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Molecular identification and functional characterization of IRF4 from common carp (*Cyprinus carpio*. L) in immune response: a negative regulator in the IFN and NF-κB signalling pathways

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Abstract

Background: The interferon (IFN) regulatory factors (IRFs) were originally identified as transcription factors playing critical roles in the regulation of IFN-related genes in the signal pathway. In mammals, IRF4 plays a vital role in both the innate and adaptive immune system. This study aims to reveal the molecular characterization, phylogenetic analysis, expression profiles and the regulatory role in the IFN and NF-κB signalling pathways of IRF4 in common carp (*Cyprinus carpio*. L) (abbreviation, ccIRF4).

Results: Here, ccIRF4 was identified and characterized, it contained a DNA binding domain (DBD) which possess five tryptophans and an IRF-associated domain (IAD). The predicted protein sequence of the ccIRF4 showed higher identities with grass carp (*Ctenopharyngodon idella*) and zebrafish (*Danio rerio*). Phylogenetic analysis suggested that ccIRF4 has the closest relationship with zebrafish IRF4. Quantitative real-time PCR analysis showed that ccIRF4 was constitutively expressed in all investigated tissues with the highest expression level in the gonad. Polyinosinic:polycytidylic acid (poly I:C) stimulation up-regulated the ccIRF4 expressions in the liver, spleen, head kidney, skin, foregut and hindgut. Upon *Aeromonas hydrophila* injection, the expression level of ccIRF4 was up-regulated in all tissues with the exception of spleen. In addition, ccIRF4 was induced by lipopolysaccharide (LPS), peptidoglycan (PGN) and Flagellin in head kidney leukocytes (HKLs). Overexpression of the ccIRF4 gene in epithelioma papulosum cyprini cells (EPC) down regulated the expressions of IFN-related genes and proinflammatory factors. Dual-luciferase reporter assay revealed that ccIRF4 decreased the activation of NF-κB through MyD88.

Conclusions: These results indicate that ccIRF4 participates in both antiviral and antibacterial immune response and negatively regulates the IFN and NF-κB response. Overall, our study on ccIRF4 provides more new insights into the

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innate immune system of common carp as well as a theoretical basis for investigating the pathogenesis and prevention of fish disease.

Keywords: Common carp (*Cyprinus carpio*. L), Interferon regulatory factor 4 (IRF4), Poly (I:C), *Aeromonas hydrophila*, Signalling pathway

Background

The innate immunity is the only defense mechanism among invertebrates, and a fundamental defense mechanism in vertebrate animals as well, especially in lower vertebrates, such as fishes, whose adaptive immunity is relatively less developed [1]. Interferon (IFN) regulatory factors (IRFs) were originally identified in the 1980s, as the transcriptional regulatory factors play important roles in regulating the expression of type I IFN and IFN-stimulated genes (ISGs) in the related signalling pathways [2]. They play multifuctions in the antiviral defense, innate and adaptive immunity, cell growth regulation and apoptosis [3]. To date, a total of 11 members have so far been reported in vertebrates, IRF10 was found absent in humans and mice, whereas IRF11 has only been identified in fish. Based on the C-terminal region and molecular phylogenetic analyses, IRF proteins can be divided into four subfamilies: IRF1 (IRF1, 2, and 11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9, and 10) and IRF5 (IRF5 and 6) subfamilies [4]. Each IRF has distinct functional roles, transcriptional activators (IRF1, IRF3, IRF7 and IRF9) and bi-functional factors that both activate and repress transcription depending on the target gene (IRF2, IRF4, IRF5 and IRF8) [5]. All IRF family members share a highly conserved DNA-binding domain (DBD) of approximately 115 amino acids which contain five or six tryptophan repeats at the N-terminal region, that binding to the core recognition sequence, A/GNGAAANNGAAACT, termed the IFN stimulated response element (ISRE). The C-terminal region of IRFs generally possesses the IRF associated domain (IAD), with the exception of IRF1 and IRF2 which possess a very similar IAD2. The IAD interacts with other proteins to form homo- or hetero-dimers which are required for accurate promoter targeting and regulation of transcription [6].

IRF4 (also known as MUM1, PIP, LSIRF or LCSAT) plays multifunction in the innate and adaptive immunity [7]. In mammals, IRF4 is found to be expressed in various immune cells, including T and B cells, macrophages, and dendritic cells and plays crucial roles in their development and differentiation, but its function in the myeloid lineage is not well characterized [8, 9]. IRF4 mediates immune response by activating the expression of other genes during cell differentiation [10, 11]. Regulation of cell development processes include B cell receptor editing, Ig class switching, plasma cell generation, the germinal centre reaction and Th1/Th2 immune responses [12–16]. Besides, IRF4 is a key molecule for interleukin (IL)-17 production induced by IL-21 and the development and stability of the Th17 phenotype mediated by IL-6/TGFβ [17, 18]. However, IRF4 can compete with IRF5 for Myd88 interaction and act as a negative transcription factor of Toll-like receptor (TLR) signalling [19]. In the IRF4 deficient peritoneal macrophages from mice, production of the TLR-dependent proinflammatory cytokines is significantly enhanced [19]. IRF4 negatively regulate the expression of IRF5 that overexpression of IRF4 inhibits IRF5 expression whereas IRF4 knockdown increases IRF5 expression. Moreover, IRF4 binds to IRF5 promoters and negatively regulates IRF5 promoter reporter activities [20]. In mammals, IRF1, IRF5, IRF7, IRF8 and IRF9 can be induced by poly I:C except IRF4 [21]. However, teleost IRF4 can be induced by virus, bacteria or pathogen-associated molecular patterns (PAMPs, such as poly I:C or LPS) and it has been identified in mandarin fish (*Siniperca chuatsi*) [22], Asian swamp eel (*Monopterus albus*) [23], orange-spotted grouper (*Epinephelus coioides*) [24], rainbow trout (*Oncorhynchus mykiss*) [25], Japanese flounder (*Paralichthys olivaceus*) [26], large yellow croaker (*Larimichthys crocea*) [27], turbot (*Scophthalmus maximus*) [28], rock bream (*Oplegnathus fasciatus*) [29], half-smooth tongue sole (*Cynoglossus semilaevis*) [30], zebrafish (*Denio rerio*) [31], Atlantic salmon (*Salmo salar*) [32], channel catfish (*Ictalurus punctatus*) [33], blunt snout bream (*Megalobrama amblycephala*) [34] and miiuy croaker (*Miichthys miiuy*) (unpublished data). Almost all of these studies have concentrated on the structural characteristics and expression analysis of IRF4 in fishes. However, the functional roles of fish IRF4 remain largely unknown. The characterization of IRF4 in more vertebrates will certainly enable the understanding of its evolution and its immune function.

Common carp (*Cyprinus carpio*. L) is one of the most important aquaculture species in China, as well as in other Asia and European countries. Up to now, IRF1, IRF2, IRF3, IRF5, IRF7, IRF9 and IRF10 were reported in common carp [35–40]. In this study, we aimed to determine the protein structure and function of IRF4 in common carp (named ccIRF4). We identified the full-length cDNA sequencing and characterization of ccIRF4. Apart from investigating their tissue distribution in healthy common carp, we also evaluated the responsiveness of

ccIRF4 upon viral or bacterial stimulation both *in vivo* and *in vitro*. Furthermore, we determined the regulatory role of ccIRF4 in the IFN and NF-κB signalling pathways. These results will contribute to the understanding of fish innate immune response against pathogens.

Materials and methods

Fish and cell lines

Healthy common carp specimens (approximately 200 g per fish) were purchased from a local fish farm and cultured in recirculating tap water at 25 °C. Fish were fed daily to satiation with commercial fish feed for more than one week prior to experimental use.

Epithelioma papulosum cyprini (EPC) cells and 293 T cells were stored in our laboratory. EPC cells were maintained at 25 °C in M199 (HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco). 293 T cells were maintained at 37 °C, 5.0% CO_2 in DMEM (HyClone) supplemented with 10% FBS, 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco). Transfection was performed according to a previous report [41].

Molecular cloning and sequencing of the ccIRF4

Partial cDNA sequence of ccIRF4 was obtained using a pair of degenerate primers IRF4-F/IRF4-R (Table 1) designed on the basis of the known IRF4 sequences download from NCBI data base. Then, the full-length cDNA of ccIRF4 was obtained by RACE (rapid amplification of the cDNA ends) method using 3′-Full RACE and 5′- Full RACE Core Set Kit (TaKaRa).

Table 2 Protein length and Genbank accession numbers of IRF4 in different species

Species	Protein length (aa)	GenBank No.
Danio rerio	460	NP_001116182
Paralichthys olivaceus	456	AEY55358
Miichthys miiuy	462	AHB59738
Ictalurus punctatus	433	AHH38752
Oplegnathus fasciatus	462	AFU81291
Cynoglossus semilaevis	457	XP_008332518
Larimichthys crocea	463	ATE88516
Monopterus albus	451	AFQ22942
Salmo salar	468	ACI33264
Ophiophagus hannah	437	ETE71355
Gallus gallus	445	AAK08199
Mus musculus	450	AAI37715
Homo sapiens	451	AAH15752
Branchiostoma belcheri tsingtauense	581	AJA02099

Table 1 Primers used in this study

Primer	Sequence(5′-3′)	Application
ccIRF4-F	GGAGCCAGCTGGACATCTC	Cloning for IRF4
ccIRF4-R	CAGGAGCTGCCTGGCGAAC	Cloning for IRF4
ccIRF4-5Rout	CGTCATCTGAGGCTGTAGAGGAGG	Cloning for IRF4
ccIRF4-5Rin	GAGCCTCTCTTGGCTCCTTCTGG	Cloning for IRF4
ccIRF4-3Fout	GGACACGCAGCAGTTCCTCTCAG	Cloning for IRF4
ccIRF4-3Fin	GCCACGCTCTCAGGTGGTGCTGTG	Cloning for IRF4
ccIRF4-Frt	CCAATATGAGATCCGCCGAAGCC	Rea-ltime PCR
ccIRF4-Rrt	CCTGGAGACGAAGAGGAGGAGATG	Rea-ltime PCR
ccS11-F	CCGTGGGTGACATCGTTACA	Rea-ltime PCR
ccS11-R	TCAGGACATTGAACCTCACTGTCT	Rea-ltime PCR
ccIRF4-F-EI	CCGGAATTCATGAACTTAGATGGGGACAGCAGC	Recombinant plasmid
ccIRF4-R-SII	TCCCCGCGGCACCTGCAAGTGCTGGATGCT	Recombinant plasmid
EPC-IFN-F	CGCTAAGGTGGAGGACCAGGTTA	Rea-ltime PCR
EPC-IFN-R	TTAGGTTCCATTGTGCTGCGTTCA	Rea-ltime PCR
EPC-viperin-F	AAGACTTCCTGGACCGCCATAAGA	Rea-ltime PCR
EPC-viperin-R	CCTCTCGGCAATCCAAGAAGCG	Rea-ltime PCR
EPC-PKR-F	TGGAGACTTCGGCCTCGTGACT	Rea-ltime PCR
EPC-PKR-R	TCGCTTGCTCCGGGCTCATGTA	Rea-ltime PCR
EPC-IL-1β-F	CCCAGACCAATCTCTACCTCGCT	Rea-ltime PCR
EPC-IL-1β-R	GAGGAGGTTGTCATTCTGGTCACC	Rea-ltime PCR
EPC-β-actin-F	GCCGTGACCTGACTGACTACCT	Rea-ltime PCR
EPC-β-actin-R	GCCACATAGCAGAGCTTCTCCTTG	Rea-ltime PCR

PCR products were analysed by electrophoresis on a 1% agarose gel and the anticipated fragments were purified from agarose gels. These fragments were ligated into the pMD18-T vector (TaKaRa) and transformed into competent *Escherichia. coli* DH-5α competent cells, and subsequently recombinants were identified and sequenced (Invitrogen).

Domains of deduced amino acid sequence search were performed with the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de) and the conserved domain search program of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was performed by the ClustalX program. Phylogenetic analysis was performed with ClustalX and the neighbor-joining methods with 1000 replicates of MEGA 5.0.

Immune challenge *in vivo* and sample collection

For the challenge groups, 50 healthy common carp were intraperitoneally injected with 500 µl phosphate-buffered saline (PBS 7.4) containing polyinosinic:polycytudylic acid (poly I:C, 2.6 mg/ml, Sigma) or *Aeromonas hydrophila* (at a dose of 2.0×10^8 cells). Before the experimental challenge in common carp, the bacteria were reactivated and cultured on Luria-Bertani (LB) medium in a shaker incubator at 28 °C overnight. The *A. hydrophila* was inactivated in 0.5% formalin at 37 °C for 36 h and then resuspended in PBS, followed by collection by centrifugation (3300 *g* for 10 min) and two washes with PBS. Whereas fish from the control group were injected with 500 µl of sterile PBS per fish.

The fish were anaesthetized with MS-222 (100 mg/L) and then sacrificed to collect experimental tissues. For both challenge experiments, three fish were sampled at 3, 6, 12, 24, 48 and 72 h post-injection from each group. Tissues were collected from each fish for total RNA extraction.

Head kidney leukocytes (HKLs) isolation and immunity challenge *in vitro*

Common carp HKLs were prepared by Percoll (Sigma) gradients according to a previous report [38]. In short, head kidney tissue from freshly killed fish was passed through 100 µm stainless steel screens, and the resulting suspension was loaded onto freshly prepared 51/34% non-continuous Percoll density gradients and separated via centrifugation at 650 g for 30 min. After overnight recovery at 25 °C, 1×10^6 cells were maintained in a 24-well tissue culture plate with poly I:C (500 µg/ml, SIGMA), LPS (1 mg/ml, SIGMA), peptidoglycan (PGN) (10 mg/ml, SIGMA), and flagellin (10 ng/ml, SIGMA).

Construction of overexpression vectors

The full-length open reading frame (ORF) region of ccIRF4 was generated by PCR using Phusion HighFidelity DNA polymerase (PrimeSTAR) with the specific primers (Table 1) and then digested with the EcoRI/SacII restriction enzymes. Purified fragments ligated into the pcDNA3.1-EGFP vector (Invitrogen) and transformed into *E. coli* Top10 cells for adequate recombinant plasmid purification. The overexpression plasmid and vector-only pcDNA3.1-EGFP plasmid were extracted using an endotoxin-free plasmid isolation kit (TIANGEN) following manufacturer's instructions. The extracted plasmids were dissolved in sterile ultrapure water, their concentrations were estimated by measuring the OD260, and they were then preserved at −20 °C for further analysis. The overexpression vector of ccMyD88 (abbreviation, pMyD88) was constructed using the same method and ligated into the fugw-2flag vector. The resulting overexpression vector pcDNA3.1-IRF4-EGFP (abbreviation, pIRF4) and pMyD88 were verified by sequencing.

Transfection of EPC cells with pIRF4 overexpression plasmid

EPC cells were seeded in 24-well plates with 500 µL in each well at a concentration of 4×10^5 cells/mL one day prior to transfections. Cells were transfected with the pIRF4 or empty vector once they reached about 80% confluency the following day with plasmids at 1 ug/well using X-tremeGENE HP DNA Transfection Reagent at 2 µl/well following the manufacturer's instructions. EPC cells were collected after 48 h transfection with the pIRF4 or empty vector for quantification of associated immune molecule expressions (IFN, PKR, Viperin and IL-1β). The primers are listed in Table 1.

RNA isolation and real-time PCR analysis

Total RNA was extracted from various tissues and EPC cells using an TRIzol reagent (TIANGEN). First-strand cDNA was synthesized using FastQuant RT kit (TIANGEN) in accordance with the manusfacture's instructions. Real-time PCR was performed in a Rotor-Gene Q PCR instrument (Qiagen) with TransStart Tip Green qPCR SuperMix (Transgen). Real-time PCR conditions were 94 °C for 30 s, followed by 94 °C for 5 s, 60 °C for 30 s, and 70 °C 50 s for 40 cycles. Reactions were performed in 20 µl volume containing 10 µl SYBR green real-time PCR master mix, 6.8 µl double-distilled water, 0.6 µl of each primer, and 20 ng (2 µl) cDNA template. All samples were analyzed in triplicates and the expression value of all genes in common carp was calculated as relative to 40S ribosomal protein S11 gene or β-actin

of EPC cells with the $2^{(-\Delta\Delta C(T))}$ method [42]. The primers were listed in Table 1.

Luciferase activity assay

Dual-luciferase reporter assays to detect the effects of ccIRF4 on the activation of NF-κB were performed in 293 T cells by using pIRF4, pMyD88 or empty vector together with a luciferase-linked NF-κB. Transfection assays were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The 293 T cells in 96-well plates were transfected with reporter gene plasmids, pGL-NF-κB-Luc, pGL-Renilla-luc plasmid (Promega), and the correct amount of expression plasmids or empty expression vectors (as control). The pGL-Renilla-luci plasmid was used as internal control. At 48 h post transfection, the Dual-Glo® Luciferase Reagent (Promega) was used to measure the activity of firefly and Renilla luciferase according to the manufacturer's instructions with each experiment done in triplicates.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6.0 software. The relative gene expression upon immune challenges was acquired using the $2^{(-\Delta\Delta C(T))}$ method. Data were expressed as the means standard deviation (SD) from at least three independent triplicated experiments. Significance differences were analysed using Student's t-test for paired comparisons. Multiple comparison analysis was performed using one-way analysis of variance (ANOVA). All the data were homogeneous and normal, and p value of <0.05 was considered to be statistically significant.

Results

cDNA cloning and molecular characterization of the ccIRF4

The full-length cDNA of ccIRF4 was found to consist of 1885 bp. The ccIRF4 cDNA (GenBank accession No. OL365854) contains a 60 bp 5′-untranslated region (UTR), a 439 bp 3′-UTR containing mRNA instability motifs (^{1857}AATAA^{1863}), and an ORF of 1386 bp that translates into a 462 amino acid putative peptide with a predicted molecular mass of 52.3 kDa, the theoretical isoelectric was 6.105. The protein structure of ccIRF4 was predicted by SMART analysis. The deduced protein exhibited a DBD which possess five tryptophans (Trp21, Trp36, Trp48, Trp68, Trp87) and an IAD.

The multiple alignments of IRF4 between common carp and other species revealed conserved areas in all vertebrate groups. Significant homology was found in the putative DBD and IAD (Fig. 1). Furthermore, the phylogenetic tree including IRF4 sequences from all known species was constructed using the neighbor-joining method, which can be divided into several branches (teleosts, amphibia, birds, mammals and appendicularia). CcIRF4 had the closest relationship with zebrafish IRF4 (Fig. 2).

Expression profile analysis of the ccIRF4 in healthy common carp

Real-time PCR was performed to examine the tissue distribution of ccIRF4 under normal physiological conditions in eleven tissues (liver, spleen, head-kidney, foregut, hindgut, gills, gonad, skin, muscle, oral epithelial and brain) of common carp. As a result, ccIRF4 mRNA expression was detected in all examined tissues, and broadly expressed in gonad, brain and spleen, moderately expressed in skin, muscle and gills, and weakly expressed in oral epithelial, hindgut, liver, foregut and head-kidney (Fig. 3).

Expression profiles of ccIRF4 following poly I:C and *A. hydrophila* injection *in vivo*

To investigate the role of ccIRF4 in immune response, the expression pattern of these genes in the immune-related tissues was examined after intraperitoneal injection with poly I:C and inactivated *A. hydrophila* at different time points. The expression profile of ccIRF4 after poly I:C injection is shown in Fig. 4. The peak expression of ccIRF4 appeared at 3 h post injection (hpi) in the liver, spleen, foregut and hindgut, with 7.0, 7.5-, 6.5- and 5.5-fold induction respectively. Meanwhile, the expression of ccIRF4 reached the highest level at 48 hpi in the head kidney (10.5-fold) and skin (45.7-fold).

The above results indicated that ccIRF4 might be involved in the antiviral immune response. Whether ccIRF4 participates in antibacterial immunity was also investigated. In *A. hydrophila* infected fish, expression of ccIRF4 was induced in the head kidney (1.4-fold) and foregut (1.8-fold) at 3 hpi and then down-regulated. In the hindgut, ccIRF4 exhibited the highest level at 6 hpi (1.3-fold). In contrast, the transcripts of ccIRF4 was reduced in the spleen (0.34-fold) (Fig. 5).

Expression profiles of ccIRF4 upon immune stimulation in isolated HKLs

Real-time PCR was used to examine the ccIRF4 transcription levels in isolated HKLs after stimulation with poly I:C,

(See figure on next page.)

Fig. 1 Multiple alignment IRF4 protein sequences in different species. The sequences were aligned using the ClustalW method. Identical (*) and similar (: or .) residues are indicated; the predicted domains from SMART Server software have been indicated by colored boxes: the yellow denotes the DBD and the blue denotes the IAD. Five tryptophan (W) residues are boxed in red. The GenBank accession numbers of the genes are listed in Table 2

Fig. 1 (See legend on previous page.)

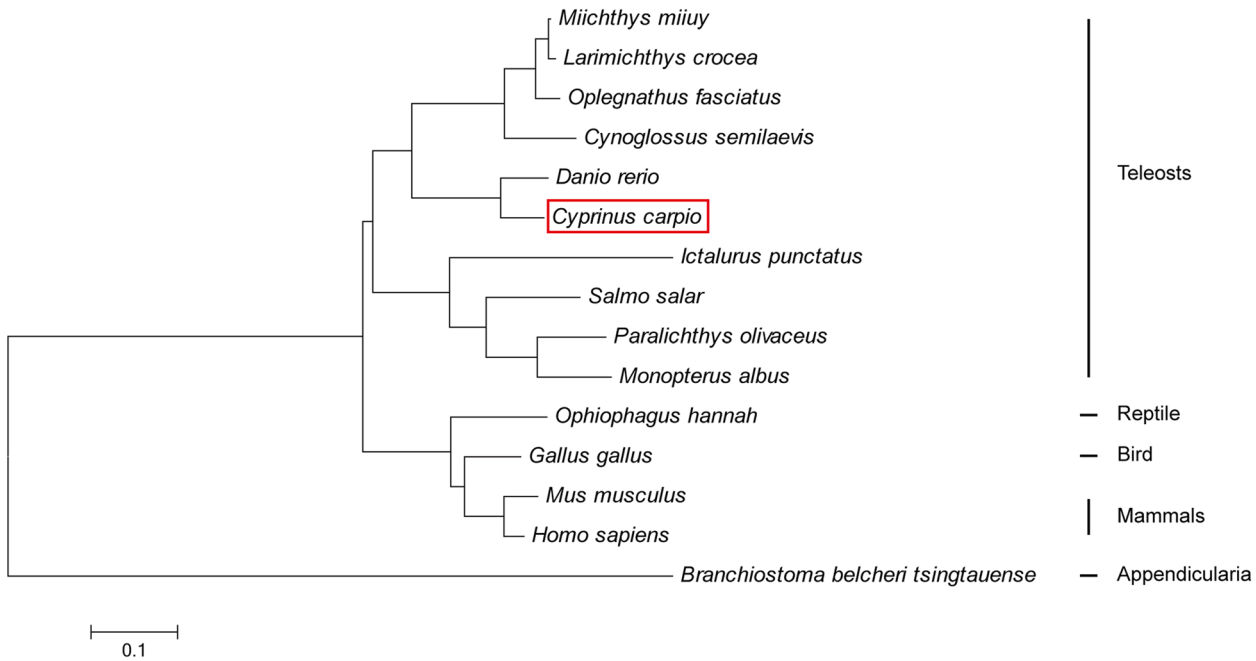

Fig. 2 Phylogenetic analysis of IRF4 protein sequences from different species. The phylogenetic tree was produced by the neighbour-joining method in MEGA 5.0. The red frame shows the common carp IRF4

LPS, PGN and flagellin. As shown in Fig. 6, ccIRF4 expression was up-regulated by LPS (1.3-fold), PGN (2.1-fold), and flagellin (1.4-fold) at 24 h, but down-regulated by poly I:C with about 0.3-fold at 3 h.

The mRNA expression of downstream IFN-associated factors in EPC cells overexpressing ccIRF4

Real-time PCR was used to detect the mRNA expression of IFN, PKR, Viperin and IL-1β genes after the empty vector and pIRF4 transfected EPC cells for 36 h. The result showed that the expression of IFN (0.58-fold, $P<0.05$), PKR (0.69-fold, $P<0.05$), Viperin (0.43-fold, $P<0.05$), and IL-1β (0.51-fold, $P<0.05$) genes were all down-regulated in EPC cells transfected with pIRF4 (Fig. 7).

Regulation of NF-κB expession by ccIRF4

To determine whether ccIRF4 could promote activation of NF-κB genes, dual-luciferase reporter assays were performed. Luciferase activity assays showed that NF-kB activity was not enhanced by overexpression of ccIRF4. While, NF-κB was significantly activated by overexpression of ccMyD88 (21.2-fold). However, co-transfection of ccIRF4 and ccMyd88 in 293 T cells inhibited activity of NF-κB compared to transfection of ccMyd88 alone (Fig. 8). These results suggest that ccIRF4 function as a transcription repressor in the NF-κB signalling pathway through ccMyD88.

Discussion

IRF family plays an essential role in the host innate and adaptive immune responses in mammals and fish [43]. With the development of aquaculture, which is constantly challenged by outbreaks of infectious diseases

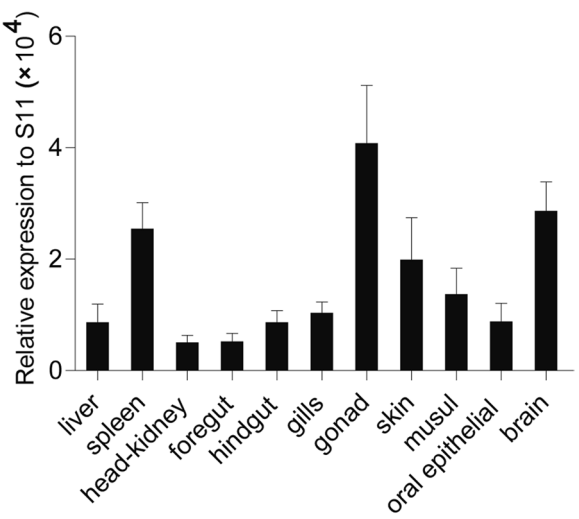

Fig. 3 Tissue-specific expression of ccIRF4 under normal physiological conditions. CcIRF4 mRNA expression in the liver, spleen, head kidney, gills, skin, foregut, hindgut, buccal epithelium, gonad, muscle and brain were determined by real-time PCR. Gene expression levels were normalized using the 40S ribosomal protein S11 mRNA, $n=3$

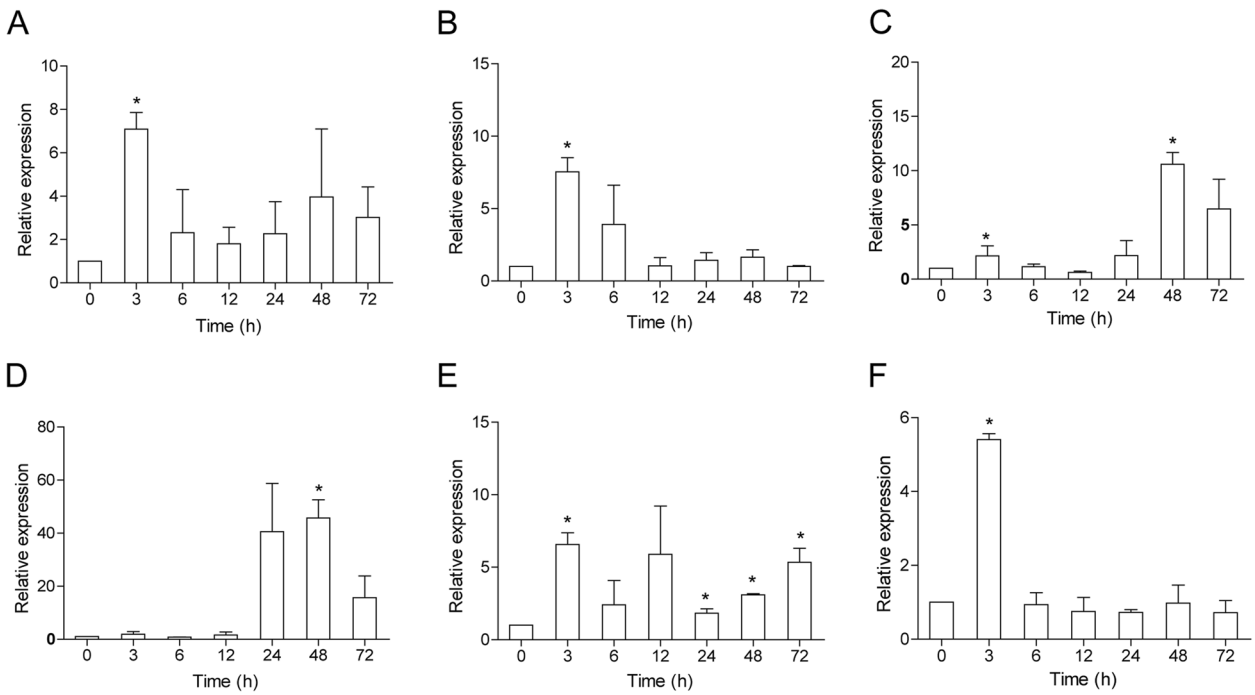

Fig. 4 Expression analysis of ccIRF4 in response to Poly I:C challenge *in vivo*. Total RNA was extracted from the liver (**A**), spleen (**B**), head kidney (**C**), skin (**D**) foregut (**E**) and hindgut (**F**) at 0 (as control), 3, 6, 12, 24, 48 and 72 h post injection for real-time PCR. Expression was normalized using the 40S ribosomal protein S11 ($n = 3$, mean ± SD, $^*P < 0.05$). The y-axis reveals fold change value relative to day 0, which has a different scale in Figure. 4 **A**-**F**

inflicting considerable damages on this industry, increasing numbers of fish IRF genes are being identified and studied to investigate the immune system [44]. More recently, common carp IRF2 and IRF10 have been cloned [35, 40]. IRF4 has been extensively studied in mammalian species and is known to modulate anti-viral and anti-bacterial activity; however, information related to its functions in fish is very limited. In this study, IRF4 gene was cloned and its functional characteristics in immune response were investigated for the first time in common carp. Structural analysis revealed that ccIRF4 had two conserved functional domains, the N-terminus DBD and the C-terminus IAD. Multiple alignments of fish and vertebrate IRF4 sequences revealed a high degree of identity and the high sequence homology that exists in the DBD and IAD, suggesting that the functions of the IRF4 gene is likely to be conserved throughout the vertebrates. The DBD contains a five tryptophan repeat, which are highly conserved throughout the IRF family [6]. This region can form a helix-turn-helix motif and bind to the IFN stimulating response element (ISRE) and IRF regulatory element (IRF-E) in target promoters [45, 46]. In addition to the DBD, all IRFs (except IRF1 and IRF2) possess an IAD at the carboxyl-terminus, which is another conserved domain. This region is responsible for homo/hetero-dimers interactions of the IRFs and association with other transcription factors by the formation of transcriptional complexes [6]. These features are helpful for understanding the functions of IRF family in antiviral defense and immune regulation.

The phylogenetic tree of all known IRF4 amino acid sequences from different species was constructed using the neighbour-joining method. In the phylogenetic tree, IRF4s of teleosts, amphibia, birds and mammals were on a branch, while IRF4 of appendicularia was separate. Furthermore, ccIRF4 clustered with other fish IRF4 and demonstrated the closest phylogenetic relationship with that of zebrafish. The results match the established evolutionary relationships among the teleosts and other vertebrate species, supporting the authenticity of the nomenclature for the common carp IRF4 and suggest that ccIRF4 might exert similar functions as IRF4 in other fishes.

The ccIRF4 transcripts were detected in all the eleven tissues of healthy carps, indicating a constitutive transcription of IRF4. This ubiquitous tissue expression pattern supports previous studies of IRF4 in teleosts, including Asian swamp eel [23], rainbow trout [25], turbot [28], rock bream [29], half-smooth tongue sole [30], zebrafish [31] and blunt snout bream [34]. CcIRF4

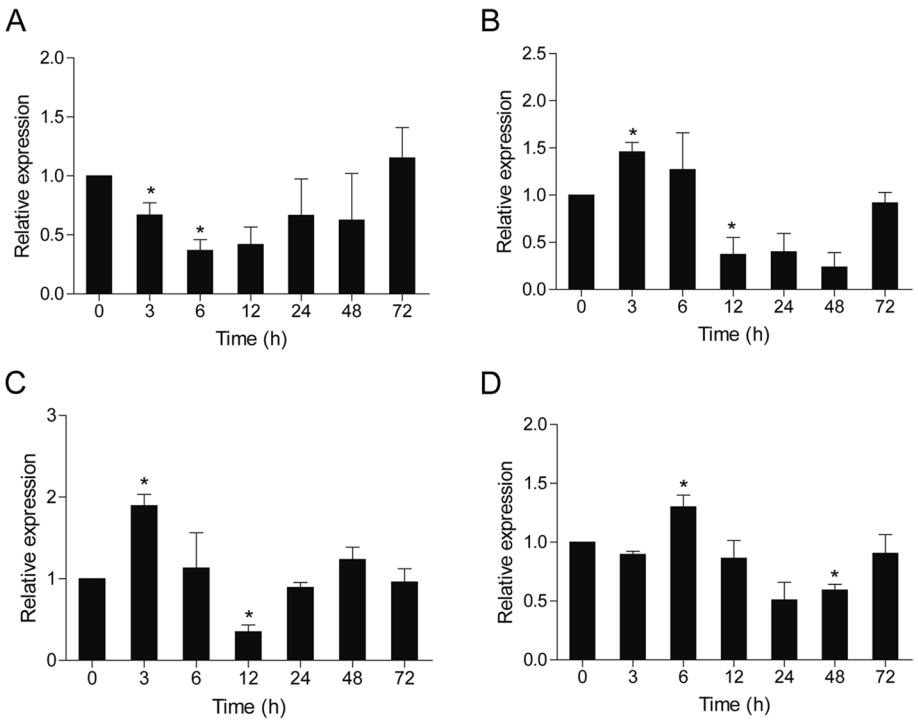

Fig. 5 Expression analysis of ccIRF4 in response to *A. hydrophila* challenge *in vivo*. Total RNA was extracted from the spleen (**A**), head kidney (**B**), foregut (**C**) and hindgut (**D**) at 0 (as control), 3, 6, 12, 24, 48 and 72 h post injection for real-time PCR. Expression was normalized using the 40S ribosomal protein S11 ($n = 3$, mean ± SD, $^*P < 0.05$)

appears to have higher transcript expression in spleen, an important tissue of the teleost immune system, which is a major site for the trapping and presentation of antigens for recognition by lymphocytes [47]. Surprisingly, the expression level of ccIRF4 in the gonad and brain was also high, implying that IRF4 may be involved in regulating the reproductive and nervous system of fish. However, ccIRF4 was expressed at very low levels in other tissues. Such expression pattern of ccIRF4 gene was similar to that in Atlantic cod, eel, chicken and mice, reflecting the possible similarity in protein function of IRF4 from different species [23, 47–49].

IRFs play a critical role in antiviral and antibacterial immunity. Previous studies in common carp showed that expression of IRF1, IRF2, IRF3, IRF5, IRF7, IRF9 and IRF10 were up-regulated upon stimulation with poly I:C, viruses and/or bacteria [35–40]. Spleen and head kidney were selected to perform the study, as these organs represent lymphoid and myeloid tissues, which are important in fish immune system. We also performed the study in the foregut and hindgut in order to determine whether ccIRF4 participate in regulating the mucosal immune system. Poly I:C, a well-established inducer of fish type I IFNs and ISGs, is a synthetic mimic of dsRNA which recognized by TLR3 and TLR22 [50, 51]. Similarly with the expression profile in eel and large yellow croaker [23, 27], in our present study, ccIRF4 expression was enhanced after stimulation with poly I: C in all the six tissues. The maximum induction of ccIRF4 in liver, spleen, foregut and hindgut occurred at 3 hpi, earlier than that in head kidney and skin (at 48 hpi), maybe because those organs are the first sites against the invading antiviral pathogens. The induction in the skin (45.7-fold) was much stronger than that in the other tissues (5.5- to 10.5-fold), revealing the important role of ccIRF4 in the mucosal immune system response to poly I:C. Besides, the expression of rock bream IRF4 was induced in the head kidney post-injection with poly I:C and rock bream iridovirus [29]. Furthermore, two isoforms, IRF4a and IRF4b, have been identified in half-smooth tongue sole, and both of them were up-regulated upon megalocytivirus infection in the spleen, head kidney and liver [29]. However, poly I:C has no impact on the expression of human IRF4, indicating the different role of IRF4 in human and fish [21].

It should be noted that *A. hydrophila* (a well-known fish bacterial pathogen) infection up-regulated the expression of ccIRF4 in the head kidney, foregut and hindgut, but its expression in the spleen was down-regulated. This is inconsistent with the studies that *Vibro harveyi* and *Edwardsiella Tarda* down-regulated the expression of

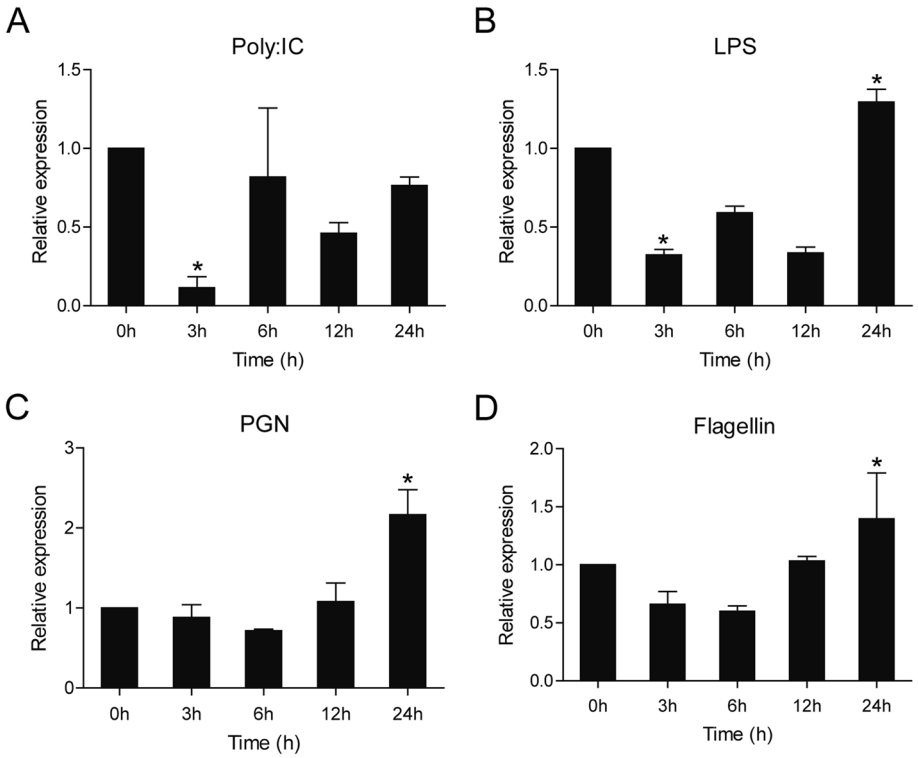

Fig. 6 Expression levels of ccIRF4 in the HKLs upon different stimulation. The cells were collected at 0 (as control), 3, 6, 12 and 24 h post-infection for RNA extraction and real-time PCR analysis. Expression was normalized using the 40S ribosomal protein S11 ($n = 3$, mean ± SD, $^*P < 0.05$)

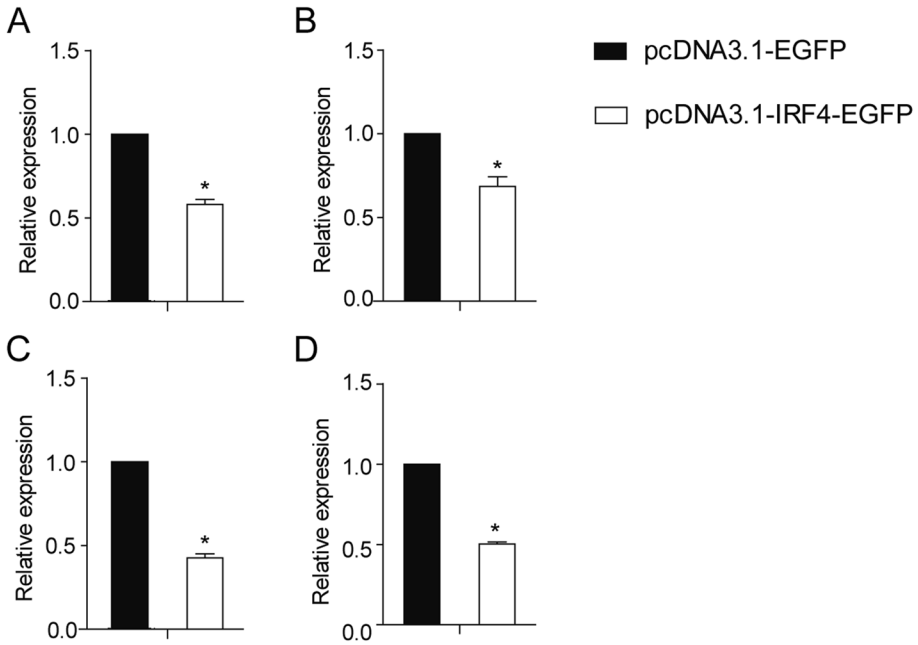

Fig. 7 Relative expression of IFN (**A**), PKR (**B**), Viperin (**C**) and IL1-β (**D**) in ccIRF4-transfected EPC cells. Expression was normalized to β-actin ($n = 3$, mean ± SD, $^*P < 0.05$)

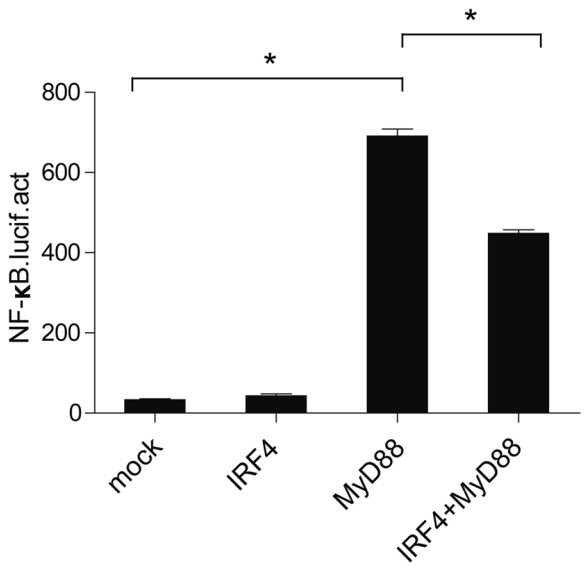

Fig. 8 CcIRF4 negatively regulates the NF-κB pathway through MyD88. 293T cells were cotransfected with the NF-κB reporter gene, together with ccIRF4 and MyD88 expression plasmids. Luciferase activity was measured after 48 h and determined against Renilla luciferase activity (mean ± SD, *$P < 0.05$)

half-smooth tongue sole IRF4a [30] and LPS down-regulated the expression of rock bream and rainbow trout IRF4 [25, 29]. Eel IRF4 had whereas relatively lower fold change induced by LPS and *A. hydrophila* [23]. Besides, half-smooth tongue sole IRF4b was up-regulated after bacterial infection [30]. These results suggest that IRF4 in different species may respond to different pathogens. However, further studies should be conducted to investigate the mechanism of its regulatory role during the bacterial and viral infection.

HKLs consist of heterogeneous cells and are widely used as an experimental system to study immune responses [52, 53]. Thus, we isolated the leukocytes from head kidney for further investigations to gain a better understanding of the antimicrobial mechanisms of ccIRF4. According to the observed expression level of ccIRF4, which was increased after stimulation with different ligands (LPS, PGN and Flagellin) in HKLs, it could be further confirmed that ccIRF4 may play substantial roles to protect the host from bacteria. However, ccIRF4 in HKLs was down regulated by poly I:C. IRF4 gene expression in channel catfish was found to be up-regulated by poly I:C in a mixed macrophage/T cell culture and down regulated in B cells, indicating whether poly I:C can induce IRF4 expression depends on the cell types [54]. What's more, IRF4 is expressed at low levels in early B cell development stages and markedly up-regulated in later stages. All the above may explain the low sensitivity of ccIRF4 to poly I:C treatment in HKLs, which are consist of T cells, B cells, granulocytes, monocytes and macrophage. Even so, it could be suggested that ccIRF4 participate in both antibacterial and antiviral innate immunity.

To date, very few studies on the regulatory roles of the IRF4 subfamily members in the IFN or NF-κB signalling pathway have appeared in the literature. In the current study, we detected the mRNA expression of Type I IFN, ISGs (PKR, Viperin), and IL-1β in transfected EPC cells. Zebrafish IRF4 induced the IFN promoter activity [31]. On the contrary, our results showed that overexpression of ccIRF4 in EPC cells down-regulated the production of type I IFN, PKR, Viperin and IL-1β. In mammals, IRF4 possesses the ability to negatively regulate TLR signalling pathway by competing with IRF5 for binding to Myd88, which is a signalling adaptor molecule [19]. In the present study, dual luciferase reporter assays showed that ccIRF4 inhibited the NF-κB signal pathway mediated by MyD88. The results demonstrated that ccIRF4 has a similar function to that of mammalian IRF4, which can negatively regulate the TLR signalling pathway. Whereas, its regulatory role in the IFN system is not conserved in fish.

Conclusions

In summary, we have identified and characterized the ccIRF4 from common carp. The mRNA expression profile showed that ccIRF4 was expressed in all the eleven tissues. In addition, ccIRF4 was found to participate in the antiviral and antibacterial immunity both *in vivo* and *in vitro*. Moreover, ccIRF4 has been identified as a negative regulator in both of the IFN and NF-κB signalling pathways. The results obtained in this study provide a basis for further, more detailed investigations into the functions of fish IRF4.

Abbreviations

IFN: Interferon; IRF: Interferon regulatory factor;; DBD: DNA binding domain; IAD: IRF-associated domain; poly (I:C): polyinosinic:polycytidylic acid; LPS: Lipopolysaccharide; PGN: Peptidoglycan; ISG: IFN-stimulated gene; ISRE: IFN stimulated response element; IL: Interleukin; TLR: Toll-like receptor; PAMP: Pathogen-associated molecular pattern; EPC: Epithelioma papulosum cyprinid; FBS: Fetal bovine serum; RACE: Rapid amplification of the cDNA ends; SMART: Simple modular architecture research tool; PBS: Phosphate-buffered saline; LB: Luria-Bertani; HKL: Head kidney leukocyte; ORF: Open reading frame; ANOVA: One-way analysis of variance; Trp: Tryptophans; NJ: Neighbor-joining; hpi: Hour post injection; ISRE: IFN stimulating response element; IRF-E: IRF regulatory element.

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Authors' contributions

Y.Z. performed the experiments, analyzed the data and wrote the paper. G.Y. conceived and designed the experiments. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The dataset supporting the conclusions of this article is available in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore/OL365854) and the accession number is OL365854.

Declarations

Ethics approval and consent to participate
The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University, and all methods were performed in accordance with the relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

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