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The CC and CXC chemokine receptors in channel catfish (*Ictalurus punctatus*) and their involvement in disease and hypoxia responses

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ABSTRACT

Chemokines are vital regulators of cell mobilization for immune surveillance, inflammation, and development. Chemokines signal through binding to their receptors that are a superfamily of seven-transmembrane domain G-coupled receptors. Recently, a complete repertoire of both CC and CXC chemokines have been identified in channel catfish, but nothing is known about their receptors. In this study, a set of 29 CC chemokine receptor (CCR) genes and 8 CXC chemokine receptor (CXCR) genes were identified and annotated from the channel catfish genome. Extensive phylogenetic and comparative genomic analyses were conducted to annotate these genes, revealing fish-specific CC chemokine receptors, and lineage-specific tandem duplications of chemokine receptors in the teleost genomes. With 29 genes, the channel catfish genome harbors the largest numbers of CC chemokine receptors among all the genomes characterized. Analysis of gene expression after bacterial infections indicated that the chemokine receptors were regulated in a gene-specific manner. Most differentially expressed chemokine receptors were up-regulated after *Edwardsiella ictaluri* and *Flavobacterium columnare* infection. Among which, CXCR3 and CXCR4 were observed to participate in immune responses to both bacterial infections, indicating their potential roles in catfish immune activities. In addition, CXCR3.2 was significantly up-regulated in ESC-susceptible fish, and CXCR4b was mildly induced in ESC-resistant fish, further supporting the significant roles of CXCR3 and CXCR4 in catfish immune responses. CXCR4b and CCR9a were both up-regulated not only after bacterial infection, but also after hypoxia stress, providing the linkage between bacterial infection and low oxygen stresses. These results should be valuable for comparative immunological studies and provide insights into their roles in disease and stress responses.

1. Introduction

Chemokines are a superfamily of chemotactic cytokines exerting pivotal roles in regulating cell migration under both inflammatory and physiological conditions (Nomiyama et al., 2008; Peatman and Liu, 2007; Zlotnik and Yoshie, 2000). They are key immune regulators, acting as a bridge between innate and adaptive immunity (Alejo and Tafalla, 2011; Raman et al., 2011). Specifically, chemokines promote leukocyte mobilization and regulate the immune responses and differentiation of the recruited cells (Alejo and Tafalla, 2011; Esche et al., 2005). In addition to their roles in immunity, chemokines also participate in angiogenesis (Arenberg et al., 1997; Keane et al., 1998), neurological development (Belmadani et al., 2006; Gordon et al., 2009), organogenesis and germ cell migration (Doitsidou et al., 2002; Knaut et al., 2003), hypoxia responses (Bosco et al., 2006; Schioppa et al., 2003), and normal development and growth (Baoprasertkul et al., 2005; David et al., 2002; Zeng et al., 2016). The chemokine superfamily includes a large number of ligands binding to a smaller number of receptors (Zlotnik and Yoshie, 2000).

Chemokine receptors are a large superfamily of G protein-coupled receptors (GPCRs) containing 7 transmembrane domains (7TM), predominantly on the surface of leukocytes (Kakinuma and

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Hwang, 2006). They are composed of about 350 amino acids that are divided into 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 which act as phosphorylation sites during receptor regulation (Murdoch and Finn, 2000). The N-terminal end of a chemokine receptor is important for ligand specificity. G-proteins couple to the C-terminal end, which is important for receptor signaling following ligand binding (Murdoch and Finn, 2000). Chemokine receptors are also grouped into four subfamilies, CXC, CC, CX3C and XC chemokine receptors, which correspond to the four subfamilies of chemokine ligands they bind (Zlotnik et al., 2006). Four subfamilies of chemokine receptors differ in spacing of cysteine residues near N-terminal of the receptor (Kakinuma and Hwang, 2006).

To date, 18 chemokine receptors with standard chemotactic functions have been identified in human and mouse genome: 6 CXCR genes, 10 CCR genes, 1 XCR gene, and 1 CX3CR gene (Nomiyama et al., 2011; Zlotnik and Yoshie, 2012). Like their ligand counterpart genes, the chemokine receptor genes are also clustered together in the genome. For example, a large cluster of chemokine receptors is located in human chromosome 3, suggesting a rapid evolution through tandem gene duplications (Zlotnik and Yoshie, 2012). In addition, five atypical chemokine receptors have also been described, which are regarded as the scavengers for excess chemokines (Zlotnik and Yoshie, 2012). Compared to chemokine ligands, chemokine receptors are relatively conserved among species especially for mammals (Nomiyama et al., 2011). Chemokine receptors often bind more than one chemokine ligands and a single chemokine ligand often binds more than one receptor. Such binding promiscuity in one of the characteristic features of the chemokine system and is mostly observed between inflammatory chemokines and their receptors. In contrast, homeostatic chemokines and their receptors show more specific relationships (Nomiyama et al., 2011).

Channel catfish (*Ictalurus punctatus*) is the major aquaculture species in the United States (Naylor et al., 2000), but its sustainable production is threatened by severe disease outbreaks. In particular, the enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri*, columnaris disease caused by *Flavobacterium columnare,* and the emerging Aeromonas disease caused by *Aeromonas hydrophila*, all bacterial diseases, cause major economic losses to the catfish industry. The disease incidents were increased with exposures to environmental stresses, especially when exposed to hypoxia (Geng et al., 2014; Wang et al., 2017a; Zhong et al., 2017). Understanding of molecular linkage between disease and hypoxia responses should help strategic thinking of solving these problems in genetic enhancing programs. We previously identified 64 CC and 17 CXC chemokine ligands from catfish genome and analyzed their responses to bacterial infection and hypoxia stress (Bao et al., 2006; Baoprasertkul et al., 2005; Fu et al., 2017a, 2017b; He et al., 2004; Peatman et al., 2006). However, systematic identification of catfish chemokine receptors has not been conducted. The major objective of the present study was to determine the CC and CXC chemokine receptors of catfish, to establish their othologies through phylogenetic and syntenic analysis. In addition, even though previous studies investigated CC and CXC chemokine receptors of several teleost fish species such as zebrafish, medaka and tetraodon (Liu et al., 2009; Nomiyama et al., 2011), the involvements of fish CC and CXC chemokine receptors in response to bacterial infection and hypoxia stress have not been fully understood. Here we also report expression of CC and CXC chemokine receptors after bacterial infections and hypoxia challenges.

2. Materials and methods

2.1. Gene identification and sequence analysis

To identify the sequences of channel catfish CC and CXC chemokine receptors (CCR and CXCR), TBLASTN was conducted against catfish transcriptome datasets (Li et al., 2012; Liu et al., 2012; Sun et al., 2012), with a cutoff E-value of $1e^{-5}$, using all available sequences of CCR and CXCR from zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*), fugu (*Takifugu rubripes*), frog (*Xenopus laevis*), chicken (*Gallus gallus*), mouse (*Mus musculus*) and human (*Homo sapiens*) retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/), Ensemble (http://www.ensembl.org), uniport (http://www.uniprot.org/) and ZFIN (http://zfin.org/). After that, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to eliminate duplicates in the initial sequence pool. The coding sequences were predicted using ORF (opening reading frames) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), which were further validated by BLASTP against NCBI non-redundant (nr) protein database. BLASTN was performed against the channel catfish genome sequence (Liu et al., 2016) to verify copy number and sequence accuracy, using the initial pool of mRNA sequences as queries, with a cutoff E-value of $1e^{-10}$. Fgenesh program of Molquest software (Softberry Int.) was used to predict the genes from retrieved genomic scaffold sequences (Solovyev et al., 2006). The characteristic functional domains were identified using the simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de) and further endorsed by conserved domain predicted through BLASTP.

2.2. Phylogenetic analysis

Phylogenetic analysis was conducted using the amino acid sequences of CC and CXC chemokine receptors including human, mouse, chicken, frog, channel catfish, zebrafish, fugu, tilapia and medaka. MUSCLE (multiple sequence comparison by log-expectation) was used to conduct alignment of multiple amino acid sequences with default parameters (Edgar, 2004). MEGA 6 was used to construct the unrooted phylogenetic tree of CCR and CXCR using the maximum likelihood method (Tamura et al., 2013), respectively. Based on the alignment results, JTT (Jones-Taylor-Thornton) + I (invariant sites) + G (gamma distribution for modeling rate heterogeneity) model was selected (Darriba et al., 2011). The bootstrapping with 1000 replications was performed to test the phylogenetic tree, and gaps/missing data treatment was analyzed using all sites.

2.3. Synteny and schematic genomic organization analysis

Syntenic analysis was conducted to provide extra evidence to the orthologies of channel catfish CC and CXC chemokine receptors, basing on the comparison the neighboring genes of CCR and CXCR in channel catfish with zebrafish. BLASTN was conducted using the coding sequences of catfish CCR and CXCR as queries against the catfish genome sequence database (Liu et al., 2016), with a cutoff E-value of $1e^{-10}$. The flanking genes of catfish CC and CXC chemokine receptors were predicted from the catfish genomic scaffolds using Fgenesh program Molquest software (Softberry Int.) (Solovyev et al., 2006) and validated by running BLASTP against NCBI non-redundant database. The genomic pattern of CC and CXC chemokine receptor genes and their neighboring genes in zebrafish were obtained from NCBI database. For the nomenclatures of channel catfish CC and CXC chemokine receptors, we named them after zebrafish whenever possible based on orthologues which determined by phylogenetic and syntenic analysis because zebrafish is