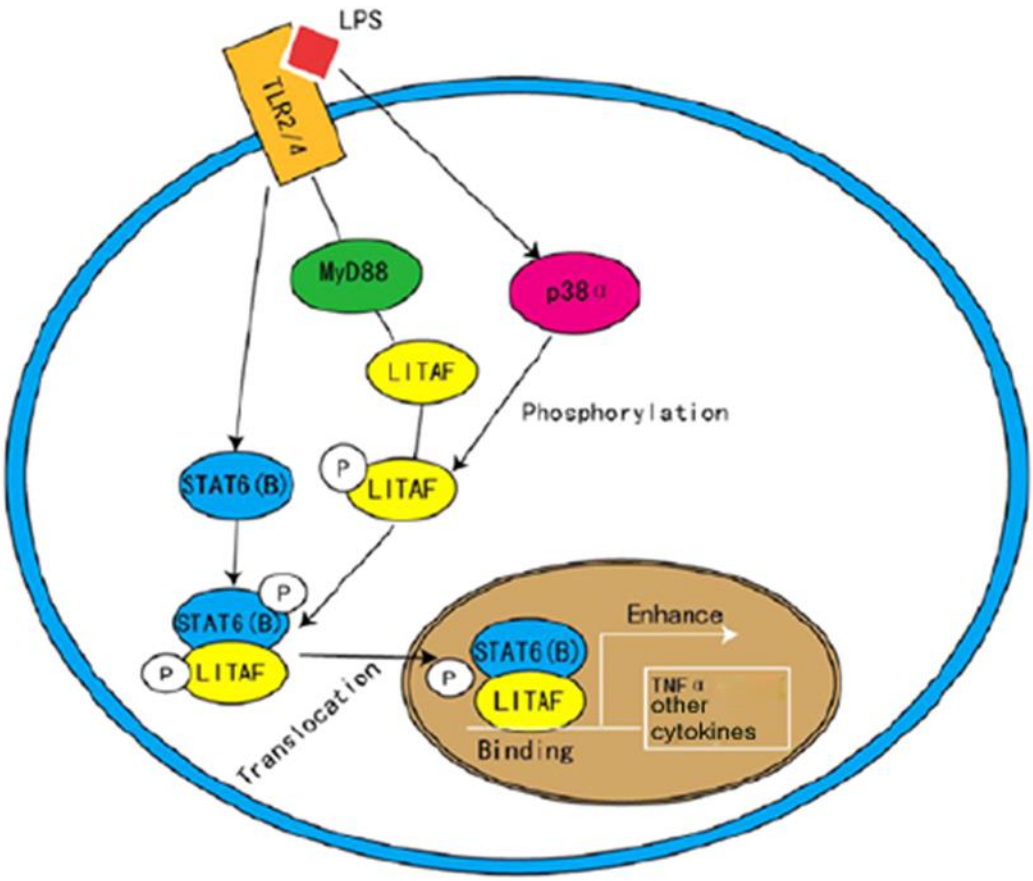

Fig.1 Regulatory mechanisms of cytokine expression by mammalian LITAF.

1.4 Tumor Necrosis Factor Alpha

TNFα is one of the ancient cytokine; the presence of functional homologues was reported from invertebrates. Teleost TNFα genes have been widely studied for their importance in immune defense and for evolutionary understanding. Previous studies showed that multiple TNFα isoforms are present in fish, which can be divided into three categories, the type I TNF-α, the type II TNF-α group and the TNF-N group. Moreover, some teleost fish such as, rainbow trout, Atlantic salmon goldfish and common carp were reported to possess multiple copies of type I or II TNF-α[9].

In cells, TNFα is present in two forms: Transmembrane TNFα and soluble TNFα. Transmembrane TNF-α has a precursor region, including the N-terminal intracellular domain and transmembrane region. The removal of the precursor region forms soluble TNF-α with the participation of the TNF-α-converting enzyme (TACE) that catalyze the cleavage of the proTNF-α protein at a specific site. Pathogenic infection in fish induce early TNF-α expression and involved in inflammation. Fish TNF-αs were shown to be involved in activation of macrophages/phagocytes to kill the microbes. In vitro treatment of primary trout headkidney leucocytes and monocytes/macrophages with TNF-α induced expression of several immune genes involved in inflammation, including IL-1β, IL-8, IL-17C, COX-2, and genes participating in antimicrobial defense. TNF-α is also involved with the NF-κB signaling pathway. Grass carp leucocyte cells treated with TNF-α showed enhanced NF-kB activity. Fish TNF-α protein enhances the phagocytic activity of leucocytes. In zebrafish infected with *Mycobacterium marinum*, TNF-α was shown to increase macrophage survival and also control bacterial growth in infected macrophages. Early studies suggested that Fish TNF-αs might be involved in the regulation of leucocyte proliferation and migration. Significant TNF-α expression was detected in trout thymus, and it could be involved in thymocyte growth. Trout TNF-αs also promote migration of primary headkidney macrophages. An in vivo

study showed that intraperitoneal injection of seabass with TNF-α results in rapid recruitment of phagocytic granulocytes to the peritoneal cavity [9].

Fish TNF-α is associated with pathogenesis of several chronic diseases. TNF-α, was shown to be induced in the salmonid heart during pathology of pancreas disease. Turbot fish infected with *Enteromyxum scophthalmi* had increased number of the TNF-α positive cells in the intestine which resulted in the infiltration of inflammatory cells, showing clinical signs such as development of the lesions, epithelial shedding and intestinal barrier dysfunction[9].

1.5 Aim of the study

The main objective of the study is to understand expression profile of mullet LITAF and TNFα genes and their interactions and involvement in apoptosis and immune related functions.

2. Methodology

2.1 Transcriptome library construction

The cDNA library of mullet *L. haematocheila* was constructed by *de novo assembly*. Briefly, five individuals of mullets were sacrificed and the total RNA was isolated from liver, spleen, headkidney, kidney, heart, intestine, stomach, brain eye, gill, skin, and blood tissues. The extracted RNA was then sent to Insilicogen, Korea where sequencing reactions were performed on a Pacbio platform.

2.2. Experimental fish and tissue collection

Adult mullet fish with an average body weight of 100 g were purchased from a fish farm (Sangdeok fishery) in Hadong, Jeju, and transported with aeration to the laboratory aquaria at Jeju National University. The fish were maintained in aerated water tanks at 20°C for a week for acclimatization prior to experiments. Five individuals were sampled for tissue distribution analysis. Tricaine Methanesulfonate (MS-222; 40 mg/L) was administered as anesthetic agent and blood was collected using sterile syringes treated with heparin sodium salt (USB, USA). Then, peripheral blood cells were isolated by centrifugation at 3,000×*g* for 10 min at 4°C. Tissue samples including, liver, spleen, head kidney, kidney, gill, heart, brain, muscle, intestine, stomach and skin were obtained, immediately snap-frozen in liquid nitrogen and then stored at −80°C.

For the immune challenge experiments, four aquatic tanks were stocked with 85 individual fish. Five unchallenged healthy fish were sampled for 0h control. Twenty fish from each tank were intraperitoneally challenged with LPS (1.25 μg/ μl), Poly I:C (1.5 μg/ μl), *Lactococus garvieae* (1×10^3 CFU/μl) and PBS, volume of 100 μl per each . Five individuals were sampled from each

experimental group at 6, 24, 48, 72 hours post-injection. Gill, spleen and head kidney samples were obtained following dissection.

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from a pool of tissue samples (n=5 for tissue distribution; n=5 for immune challenge) by RNAiso plus (Takara) following a clean-up with RNeasy spin column (Qiagen). RNA quality was determined by 1.5% agarose gel electrophoresis and the concentration was measured at 260 nm in μDrop Plate (Thermo Scientific). First-strand cDNA was synthesized from 2.5 μg of RNA with reaction mixture volume of 20 μl using PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara). The synthesized cDNA was further diluted 40-fold in nuclease-free water and stored in a freezer at −80 °C.

2.4 Expression analysis by quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out using a Thermal Cycler Dice™ TP950 (Takara) in a 10 μl reaction volume containing 3 μl of diluted cDNA template, 5 μl of 2× TaKaRa Ex Taq™ SYBR premix, 0.4 μl of each of the forward and reverse primer (10 pmol/μl) and 1.2 μl of H_2O. The qRT-PCR cycle profile included one cycle of 95°C for 10 s, followed by 45 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 20 s, and a final single cycle of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Each assay was conducted in triplicates to increase the credibility. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression. Mullet Elongation Factor 1 alpha (EF1α) was used as the internal control gene. All the data are presented as relative mRNA expression means ± standard deviation (SD). To determine statistical significance, the obtained data were subjected to statistical analysis and unpaired sample one-way analysis of variance (ANOVA) and *t*-test, using SPSS program.

2.5 Construction of the recombinant vector

Specific primers were designed for cloning mullet LITAF into pCDNA3.1+ and pEGFPN-1 vector and TNFα genes into pCDNA3.1+vector. The coding sequence of LITAF and TNFα genes were amplified with cDNA synthesized from tissues showing higher expression in tissue specific qPCR analysis. The PCR reaction was performed in a total volume of 50 µL containing 4 µL template, 5 µL 10 × Ex Taq buffer, 4 µL of 2.5 mM dNTP, 2 µL of each 10 pmol forward and reverse primers and 0.2 µL of 5 U of Ex Taq polymerase (Takara, Japan). The PCR condition was as follows: 1cycle of 94 ºC for 5 min, 30 cycle of 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 30 s and final extension of 72 ºC for 5 min for LITAF genes and 1cycle of 94 ºC for 5 min, 30 cycle of 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 45 s and final extension of 72 ºC for 5 min for TNFα genes. PCR products of *LITAF and TNFalpha genes* and plasmids (pCDNA3.1(+) and pEGFP-N1) were restriction digested with corresponding enzymes and gel purified using Accuprep^{TM} purification kit (Bioneer Co., Korea). Ligation reaction was performed using Mighty Mix (Takara, Japan) by incubation at 16 °C for 30 min. The recombinant vectors were transformed into *Escherichia coli* DH5α competent cells and positive clones were sequence confirmed with (Macrogen, Korea).

2.6 Subcellular localization analysis

For the subcellular localization analysis mullet kidney cells were cultured in Leibovitz's L-15 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 25 °C. Cells were seeded in 24-well plates at a cell density of 2×10^5 cells/well (80% confluence). One microgram of plasmids were transfected into mullet kidney cells using XtremeGENE™ 9 DNA transfection reagent (Roche, Germany) following the manufacturer's instructions. After 48 h, the

transfected cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were stained with 4, 6-diamino-2-phenylindole (DAPI) for 5 min and observed by fluorescence microscopy (Leica, Germany).

2.7 Hoechst staining.

To detect the effects of LhLITAF expression on the fish cells, FHM cells and mullet kidney cells were transfected with pcDNA-LhLITAF1, pcDNA-LhLITAF2, pcDNA-LhLITAF3 and pcDNA 3.1+ individually. Following transfection for 48 h, cells were washed with PBS, and then stained with Hoechst 33342 at a final concentration of 1 lg/ml to visualize nuclear morphology. The cells were observed under fluorescence microscopy.

2.8 Caspase-3 activity

FHM cells were seeded in 24 well-plate (2×10^5 cells/well), One microgram of plasmids were transfected into mullet kidney cells using XtremeGENE™ 9 DNA transfection reagent (Roche, Germany) following the manufacturer's instructions. After 48 h, the transfected cells were washed with cold PBS. Cells were lysed with 200 µL lysis buffer (CaspACE™ Assay System, Colorimetric) in ice and caspase-3 activity was performed following manufactures instruction.

2.9 TNFα mRNA expression analysis

For the mullet TNFα expression analysis, mullet kidney cells were seeded in 6 well plate (4×10^5 cells/well) and transfected with pcDNA-LhLITAF1, pcDNA-LhLITAF2, pcDNA-LhLITAF3 and pcDNA 3.1+ individually. Following transfection for 48 h, cells were lysed, mRNA was extracted and cDNA was synthesized as mentioned above. qPCR reactions were performed for TNFα isoforms and EF1α. Relative mRNA expression values were compared for pcDNA-LhLITAF1, pcDNA-LhLITAF2, pcDNA-LhLITAF3 and pcDNA 3.1+ transfections.

3. Results and Discussion

3.1 In silico analysis

Three LITAF genes and three TNF α genes were identified from the mullet transcriptome data base. The domains were analyzed for all the genes and discussed with multiple sequence analysis.

The LhLITAF1 gene consist of an ORF of 468bp encoding 155 amino acids. The length of LhLITAF2 sequence had an ORF of 450 bp encoding for 149 amino acids. The LhLITAF3 contained ORF of 552 bp encoding 183 amino acids (Fig 2). The LhTNF alpha was identified with an ORF of 750bp encoding 249 amino acids. Both LhTNFα2 isoform1 and LhTNFα2 isoform2 are same size with an ORF of 717 bp encoding 238 amino acids (Fig 3).

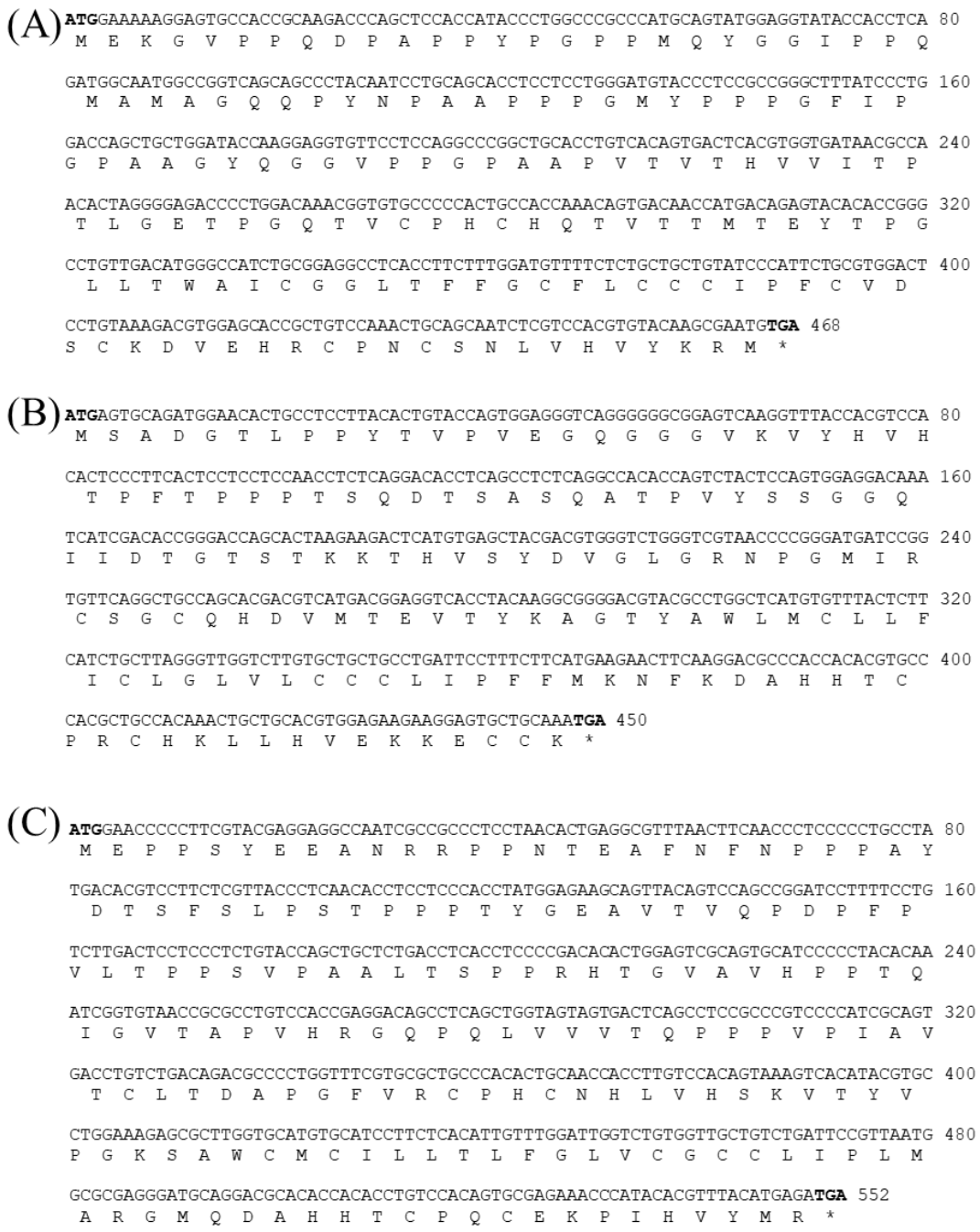

Fig2. The nucleotide and deduced amino acid sequences of LhLITAF genes (A) LhLITAF1, (B) LhLITAF2 and(C) LhLITAF (3).

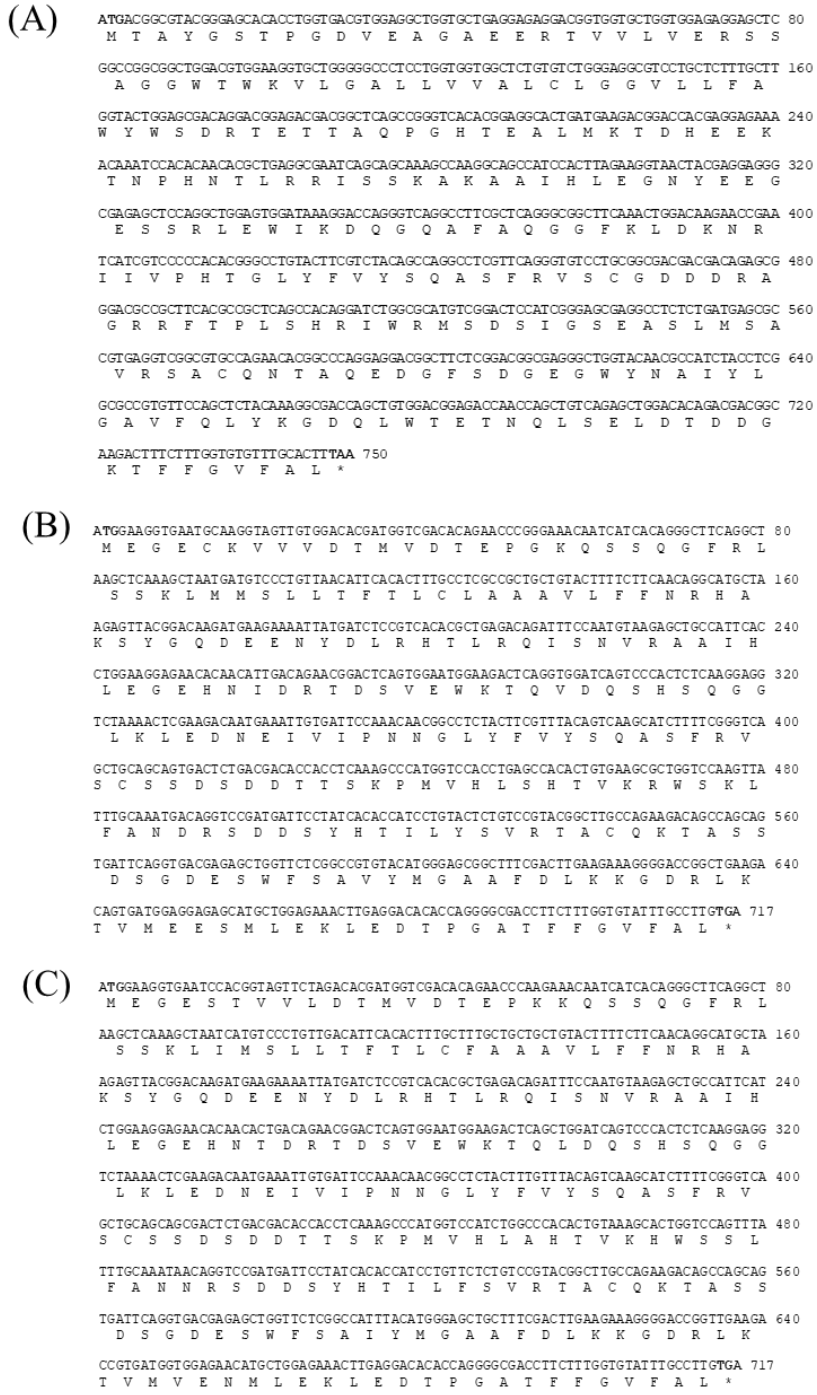

Fig3. The nucleotide and deduced amino acid sequences of LhTNFα genes (A) LhTNFα 1, (B) LhTNFα 2 isoform1and (C) LhTNFα 2 isoform2

3.2 Multiple sequence alignment

Multiple sequence alignment of LhLITAF genes with other fish LITAF genes showed that LITAF genes are less conserved in the N- terminal region than the C-terminal region containing LITAF domain. Nevertheless, the conserved LITAF domain of fish LITAF revealed that LITAF domains are conserved with strictly conserved CXXC motifs of the LITAF domain (Fig.4).

Fish TNFα genes are highly conserved (Fig.5). They possessed a transmembrane region, TACE cleavage site and TNF domain. TACE cleavage sites of fish TNFα were highly conserved. From the TACE cleave site to C-terminal region amino acid sequence are highly conserved which are the part of matured soluble TNFα form. Moreover, conserved cysteine residues in mammals involved in formation of tertiary structure is conserved in fish including mullet TNFα[9][11].

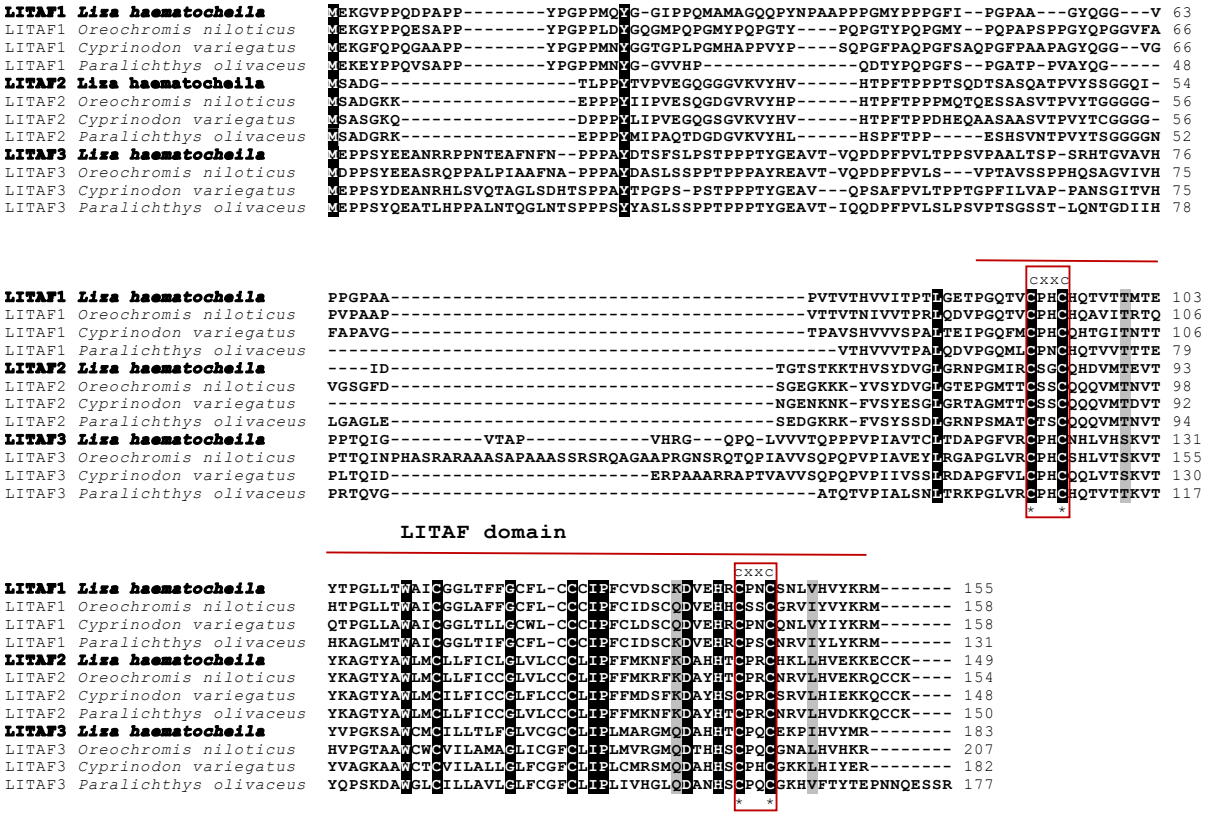

Fig.4. Multiple sequence alignment of LITAF genes

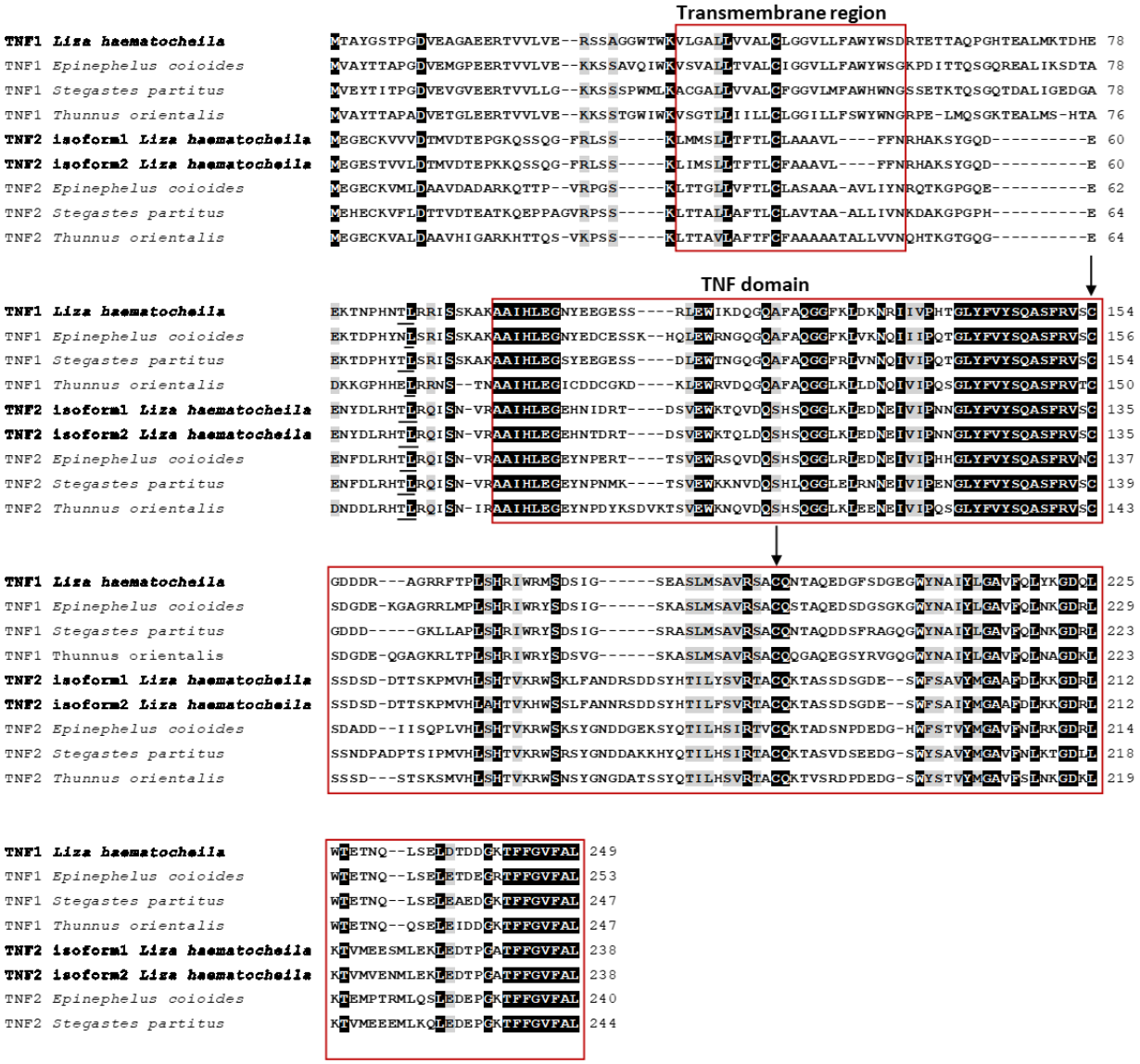

Fig.5. Multiple sequence alignment of TNFα genes

3.3 Phylogenetic analysis

Phylogenetic analysis revealed that fish LITAF genes are clustered together while other amphibians, reptiles, birds and mammals forming separate clusters. Fish LITAF genes were divided into 2 major clusters LITAF1 and another cluster contains both LITAF2 and LITAF3. Then LITAF2 and LITAF3 are divided further into subclusters. LITAF orthologs from Perciform fish *Oreochromis niloticus* showed closer relationship with LITAF from mugillid *L.heamatocheila* (Fig.6).

Phylogenetic analysis with selected TNFα orhologs showed vertebrate TNFα are divided in to two major clusters: fish TNFα and other tetrapod TNFα. Fish TNFα further divided into two sub clusters: type I TNFα and type II TNFα. The two type II TNFα isoforms from Liza haematocheila showed closer relationship with bootstrap value of 100% (Fig.7).

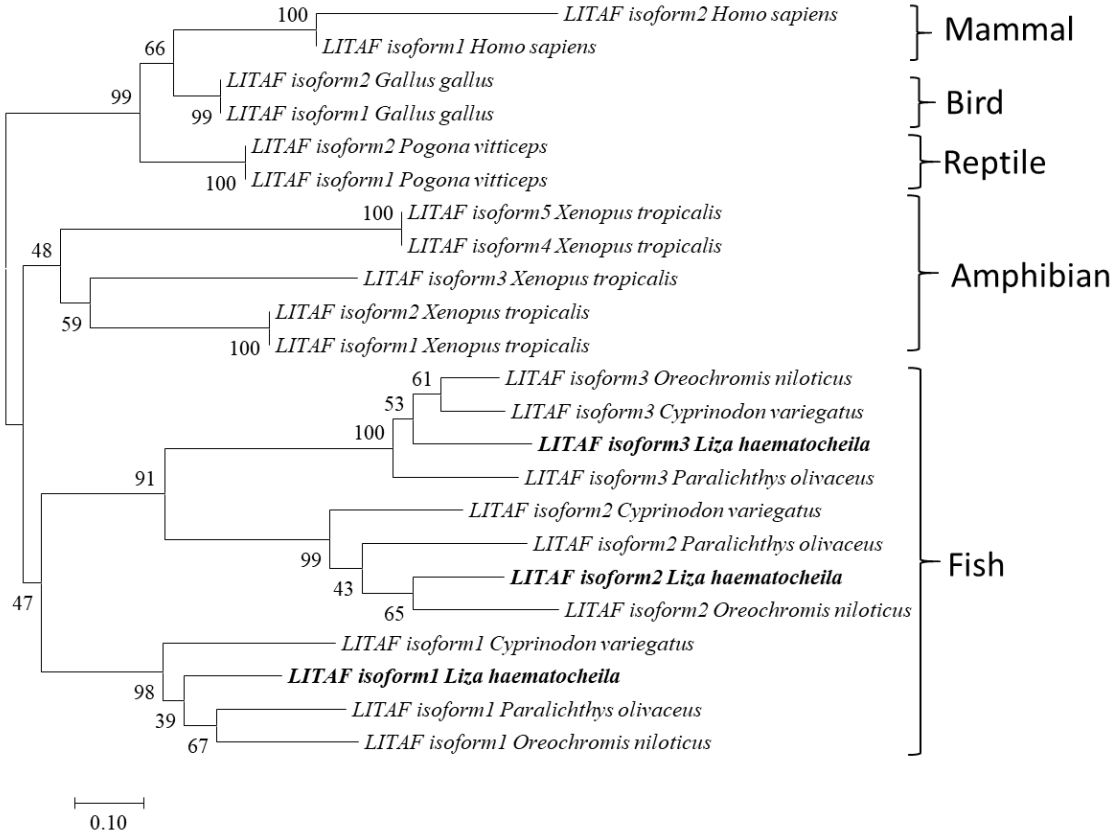

Fig.6. phylogenetic analysis of LITAF genes

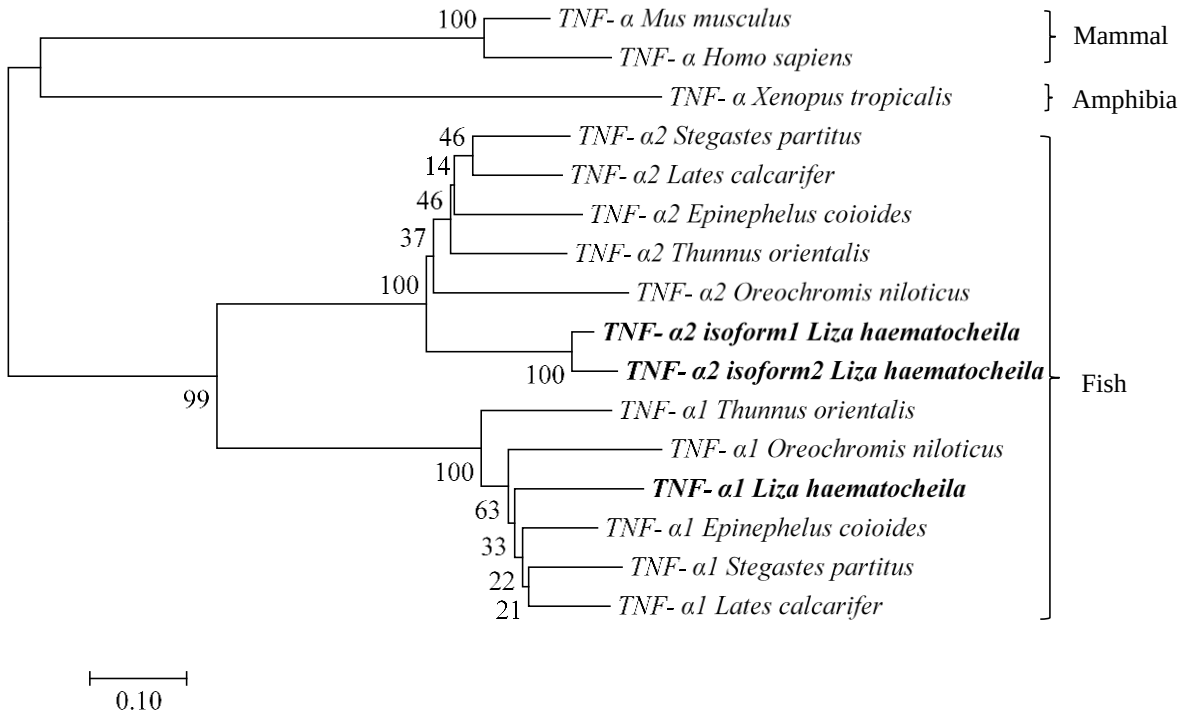

Fig.7. phylogenetic analysis of TNFα genes

3.4 Tissue specific expression

i. LITAF gene

All three LhLITAF genes were ubiquitously expressed in all the tissues analyzed (Fig.8). For all three LIITAF genes, the lowest level of mRNA expression was recorded in liver tissue. While LhLITAF1 gene showed higher expression in intestine, LhLITAF2 and LhLITAF3 had the highest expression in gills. While LhLITAF2 and LhLITAF3 had similar expression profile in immune tissues with different fold change, the expression pattern of LhLITAF1 showed considerable difference from LhLITAF2 and LhLITAF3. Previous studies have showed that tissue specific expression profile of LITAF genes differ from species to species. In flounder *Paralichthys olivaceus*, LITAF gene expression was shown relatively high in skin, blood and gill as well as low expression from spleen, kidney and head kidney[12]. However, LITAF gene from Snout Bream *Megalobrama amblycephala* showed higher expression in liver, headkidney and spleen while showing lower expression in gill and kidney[13]. In orange-spotted grouper *Epinephelus coioides*, ubiquitous expression of LITAF was reported with low expression from liver and muscle tissues[14]. In rock bream, *Oplegnathus fasciatus*, two LITAF isoforms were reported; both isoforms showed the lowest expression in peritoneal blood lymphocytes while the highest expression of rock bream LITAF1 and LITAF2 was from spleen and gill, respectively [15]. Hence, tissue specific LITAF gene expression differ among different isoforms as well as among different teleost species.

ii. TNFα

Mullet TNFα isoforms showed different tissue specific expression pattern in various tissues examined (Fig.9). While both LhTNFα1 and LhTNFα2 isoform1 showed higher expression in

spleen LhTNFα2 isoform2 showed highest expression in skin. However, the lowest expression of LhTNFα1, LhTNFα2 isoform1 and LhTNFα2 isoform2 were recorded from liver, headkidney and stomach, respectively. In rainbow trout *Oncorhynchus mykiss*, the tissue specific expression profile of three TNFα genes were analyzed; all three genes were highly expressed in gills while TNFα1 and TNFα3 showed lowest expression in liver and TNFα2 in muscles[11]. However, two of the TNFα from *Argyrosomus regius* expressed higher expression in spleen while the lowest expression was in gut tissues[16]. Zebrafish TNFα isoforms showed higher expression in intestine and lower expression in skin[17]. These previous studies showed that tissue specific expression profile of TNFα isoforms differs remarkably among different isoforms in different fish species.

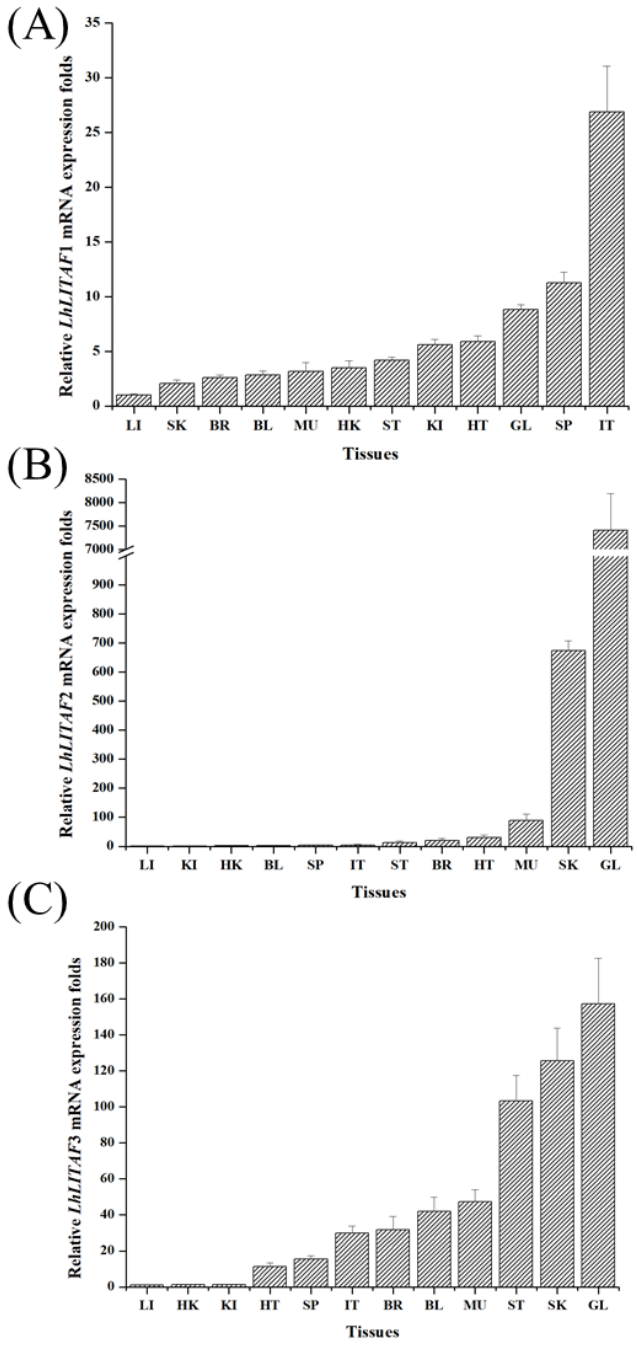

Fig.8. Tissue specific mRNA expression of LhLITAF genes (A) LhLITAF1, (B) LhLITAF2 and(C) LhLITAF (3).

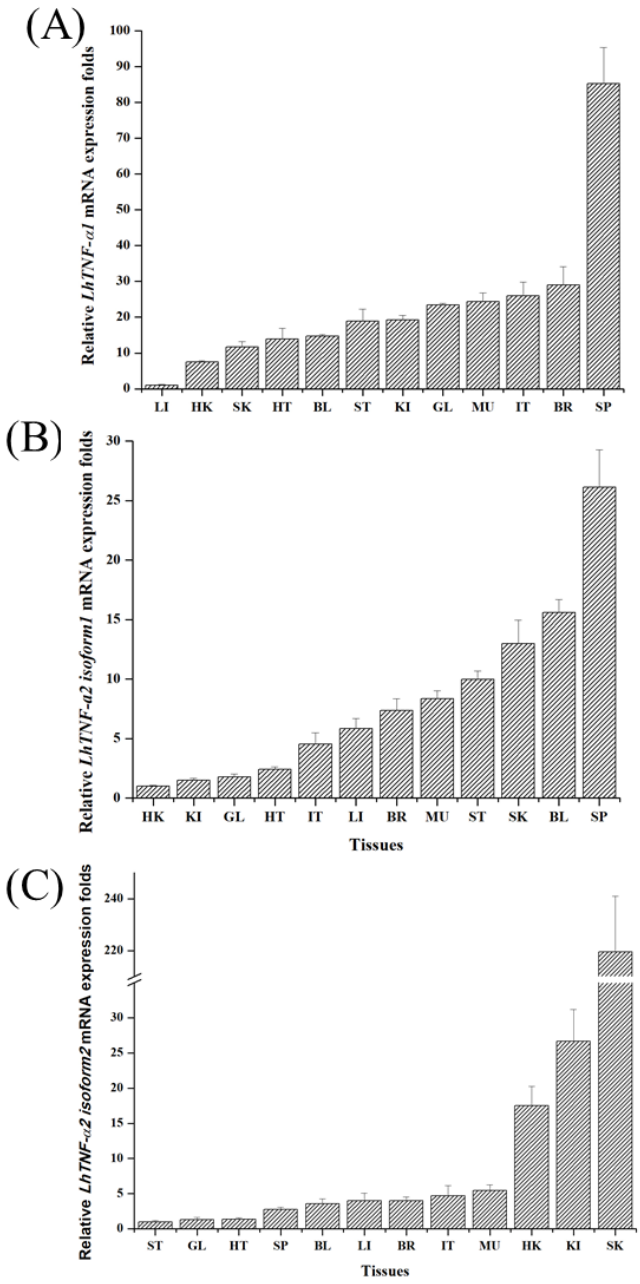

Fig.9. Tissue specific mRNA expression of LhTNFα genes (A) LhTNFα 1, (B) LhTNFα 2 isoform1and (C) LhTNFα 2 isoform2

3.5 Challenge experiment

i. LITAF gene

In Spleen LhLITAF1 and LhLITAF3 showed peak expression post 48hours while LhLITAF2 after 24hours. Challenge experiment with Poly I:C showed early upregulation of LhLITAF1after 6h, but, LhLITAF2 and LhLITAF3 showed late peak expression at 48 hours. In headkidney, LhLITAF1 showed early upregulation for LPS and Poly I:C injection. But, LhLITAF2 and LhLITAF3 showed downregulated expression even though LhLITAF2 showed upregulation for LPS and L.gaeviae after 72hours. In gill, LhLITAF1 and LhLITAF2 showed upregulation with all three PAMP injection, LITAF3 showed upregulation only with LPS. LhLITAF genes showed late upregulation following challenge experiment with L.gaeviae (Fig 10, 11, and 12).

Rock bream genes LITAF1 and LITAF2 were significantly upregulated in spleen with peak expression after 5days post challenge with gram-negative, gram-positive bacterial and viral injections[15]. In kidney tissue, Rock bream LITAF1 gene showed early upregulation for gram-negative bacterial and viral challenge experiment while gram positive bacterial injection didn't change the LITAF1 expression pattern significantly. Rock bream LITAF2 expression was downregulated for all the time point analyzed in kidney. But, rock bream LITAF1 also show significant downregulation during the late phase of the challenge. LITAF gene from flounder *Paralichthys olivaceus* and snoutbream *M. amblycephala* showed early upregulation as early as 2 hours after LPS injection with post 4 hour downregulation[12][13]. Similarly, LITAF gene of *Paralichthys olivaceus* and *Ctenopharyngodon idella* expressed early upregulation after

challenging with viral pattern associated molecular patterns[12][18]. Hence, LhLITAF genes considerably differ in expression pattern from other fish species.

TNFα

LhTNFα genes showed early peak expression for Poly I:C challenge experiment. LPS challenge showed late upregulation of LhTNFα1 in all three sampled tissues, while TNFα2 isoforms showed early upregulation in headkidney but late upregulation in gills (Fig. 13, 14, and 15). While challenge experiment with *L.garviae* showed downregulated expression, LhTNFα2 isoform1 showed late upregulation in spleen and headkidney and LhTNFα isoform2 showed early upregulation in headkidney and gill. In trout *O.mykiss*, primary headkidney macrophage cells treated with LPS and Poly I:C showed early upregulated expression of all three TNFα genes[11]. Meagre *Argyrosomus regius* showed upregulated expression after intraperitoneal injection as well as cells treatment following LPS and poly I: C. viral challenge experiments in *Scophthalmus maximus* [19] and *Siniperca chuatsi* [20] showed upregulated expression of TNFα. The expression profile of TNFα showed significant difference among species.

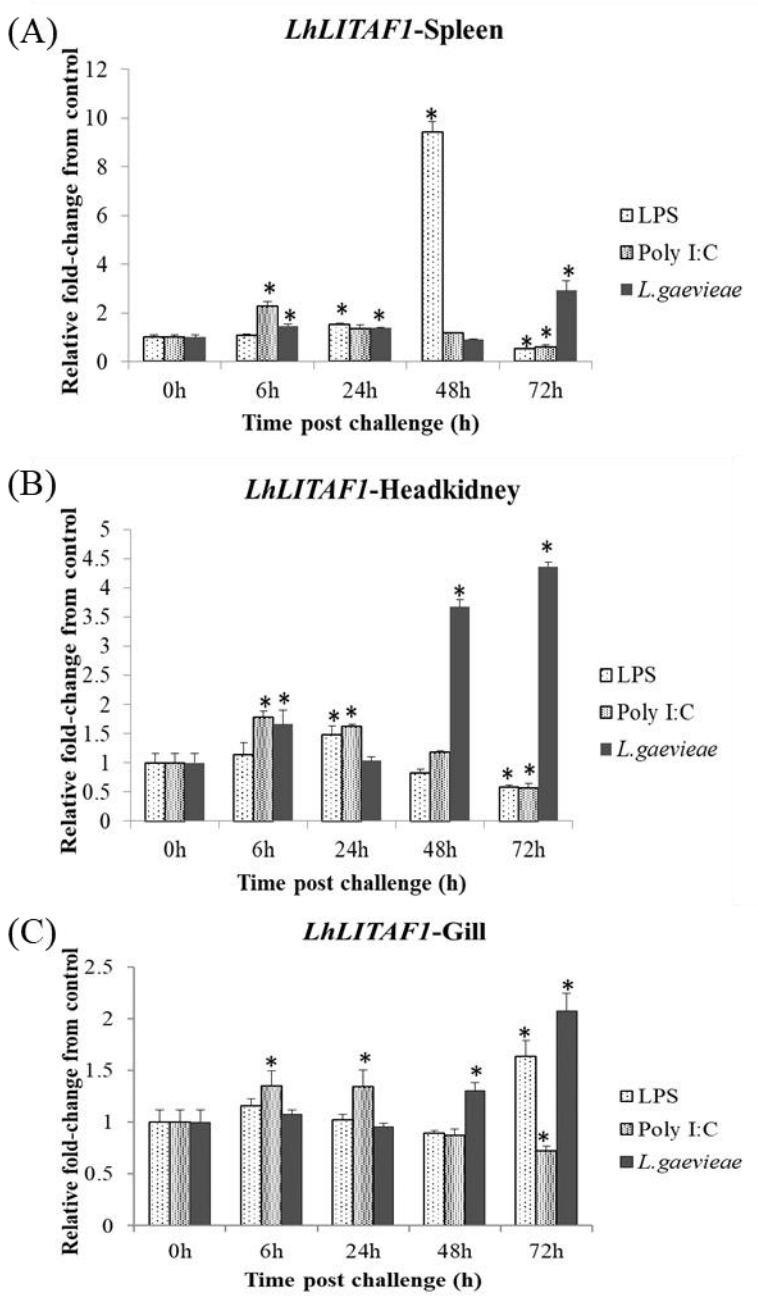

Fig.10. mRNA expression of LhLITAF1 gene after immune challenge

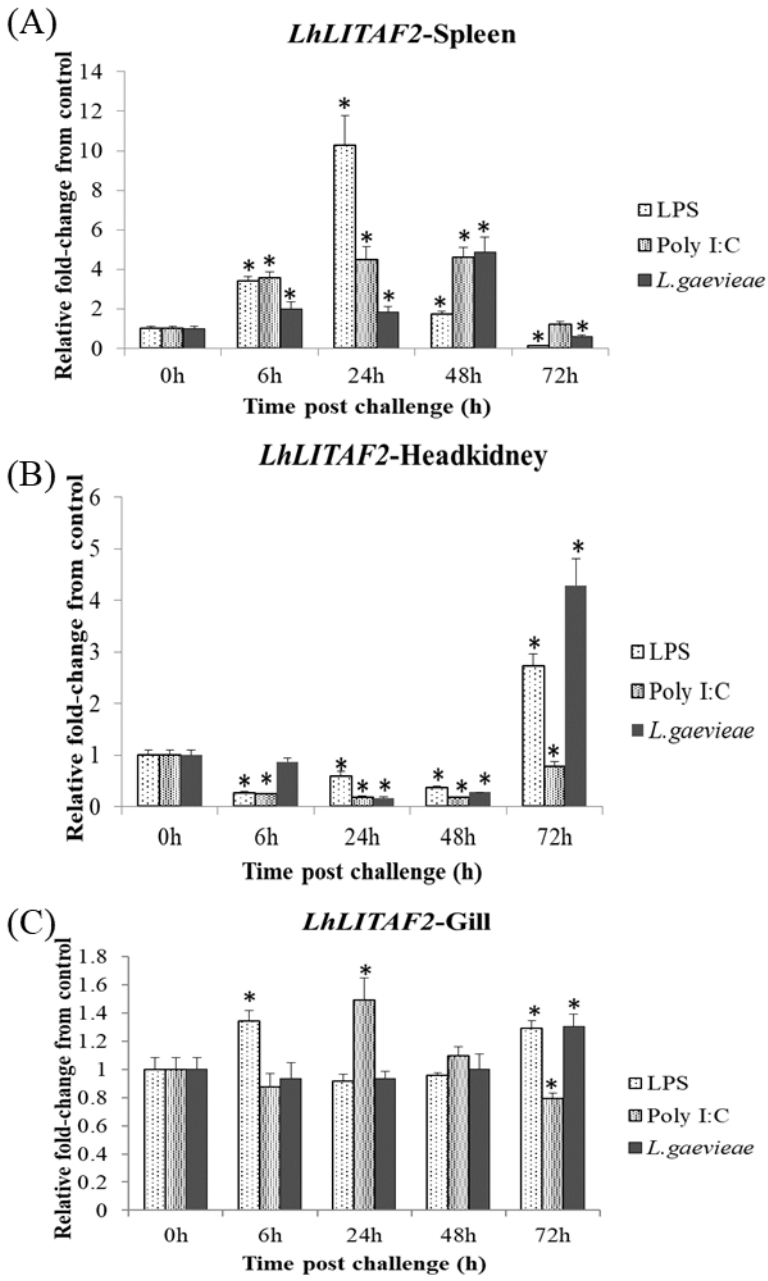

Fig.11. mRNA expression of LhLITAF2 gene after immune challenge

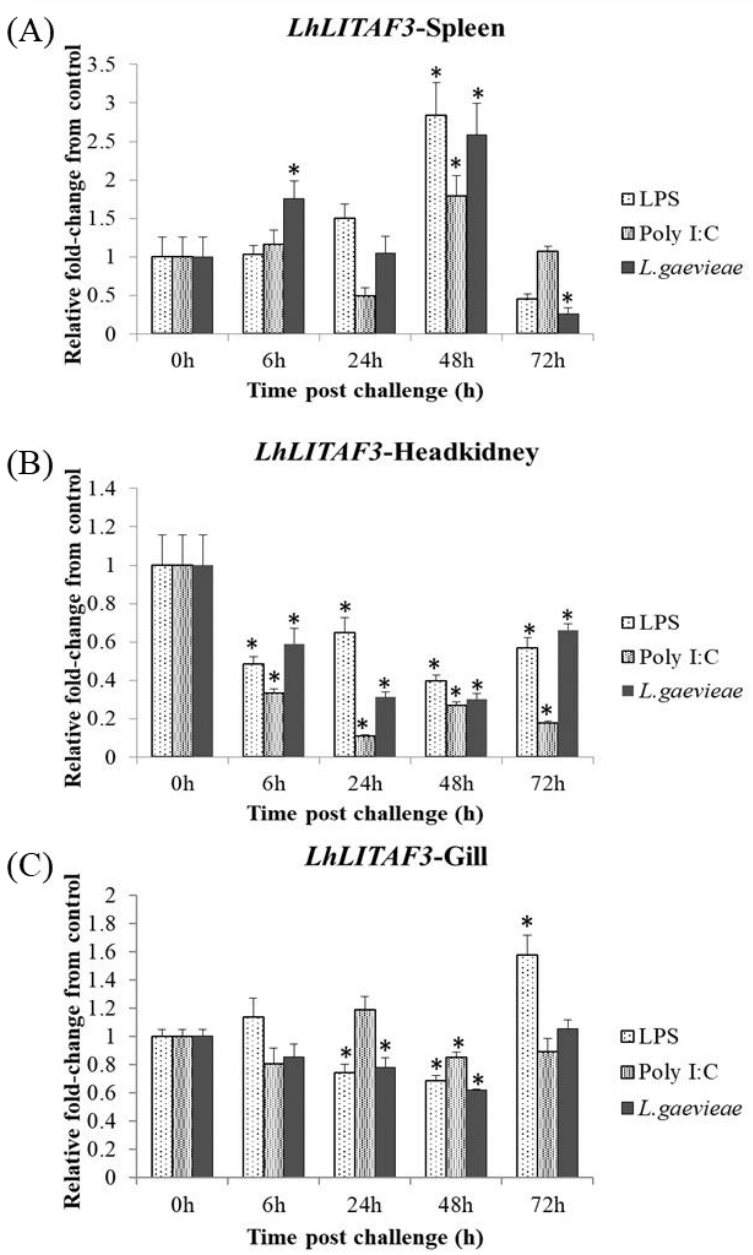

Fig.12. mRNA expression of LhLITAF3gene after immune challenge

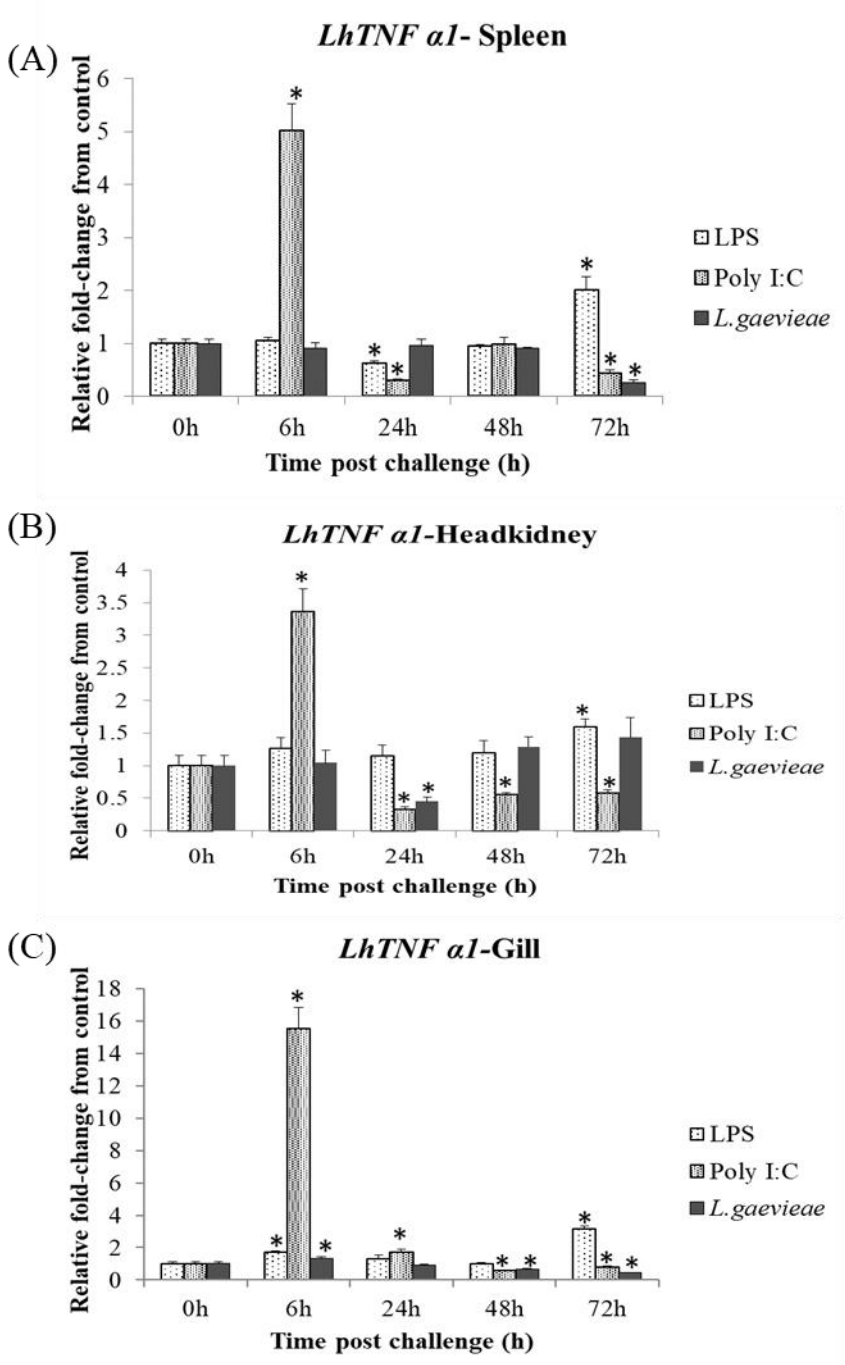

Fig.13. mRNA expression of LhTNFα1 gene after immune challenge

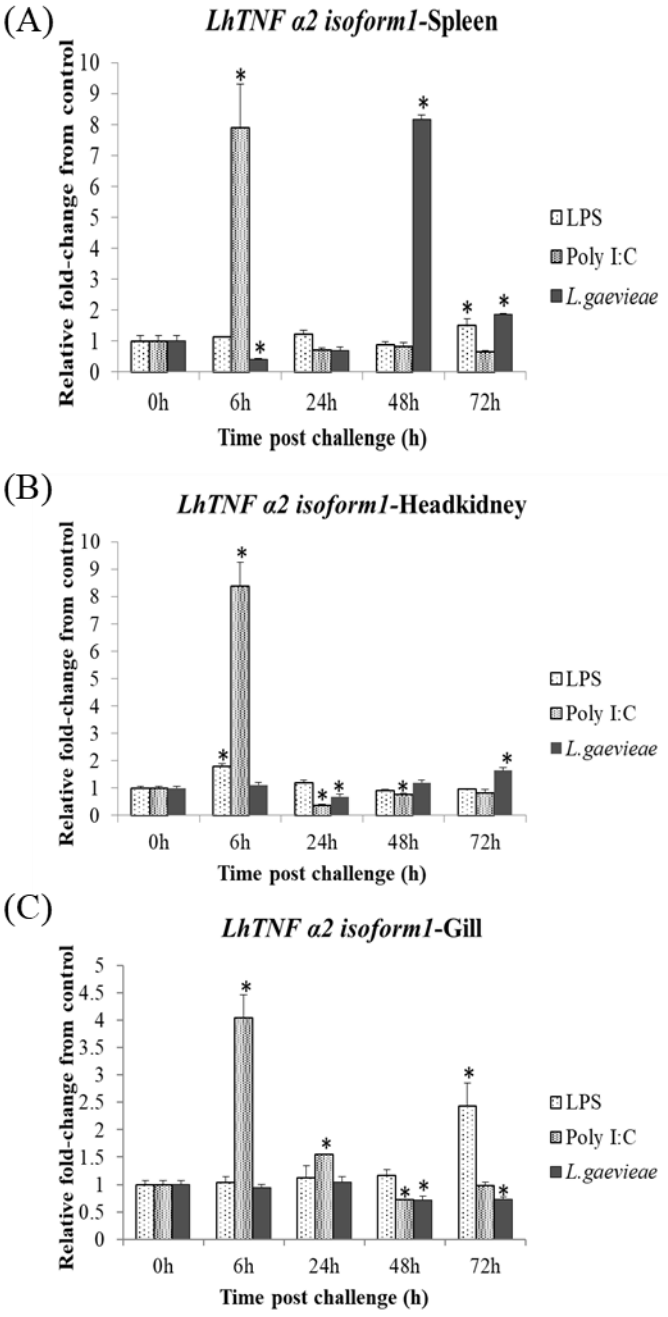

Fig.14. mRNA expression of LhTNFα2 isoform1 gene after immune challenge

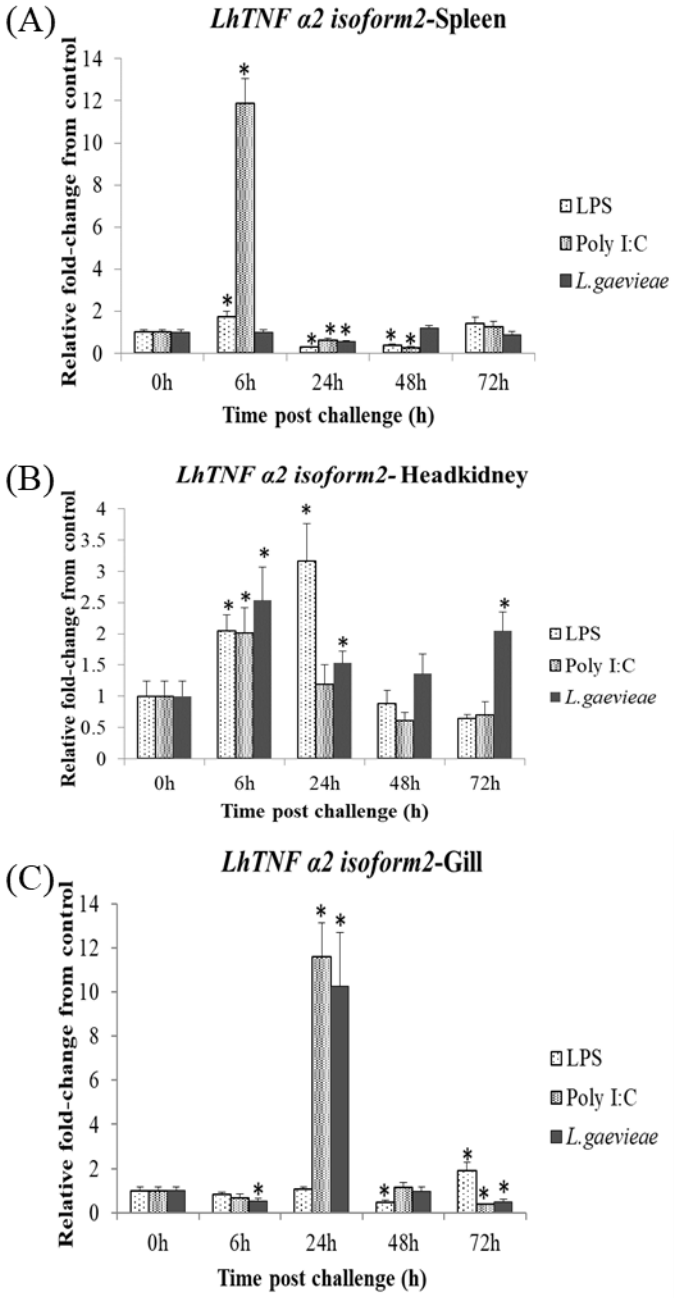

Fig.15. mRNA expression of LhTNFα2 isoform2 gene after immune challenge

3.6 Subcellular localization

Subcellular localization studies showed that all three LhLITAF genes were localized in cytoplasm under normal condition (Fig.16). A similar observation was reported with grouper Epinephelus coioides. Since treatment with LPS or virus was shown to change the localization pattern in other animal species, further studies required for the understanding of how virus or LPS change LhLITAF localization pattern.

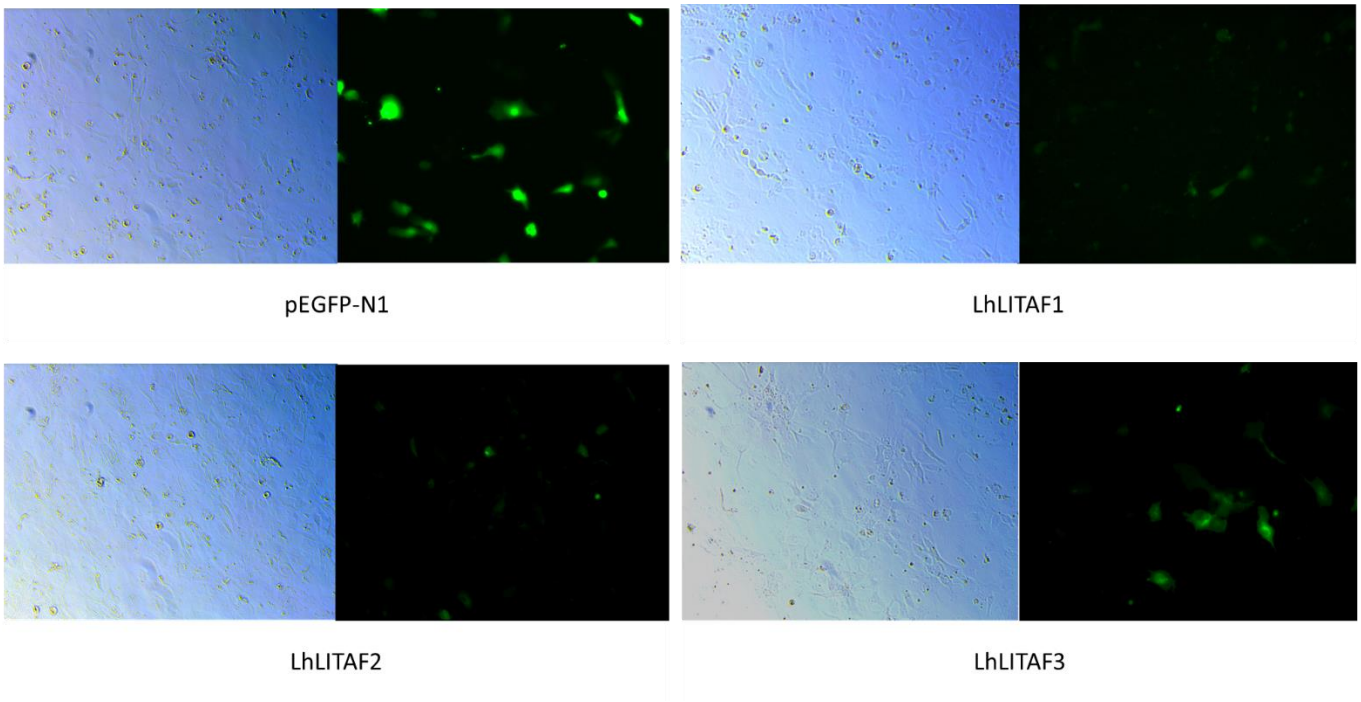

Fig.16. Subcellular localization of LhLITAF genes in primary kidney cells

3.7 Hoechst staining

The over expression of LITAF genes were shown to induce apoptosis in previous studies. So mullet kidney cells were transfected with LITAF genes were compared for the presence of apoptotic bodies. While there were few cells were shown to possess apoptotic bodies, a significant number of nuclei showed apoptotic signs such as half-moon shape or crescent shape nuclei (Fig.17). Hence, the same study was repeated with FHM cells showed significantly higher number of apoptotic bodies present in LhLITAF gene transfected cells than control (Fig.18 and Table.2).

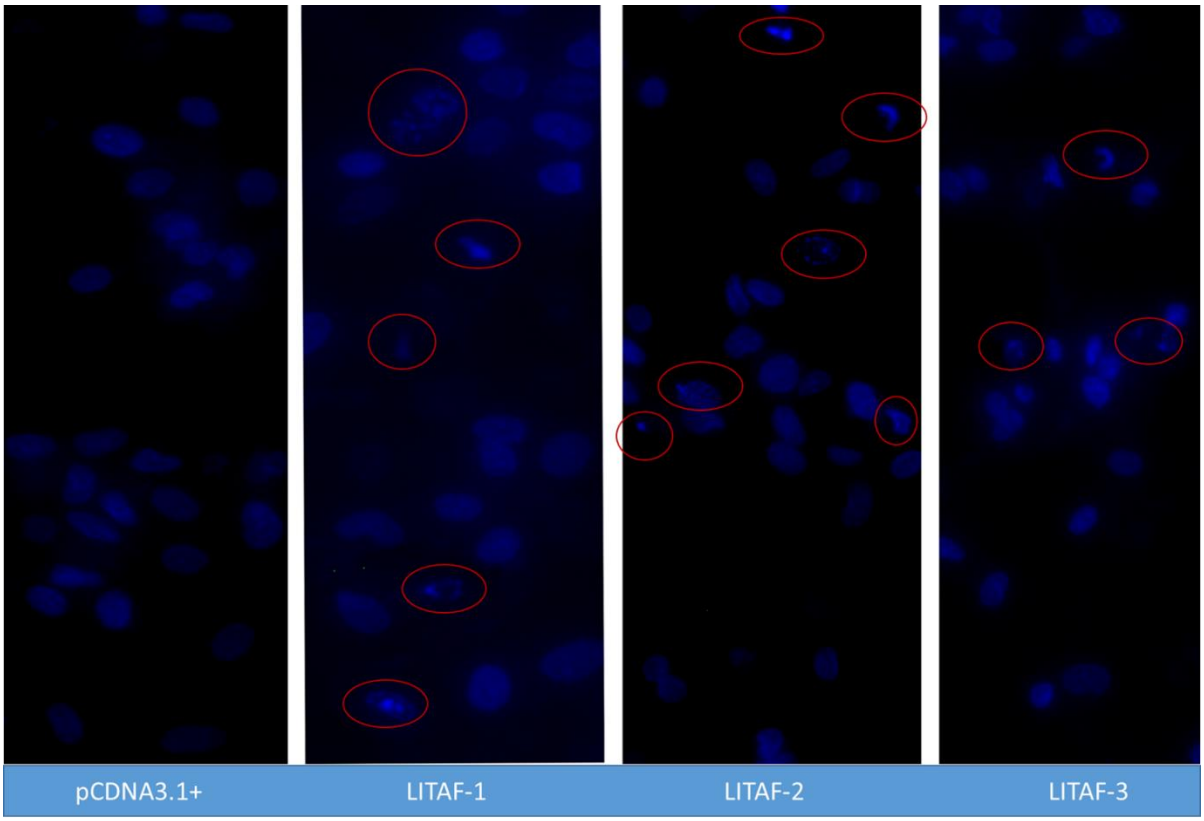

Fig.17. Hoechst staining of mullet cells 48hour post transfection with LITAF genes and pCDNA3.1+ vector

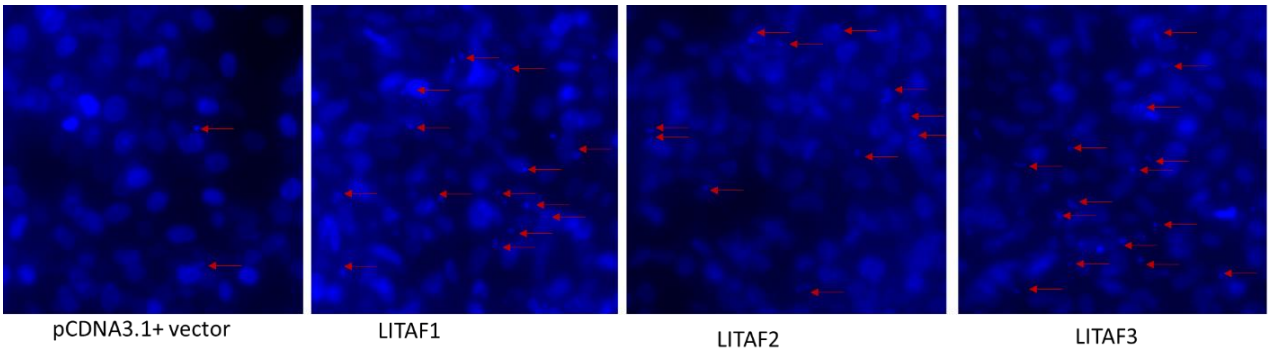

Fig.18. Hoechst staining of FHM cells 48hour post transfection with LITAF genes and pCDNA3.1+ vector

Table2. Percentage of apoptotic cells present in FHM cells transfected with LITAF genes and pCDNA3.1 vector

Transfection	Number of cells counted	Percentage of apoptosis
pCDNA3.1 + vector	14/605	2.31%
LhLITAF1	73/652	11.20%
LhLITAF2	49/584	8.39%
LhLITAF3	77/633	12.16%

3.8 Caspase-3 activity

LITAF genes were shown to induce the caspase-3 expression since they are involved in apoptotic pathway. Caspase-3 activity was detected after LITAF transfection. Higher caspase-3 activity was measured with LhLITAF1 gene followed by LhLITAF2 and LhLITAF3.This might be because LITAF1 having higher homology with mammalian LITAF genes than other two LITAF genes(Fig.16).

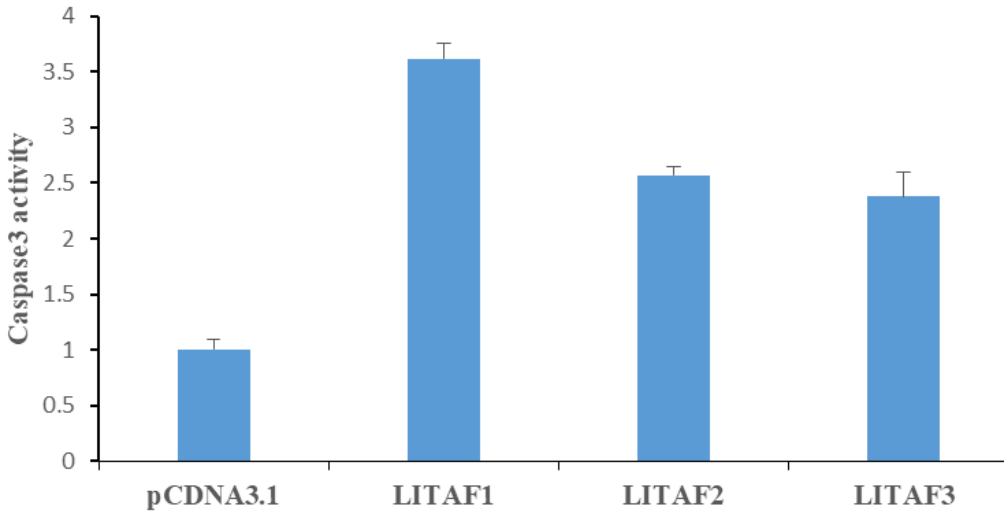

Fig.16. Caspase3 activity measurements for pCDNA3.1, and pCDNA-LITAF genes

3.9 TNFα mRNA expression profile

TNFα mRNA expression profile was analyzed for transfected cells with LhLITAF1, LhLITAF2, LhLITAF3 genes and pCDNA3.1 vector. The results showed that all three LITAF genes induce upregulation of all three LhTNFα isoforms compared to control pCDNA3.1+ empty vectors(Fig.17).

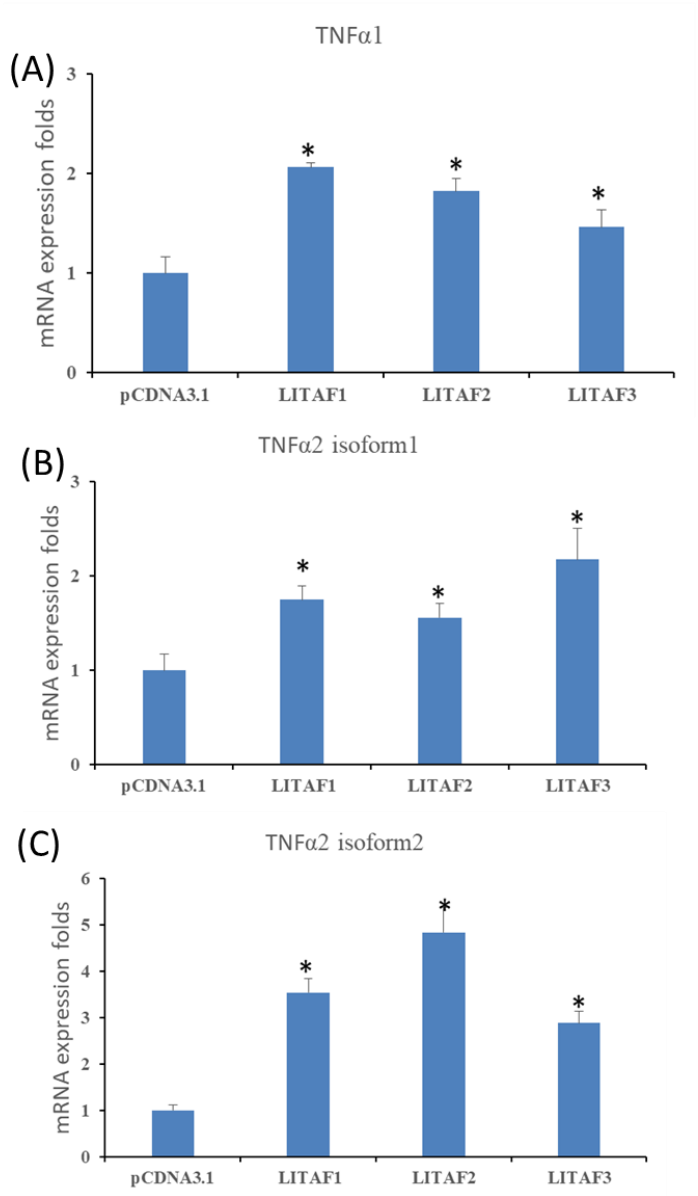

Fig.17. mRNA expression profile of TNFα post LITAF transfection

4. Conclusion

LhLITAF genes and TNFα genes were significantly upregulated in immune tissues following infection with pathogen associated molecular patterns. LhLITAF genes were ubiquitously expressed and significantly upregulated in all the immune tissues analyzed compared to LhLITAF2 and LhLITAF3. This might be because their higher homology with mammalian counterparts than LhLITAF2 and LhLITAF3. LhTNFα showed prominent peak with early upregulation with poly I: C. However, LPS induced expressions are late upregulated. The presence of multiple TNF alpha isoforms could support better immune defense in mullets since they inhabit wide range of habitats with different set of pathogenic profile. LhLITAF genes are involved in apoptosis in mullet and could induce TNFα expression. This observations suggest that LITAF and TNF alpha are critical immune genes in mullets.

5. References

[1] K.M. Leber, C. Lee, N.P. Brennan, S.M. Arce, C.S. Tamaru, H.L. Blankenship, R.T. Nishimoto, Stock Enhancement of Mugilidae in Hawaii (USA), (2016).

[2] I. Paperna, R.M. Overstreet, Parasites and diseases of mullets (Mugilidae), Aquac. Grey Mullet. (1981) 411–493.

[3] M. Ovcharenko, Microparasites of worldwide mullets, Ann. Parasitol. 61 (2015) 229–239. doi:10.17420/ap6104.12.

[4] W.S. Kim, J.H. Kim, M.S. Jang, S.J. Jung, M.J. Oh, Infection of wild mullet (Mugil cephalus) with Myxobolus episquamalis in Korea, Parasitol. Res. 112 (2013) 447–451. doi:10.1007/s00436-012-3075-7.

[5] J.B. Cho, S.R. Kwon, M.K. Lee, M. Do Huh, K.H. Kim, Myxobolus episquamalis (Myxosporea : Myxobolidae) on the scales of wild mullet , Mugil cephalus L , in Korea ., 1 (2006) 1–6.

[6] S. Revathy, N. Umasuthan, I. Whang, Q. Wan, B. Lim, H. Jung, J. Lee, Fish & Shell fi sh Immunology Akirin2 homologues from rock bream , Oplegnathus fasciatus : Genomic and molecular characterization and transcriptional expression analysis, Fish Shellfish Immunol. 35 (2013) 740–747. doi:10.1016/j.fsi.2013.06.006.

[7] H.M.L.P.B. Herath, D.A.S. Elvitigala, G.I. Godahewa, N. Umasuthan, I. Whang, J.K. Noh, J. Lee, Molecular characterization and comparative expression analysis of two teleostean pro-inflammatory cytokines, IL-1β and IL-8, from Sebastes schlegeli, Gene. 575 (2016) 732–742. doi:10.1016/j.gene.2015.09.082.

[8] M. Oh, N. Umasuthan, D.A.S. Elvitigala, Q. Wan, E. Jo, J. Ko, G.E. Noh, S. Shin, S. Rho, J. Lee, First comparative characterization of three distinct ferritin subunits from a teleost: Evidence for immune-responsive mRNA expression and iron depriving activity of seahorse (Hippocampus abdominalis) ferritins, Fish Shellfish Immunol. 49 (2016) 450–460. doi:10.1016/j.fsi.2015.12.039.

[9] J. Zou, C. Secombes, The Function of Fish Cytokines, Biology (Basel). 5 (2016) 23. doi:10.3390/biology5020023.

[10] S. Ceccarelli, N. Panera, M. Mina, D. Gnani, C. De Stefanis, A. Crudele, C. Rychlicki, S. Petrini, G. Bruscalupi, L. Agostinelli, L. Stronati, S. Cucchiara, G. Musso, C. Furlanello, G. Svegliati-Baroni, V. Nobili, A. Alisi, LPS-induced TNF-α factor mediates pro-inflammatory and pro-fibrogenic pattern in non-alcoholic fatty liver disease, Oncotarget. 6 (2015) 41434–41452. doi:10.18632/oncotarget.5163.

[11] S. Hong, R. Li, Q. Xu, C.J. Secombes, T. Wang, Two Types of TNF- Exist in Teleost Fish: Phylogeny, Expression, and Bioactivity Analysis of Type-II TNF- 3 in Rainbow Trout Oncorhynchus mykiss, J. Immunol. 191 (2013) 5959–5972. doi:10.4049/jimmunol.1301584.

[12] S. Li, X. Li, X. Gen, Y. Chen, J. Wei, J. Sun, Identification and characterization of lipopolysaccharide-induced TNF-alpha factor gene from Japanese flounder Paralichthys olivaceus, Vet. Immunol. Immunopathol. 157 (2014) 182–189. doi:10.1016/j.vetimm.2013.11.006.

[13] Y. Lv, X. Xiang, Y. Jiang, L. Tang, Y. Zhou, H. Zhong, J. Xiao, J. Yan, Identification and characterization of lipopolysaccharide induced TNFα factor from blunt snout bream, Megalobrama amblycephala, Int. J. Mol. Sci. 18 (2017). doi:10.3390/ijms18020233.

[14] J. Cai, Y. Huang, S. Wei, Z. Ouyang, X. Huang, Q. Qin, Characterization of LPS-induced TNFα factor (LITAF) from orange-spotted grouper, Epinephelus coioides, Fish Shellfish Immunol. 35 (2013) 1858–1866. doi:10.1016/j.fsi.2013.09.023.

[15] S.D. Hwang, S.H. Shim, M.G. Kwon, Y.S. Chae, W.J. Shim, J.H. Jung, J.W. Kim, C. Il Park, Molecular cloning and expression analysis of two lipopolysaccharide-induced TNF-α factors (LITAFs) from rock bream, Oplegnathus fasciatus, Fish Shellfish Immunol. 36 (2014) 467–474. doi:10.1016/j.fsi.2013.12.023.

[16] D.J. Milne, C. Campoverde, K.B. Andree, J. Zou, C.J. Secombes, Two types of TNFα in meagre (Argyrosomus regius): Discovery, distribution and expression modulation, Mol. Immunol. 92 (2017) 136–145. doi:10.1016/j.molimm.2017.10.007.

[17] S. Kinoshita, G. Biswas, T. Kono, J. Hikima, M. Sakai, Presence of two tumor necrosis factor (tnf)-α homologs on different chromosomes of zebrafish (Danio rerio) and medaka (Oryzias latipes), Mar. Genomics. 13 (2014) 1–9. doi:10.1016/j.margen.2013.10.004.

[18] H. Wang, X. Shen, D. Xu, L. Lu, Lipopolysaccharide-induced TNF-α factor in grass carp (Ctenopharyngodon idella): Evidence for its involvement in antiviral innate immunity, Fish Shellfish Immunol. 34 (2013) 538–545. doi:10.1016/j.fsi.2012.11.045.

[19] M.C. Ordás, M.M. Costa, F.J. Roca, G. López-Castejón, V. Mulero, J. Meseguer, A. Figueras, B. Novoa, Turbot TNFα gene: Molecular characterization and biological activity of the recombinant protein, Mol. Immunol. 44 (2007) 389–400. doi:10.1016/j.molimm.2006.02.028.

[20] J. Xiao, Z.C. Zhou, C. Chen, W.L. Huo, Z.X. Yin, S.P. Weng, S.M. Chan, X.Q. Yu, J.G. He, Tumor necrosis factor-alpha gene from mandarin fish, Siniperca chuatsi: Molecular cloning,

cytotoxicity analysis and expression profile, Mol. Immunol. 44 (2007) 3615–3622. doi:10.1016/j.molimm.2007.03.016.

6. Appendix.1

Table.3. Cloning primers used in the study

Primers	
GAGAGAaagcttATGGAAAAAGGAGTGCCACCGCAA	LITAF1 pCDNA3.1, forward
GAGAGAgaatccTCACATTCGCTTGTACACGTGGACG	LITAF1 pCDNA3.1 reverse,
GAGAGAaagcttATGAGTGCAGATGGAACACTGCCT	LITAF2 pCDNA3.1 forward
GAGAGAgaatccTCATTTGCAGCACTCCTTCTTCTCCA	LITAF2, pCDNA3.1 reverse,
GAGAGAaagcttATGGAACCCCCTTCGTACGAG	LITAF3 pCDNA3.1, forward
GAGAGAgaatccTCATCTCATGTAAACGTGTATGGGTTTCTCG	LITAF3 pCDNA3.1 reverse
GAGAGAaagcttATGGAAAAAGGAGTGCCACCGCAA	LITAF1 pEGFP-N1, forward
GAGAGAggatccCATTCGCTTGTACACGTGGACGAGATT	LITAF1 pEGFP-N1, reverse
GAGAGAaagcttATGAGTGCAGATGGAACACTGCCT	LITAF2 pEGFP-N1 forward
GAGAGAggatccTTTGCAGCACTCCTTCTTCTCCAC	LITAF2 pEGFP-N1, reverse
GAGAGAaagcttATGGAACCCCCTTCGTACGAG	LITAF3 pEGFP-N1, forward
GAGAGActgcagTCTCATGTAAACGTGTATGGGTTTCTCGC	LITAF3 pEGFP-N1, reverse

Table. 4. qPCR primers used in the study

Primers	
ACAGTGACTCACGTGGTGATAACGC	LITAF1, forward
CGCAGATGGCCCATGTCAACAG	LITAF1 reverse,
TCTCAGGCCACACCAGTCTACTC	LITAF2 forward
GCAGCCTGAACACCGGATCAT	LITAF2, reverse,
AGCCTCAGCTGGTAGTAGTGACTC	LITAF3 forward
TGAGAAGGATGCACATGCACCAAG	LITAF3 reverse
CCACAGGATCTGGCGCATGT	TNFα1 forward
CGAGGTAGATGGCGTTGTACCAG	TNFα1 reverse
ACTGTGAAGCGCTGGTCCAAG	TNFα2 isoform1 forward
CTCTCCTCCATCACTGTCTTCAGC	TNFα2 isoform1 reverse
CACACTGTAAAGCACTGGTCCAGT	TNFα2 isoform2 forward
GTTCTCCACCATCACGGTCTTCAAC	TNFα2 isoform2 reverse