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The expression of two novel orange-spotted grouper (*Epinephelus coioides*) TNF genes in peripheral blood leukocytes, various organs, and fish larvae

Freda Wai-San Lam [a], Szu-Yin Wu [a], Shih-Jie Lin [a], Chin-Chou Lin [a], Yi-Ming Chen [a,b,c], Han-Ching Wang [a,b,c], Tzong-Yueh Chen [a,b,c], Han-Tso Lin [d], John Han-You Lin [a,b,c,*]

[a] *Institute of Biotechnology, National Cheng Kung University, Tainan 701, Taiwan, ROC*
[b] *Research Center of Ocean Environment and Technology, National Cheng Kung University, Tainan 701, Taiwan, ROC*
[c] *Agriculture Biotechnology Research Center, National Cheng Kung University, Tainan 701, Taiwan, ROC*
[d] *Department of Biotechnology, Ming Chuan University, Taoyuan 333, Taiwan, ROC*

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ABSTRACT

The tumour necrosis factor (TNF) super-family is a group of important cytokines involved in inflammation, apoptosis, cell proliferation, and the general stimulation of the immune system. The TNF gene has been cloned in some bony fish; however, its counterparts are still unidentified in the majority of fish species. In this study, we cloned *gTNF*-1 and *gTNF*-2 from the orange-spotted grouper (*Epinephelus coioides*), an economically important farmed fish. Both genes include 4 exons and 3 introns and encoded 253 and 241 amino acid proteins with a molecular weight of approximately 27 and 26 kDa, respectively. The identity of the putative amino acid sequences between *gTNF*-1 and *gTNF*-2 was only 38%. The positions of cysteine residues, a protease cleavage site, and a transmembrane domain sequence derived from *gTNF*-1 and *gTNF*-2 were similar to those in other fish and mammalian TNF-α. The mRNA expression levels of the 2 gTNF molecules were evaluated in unstimulated/stimulated peripheral blood leukocytes, various organs, and fish larvae. Following lipopolysaccharide (LPS) treatment, *gTNF*-2 was expressed at higher levels, was up-regulated more quickly, and was more sensitive to the immune response than *gTNF*-1. *gTNF*-1 was constitutively expressed in the thymus, brain, and spleen, but it was also expressed in the heart, head kidney, and trunk kidney after LPS stimulation. *gTNF*-2 was constitutively expressed in the thymus, head kidney, trunk kidney, spleen, and intestine; further, *gTNF*-2 was highly expressed in all organs post-LPS stimulation. Finally, the *gTNF* expression levels were evaluated at various developmental stages in grouper larvae. A higher variation of *gTNF* expression levels was observed in fish larvae from a contaminated hatchery. This study revealed the different expression patterns of *gTNF*-1 and *gTNF*-2. In addition, *gTNF*-2 was more sensitive to pathogens than *gTNF*-1; therefore, it may be an appropriate marker for pathogen invasion and the evaluation of the larval rearing environment.

1. Introduction

The orange-spotted grouper (*Epinephelus coioides*) belongs to Perciformes, Serranidae, Epinephelinae, and Epinephelus. They are distributed in the subtropical/tropical area of Indian and west Pacific oceans, preying on fish and crustaceans. Grouper is an economically important farmed fish in Southeast Asia, e.g. China, Hong Kong, Indonesia, Malaysia, the Philippines, Singapore, Taiwan, Thailand, and Vietnam [1]. The first successful commercial artificial procedure to breed grouper fry was established in Taiwan 2 decades ago [2]. However, disease outbreaks due to bacterial and viral infections causes a bottleneck in fry output, and is a considerable problem during the rearing of groupers [2]. Pathogen invasion results in terrible losses or complete annihilation of stock while rearing fish, especially at the larval stages [3]. Immunoprophylactic methods such as vaccination or immunostimulation are useful against disease outbreaks [4,5]. These methods have been widely used to prevent and control disease transmission in the poultry industry as a form of biosecurity, and are now being applied to aquaculture [6]. However, the establishment of immunoprophylaxis and biosecurity methods is based on an understanding of a specific animal's immune response and target pathogens.

The immune system includes innate and adaptive immunity. Innate immunity is a widespread non-specific immune response

* Corresponding author. Institute of Biotechnology, National Cheng Kung University, Tainan 701, Taiwan, ROC. Tel.: +886 6 2757575#65621; fax: +886 6 2766505.
E-mail address: hanyou@mail.ncku.edu.tw (J.H.-Y. Lin).

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which is associated with phagocytes [7], effector molecules [8], and cytokines. Cytokines are a group of small and soluble molecules, e.g. interleukins, interferons, and tumour necrosis factors which have a key role in the proliferation of immunocytes, apoptosis, and immune modulation [9,10,11]. Teleosts represent the foremost fish species with typical adaptive immune and innate immune responses. However, the fish adaptive immune system is different from higher vertebrates, with a lower sensitivity and complexity; indeed, the innate immune system may play a significant role in fish [12].

The tumour necrosis factor (TNF) super-family is a group of important cytokines implicated in inflammation, apoptosis, cell proliferation, and innate immune responses. TNF-α enhances phagocytosis and triggers an immune response to eliminate pathogen infections [7,13,14]. Piscine TNF-α was found to be expressed in macrophages [15] and infected trout [16]. The first fish TNF-α gene was cloned and sequenced from the Japanese flounder [17,18]. Furthermore, TNF-α genes have been cloned from a range of fish species, e.g. Atlantic salmon [19], ayu [20], catfish [21], carp [22], European sea bream [23], gilthead sea bream [24], goldfish [25], large yellow croaker [26], Pacific bluefin tuna [27], rainbow trout [28,29], tiger puffer fish [30], tilapia [31], turbot [32], trout [18], and zebrafish [30]. As observed in higher vertebrates, fish TNF-α was activated by stimulation with endotoxins, vaccinations, and pathogen molecules [28,33,34]. TNF-α has been proposed as a biomarker to monitor fish health and the efficacy of vaccination; however, TNF molecules are highly diverse between fish species and it is difficult to generally apply the information derived from TNFs from different fish species. Thereby, it is necessary to clone TNF genes from the species of interest before research and application of this information. Meanwhile, another new TNF ligand (TNF-N) was identified in zebrafish and the tiger puffer fish [30]. It was located upstream of the TNF-α gene, although its gene structure was not similar to the conventional teleost TNF-α gene, but it was similar to the mammalian lymphotoxin (LT) gene. However, the function of TNF-N is still unclear [30].

In this study, we cloned 2 orange-spotted grouper TNF genes. We characterised their gene structure, putative amino acid sequences, and their expression pattern in peripheral blood leukocytes (PBLs), various organs, and larval stages of fish in the presence and absence of stimulation. The information presented in this study will facilitate research into the grouper's immune system.

2. Materials and methods

2.1. Experiment animals

Orange-spotted groupers (*E. coioides*) were obtained from the Laboratory of Marine Biotechnology, National Cheng Kung University. Grouper fry were spawned from disinfected fertilised eggs and kept in 6 m^3 fiber reinforced plastic (FRP) tanks with aerated, sand-filtered, and UV-treated seawater. The rearing conditions of the fish larvae, including the time when fry started to feed on algae and zooplankton and the control of the seawater environmental quality were as previously described [4]. Juvenile groupers were raised from fish fry in 1 m^3 FRP tanks with the same conditions as above and they were fed twice a day with dry pellets (Uni-President, Tainan, Taiwan).

2.2. Cloning and sequencing of grouper TNF genes

Blood samples were collected from orange-spotted groupers (200–300 g) and gently mixed with an equal volume of balance buffer (0.5 mM glucose, 0.5 mM $CaCl_2$, 0.01 mM $MgCl_2$, 0.5 mM KCl, 15 mM Tris, and 126 mM NaCl, pH 7.6). The mixture was transferred onto the top of 70% Ficoll (GE Healthcare Bioscience AB, Uppsala, Sweden)/phosphate-buffered saline (PBS; 0.15 M NaCl, pH 7.2) solution in a centrifugation tube and isolated by centrifugation at 400× *g* for 30 min. The leukocytes were separated on the margin layer between the balance buffer and the Ficoll/PBS solution. The cells were collected and washed by re-suspension with PBS and centrifugation. The cells were then incubated overnight in a flask containing culture medium (L15 medium (Invitrogen, Carlsbad, CA, USA), 5% foetal bovine serum, 1‰ penicillin/streptomycin (Invitrogen)). The unattached cells were washed with PBS, but the attached cells were PBLs. The quality and quantity of PBL isolation were checked on an inverted microscope (DMIL; Leica Microsystems GmbH, Wetzlar, Germany).

PBLs were stimulated with 10 μg mL^{-1} lipopolysaccharide (LPS; Sigma Chemical, St Louis, MO, USA) in fresh culture medium for 24 h at 25 °C. Total RNA, for cloning the *TNF* genes, was extracted from stimulated PBLs by using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. cDNA was synthesised from total RNA by MMLV reverse transcriptase (Promega, Madison, WI, USA) with random hexamer primers. The partial sequence of a grouper *TNF* (*gTNF*) gene was obtained by PCR using cDNA and the degenerate primers dTNF-F/R (the sequences of these primers and the PCR conditions are listed in Table 1). The degenerate primers were designed from the consensus regions between European sea bass, Chinese perch, and red sea bream *TNF-α* gene sequences (GenBank accession numbers: DQ070246, DQ486758, and AY314010, respectively). PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells (Promega) according to the manufacturer's protocol. The 5′- and 3′-untranslated regions (5′- and 3′-UTR) of *TNF* mRNA were determined with a 5′- and 3′-rapid amplification of cDNA ends (5′- and 3′-RACE) system (GeneRacer; Invitrogen) with the specific primers gTNF1-F/R (TNF-1) and gTNF2-F/R (TNF-2) (Table 1). The PCR products were cloned into the pGEM-T Easy vector and sequenced (MB MISSION BIOTECH Ltd., Taipei, Taiwan).

Genomic DNA of *E. coioides* was obtained from muscle with the DNA Isolation Reagent (Invitrogen). The *gTNF* genes were obtained by PCR amplification with the specific primers used for the RACE reaction (Table 1). The PCR products were cloned into the pGEM-T Easy vector and sequenced as above.

2.3. gTNF amino acid sequences and phylogenetic analyses

The predicted TNF amino acid sequences from the orange-spotted grouper and several available teleosts were analysed with multiple sequence alignment by using MegAlign software (DNASTAR Inc., Madison, WI, USA) with the Clustal W method. To determine the evolutionary position of gTNF-1 and gTNF-2, we constructed a phylogenetic tree for the available teleost TNF-α, TNF-N, and LT-β proteins, and mammalian TNF-α and LT-β using the neighbour-joining (NJ) method with the MEGA4 program (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA). The human FasL gene (GenBank accession number: BC017502) was used as an outer group.

2.4. gTNF mRNA expression profiles in PBLs

Total RNA was extracted from PBLs after their induction with LPS (10 μg mL^{-1} medium) at different time points (0, 2, 4, 8, 16, and 24 h) as described as above. The Oligotex-mRNA Mini Kit (Qiagen, Hilden, Germany) was used for mRNA purification to avoid genomic DNA contamination. *gTNF* mRNA expression was detected by quantitative real-time PCR performed with the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and specific primers for TNF-1 (OsgTNF1-F/R), TNF-2 (OsgTNF2-F/R), and IL-1β