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# Functional analysis of the CXCR1a gene response to SGIV viral infection in grouper

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ABSTRACT

Chemokine receptors are a superfamily of seven-transmembrane domain G-coupled receptors and have important roles in immune surveillance, inflammation, and development. In previous studies, a series of CXCRs in grouper (*Epinephelus coioides*) was identified; however, the function of CXCR in viral infection has not been studied. To better understand the effect of the CXCR family on the fish immune response, full-length CXCR1a was cloned, and its immune response to Singapore grouper iridovirus (SGIV) was investigated. Grouper CXCR1a shared a seven-transmembrane (7-TM) region and a G protein-coupled receptor (GPCR) family 1 that contained a triaa stretch (DRY motif). Phylogenetic analysis indicated that CXCR1a showed the nearest relationship to *Takifugu rubripes*, followed by other fish, bird and mammal species. Fluorescence microscopy revealed that CXCR1a was expressed predominantly in the cytoplasm. Overexpression of CXCR1a in grouper cells significantly inhibited the replication of SGIV, demonstrating that CXCR1a delayed the occurrence of cytopathic effects (CPE) induced by SGIV infection and inhibited viral gene transcription. Furthermore, our results also showed that CXCR1a overexpression significantly increased the expression of interferon-related cytokines and activated ISRE and IFN promoter activities. Taken together, the results demonstrated that CXCR1a might have an antiviral function against SGIV infection.

## 1. Introduction

Chemokines are members of chemotactic cytokines that play an important role in cell movement and activation under inflammatory conditions [1–3]. Furthermore, chemokine superfamilies are an important bridge between innate and adaptive immunity [4]. Most chemokines generally contain four types of cysteine; according to differences in the first two cysteines, chemokines can be classified into five subtypes: CXC, XC, CX, CC and CX3C [5]. Chemokine receptors are G protein-coupled receptors containing seven transmembrane structures [6]. These receptors contain 7 helical transmembrane domains, three intracellular and three extracellular hydrophilic rings, a short acidic N-terminal and an intracellular C-terminal, with serine and threonine residues that act as phosphorylation sites in the receptor regulation process [7]. The N-terminal of a chemokine receptor is the key position that specifically binds to a ligand, whereas G proteins couple to the C-terminal end, which is an important signal pathway after ligand binds to the receptor [7]. 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 [6,8]. Most chemokines can bind to multiple receptors, and one receptor can also bind to multiple chemokines [9]. The ligand receptor complex then initiates a series of signaling pathways that regulate various physiological and pathological processes [10,11]. Although chemokine and chemokine receptors are widely studied in mammals, particularly humans, the involvement of the chemokine-receptor system in different diseases [7] and the chemokine and chemokine receptor biology of teleost fish and its involvement in fish immunity are ill-defined.

CXC chemokine receptor 1 (CXCR1) was the first defined chemokine receptor subtype, and this is the only known receptor for ELR + CXC chemokines in mammals. CXCR1 is often present on the surface of neutrophils as a receptor for il-8, and IL-8 carries out its function by binding and activating the receptor. Il-8 binds to CXCR1 to induce chemotaxis and cytotoxic reactions, such as the exocytosis of lysosomal

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enzymes and the production of superoxide anions [12–14]. Hence, CXCR1 has fundamental regulatory effects on natural immunity, inflammation, white blood cell transport, neutrophil recruitment, immune system signal transduction pathways and host anti-infection [15]. To date, CXCR1 has been identified and characterized only from a few fish species [16–23]. In our previous study, we found two CXCR1 (CXCR1a and CXCR1b) genes in orange-spotted grouper, in an SGIV-challenged experiment, CXCR1a was highly expressed in resistant fish [24]. However, the function of CXCR1a in viral infection has not been studied.

Orange-spotted grouper (*Epinephelus coioides)* is an economically valuable fish in China and Southeast Asian countries. However, in recent years, outbreaks of various viral diseases have affected the development of grouper aquaculture [25]. Specifically, Singapore grouper iridovirus (SGIV) infection causes spleen and liver hemorrhage and enlargement, resulting in more than 90% mortality in fish farms and challenge experiments [26]. As chemokine receptors play an important role in the natural immunity and anti-viral, elucidating the molecular relationship between chemokine receptors and SGIV will help to provide ideas for the treatment of SGIV infection, like increase disease resistance through enhancing CXCR1a expression.

In this study, we cloned the orange-spotted group CXCR1a gene and examined its expression in normal tissues. We investigated the subcellular localization of CXCR1a in grouper cells and then evaluated the roles of CXCR1a in SGIV infection. In addition, the effects of CXCR1a on the host interferon immune response were also elucidated. Our results will provide new insights into understanding the roles of fish CXCR in viral infection.

## 2. Materials and methods

### 2.1. Cloning of CXCR1a and sequence analysis

The full-length cDNA of CXCR1a was obtained using RACE methods based on the sequences from Wang et al., 2019 [27]. The resulting sequence was analyzed by BLAST searches of the GenBank database using the default settings, and the conserved domain was predicted by the SMART program (http://smart.emblheidelberg.de/). Multiple sequence alignments were performed using ClustalW [28]. A phylogenetic tree was generated with MEGA6.0 using the neighbor-joining method [29].

### 2.2. Tissue distribution analysis

The expression profiles of CXCR1a in healthy and SGIV infected orange-spotted grouper were examined by quantitative real-time PCR using gene-specific primers flanking the ORF. Different tissues, including the head kidney, heart, brain, liver, stomach, skin, spleen, kidney, muscle, gill, gonad and intestine, were collected for RNA extraction and further quantitative real-time PCR (qRT-PCR) analysis. qPCR was carried out using a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) with the following program: 1 cycle of 94 °C for 10 min, 40 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, with a final elongation step of 10 min at 72 °C. We analyzed relative gene expression using the typical Ct method ($2^{-\triangle\triangle}$Ct method) [30].

### 2.3. Viral infection

Grouper spleen (GS) cells used in this study were cultivated in Leibovitz's L15 medium supplemented with 10% fetal bovine serum (FBS) at 25 °C [31]. SGIV stocks were prepared as mentioned above and were placed in a refrigerator at −80 °C for further use [32]. For viral infection, GS cells were infected with SGIV for the indicated length of time. At the specified time after infection, the morphology of the cells was observed or imaged under an optical microscope, and cells with viral infection were collected for further analysis.

### 2.4. Plasmid constructions

CXCR1a was subcloned into pcDNA3.1-3 × Flag and pEGFP-C1 expression vectors to study the potential function of CXCR1a *in vitro*. The primers used for plasmid construction are listed in Supplementary File 1. The recombinant plasmids were confirmed by DNA sequencing.

### 2.5. Cell transfection

Cell transfection was performed using a transfection reagent (TA), Lipofectamine 2000 (Invitrogen), as described previously [33]. In short, GS cells were seeded into a 24-well cell culture dish at a concentration of 60–70%. The next day, the GS cells were transiently transfected with plasmids using Lipofectamine™ 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Six hours later, the medium and cell culture were replaced at 25 °C for further study.

### 2.6. Western blot and protein concentration analysis

The western blot was performing as previous study [34]. The protein was separated on a 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride membranes. The rabbit *anti*-MCP antibody and beta-actin (proteintech) was diluted at a ratio of 1:1000 and 1:2000 respectively. Quantification of the protein blots was performed using the Quantity One 1-D software (version 4.4.0) (Bio-Rad Laboratories, Inc.).

### 2.7. Fluorescent microscopy

To determine the subcellular localization of CXCR1a, pEGFP-C1 and pEGFP- CXCR1a plasmids were transfected into GS cells as described above. After 48 h of culture, the cells were fixed with 4% paraformaldehyde and were then stained with 6-diamidino-2-pheny-lindole (DAPI) (1 mg/ml) for 15 min. Finally, the cells were rinsed with PBS, mounted with 50% glycerol, and observed using fluorescence microscopy (Leica, Germany).

### 2.8. RNA extraction and qRT-PCR analysis

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Real-time PCR analyses were performed on a Roche Light Cycler 480 real-time PCR system using SYBR Green I Master (Roche) according to the manufacturer's instructions. The PCR conditions were as follows: 95 °C for 5 min for activation, followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s and 72 °C for 20 s. The β-actin gene was used as the internal control. The expression level of each target gene was normalized to the geometric mean of the mRNA level of this housekeeping gene by the $2^{-\Delta\Delta Ct}$ method [29]. The primers are listed in Supplementary File 1. The data are presented as the mean ± SD, and the statistical significance level was defined as $p < 0.05$ (*).

### 2.9. Reporter gene assay

GS cells were seeded into 24-well plates for 18 h, and then CXCR1a luciferase reporter constructs and luciferase plasmids, including ISRE-Luc and IFN1-Luc, were used for cotransfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After an additional 18 h of incubation, samples were prepared for luciferase assay using a commercial Luciferase Assay System (Promega), and


**Fig. 1. The nucleotide and deduced amino acid sequences of CXCR1a**. Deduced transmembrane regions was shaded in gray. The G-protein coupledbreceptors family 1 signature is boxed in which the DRY motif is shown in red letter. The polyadenylation signals are in boldface and underlined. Potential N-glycosylation sites are shaded with yellow. The C-termimus sequence rich in Ser and Thr is delineated by break line. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

luminescence was measured immediately using a Victor *X*5 Multilabel plate reader (PerkinElmer). The results are representative of three independent experiments, and each independent experiment was performed in triplicate.

## 3. Results

### *3.1. Cloning and sequence analysis of CXCR1a*

The full-length cDNA of CXCR1a was 1676 bp in length with an open reading frame of 981 bp encoding a polypeptide of 326 amino acids, a 137-bp 5′ un-translated region (UTR), a 558-bp 3′ UTR with a 29-bp poly (A) tail and a consensus polyadenylation signal sequence, AATAAA. Motif Scan detected a G protein-coupled receptor (GPCR) family 1 profile in the CXCR1a primary structures, in which the Prosite program located a GPCR signature. A triaa stretch (DRY motif), which is a characteristic feature of GPCRs, was present within the GPCR signature of CXCR1a. Examination of CXCR1a with TMHMM revealed that it was composed of a seven-transmembrane region, 7 TM (Fig. 1). In the phylogenetic analysis, the orange-spotted group CXCR1a clustered in the teleostean CXCR1 group and was found to be closely related to *Takifugu rubripes* CXCR1a (Fig. 2).

### *3.2. The expression level of CXCR1a in various tissues of healthy and SGIV infected grouper*

We then examined the tissue distribution patterns of CXCR1a in healthy and SGIV infected orange-spotted group using real-time quantitative PCR. As shown in Fig. 3, the highest levels of CXCR1a mRNA were detected in the head kidney, and moderate levels were observed in the kidney and spleen. Furthermore, the expression of CXCR1a was significantly increase induced by SGIV infected fish throughout in head kidney, kidney, skin and spleen tissues.

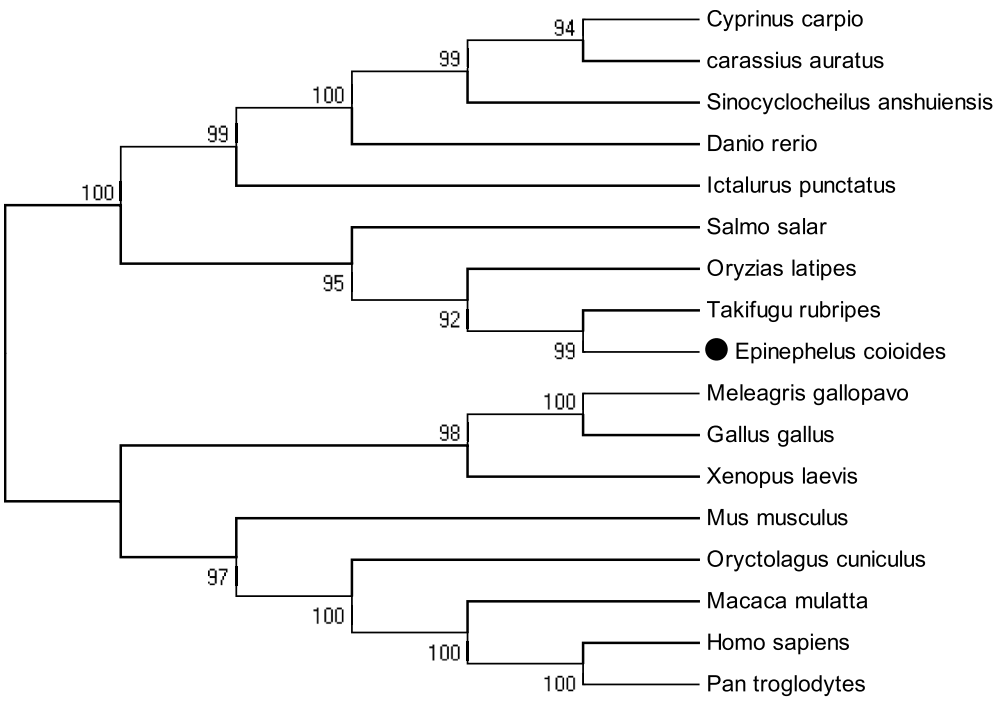


**Fig. 2. Phylogenetic analysis of CXCR1a using amino acid sequences**. The phylogenetic tree was constructed by MEGA 6 using the neighbor-joining method. Data were resampled with 1000 bootstrap replicates. Bootstrap values lower than 75 were removed. Amino acid sequences of used for alignment are as follows: *Homo sapiens,* CXCR1, NM_000634; *Pan troglodytes,* CXCR1, NP_001035537.1; *Mus musculus,* CXCR1, NM_178241; *Oryctolagus cuniculus,* CXCR1, NP_001164553.1; *Macaca mulatta*, CXCR1, NP_001035510.1; *Meleagris gallopavo*, CXCR1-like, XP_003207686.2; *Gallus gallus,* CXCR1, AJ973196; *Xenopus laevis*, CXCR1, NP_001082234.1; *Danio rerio*, XP_021334740.1; *Oryzias latipes* CXCR1-like, XP_004066792.1; *Takifugu rubripes*, IL8R1, NP_001072110.1; *Ictalurus punctatus*, XP_017325250.1, *Salmo salar*; XP_014007719.1. *Sinocyclocheilus anshuiensis*, XP_016342297.1. *Cyprinus carpio,* KTG37669.1; *carassius auratus*, XP_026126994.1.

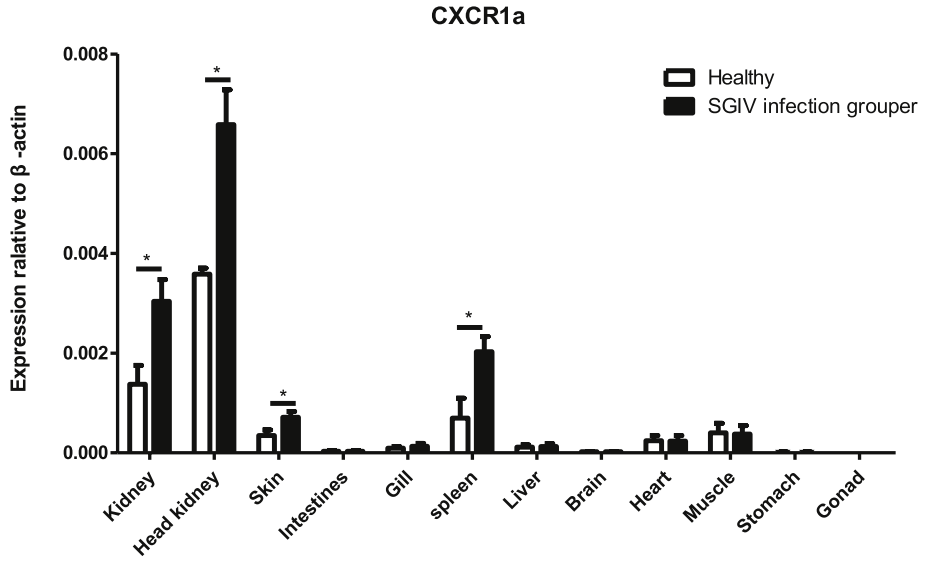


**Fig. 3. Tissue expression patterns of CXCR1a in healthy and SGIV infected grouper by qRT-PCR analysis**. The amplification of β-actin was used as the housekeeping gene control. Data are represented as the mean ± SEM (n = 3). * represent statistical significant difference.

### 3.3. Subcellular localization of CXCR1a in the orange-spotted group

To explore the localization of CXCR1a *in vitro*, pEGFP-CXCR1a was transfected into GS cells, and the fluorescence was observed under a fluorescence microscope. As shown in Fig. 4, bright green fluorescence was distributed mainly in the cytoplasm. In pEGFP-C1 transfected cells, the fluorescence signal was observed in both the cytoplasm and nucleus (Fig. 4).

### 3.4. CXCR1a overexpression inhibited SGIV replication in vitro

To determine the roles of CXCR1a in SGIV replication, we transfected CXCR1a into GS cells and then infected SGIV. As shown in Fig. 5, after SGIV infection, the transcription of the SGIV ICP18, MCP, LITAF and VP19 genes was significantly decreased in SGIV-infected CXCR1a-overexpressing cells compared to the control vector-transfected cells. Furthermore, western blot result and protein concentration analysis show MCP protein was decreased in SGIV-infected CXCR1a-overexpressing cells compared to the control vector-transfected cells (Fig. 6). These data suggested that CXCR1a exerted antiviral action against SGIV infection.

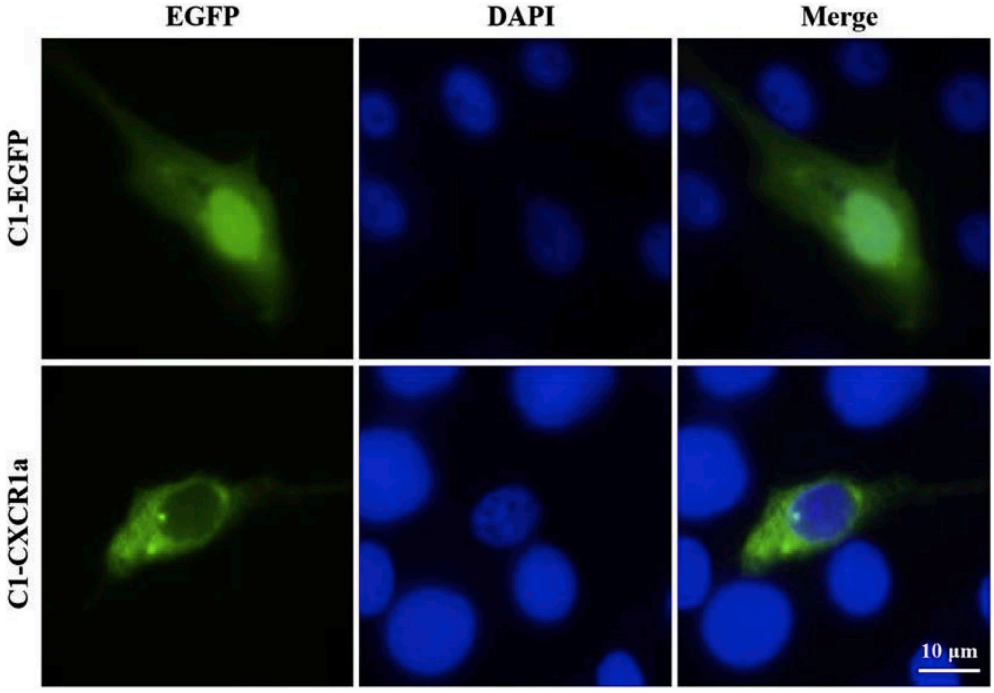


**Fig. 4. Cellular localization of CXCR1a**. GS cells were transfected with paEGFP-CXCR1a plasmid or pEGFP-C1 plasmid. After 48 h, fixed cells on the coverslips were stained with DAPI and imaged by fluorescence microscopy.

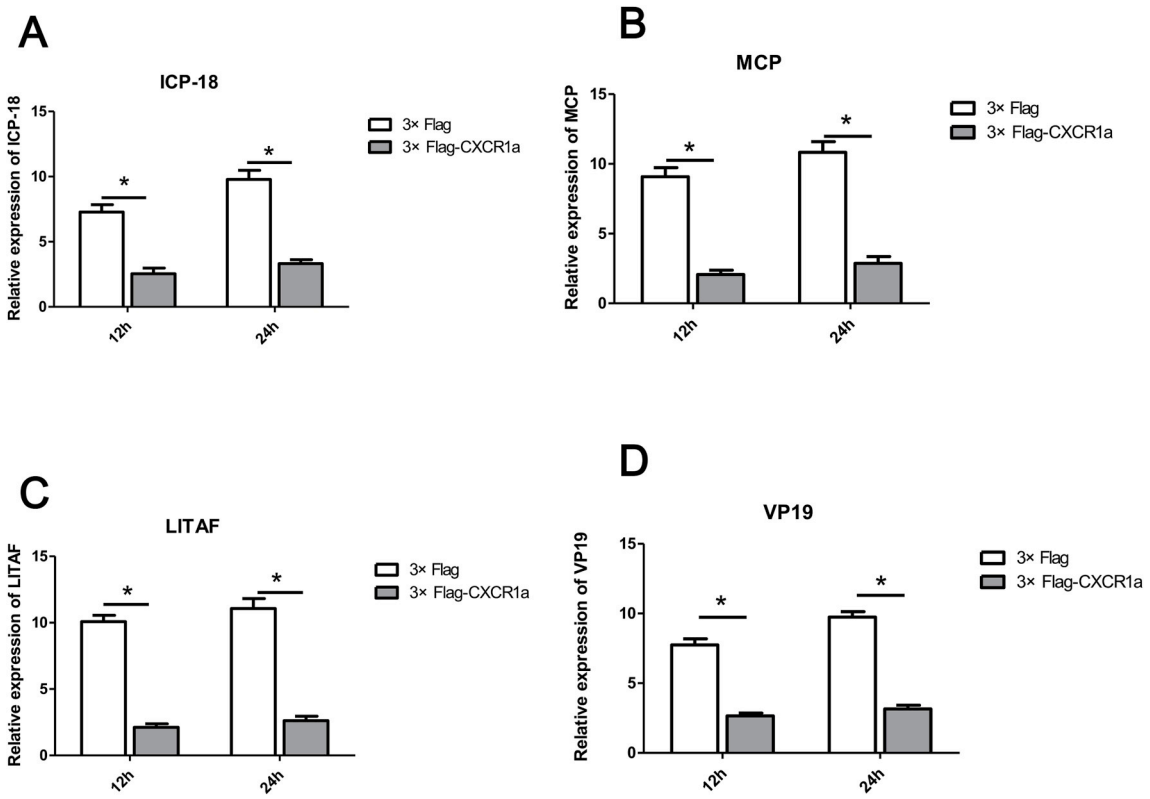


**Fig. 5. The viral gene transcription of SGIV in CXCR1a overexpressing cells**. After transfection with CXCR1a, GS cells were infected with SGIV, the relative expression of viral genes, including ICP-18 (A), MCP (B), LITAF (C) and VP19 (D) were examined using qRT-PCR. Error bars represent the mean ± SD; * represent statistical significant difference.

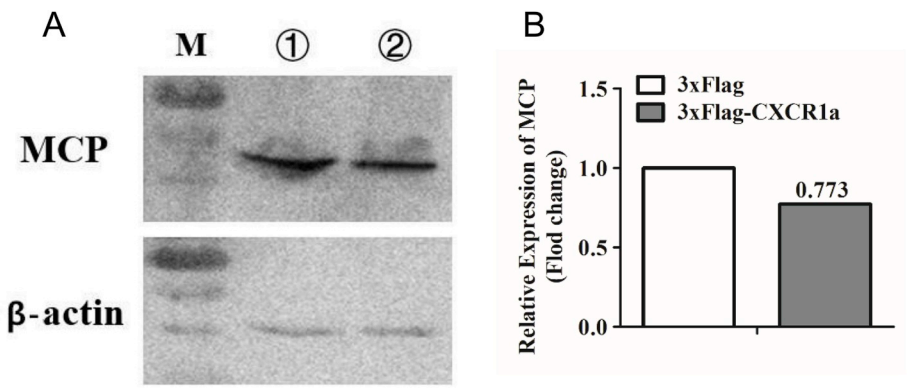


**Fig. 6. Western blot and protein concentration analysis of SGIV in CXCR1a overexpressing cells.** After transfection with CXCR1a, GS cells were infected with SGIV, the relative expression of viral protein MCP was examined using Western blot. A: Western blot analysis of MCP protein expression. ①: GS cells were transfection with 3 × Flag plasmid; ②: GS cells were transfection with 3 × Flag-CXCR1a plasmid; B: The protein concentration analysis of MCP.

### *3.5. Ectopic expression of CXCR1a enhanced the interferon immune response*

To clarify the potential molecular mechanism of the antiviral effect of CXCR1a, we examined the effect of CXCR1a overexpression on the host interferon immune response. As shown in Fig. 7, the transcripts of IRF1, IRF3, IRF7, IRF9, IFNr, IFNh, MDA5, viperin, IL-8, MXI and MXII were all significantly increased in CXCR1a-overexpressing cells compared to the control vector-transfected cells. Thus, overexpression of CXCR1a was speculated to positively regulate the interferon immune response *in vitro*.

### *3.6. Effects of CXCR1a on ISRE and IFN promoter activity*

To clarify whether IFN and ISRE was involved in the CXCR1a-induced interferon immune response, we assessed the interferon promoter activity in CXCR1a-overexpressing cells. As shown in Fig. 8, the overexpression of CXCR1a significantly increased the luciferase IFN and ISRE promoter compared to the control vector-transfected cells. These results suggest that CXCR1a promotes the activity of IFN and ISRE promoter.

## 4. Discussion

The role of chemokine receptors in the vertebrate immune response has attracted much attention in recent years [35,36], and understanding the role of chemokine receptors in pathogen defense is critically important. In this study, we obtained the full length of the CXCR1a gene from orange-spotted grouper. Typical features of CXCRs, such as seven hydrophobic membrane-spanning domains, the most highly conserved DRY motif, were found in CXCR1a. This motif is important for the ability of CXCR to signal upon ligand binding and G-protein interaction [37,38]. A phylogenetic tree was constructed based on the amino acid sequence of CXCR1a and CXCR1 from other known species. The results showed that the orange-spotted group CXCX1a clustered only with other fish CXCR1 and formed distinct clades. The overall topology of the tree was consistent with traditional taxonomy and phylogenetic transition.

Previous study showed that CXCR plays an important role in viral infection and that CXCR1, as an important molecule in CXCR, also plays important roles in viral pathogenesis [39–42]. In mammals, CXCR1 plays an important role in resisting viral infection [43,44], and CXCR1 plays an important role in acute inflammation and innate immunity and is a prototype receptor for inflammatory (induced) cytokines [45]. In rock bream, *Oplegnathus fasciatus*, the magnitude of the CXCR1 transcripts in head kidney and spleen was increased after in vivo injection with rock bream irido virus, showing the inhibitory effects of CXCR1 on viral replication [46]. In addition, mandarin fish CXCR1 was also shown to inhibit viral replication [47]. A previous study conducted by our group showed that CXCR plays an important antiviral role when SGIV infection occurs in vivo [24]. In this study, CXCR1a overexpression significantly inhibited SGIV replication. The results indicate that CXCR1a may be one of the antiviral factors acting against viral infection.

Research in humans has shown that TNF-α can induce the downregulation of human CXCR1 [48]. In chickens, CXCR1 plays a role in activating the IFN α/β system by binding to various transcription factors in the host response during viral infection [49,50]. In this study, we also found that overexpression of CXCR1a in grouper cells significantly increased the expression of IFNr and IFNh. In mammals, IL8/CXCR1 signaling is implicated in autoimmunity and human immunodeficiency virus-1 (HIV-1) infection [51,52]. Targeting the IL8/CXCR1 axis therefore represents an attractive therapeutic approach for various diseases. In our study, we found that overexpression of CXCR1a in grouper cells also increased the expression of IL8, This suggest that CXCR1a play an antiviral role in grouper may also through IL8/CXCR1 axis. In mandarin fish, the antiviral function of CXCR1 is mostly due to interactions with interferon and interferon-inducible genes, such as IRF and NF-κB transcription factors [41]. Here, overexpression of CXCR1a in grouper cells significantly increased the expression of interferon regulation factors, including IRF1, IRF3, IRF7, IRF9, and interferon-induced/stimulated genes, including viperin, MXI and MXII. Moreover, the ectopic expression of CXCR1a significantly increased the promoter activity of ISRE and IFN induced by SGIV infection. We speculated that the CXCR1a-induced interferon immune response might contribute greatly to its inhibitory effect on SGIV replication.

In summary, full-length cDNA cloning, analyses of the cellular localization, expression, and function of CXCR1a in response to grouper viral infection were performed. Our results indicated that CXCR1a encodes a cytoplasmic protein. Furthermore, ectopic expression significantly inhibited SGIV replication *in vitro*, and this inhibition was due to its enhancing effect on the host interferon immune response. Our results will contribute greatly to understanding the roles of fish CXCR in viral infection.

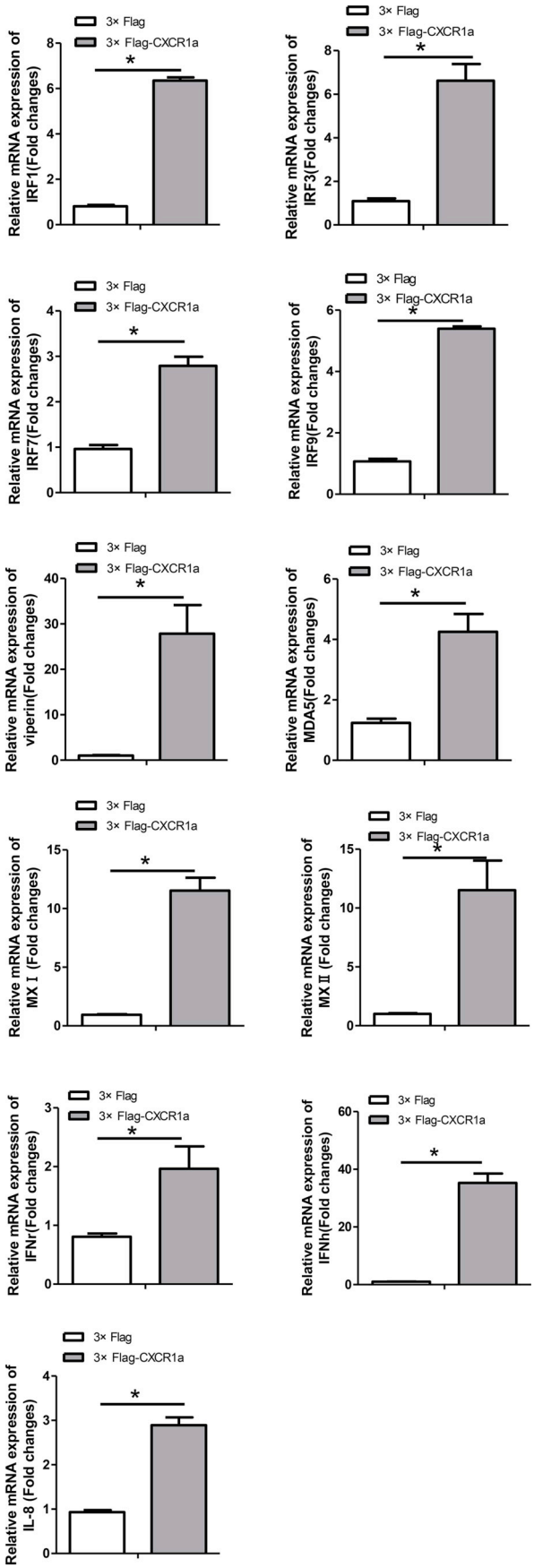


**Fig. 7. The ectopic expression of CXCR1a increased the expression of interferon related immune genes**. GS cells were transfected with CXCR1a and empty vector, then cells were collected for RNA extraction and qRT-PCR. The expression levels of IRF1, IRF3, IRF7, IRF9, IFNr, IFNh, MDA5, viperin, MXI, MXII and IL-8 were examined. * represent statistical significant difference.

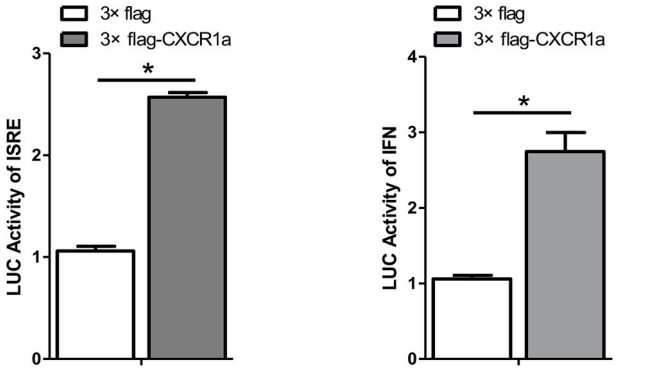


**Fig. 8. CXCR1a overexpression evoked the ISRE and IFN promoter activity**. Interferon promoter activity was evoked by CXCR1a overexpression. GS cells were co-transfected with CXCR1a and different interferon promoter plasmids ISRE-Luc and IFN-Luc, then the luciferase activities were determined using reporter gene assay. * represent statistical significant difference.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.046.

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