Fish & Shellfish Immunology 31 (2011) 439–445

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

# Molecular cloning, characterization and expression analysis of a miiuy croaker (*Miichthys miiuy*) CXC chemokine gene resembling the CXCL9/CXCL10/CXCL11

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ARTICLE INFO

*Article history:*
Received 22 March 2011
Received in revised form
12 June 2011
Accepted 14 June 2011
Available online 21 June 2011

*Keywords:*
*Miichthys miiuy*
CXC chemokine
Genomic structure
*Vibrio anguillarum*
Gene expression

ABSTRACT

Chemokines are a large family of chemotactic cytokines playing crucial roles in the innate immune response. In the present study, we report the cloning of a CXC chemokine gene resembling the closely related CXCL9/CXCL10/CXCL11 from the miiuy croaker *Miichthys miiuy* (MimiCXC). Both 5′-RACE and 3′-RACE were carried out in order to obtain the complete cDNA, which consists of a 73 bp 5′-UTR, a 369 bp open reading frame encoding 122 amino acids and a 715 bp 3′-UTR. The deduced MimiCXC contains a 19-aa signal peptide and a 103-aa mature polypeptide, which possesses the typical arrangement of four cysteines as found in other known CXC chemokines. It shares 4.8%–65.6% sequence identities to mammalian CXC chemokines and the highest sequence identity of 65.6% is between MimiCXC and CXCL10 chemokine. Three exons and two introns were identified in MimiCXC gene. The MimiCXC gene was constitutively expressed in all tissues tested, although at different levels. Upon induction with *Vibrio anguillarum*, MimiCXC gene expression was up-regulated in kidney and spleen, however, down-regulated in liver. These results indicate that MimiCXC may be involved in immune responses as well as homeostatic processes in miiuy croaker.



## 1. Introduction

Chemokines are traditionally viewed as a superfamily of chemotactic cytokines involved in the recruitment, activation and adhesion of a variety of leukocyte types to inflammatory loci [1,2]. Chemokines have pleiotropic effects in regulating immunity and angiogenesis, and stem cell trafficking appears to play a central role in linking innate and acquired immune regulation [3,4]. Functionally, chemokines fall into two main categories. Some are homeostatic and are produced and secreted. These are generally involved in lymphocyte trafficking, immune surveillance and localization of lymphocytes with antigen in the lymphatic system [5]. Other are only produced by cells during inflammation to prompt the migration of leukocytes to an injured or infected site and also activates cells to raise an immune response and commence the wound healing process [1]. Chemokine ligands bind to 7-transmembrane G-protein coupled receptors, thereby transducing signals to increase integrin adhesion receptor function and direct migration of lymphocytes [6].

Chemokines are classified into four groups C, CC, CXC, and $CX_3C$ based on the arrangement of invariant cysteine residues that determine their tertiary structure [7]. The major subfamilies are the CC and CXC chemokines. Among mammalian CXC chemokines, 16 proteins have been designated CXCL1–CXCL16. The mammalian CXC chemokines are further classified by the presence or absence of an ELR motif preceding the CXC motif [8,9]. The ELR subgroup includes CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15. They are specifically attracted neutrophils that express CXCR1 and CXCR2 and are produced by a wide variety of cells in response to many stimulants [1,10]. Their major function is to promote the adherence of neutrophlis to endothelial cells and subsequent migration along a gradient of chemokines associated with matrix proteins and cell surface toward inflammatory sites. The non-ELR subgroup includes CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, and CXCL16, which attract lymphocytes and monocytes, with poor chemotactic ability for neutrophils [10]. Additionally, most members of this subgroup are involved in angiostatic function and possess anti-angiogenic properties.

In fish, only a few orthologues to the mammalian CXC chemokines have been identified, such as CXCL8-like chemokines in catfish, haddock, carp, rainbow trout, flounder, and zebrafish [11–16], CXCL10-like in chemokines in catfish, carp, and rainbow trout [17–19]. Recently, homeostatic chemokines, such as CXCL12, CXCL13 and CXCL14, have been identified in catfish, zebrafish, large yellow croaker, flounder and carp [20–23], and these chemokines are consistently expressed in several tissues, including those not traditionally associated with the immune response. However,

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doi:10.1016/j.fsi.2011.06.016

understanding of the function of fish CXC chemokines in the immune response is limited.

Miiuy croaker, *Miichthys miiuy*, is a marine fish that is mainly distributed from the western Japan Sea to the East China Sea. In China, it is very important food fish specie with a worldwide market demand, due to its good taste, high nutritive and medicinal value, and has been widely cultured since the late 1990s [24]. Infectious disease is a significant factor hindering miiuy croaker aquaculture. To improve fish health, a better understanding of the teleost immune system is required [25]. An important step toward deciphering immune mechanisms is the identification, characterization and function analysis of genes that are regulated in response to vaccination and pathogen exposure. In the present study, we identified a CXC chemokine from miiuy croaker immune organ cDNA library. The isolation and structural analysis of the MimiCXC chemokine gene were reported. A phylogenetic analysis was carried out trying to establish its orthology. In addition, the tissue distribution and expression pattern upon stimulation with *Vibrio anguillarum* of MimiCXC was examined.

## 2. Materials and methods

### 2.1. Fish and challenge experiments

Miiuy croakers (mean weight 800 g) were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China). Fish were maintained in aerated water tanks with a flow-through seawater supply. After one week of acclimatizing, these fish were used for the challenge experiments. The bacteria *V. anguillarum* isolated from diseased miiuy croakers in Zhoushan Fisheries Research Institute was shown to be pathogenic to miiuy croaker. Briefly, *V. anguillarum* were cultured at 28 °C to mid-logarithmic growth in medium 2216E, and then resuspended after centrifugation to approximately $3 \times 10^7$ CFU/ml in phosphate-buffered saline. Fish were anaesthetized by immersion in MS222 and injected intraperitoneally with 1 ml bacterial suspension. Uninfected fish were maintained in separate tanks as control. The infected fish were killed at 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h after injection, respectively. Tissues (liver, spleen, kidney and intestine) were removed and kept at −80 °C until use. Ten tissues (liver, spleen, kidney, intestines, heart, muscle, gill, brain, eye, and fin) of uninfected miiuy croaker were also removed and kept at −80 °C until use.

### 2.2. Primer design

Two gene specific primers (5′-GSP and 3′-GSP) were designed according to the MimiCXC chemokine partial cDNA sequence in order to carry out rapid amplification of the cDNA ends (5′-RACE and 3′-RACE). 5′-GSP primers were used for amplification of the 5′ end, and 3′-GSP primers were used for amplification the 3′ end, and the universal primers mix (UPM) used for 5′-RACE and 3′-RACE according to the manufacture's instructions (Clonetech). Two pairs of primers (MimiCXC-RT-F/R and β-actin-RT-F/R) were designed to study the expression of MimiCXC gene. Additionally, two pairs of primers (MimiCXC-intron1-F/R and MimiCXC-intron2-F/R) were designed to amplify the introns from miiuy croaker genome. The above primers were listed in Table 1.

**Table 1**
Primers used in this study.

| Name | Sequence |
|---|---|
| MimiCXC-GSP5′ | CAGGATGAGTTTTCCGAGTGGTGAGCGT |
| MimiCXC-GSP3′ | GGCAAAATCAAACCACAAGACATCAAGG |
| MimiCXC-intron1-F | GCTGTTGCCTTTCTCGTCTGC |
| MimiCXC-intron1-R | CCCTGCCTTGATGTCTTGTGG |
| MimiCXC-intron2-F | CAACAACAGAAAATAGGGAGAA |
| MimiCXC-intron2-R | TGTCGTATTCACAGTTGCATTT |
| MimiCXC-RT-F | GCCTTTCTCGTCTGCCTGCTT |
| MimiCXC-RT-R | CCGAGTGGTGAGCGTGGATT |
| β-actin-RT-F | GTGATGAAGCCCAGAGCA |
| β-actin-RT-R | CGACCAGAGGCATACAGG |

### 2.3. DNA and RNA isolation, cDNA synthesis

Genomic DNA was extracted from fin samples of miiuy croaker with the method of phenol-chloroform as descried [26]. Total RNA was extracted from various tissues of adult individuals using Trizol reagent (Qiagen) according to the manufacture's instructions. cDNA was synthesized using BD Smart™ RACE cDNA amplification kit (Clontech) according to the manufacture's instructions.

### 2.4. Cloning of MimiCXC full-length cDNA

One EST (GW670971), similar to CXCL10 chemokines in *Psetta maxima* and other fish species, was obtained from the spleen cDNA library of miiuy croaker by EST analysis [27]. To obtain full length cDNA sequence of MimiCXC gene, RACE-PCR was performed using a Smart RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. Touchdown polymerase chain reaction (PCR) was used for RACE amplification: 94 °C for 2 min, 94 °C for 1 min, 70 °C for 50 s, 72 °C for 1 min, for 5 cycles; 94 °C for 1 min, 65 °C for 50 s, 72 °C for 1min, for 30 cycles; and 72 °C for 10 min for elongation. The PCR product was cloned into PMD19-T vector (TaKaRa, Dalian, China). At least three clones were sequenced by M13+/− sequencing primers.

### 2.5. Amplification of MimiCXC chemokine gene

Two pairs of primers (MimiCXC-intron1-F/R and MimiCXC-intron2-F/R) were used to amplify intron sequences of the MimiCXC chemokine gene. PCR was performed on a PTC-200. The conditions of the PCR are as follows: predenaturalization at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 60 °C for 60 s, extension at 72 °C for 2–3 min; and final extension at 72 °C for 10 min. The PCR products were cloned into vector, propagated in TOP-10 cells, and sequenced.

### 2.6. Sequence and phylogenetic analysis

Signal peptide prediction was made using SignalP 3.0 Server [28]. The theoretical pI value and molecular weight of MimiCXC mature protein was calculated using Compute pI/Mw tool program [29]. Alignment of putative amino acid sequences of miiuy croaker and other known vertebrates was performed using the Cluster W program. The sequence identities among MimiCXC and mammalian CXC chmokines were calculated by the MEGALIGN program of DNASTAR [30]. To analyze the evolutionary relationships between MimiCXC and other vertebrate chemokines, a phylogenetic tree was constructed using amino acid sequences via the neighbor-joining method [31].

### 2.7. Expression analysis of CXC chemokine gene

The primers MimiCXC-RT-F and MimiCXC-RT-R were used for amplifying MimiCXC chemokine gene fragment. Real-time quantitative PCR was conducted on a 7500 Real time PCR system (Applied Biosystems, USA) using a SYBR® *premix ExTaq*™ kit (TaKaRa). The reaction carried out without the template was used as blank control. Cycling conditions were as follows: 10 s at 95 °C, followed