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Characterization and role of suppressor of cytokine signaling 1a (SOCS1a) in a teleost fish, *Miichthys miiuy*

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ABSTRACT

The suppressor of cytokine signaling 1 (SOCS1) is a crucial regulator in the immune systems of mammals, which functions classically as a negatively regulator in the IFN signaling pathways. However, data on functional characterization of SOCS1 in lower vertebrates is limited. In this study, we identified and characterized the full-length SOCS1a gene of miiuy croaker (*Miichthys miiuy*). The sequence analysis results showed that miiuy croaker SOCS1a (mmSOCS1a) shared some conserved motifs with other vertebrates. To further study the function of fish SOCS1, we identified mmSOCS1a and determined its potential ability to perceive poly(I:C) stimulation. Induction experiments with poly(I:C) indicated the significant expression levels of mmSOCS1a in liver and kidney. In addition, mmSOCS1a could inhibit poly(I:C)-induced or IFNs-induced ISRE reporter gene activation. In a word, we systematically and comprehensively analyzed evolution and function of mmSOCS1a, which will provide the basis for future research on fish SOCS family.

1. Introduction

Cytokines are pleiotropic molecules that have crucial roles in the proliferation, differentiation and immune regulation through different signaling mechanisms (Jin et al., 2008). Cytokines that included interleukins, interferons (IFNs) and haematopoietic growth factors, which could activate the Janus kinase–signal transducer and activator of transcription (JAK–STAT) signal transduction pathway to elicit downstream effects in responsive cells (Yoshimura et al., 2007). JAK–STAT signal transduction pathway plays an extremely important role in controlling immune responses (Shuai and Liu, 2003). While, excessive cytokine signaling may disorder the normal homeostasis and cellular functions. Therefore, to maintain homeostasis, corresponding genes evolved to regulate JAK-STAT pathway.

So far, the intracellular suppressors of cytokine signaling (SOCS) family is composed of 8 members, including CISH, SOCS1–7 of SOCS family, has been identified in mammals (Croker et al., 2008; Shuai and Liu, 2003). However, fish had at least 12 SOCS family members, which included 4 fish-specific members SOCS3b, SOCS5b, SOCS8, and SOCS9, except 8 known mammalian counterparts (SOCS1 to SOCS7, and CISH) (Jin et al., 2008). SOCSs share a similar structure, including a central SH2 domain, a variable N terminal region and a C-terminal SOCS box (Sepulveda et al., 1999). The SOCS family members are emerging as one of the most important regulators to regulate the JAK-STAT pathway (Yoshimura et al., 2007). And most SOCS proteins are induced by cytokines and act as important inhibitors to negative-feedback signal transduction (Wang et al., 2011). Among mammalian SOCS proteins, SOCS1 plays an important role in innate and adaptive immunity in mammalian SOCS proteins (Dimitriou et al., 2008; Endo et al., 1997; Naka et al., 1997).

SOCS1 could negatively regulate a broader range of immune-relevant signaling pathways, such as NF-κB, JNK, and p38 (He et al., 2006; Ryo et al., 2003). However, the most traditional function of SOCS1 could negatively regulate the IFN signaling pathway, which reduces the responsiveness of lots of cell types to IFN-α, -β, and-γ (Fenner et al., 2006; Qing et al., 2005). For example, SOCS1 is an inhibitor of IFN-mediated JAK-STAT signaling in Atlantic salmon (Skjesol et al., 2014). Similarly, SOCS1 also have the inhibitory role of in Atlantic salmon antiviral immunity (Sobhkhez et al., 2017). In addition, the inhibitory effect of zebrafishes SOCS-1s was found in IFN signaling pathway (Nie et al., 2014). To further study the function of SOCS1 in teleost fish, we choice miiuy croaker as subject investigated to confirm the role of SOCS1. Miiuy croaker is an economically important marine fish species. The study of this species have been conducted in-depth from transcriptome (Chu

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et al., 2015, 2017a), whole-genome (Xu et al., 2016) to functional genes (Chu et al., 2017b; Wang et al., 2016a,b; Zhao et al., 2017), which left miiuy croaker as an excellent model for studying the functions of some immune-related genes. Because of the fish-specific genome duplication (FSGD), the SOCS1 gene is differentiated into two genes in fish, named SOCS1a and SOCS1b respectively. In this study, the full-length of SOCS1 gene was identified in miiuy croaker and named as SOCS1a, which further study their genomic organizations, gene structures, gene synteny, expression analysis. In addition, the cellular localization and the regulate effect of SOCS1a for poly (I:C) or IFN2b stimulation induced signal transduction pathway were also reported to enrich the knowledge about the functions of SOCS1 gene in teleost fish. Furthermore, this study not only enriches the current knowledge of SOCS proteins in IFN signaling regulation, but also provides an evidence about the negatively regulation of IFN signaling pathway in teleost fish.

2. Materials and methods

2.1. Samples and challenge experiments

Miiuy croakers were temporarily reared in the seawater tanks with a flow-through seawater supply at ambient temperature 25 °C for before experimental manipulation, and the selected non-deformed healthy individual (mean weight of 750 g) with abundant physical strength and no technical damage was utilized for pathogen infection experiment (Xu et al., 2010). The healthy fishes were randomly divided into two groups including control group and injection group. For the purpose examining the stimulation experiment, we conduct immune stimulation. In the injection group, 1 ml poly (I:C) (2.5 mg/ml, InvivoGen) was injected. After injection, fish samples were killed at 6 h, 12 h and 36 h from miiuy croaker.

2.2. Isolation of macrophages

To extract macrophages, miiuy croaker kidney from three individuals respectively were removed aseptically and placed in ice-cold L-15 cell culture medium containing antibiotics. The tissue were filtered aseptically using a 100 μm pore size cell strainer in L-15 medium with streptomycin (100 IU/ml), penicillin (100 μg/ml), 2% fetal bovine serum (FBS), and heparin (20 U/ml). The suspension was placed on a 51% discontinuous Percoll gradient and centrifuged at 400 g for 40 min at 4 °C. Then, the cells at the interface were collected and washed twice in L-15 medium. Macrophages were seeded in 6-well plates at a density of 4×10^7 cells per well in L-15 containing 0.1% FBS, and then cultured at 26 °C, 4% CO2. The following day, the cells were cultured with fresh complete L-15 medium (with 20% FBS), and 6 h later, the cells were stimulated with poly (I:C) (5 μg/ml, SIGMA). The cells were collected after 6 h, 12 h and 24 h stimulation for RNA extraction and qRT-PCR analysis. Cells with no stimulation were collected as the control for the experiment; each treatment and control had three replicates.

2.3. Identification of mmSOCS1a

To obtain cDNA and genomic sequences of mmSOCS1a, the BLASTn and tBLASTn programs were used to search a series of the closely related species SOCS1 gene sequences from GenBank, which search against the miiuy croaker transcriptome assembled sequences (Che et al., 2014) and a database of miiuy croaker whole-genome sequences (Xu et al., 2016) were applied to search for SOCS1a cDNA sequences and gene sequences via local BLASTn and tBLASTn programs. The cDNA sequence was aligned with the genome scaffolds by MAFFT to determine the genomic structure of mmSOCS1a. On the side, the desired SOCS cDNA sequence found in the cDNA database was aligned with the selected genomic sequence to decide the genomic structure of mmSOCS1 by using MAFFT (Katoh and Standley, 2013).

2.4. Sequence and phylogenetic analysis

The deduced protein, the molecular weight and isoelectric point (pI) of mmSOCS1a were conducted by using the software ExPASy (http://expasy.org/tools/). The result of multiple alignment was acquired by using MEGA 5 software (Tamura et al., 2011) and DNAman (Woffelman, 1994). To understand the evolutionary relationship mmSOCS1a in vertebrate SOCS1, MEGA 5 was applied to establish the phylogenetic trees, which was run with 10 000 generations with 25% of trees burned. The SOCS sequences used in the analysis, including fish and representative animal, were retrieved from GenBank (Table S1).

2.5. Real-time quantitative PCR analysis

Total RNA was extracted from adult miiuy croaker various tissues by using Trizol reagent (Takara). The purity and concentration of total RNA were determined by using Nanodrop 2000 spectrophotometer (Thermo scientific). According to the manufacturer's instructions, the cDNA template was synthesized utilizing a QuantScript RT kit (with gDNase to eliminate genomic contamination). Two pairs of primers were used to explore expression patterns of SOCS1a genes (Table S2). Before quantitative real-time PCR (qRT-PCR), the specificity and amplification efficiency of these primers need to be tested. The mRNA expression level of mmSOCS1a gene in different tissues (liver and kidney) of uninfected and infected miiuy croakers were tested by qRT-PCR. qRT-PCR was conducted on a 7300 real time PCR system (Applied Biosystems, USA) using a RealMasterMix kit (TIANGEN). The PCR was carried out in a total volume of 20 μl, which included 9 μl SYBR Green Real-time PCR master mixtures, 1 μl cDNA sample, 1 μl sense primer, 1 μl antisense primer and 8 μl ddH_2O. Every expression was performed in triplicate wells and cycling conditions were carried out as followed: 10 s at 95 °C, then followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Dissociation curve analysis was implemented after each assay to determine target specificity. The amplification efficiency was close to the theoretical value. The data was analyzed by using SPSS V13.0 software and 7300 System SDS Software v1.3.0 (Applied Biosystems, USA).

2.6. Plasmid construction

To construct the expression plasmid, the full-length CDS region of mmSOCS1a was amplified by PCR with primers and cloned into the *BamH* I and *Xba* I restriction enzyme cutting sites of pCDNA3.1 (Invitrogen) with Flag tag (Table S2). The constructed recombinant plasmid was confirmed by sequencing. The plasmids were extracted using Endotoxin-Free Plasmid DNA Miniprep Kit (TIANGEN) for cell transfection. In addition, human TLR3 (hTLR3) plamsid was purchased from PPL.

2.7. Cell culture, transient transfection and luciferase reporter assays

HEK293 and Hela cells were cultured in Dulbecco's modified Eagle's medium(DMEM), supplemented with 10% FBS (Fetal Bovine Serum, Gibco), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin under humidified conditions with 5% CO2 at 37 °C. The proportion of the amount plasmid of expression plasmids: pRL-TK: NF-κB or ISRE reporter gene is 1:10. The control