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Short communication

Comparative analysis of the expression patterns of eight suppressors of cytokine signaling in tongue sole, *Cynoglossus semilaevis*

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A B S T R A C T

Suppressor of cytokine signaling (SOCS) family members are inhibitors of cytokine signaling pathways and key regulators of immunological homeostasis. They have been extensively studied in mammalian models, but systematic analyses of SOCS in fish are limited. In the current study, a total of eight SOCS genes from tongue sole (*Cynoglossus semilaevis*) were characterized. All eight CsSOCS exhibit conserved structures of SOCS and were phylogenetically grouped together with the respective SCOS members known in mammalian and teleost species. Under normal physiological conditions, the expressions of the eight CsSOCS genes were detected at varied levels in nine major tissues, with most CsSOCS highly expressed in kidney. Following challenge with intracellular and extracellular bacterial pathogens, the majority of CsSOCS genes exhibited distinctly different expression profiles in a time-, tissue-, and pathogen-dependent manner. In general, intracellular pathogen caused wider and higher levels of CsSOCS expressions than extracellular pathogen. These results suggest that different members of SOCSs in teleost may play different roles in the infection processes of different bacterial pathogens.

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1. Introduction

Cytokines activate cell surface receptors and regulate a wide range of biological processes including survival, proliferation, differentiation, and immune response [1,2]. Cytokines signal by interaction with cognate receptors in cell membrane and activation of the receptor-associated Janus kinases (JAKs); JAKs phosphorylate the cytoplasmic domain of the receptor, which recruits signal transducer and activator of transcription factors (STATs) [3]. Subsequent phosphorylation of STATs by JAKs enables nuclear translocation of STATs and gene expression [4]. However, excessive cytokine signaling can disrupt the normal homeostasis and cellular functions [5]. As a result, cytokine signaling is controlled by a number of negative regulators, one of which is the suppressors of cytokine signaling (SOCS) family, a group of important feedback inhibitors of cytokine signaling [6].

Eight SOCS members, i.e. CISH and SOCS1–7, have been identified in mammals, all which are structurally characterized by a central Src-homology 2 (SH2) domain and a conserved C-terminal motif named SOCS box [7]. SOCS1 is known to function in interferon signaling in fish models and human cells [8]. Several studies indicate that SOCS2 plays an important role in the regulation of metabolism, immune response, mammary gland development, cancer, and other cytokine-dependent signaling pathways [9,10]. SOCS3 protein is the key physiological regulator in immune homeostasis, and dysregulation of SOCS3 can cause a variety of diseases, including allergy, autoimmune diseases, inflammation, and cancer [11]. SOCS4 is a critical regulator of anti-viral immunity, and SOCS4-deficient animals suffered dysregulated production of proinflammatory cytokines and chemokines and delayed viral clearance [12]. SOCS5 has been reported to be implicated in T-cell differentiation [13]. SOCS6 and SOCS7 may have a redundant role in insulin signaling [14].

Although the functions of the SOCS family have been well studied in mammals, similar studies are scarce in fish. To date, identification and analyses of SOCS in fish have been reported in *Tetraodon nigroviridis* [15–17], *Danio rerio* [15–17], *Takifugu rubripes* [15–17], *Gasterosteus aculeatus* [16,17], *Cyprinus carpio* L. [18], *Scophthalmus maximus* [19], *Carassius auratus* L. [20], *Perca flavescens* [21], *Salvelinus alpinus* [22], *Oncorhynchus mykiss* [23–25]

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and *Ictalurus punctatus* [26]. In some of these reports, the expression of SOCS genes in response to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) and poly I:C, has been examined [15,17,25].

Tongue sole (*Cynoglossus semilaevis*) is an important aquaculture species in China [27]. In this study, we aimed to investigate the expression patterns of different SOCS members in tongue sole under different conditions. For this purpose, we analyzed, in a comparative manner, the mRNA profiles of six SOCS genes in the presence and absence of experimental infection with different bacterial pathogens.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (average 14.2 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before experiment, fish were acclimatized in the laboratory for two weeks and confirmed to be absent of bacterial and viral pathogens in liver, kidney, and spleen by plate count as reported previously [28]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA).

2.2. Sequence analysis

The amino acid sequences of CsCISH, CsSOCS1, CsSOCS2, CsSOCS3, CsSOCS4, CsSOCS5, CsCsSOCS6, and CsSOCS7 (GenBank accession numbers: XP_008317825.1, XP_008328399.1, XP_008313498.1, XP_008327772.1, XP_008335216.1, XP_008320412.1, XP_008305817.1, and XP_008314403.1, respectively) and were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the conserved domain search program of NCBI. Multiple sequence alignment was created with DNAMAN. Phylogenetic analysis was performed with ClustalX and the Neighbor-joining algorithm of MEGA 4.0. Gaps were removed by pair-wise deletion and 1000 bootstrap replicates were performed in phylogenetic analysis.

2.3. Quantitative real time reverse transcription-PCR (qRT-PCR) analysis of gene expression in the presence and absence of bacterial infection

qRT-PCR was performed as reported previously [29]. Briefly, to examine gene expression in the absence of bacterial infection, the tissues of brain, gill, liver, blood, heart, spleen, kidney, intestine, and muscle were taken aseptically from five tongue sole and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). The primers used for qRT-PCR are listed in Table 1. The expression levels of CsSOCS were analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference [30]. To examine gene expression in the presence of bacterial infection, *Edwardsiella tarda* and *Vibrio harveyi* were cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 2×10^6 CFU (colony forming unit)/ml and 2×10^7 CFU/ml, respectively. Tongue sole were divided randomly into three groups (20/group) and injected intraperitoneally with 50 μl *E. tarda*, *V. harveyi*, or PBS. At 6 h, 12 h, 24 h, and 48 h post-infection, spleen, kidney, and liver were taken from five fish, and CsSOCS expression was determined by qRT-PCR as above with 60S ribosomal protein L18a (for spleen), ACTB (for kidney), and 18s rRNA (for liver) as internal controls [30]. All experiments were performed three times, and statistical analyses were carried out with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

Table 1
Primers used for qRT-PCR in this study.

Primers	Sequences (5′–3′)	Target gene
CISH-RT-F	CCCGAGCCCTAGAACTCTTC	CsCISH
CISH-RT-R	TTTGTGGGGTCCCATGTTGG	
SOCS1-RT-F	GTATCATGGAGCTGTGCGGT	CsSOCS1
SOCS1-RT-R	GGTGTACAAAGTTGACGCGG	
SOCS2-RT-F	TCACACTGTCCCGAACACAC	CsSOCS2
SOCS2-RT-R	CTGCACCTGCTTGGTCCTAC	
SOCS3-RT-F	GCTGCCTCAGCGTTACAAGA	CsSOCS3
SOCS3-RT-R	GGTGCCATTGGACTCGTTTG	
SOCS4-RT-F	TGACAGGGCTGTTGAGACAC	CsSOCS4
SOCS4-RT-R	GAGGCAGTGGCAGGTTGTTA	
SOCS5-RT-F	TTGTTGCAGGTCCATCAGGG	CsSOCS5
SOCS5-RT-R	ACTTTCCAGGGTCCAGGTTG	
SOCS6-RT-F	AGTAGAGGTTCCCCCTCCTC	CsSOCS6
SOCS6-RT-R	CCCACTGCTTTGGACTGAAT	
SOCS7-RT-F	TGGACGCAGAAAACGGGAATCT	CsSOCS7
SOCS7-RT-R	GCTCGACGCCCGCAG	

3. Results

3.1. Sequence analysis of tongue sole SOCS proteins

CsCISH, CsSOCS1, CsSOCS2, CsSOCS3, CsSOCS4, CsSOCS5, CsCsSOCS6, and CsSOCS7 consist of 226, 293, 209, 204, 380, 557, 535, and 850 amino acid residues, respectively. They all possess a central SH2 domain, a SOCS box, a phosphotyrosine binding pocket, and a hydrophobic binding pocket (Fig. S1). In addition, CsCISH, CsSOCS2, CsSOCS3, CsSOCS4, and CsSOCS5 also have an elongin B/C interaction site (Fig. S1).

3.2. Phylogenetic analysis of CsSOCS

Multiple sequence alignments revealed that CsCISH, CsSOCS1, CsSOCS2, CsSOCS3, CsSOCS4, CsSOCS5, CsCsSOCS6, and CsSOCS7 share 69%–85%, 66%–74%, 70%–74%, 79%–87%, 66%–78%, 84%–91%, 75%–94%, and 60%–88% overall sequence identities, respectively, with the corresponding SOCSs in other teleost species, and 65%, 59%, 53%, 53%, 41%, 64%, 72%, and 62% overall sequence identities, respectively, with their human counterparts (Fig. S2). Phylogenetic analysis of the SOCS homologues of teleost and mammals indicated that two main clades were generated, one containing CsCISH, CsSOCS1, CsSOCS2, and CsSOCS3, and the other containing CsSOCS4, CsSOCS5, CsCsSOCS6, and CsSOCS7 (Fig. 1). In the first clade, SOCS1 and SOCS3 were clustered into a group which is distinct from that formed by CISH and SOCS2; in the other clade, SOCS4 and SOCS5 were clustered into a group which is distinct from that formed by SCOS6 and SOCS7.

3.3. Expression profiles of the CsSOCS under normal physiological conditions

qRT-PCR analysis was conducted to examine the expression of CsSOCS under normal physiological conditions in nine tissues (gill, blood, brain, heart, liver, muscle, spleen, intestine, and kidney). The results showed that all CsSOCS expressed in the examined tissues, but the expression patterns differed. Except for CsCISH and CsSOCS2, which displayed highest levels of expression in liver, all

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