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The CXC chemokines and CXC chemokine receptors in orange-spotted grouper (*Epinephelus coioides*) and their expression after *Singapore grouper iridovirus* infection

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ABSTRACT

Chemokines comprise a group of small molecular weight (6–14 kDa) cytokines; chemokine receptors are a superfamily of seven transmembrane domain G-coupled receptors. Both chemokines and their receptors have important roles in immune surveillance, inflammation, and development. Recently, 9 CXC chemokine ligands (CXCLs) and 8 CXC chemokine receptors (CXCRs) were identified and cloned from orange-spotted grouper (*Epinephelus coioides*) and annotated by phylogenetic and syntenic analyses. We detected mRNA transcripts for CXCLs and CXCRs in healthy tissues of *E. coioides*. Our data show that CXCL genes are highly expressed in the spleen, kidney and liver and that CXCR genes are ubiquitously expressed, rather than being expressed only in immune organs. Analysis of gene expression after *Singapore grouper iridovirus* infection indicated that CXCL and CXCR genes are regulated in a gene-specific manner. CXCL8 and CXCL12a were significantly upregulated in the spleen, kidney and liver of resistant fish, indicating potential roles in immunity against the pathogen. Additionally, CXCR4a was upregulated in all three organs in resistant fish, suggesting that CXCL8 or CXCL12a may participate in the immune response via interaction with CXCR4a. In addition, the new orange-spotted grouper receptor CXCR1b was found to be upregulated in the spleen and kidney of resistant fish, indicating that this receptor plays an important role in immune responses to viral infection. These results are valuable for comparative immunological studies and provide insight into the roles of these genes in viral infection.

1. Introduction

Chemokines are a superfamily of chemotactic cytokines that play important roles in regulating cell migration and activation under inflammatory conditions (Nomiyama et al., 2008; Peatman and Liu, 2007; Zlotnik and Yoshie, 2000), such as angiogenesis (Arenberg et al., 1997; Keane et al., 1998), neurological development (Gordon et al., 2009; Belmadani et al., 2006), organogenesis and germ cell migration (Doitsidou et al., 2002; Knaut et al., 2003). Moreover, the chemokine superfamily consists of important immune components that link innate and adaptive immunity (Alejo and Tafalla, 2011). Specifically, chemokines promote leukocyte mobilization and regulate immune responses and differentiation in recruited cells (Alejo and Tafalla, 2011; Esche et al., 2005). Chemokines are structurally related small peptides, and the majority of these peptides contain four conserved cysteine residues (Peatman and Liu, 2007; Chen et al., 2013). Chemokines are divided into four subfamilies according to the positions of these four conserved N-terminal cysteines: CXC, CC, C and CX3C (Bao et al., 2006). The CXC subfamily is highly important immunologically.

Chemokine receptors are a large superfamily of G protein-coupled receptors containing 7 transmembrane domains that are predominantly localized on the surface of leukocytes (Kakinuma and Hwang, 2006). These receptors contain a short acidic N-terminal end, seven helical transmembrane domains with three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites during receptor regulation (Murdoch and Finn, 2000). The N-terminal end of a chemokine receptor is key for ligand specificity, whereas G-proteins couple

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to the C-terminal end, which is important for receptor signaling after ligand binding (Murdoch and Finn, 2000). Chemokine receptors are also divided into four subfamilies, CXC, CC, CX3C and XC, based on the spacing of cysteine residues near the N-terminal of the receptor (Zlotnik et al., 2006; Kakinuma and Hwang, 2006).

CXC chemokine ligands (CXCL) can be classified as ELR^+ or ELR^- according to the presence or absence of a tri-amino acid motif (Glu-Leu-Arg or E-L-R) preceding the first conserved cysteine at the N-terminus (Pisabarro et al., 2006; Matloubian et al., 2000; Wang et al., 2005). CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 and CXCL15 are ELR^+ proteins in mammals. They play an important role in promoting adhesion of neutrophils to endothelial cells; these cells are then transferred to inflammatory sites along the gradient of chemokines associated with the matrix protein and cell surface (Laing and Secombes, 2004). The ELR^- chemokine group includes CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16. Most ELR^- CXC chemokines are angiostatic, with anti-angiogenic characteristics; they also attract lymphocytes and monocytes (Oppenheim et al., 2000; Fernandez and Lolis, 2002). In teleost fish, the ELR motif is sometimes replaced by a DLR motif (Asp-Leu-Arg). Although it was initially thought that this conservative change from E to D did not affect function (Hebert et al., 1991), recent studies have shown that the DLR motif is not necessary for neutrophil attraction to CXC chemokines (Cai et al., 2009).

To date, six different CXC clades have been found in teleost fish: CXCa, CXCb, CXCc, CXCd, CXCL12 and CXCL14 (Huising et al., 2003, 2004). However, not all fish contain these six clades of CXC chemokine genes. For example, CXC chemokines belonging to the CXCc clade are only found in carp (*Cyprinus carpio*) (Chen et al., 2013; Huising et al., 2003), whereas rainbow trout (*Oncorhynchus mykiss*) possesses CXC chemokines belonging to the CXCa and CXCb, CXCL12, and CXCL14 clades as well as the fish-specific CXCd clade (Chen et al., 2013; Wiens et al., 2006). In contrast, over 100 chemokine genes have been reported in zebrafish (Nomiyama et al., 2008). Compared to chemokine ligands, chemokine receptors are relatively conserved among species, especially among mammals (Nomiyama et al., 2011), with only 6 CXC chemokine receptor (CXCR) genes have been identified in the human and mouse genomes (Zou et al., 2015; Nomiyama et al., 2011; Zlotnik and Yoshie, 2012). In contrast, 8 CXCR genes have been reported in zebrafish and channel catfish (Zou et al., 2015; Fu et al., 2017a, 2017b). Chemokine receptors can often bind more than one chemokine ligand, and a single chemokine ligand often binds to more than one receptor. This binding promiscuity is one of the characteristics of the chemokine system and is primarily observed for inflammatory chemokines and their receptors. Therefore, interaction between chemokines and receptors plays an important role in immune cell differentiation, development and directional migration.

Orange-spotted grouper (*Epinephelus coioides*) is a coral reef fish with a high commercial value in China and Southeast Asian countries. However, in recent years, various outbreaks of viral diseases have affected the grouper aquaculture industry (Wei et al., 2010). Specifically, *Singapore grouper iridovirus* (SGIV) infection causes spleen and liver hemorrhage and enlargement, resulting in more than 90% mortality in fish farm and challenge experiments (Qin et al., 2003). As chemokines and their receptors play important roles in inflammation, elucidation of the molecular link between SGIV and chemokine responses should help solve such problems in genetic enhancement programs. Previous studies reported two members of the CXC chemokine family (CXCL8 and CXCL12) and one receptor (CXCR4) in orange-spotted grouper (Hu et al., 2010; Wu et al., 2015; Lin et al., 2012), though systematic identification of orange-spotted grouper chemokines and their receptors has not been conducted, and the responses of these genes in SGIV infection have not been fully elucidated. Therefore, the objective of this work was to identify all possible members of the CXC chemokine subfamily and their receptors in orange-spotted grouper as well as their expression in the spleen, liver and kidney after SGIV infection.

2. Materials and methods

2.1. Gene identification and sequence analysis

To identify CXC chemokine ligand and CXC chemokine receptor genes in *E. coioides*, we searched the whole-genome sequence database of this species (unpublished) and performed tblastn analysis of all available CXCL and CXCR sequences in genome databases of human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and catfish (*Ictalurus punctatus*) available in the Ensembl (http://www.ensembl.org), NCBI (http://www.ncbi.nlm.nih.gov/) and ZFIN (http://zfin.org/) databases. Each sequence was confirmed to be a unique gene according to their position information in the genome. Duplicates in the initial sequence were eliminated using Clustal Omega (http:/www.ebi.ac.uk/Tools/msa/clustalo/). ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict coding sequences, which were further validated by BLASTP against the NCBI non-redundant (nr) protein database. We cloned and sequenced the newly discovered CXC chemokine ligand and CXC chemokine receptor genes in orange-spotted grouper to verify sequence accuracy. The simple modular architecture research tool (SMART http://smart.embl-heidelberg.de) was employed to identify characteristic functional domains, and the results were further verified by conserved domains predicted in BLASTP. The orange-spotted grouper CXC chemokine ligand and CXC chemokine receptor amino acid sequences acquired in this way were used in phylogenetic analyses to determine gene identities.

2.2. Molecular cloning of new CXCL and CXCR genes

Total RNA from mixed orange-spotted grouper tissues was prepared using TRIzol (Invitrogen, USA). One microgram of isolated RNA was used to synthesize first-strand cDNA with a ReverTra Ace-α First-strand cDNA Synthesis Kit (TOYOBO, Japan). Gene-specific primers flanking open reading frames (ORFs) of orange-spotted grouper CXC and CXCR genes were designed according to the predicted sequences. All primers used in the present study are listed in Table S1. For PCR reactions, amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 52–58 °C for 15 s and 72 °C for 1–1.5 min, followed 10 min at 72 °C.

The amplification products were separated by agarose gel electrophoresis, and a band of the desired size was excised and purified using an E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA). The purified amplification products were subcloned into the pTZ57R/T vector (Fermentas, USA) and sequenced using an ABI 3700 sequencer (Applied Biosystems, USA). Three different positive clones were sequenced to confirm the sequence information.

2.3. Phylogenetic analysis

CXCL and CXCR sequences were aligned with Clustal X 1.81, and a phylogenetic tree was constructed with MEGA 6 (Tamura et al., 2013) using the neighbor-joining method. One thousand bootstrap replications were conducted. Bootstrap values lower than 50 were removed.

2.4. Genomic synteny analysis

Syntenic analysis was conducted to better support the orthology determined for the orange-spotted grouper CXCL and CXCR genes based on a comparison of CXCL and CXCR neighboring genes in orange-spotted grouper with those of zebrafish. BLASTN was conducted using the grouper CXCL and CXCR coding sequences as queries against the orange-spotted grouper genome sequence database with a cutoff E-value of $1e^{-10}$. Flanking genes were predicted from catfish genomic scaffolds using Fgenesh program Molquest software (Softberry Int.) (Solovyev et al., 2006). We then performed BLASTP analysis against the