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Expression profile of carp IFN correlate with the up-regulation of interferon regulatory factor-1 (IRF-1) *in vivo* and *in vitro*: the pivotal molecules in antiviral defense

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ABSTRACT

Interferon regulatory factors (IRFs) are a family of transcription factors that mediate the transcriptional regulation of interferon (IFN) genes and IFN-inducible genes. In this study, IRF-1 gene is cloned from the common carp, *Cyprinus carpio* L., named *Cc*IRF-1. The full-length cDNA of *Cc*IRF-1 is 1427 bp, including an open reading frame of 861 bp encoding a protein of 286 amino acids. The putative *Cc*IRF-1 is characterized by a conserved DNA-binding domain and includes a signature of six conserved tryptophan residues. The genomic sequence of *Cc*IRF-1 is described, which consists of 9 exons and 8 introns. The sequence analysis shows that *Cc*IRF-1 is clustered into IRF-1 subfamily, and has the closest relationship with the zebrafish IRF-1. *Cc*IRF-1 is found constitutively expressed in different organs of healthy common carp. The main findings are that *Cc*IRF-1 is up-regulated following stimulation with poly(I:C) in all tested tissues. Moreover, the downstream gene of IRF-1 – IFN is found to be correlated with the up-regulation of IRF-1 after injection with poly(I:C). Furthermore, we also isolate the peripheral blood leukocytes (PBLs) and find that there is a relevance between the expression profile of *Cc*IRF-1 and IFN in poly(I:C) stimulated PBLs.

1. Introduction

Interferon regulatory factors (IRFs) are a family of transcription factors that mediate in the transcriptional regulation of interferon (IFN) genes and IFN-inducible genes in mammals [1,2]. To date, nine members of IRFs family have been identified in mammals, including IRF-1, IRF-2, IRF-3, IRF-4 (also known as LSIRF, PIP or ICSAT), IRF-5, IRF-6, IRF-7, IRF-8 (also known as ICSBP) and IRF-9 (also known as ISGF3γ), which play critical roles in antiviral immune response and cell growth regulation [3]. In fish, 11 members were identified, including two novel IRFs named IRF-10 and IRF-11 [4,5].

IRF-1 is the founder member of IRFs family which was originally defined as a transcription regulator of IFN-β [6,7] and activated cellular genes inducible by IFN and other cytokines [8]. The previous studies showed both IRF-3 and IRF-7 rather than IRF-1 played critical roles in regulation of type I IFNs [2]. As the dsRNA could trigger the activation of IRF-3 and/or IRF-7, to induce the expression of type I IFNs by binding to IFN responsive elements (IRF-Es) or IFN-sensitive response elements (ISREs) within their promoters via two classes of pattern recognition receptors, TLRs and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (primarily RIG-I and melanoma differentiation-associated gene 5 [MDA5]) [9,10]. However, recent studies demonstrated that IRF-1 acted as an essential regulator of the type I IFN response in cell type-specific fashions. IFN-γ-induced IRF1 is activated by MyD88 to mediate an efficient induction of IFN-β in conventional dendritic cells (cDCs) [11]. Moreover, IRF-1 is essential for TNF mediated IFN-β production in human monocytes [12,13]. IRF-1 also plays a critical role in the induced expression of MHC class I, demonstrating that MHC class I genes could not be induced by interferon in IRF1-deficient cell line. In addition, IRF-1 is related to pro-inflammatory injury and autoimmune disease [14–17]. Besides the immune functions, IRF-1 was found to participate in regulating apoptosis, DNA damage and tumor suppression [18–21].

In fish, IRF-1 is first identified as interferon regulatory factor in

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Japanese flounder (*Paralichthys olivaceus*), the mRNA expression of which is up-regulated after injected with *Hirame rhabdovirus* (HRV) and *Edwardsiella tarda* [22]. Since then, IRF-1 gene was reported in many teleosts, including pufferfish (*Fugu rubripes*) [23], rainbow trout (*Oncorhynchus mykiss*) [24], zebrafish (*Danio rerio*) [25], turbot (*Scophthalmus maximus*) [26], sea bream (*Sparus aurata*) [26], mandarin fish (*Siniperca chuatsi*) [27], snakehead (*Channa argus*) [28], crucian carp (*Carassius auratus*) [29], Atlantic salmon (*Salmo salar*) [30], orange-spotted grouper (*Epinephelus* coioides) [31], large yellow croaker (*Larimichthys crocea*) [32], paddlefish (*Polyodon spathula*) [33] grass carp (*Ctenopharyngodon idella*) [34] half-smooth tongue sole (*Cynoglossus semilaevis*) [35] and murrel (*Channa striatus*) [36]. Fish IRF-1 is a key regulator to activate IFN promoter. In Japanese flounder, IRF-1 has a role in the induction of an antiviral state in IRF-1 overexpressing cells [37] and modulates the early immune response against hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV) in hirame natural embryo (HINAE) cell line and epithelial papillosum of cyprini (EPC) cell line [38]. zebrafish IRF1 acts as a positive IFN regulator in concert with MyD88 to activate zebrafish IFNϕ3 but not IFNϕ1 [39].

Common carp (*Cyprinus carpio* L.) is one of the most important freshwater aquaculture species in China, which is prone to be infected by virus. Considering the roles of IRF-1 in the regulation of IFN system, the identification and function studies of carp IRF-1 are important to understand the mechanism against pathogen infection. In the present study, we identified and characterized the IRF-1 cDNA and genomic structure from common carp (named *Cc*IRF-1), then analyzed its expression pattern under normal condition and altered expression after a poly(I:C) challenge in various tissues. We also examined the expression profile of IFN – the downstream gene of IRF-1 following poly(I:C) treatment. Furthermore, we isolated the peripheral blood leukocytes (PBLs) and detected the expression of *Cc*IRF-1 and *Cc*IFN in poly(I:C) stimulated PBLs. These results demonstrated that *Cc*IRF-1 might play an unanticipated role in the positive regulation of IFN.

2. Materials and methods

2.1. Fish rearing, tissue collection and immune challenge

Healthy common carp (*Cyprinus carpio* L.), with an average of 200 g, were purchased from a local fish farm and cultured at 20 °C in recirculating tap water and fed daily to satiation with commercial fish feed for more than one week prior to experimental use. Then the liver, spleen, gills, skin, muscle, head kidney, foregut, hindgut, buccal epithelium, brain and gonad were isolated for RNA extraction.

The protocol in this study was approved by the Ethics Committee on Animal Experiments of Medical School of Shandong University (Permit Number: ECAESDUSM 1420123009). All operations were performed under anesthesia, and all efforts were made to minimize suffering of the fish.

Poly(I:C) (Sigma, USA) was suspended in phosphate buffered saline (PBS) for use in immune challenge experiments. The carps were intraperitoneally injected with 500 μl poly(I:C) solution (2.6 mg/ml) per fish, while the control carps were injected with the same amount of PBS. Seven tissues including gills, head kidney, liver, spleen, foregut, hindgut and skin of three infected individuals were collected at 3, 6, 12 and 24 hpi (hours post injection), and three control individuals were sampled at 0 h.

2.2. Isolation of common carp peripheral blood leukocytes (PBLs) and stimulation

Heparinized blood samples were taken from the caudal blood vessel and then quickly diluted with an equal volume of RPMI-1640 (Hyclone). The diluted blood was placed on the top of 65% Percoll (Sigma-Aldrich) and centrifuged at 2500 rpm for 30 min. The layer of PBLs was retrieved and washed three times with PBS. The cells were resuspended in L-15 (Gibco) containing 10% calf serum (MRC). About 10^7 cells/well were seeded in 24-well plate with 500 μl complete mediums. After recovering overnight at 25 °C, drug treatment was performed using poly(I:C) (5 μg/ml). Then the cells were collected at 3, 6, 9, 12 and 24 h. Total RNA was isolated from the PBLs and qPCR was performed with the gene-specific primers (Supplementary Table 1) to evaluate the mRNA levels of IRF-1 and IFN.

2.3. RNA extraction, cDNA synthesis and genomic DNA isolation

Total RNA were extracted from various tissues using RNAsimple Total RNA Kit (TIANGEN) according to the manufacturer's instructions. Concentration and quality of total RNA were measured by ultraviolet spectrophotometry (TIANGEN). Then the first-strand cDNA was synthesized by using FastQuant RT Kit (with gDNase) (TIANGEN) following the manufacturer's protocol. Genomic DNA was purified from the liver of healthy common carp by Genomic DNA Kit (TIANGEN).

2.4. Cloning of CcIRF-1 cDNA and genomic sequence

To clone IRF-1 gene from common carp, a pair of primers (Supplementary Table 1) were designed based on the conserved region of reported IRF-1 sequences. The cDNA was synthesized by spleen-derived RNA. The PCR parameters were 94 °C for 3 min, followed by 33 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 50 s, then a further 10 min extension step at 72 °C.

To obtain the full-length cDNA sequence of *Cc*IRF-1, the rapid amplification of cDNA ends (RACE) was used. The 3′-RACE and 5′-RACE were performed using the 3'-full RACE core set (TaKaRa) and 5'-full RACE core set (TaKaRa), respectively. The procedures were followed the manufacturer's instructions and the primers and annealing temperatures were shown in Supplementary Table 1.

Genomic DNA was purified from the spleen of healthy common carp using a Genomic DNA Kit (TIANGEN). Three pairs of primers were designed according to *Cc*IRF-1 full-length cDNA sequence. Three genomic fragments of *Cc*IRF-1 gene were obtained, all of which were overlapped between neighbouring products. Cycling parameters were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s, then a further 10 min extension step at 72 °C. All the PCR primers used in this study are shown in Supplementary Table 1.

All PCR products were analyzed by electrophoresis on a 1% agarose gel and the anticipated fragments were purified by PCR purification kit (TIANGEN). These fragments were ligated into the pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH-5α competent cells, and the subsequent recombinants were identified and sequenced by BGI China.

2.5. Sequence analysis, alignment and phylogenetic analysis

The sequence homology was obtained using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment was compared with that of multiple amino acid sequences of IRF-1 reported using BioEdit. The protein domains were predicted by the InterProScan 5 tool (www.ebi.ac.uk/Tools/pfa/iprscan5/) and SMART tool (smart.embl-heidelberg.de/). Phylogenetic tree was constructed by neighbor-joining method in MEGA 6.0 software. The GenBank accession numbers for these sequences were shown in