Fish & Shellfish Immunology 47 (2015) 996–1005

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Molecular cloning and characterization of orange-spotted grouper (*Epinephelus coioides*) CXC chemokine ligand 12

Chen-Shiou Wu [a, 1], Ting-Yu Wang [a, 1], Chin-Feng Liu [a, 1], Hao-Ping Lin [a], Young-Mao Chen [a, b, c, d], Tzong-Yueh Chen [a, b, c, d, e, f, *]

[a] *Laboratory of Molecular Genetics, Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan*
[b] *Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan*
[c] *Translational Center for Marine Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan*
[d] *Agriculture Biotechnology Research Center, National Cheng Kung University, Tainan 70101, Taiwan*
[e] *University Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan*
[f] *Research Center of Ocean Environment and Technology, National Cheng Kung University, Tainan 70101, Taiwan*

ARTICLE INFO

*Article history:*
Received 5 August 2015
Received in revised form
29 October 2015
Accepted 2 November 2015
Available online 6 November 2015

*Keywords:*
*Epinephelus coioides*
Chemokine
CXCL12
Nervous necrosis virus

ABSTRACT

Chemokines are a family of soluble peptides that can recruit a wide range of immune cells to sites of infection and disease. The CXCL12 is a chemokine that binds to its cognate receptor CXCR4 and thus involved in multiple physiological and pathophysiological processes. In this study, we cloned and characterized CXCL12 from *Epinephelus coioides* (osgCXCL12). We found that the open reading frame of osgCXCL12 consists of 98 amino acid residues with the small cytokine C-X-C domain located between residues 29 and 87. Higher expression levels for *osgCXCL12* were detected at the kitting stage, compared with the prolarva and larva shape stages. The expression patterns revealed that *osgCXCL12* may play a key role in early grouper development. We detected mRNA transcripts for osgCXCL12 in healthy tissues and found the highest osgCXCL12 expression in the head kidney. Furthermore, a time-course analysis revealed significantly increased osgCXCL12 and osgCXCR4 expression levels after the nervous necrosis virus (NNV) challenge. In addition, expression of osgCXCL12 was affected by injection with microbial mimics [LPS and poly(I:C)]. These results suggest that osgCXCL12 is associated with inflammatory and developmental processes in the grouper.



## 1. Introduction

Chemokines are a superfamily of small (7–16 kDa), proinflammatory chemoattractant cytokines, which were originally recognized by their ability to induce the migration of leukocytes [1,2]. In mammals, chemokines have been divided into four subfamilies, according to the number and spacing of the first two conserved cysteine residues in the N-terminal: CXC, CC, CX3C, and XC [3]. Recently, a fish-specific CX subfamily has been identified in zebrafish [4]. In mammals, these cysteine residues mediate chemokine functions by binding to and interacting with G-protein coupled receptors [5]. Various pathways can be activated through ligand-receptor interaction, and these interactions may play roles in angiogenesis, hematopoiesis, embryologic development, and in many immune functions including dendritic cell maturation, B cell antibody class switching, and T cell activation and differentiation [6]. Therefore, an increasing number of studies have been performed to gain a deeper understanding of the role of these critical regulators in development, as well as their chemoattractant properties and contributions to immune processes that are not directly involved in leukocyte migration [7–10].

Findings in mammals suggest that the CXC chemokine ligand 12 (CXCL12), also called stroma-derived factor 1 (SDF-1), belongs to the CXC chemokine family and is expressed in α- and β-isoforms that arise from two splice variants of a single gene [11]. Initially cloned from the cDNAs of a bone marrow stromal cell line [12], CXCL12 was later identified as a growth factor for B cell progenitor cells [13] and a chemotactic factor for T cells and monocytes, and

* Corresponding author. Laboratory of Molecular Genetics, Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan.
*E-mail address:* ibcty@mail.ncku.edu.tw (T.-Y. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2015.11.001
1050-4648/

was also shown to be critical in the regulation of B cell lymphopoiesis and bone marrow myelopoiesis [14]. Although multiple roles for CXCL12 in the regulation of immunity have been described [15], it remains unclear how this chemokine exerts its actions in the orange-spotted grouper (*Epinephelus coioides*).

Nervous necrosis virus (NNV) is a betanodavirus of the family Nodaviridae [16]. Betanodavirus strains have broad host ranges, and groupers show particular susceptibility with outbreaks causing high mortality rate [17,18]. Therefore, developing prevention strategies or effective therapies against viral infection are important fields of study for grouper. Previous research has identified nodavirus infection triggers an intracellular inflammatory response that includes increased expression of proinflammatory cytokines, such as interleukin (IL)-1b, tumour necrosis factor (TNF), antiviral molecules (Mx), and type I interferon (I-IFN) [19–21]. Another possibility is that nodavirus infection causes apoptosis in grouper cells [22]. A functional study indicated that grouper chemokine receptor expression is important for the recruitment of immune cells in the early steps of the immune response to microbial infection [23]. Previously, we cloned CXCR4 from *E. coioides* and found that it is primarily expressed in the head kidney and on the surface of the intestinal villi [23]. Moreover, we have shown that CXCR4 plays a role in the early immune response to NNV infection [23]. The CXCL12 is a chemokine that binds to its cognate receptor CXCR4 and thus involved in multiple physiological and pathophysiological processes. Consequently, the understanding of the possible role of its cognate ligand CXCL12 in the immune response would be important.

The orange-spotted grouper, *E. coioides*, is a commercially important fish that is widely farmed in the tropical waters of many countries. Infection of this fish by the piscine nodavirus has led to considerable economic losses. Therefore, prevention strategies and therapies against viral infection are important fields of grouper study. In the present study, we aimed to elucidate the role of the CXCL12 gene in embryonic development stages and immune responses. Specifically, we cloned, identified, and characterised CXCL12 from *E. coioides*. Taken together, our data can provide valuable chemokine signaling insights towards achieving a clearer understanding of the role of CXCL12 in immune responses to infection and development.

## 2. Materials and methods

### 2.1. Fish, virus, and immune challenge experiments

Healthy orange-spotted groupers weighing approximately 0.3 g or 5 g and aged between 0 and 28 days were obtained from the Core Facility of Grouper Bio-resources in the Translational Center for Marine Biotechnology, An-Nan Campus, National Cheng Kung University (Tainan, Taiwan). The fish were maintained in 20-L containers at 27 °C ± 1 °C. For the NNV challenge experiment, NNV was isolated from naturally infected orange-spotted groupers collected from Jiading, Taiwan. The isolated virus was propagated in grouper fin (GF-1) cells and collected until 90% of the cells exhibited a cytopathic effect (CPE). The general procedure for virus isolation and purification was conducted as reported previously [24]. The virus-challenged grouper larvae (TBW 0.3 ± 0.1 g; TBL 1.6 ± 0.1 cm; at 30 days post hatch [dph]) were separated into two groups of six fish each. Each group was immersed in 500 mL of rearing water which contained either 50 mL of a viral solution ($10^6$ $TCID_{50}$/0.1 mL) or saline for 2 h. The fish were then transferred to a virus-free aquarium, which had been exposed to ultraviolet light for 24 h, and cultured at 27 °C ± 1 °C. The fish were sacrificed and collected as whole fish at 6, 12, 24, 48, and 72 h postimmersion to determine gene expression levels at different time points. For the polyinosinic-polycytidylic acid (poly[I:C]) and LPS challenge experiments, 108 fish (groups of six fish in 18 tanks; TBW 5.0 ± 0.2 g; TBL 5.1 ± 0.2 cm; at 60 dph) were maintained in the containers. The fish were each exposed to a challenge of approximately 50 μg of poly(I:C) (Sigma–Aldrich, St. Louis, MO, USA) or 20 μg of LPS (Sigma-Aldrich) through an intraperitoneal (IP) injection. Fish that were injected with PBS only were used as a control group. At 3, 6, 12, 24, 48, and 72 h, postexposure spleen and intestine tissues were sampled from six fish of both groups. The tissues were collected for gene expression analysis.

### 2.2. Tissue distribution analysis

Groupers naturally infected with NNV (~5 g) were collected from Linyuan fish farms in southern Taiwan. RT-PCR was then used to confirm the fish was infected by NNV. RNA2 encodes the capsid protein [25]. The NNV RNA2 primers were designed as described by Kuo et al. [24], and the sequences are listed in Table 1. Healthy and naturally infected fish (n = 6 per group) were killed and tissues (brain, eye, fin, gill, spleen, head kidney, intestine, skin, and heart) collected for gene expression analysis.

### 2.3. Total RNA extraction and cDNA synthesis

For total RNA extraction, fish samples were homogenized in TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) using a MagNALysis homogenizer (Roche, Basel, Switzerland), following the manufacture's recommendations. To ensure complete removal of traces of genomic DNA, 1 mg of total RNA was incubated with 1 unit of RNase-free DNase I (TaKaRa, Tokyo, Japan) for 30 min at 37 °C. RNA were quantified using an Ultrospec 3300 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). cDNA was synthesized with 2 μg RNA, 0.1 μM oligo(dT) primer, 12.5 μM dNTP (Bioman Scientific Co., Ltd., Taipei, Taiwan), and 50 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 1 h.

### 2.4. Rapid amplification of cDNA ends (RACE)

All primers used in this study were synthesized by Genomics BioSci & Tech (Taipei, Taiwan) (Table 1). Full-length cDNA was obtained by 5′/3′ RACE, which was performed with a 5′/3′ RACE Kit (Roche Diagnostics GmbH, Mannheim, Germany). Gene-specific primers for 5′ and 3′ RACE were designed based on the partial *osgCXCL12* sequence. For 5′ RACE, mRNA was transcribed by MMLV reverse transcriptase (Sigma-Aldrich) with the primers *osgCXCL12-5SP1*, *osgCXCL12-5SP2*, and *osgCXCL12-5SP3*. 3′ RACE was performed using primers for *osgCXCL12-3SP1* and *osgCXCL12-3SP2*. Polymerase chain reaction (PCR) conditions for initial denaturation were 95 °C for 3 min, followed by 35 cycles each at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The primers *osgCXCL12-FL-F* and *osgCXCL12-FL-R* were used to amplify the full-length *osgCXCL12* cDNA.

### 2.5. Cloning of grouper CXCL12 introns

The positions of introns were determined by comparison of the size of products obtained by PCR amplification of genomic DNA with the cDNA sequence. Intron 1, 2, and 3 were amplified using primer pairs (Table 1). The amplified fragment was verified by subcloning in pGEM-T vectors (Promega) for sequencing.

### 2.6. Bioinformatic analysis

The resulting cDNA sequence was analysed for the presence of a