Iranian Journal of Fisheries Sciences 19(6) 2846-2864 2020
DOI: 10.22092/ijfs.2020.122658

Research Article

Expression differences of interferon regulatory factor 3 and non-specific cytotoxic cell receptor protein-1 in grass carp (*Ctenopharyngodon idella*) after challenges with two genotypes of grass carp reovirus, and analysis of antiviral signaling pathways

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Received: April 2018 Accepted: November 2018

Abstract

Grass carp reovirus (GCRV), with high genetic diversity, seriously affects grass carp farming. Meanwhile, interferon regulatory factor 3 (IRF3) and non-specific cytotoxic cell receptor protein-1 (NCCRP-1) play crucial roles in immunity system of fish. In this paper, we took IRF3 and NCCRP-1 as molecular markers in order to explore the differences in immune responses of grass carp infected with different GCRV strains, which is helpful in better understanding of the GCRV pathogenesity. Accordingly, we analyzed the expression differences of IRF3 and NCCRP-1 in grass carp after challenges with GCRV GD108 (genotype III) and GCRV 096 (genotype I) by qRT-PCR. We found IRF3 and NCCRP-1 was up-regulated in all tested tissues after challenges of GCRV GD108 and GCRV 096. However, the expression levels of IRF3 and NCCRP-1 depended on the GCRV strain, and the up-regulation changes were qualitatively similar but quantitatively different after challenges of GCRV GD108 and GCRV 096, that is, NCCRP-1 was up-regulated more, earlier, and more broadly than IRF3 after GCRV challenge. Besides, the NCCRP-1 was more rapidly up-regulated to a greater extent after challenges with GCRV 096 than GCRV GD108. In order to clarify whether the antiviral immune response and development of grass carp hemorrhage in grass carp differed depending on GCRV genotypes needs more investigation. Moreover, we speculated that the GCRV-induced signaling pathways in grass carp were enriched with measles- and influenza A-induced signaling pathways, and found 13 types of new genes in the GCRV-induced signaling pathway.

Keywords: Interferon regulatory factor 3 (IRF3), Non-specific cytotoxic cell receptor protein-1 (NCCRP-1), Grass carp (*Ctenopharyngodon idella*), Grass carp reovirus (GCRV)

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Introduction

GCRV causes grass carp hemorrhage and has seriously affected the grass carp farming industry (Zhang *et al*., 2010; Wang *et al*., 2012; Yan *et al*., 2014). Diseased grass carp had different hemorrhagic symptoms, "red fins and opercula, red muscles, or red intestines" (Chen *et al*., 2010). However, recent epidemiology (from 2012.04 to 2015.06) of grass carp hemorrhage indicated that all the detected sick grass carp showed the symptom of "red intestines", much less symptoms of "red muscles" or "red fin and opercula" (Huang *et al*., 2016). In addition, most of the detected GCRV isolates were attributed to GCRV genotype III (Huang *et al*., 2016).

GCRVs and antiviral immune responses of grass carp have been studied extensively (Chen *et al*., 2010; Zhang *et al*., 2010; Wang *et al*., 2012; Yan *et al*., 2014; Xu *et al*., 2015; Ou *et al*., 2017). However, the pathogenesis of GCRVs is not completely understood, and the prevention and treatment of grass carp hemorrhage are still difficult, in part owing to the high genetic diversity of GCRVs. Three of four GCRV genotypes are found in China, where GCRV strains of genotype I and genotype III are common. Substantial differences among GCRV genotypes have been reported (Wang *et al*., 2012; Yan et al., 2014). Thus, we hypothesized whether different GCRV genotypes are related to the antiviral immune responses of grass carp. Moreover, studies on the differences in the immune response of grass carp after challenges with GCRVs are necessary for development of prevention and treatment strategies for grass carp hemorrhage, and pathogenesis of GCRV.

The innate immune response of the host is the first line of defense against viral infection by involving in the subsequent activation of the adaptive immune response (Feng *et al*., 2011). Among the major immune mechanisms against viral infections in fish, the interferon (IFN) pathway and non-specific cytotoxic cell (NCC) activity are critical. IRF3 and NCCRP-1 respectively play crucial roles in the IFN (interferon) pathway and NCC activity (Jaso-Friedmann *et al*., 2001; Kallio *et al*., 2011; Tremblay *et al*., 2016).

In the induction of type I IFNs following virus infection, IRF3-mediated IFN-β production and the antiviral innate immune response are regulated by the IRF3 nuclear translocation, activation, polyubiquitination, phosphorylation, and so on (Wang *et al*., 2015; Song *et al*., 2016; Tremblay *et al*., 2016; Wang *et al*., 2016; Bakshi *et al*., 2017). Viruses have evolved multiple strategies to abrogate or inhibit the activation of IRF3 to counteract IFN-mediated responses and successfully infect the host (Kuo *et al*., 2016; Zhang *et al*., 2016). Fish possess an IFN system similar to that of mammals (Sun *et al*., 2010; Avunje *et al*., 2011; Yao *et al*., 2012). For example, IRF3 also plays essential roles in controlling the IFN response during viral infection in fish. IRF3 is expressed broadly in various tissues in fish and is transcriptionally regulated by viral stimulation (Sun *et al*., 2010; Yao *et al*.,

2012; Briolat *et al*., 2014; Xu *et al*., 2015; Gu *et al*., 2016).

NCCRP-1 also functions in the NCC immune response in teleosts (Evans and Jaso-Friedmann, 1992; Jaso-Friedmann *et al*., 2001). NCCs play a number of important roles in immune responses by lysing tumor target cells, protozoan parasites, and virus-infected cells (Jaso-Friedmann *et al*., 2000). Moreover, NCCRP-1 is a type III protein receptor on NCCs, the only known receptor on NCCs, with an antigen-binding domain, signaling domain, a transcriptional activation domain, and F-box-associated (FBA) domain. NCCRP-1 may play dual roles in the activation of NCC, i.e., the recognition and binding to target cells and the activation of the JAK/STAT signaling pathway (Jaso-Friedmann *et al*., 2004; Ye *et al*., 2012).

Furthermore, GCRV GD108 belongs to GCRV genotype III and causes 60–80% mortality in grass carp fingerlings (about 10 cm in length) with "red intestines and without obvious cytopathic effects in their kidney cells (Tamura *et al*., 2007). GCRV 096 also belongs to GCRV genotype I, and causes 20–30% mortality in grass carp fingerlings with "red muscle", and with obvious cytopathic effects in their kidney cells (Yan *et al*., 2014). Therefore, in the present study, we examined the expression differences in IRF3 and NCCRP-1 of grass carp after challenges with GCRV GD108 and GCRV 096 in order to verify our hypothesis. Moreover, we predicted the signaling pathways involved in grass carp to GCRV infection.

Materials and methods

Virus

GCRV 096 was isolated from grass carp with hemorrhagic disease in Hubei Province of China and GCRV GD108 was isolated from grass carp with hemorrhagic disease in Guangdong province of China. Both viruses were stored in our laboratory (Ye *et al*., 2012; Yan *et al*., 2014).

Fish and viral infection

All grass carps (200±15 g) were obtained from the Hedi reservoir in Guangdong Province. We examined these fishes for pathogens by PCR, RT-PCR, microscope, etc. and did not find any pathogens or disease symptoms in them. So, we assumed the grass carps were healthy. The fish were acclimated in an aerated fresh water tank at 26±0.5 °C under natural photoperiod and fed for 2 weeks to adjust to the laboratory conditions before GCRV infection. The water quality parameters DO, NH3, NO2 and pH were measured as 8.1 mg L^{-1}, 0.1 mg L^{-1}, 0.03 mg L^{-1} and 7.5, respectively. The fishes in the infection group were intraperitoneally injected with 200 μl of GCRV GD108 or GCRV 096 suspension (approximately 2000 μg ml^{-1}) (Hiscott, 2007; Yan *et al*., 2014), and the same amount of 0.7% saline solution was administered to the fish of the control group.

Sample collection

After the experiment was set up, three grass carps from each of the infection groups and the control group were captured at days 0.5, 1, 2, 3, 4, 5, 6, and

7 after the infection induction, and tissues samples were collected from the kidney, spleen, liver, heart, brain, skin, gill, intestine, and muscle (3 specimens at each time in order to calculate the mean value of the measured parameters for each tissue). These samples were immediately stored at -80 °C until total RNA was extracted for dynamic expression analyses.

Total RNA extraction

All collected samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNAs were isolated according to the manufacturer's instructions. The quality and concentration of total RNA were analyzed by agarose gel electrophoresis and ultraviolet spectrophotometry based on the absorbance at 260 and 280 nm. Total RNA was incubated with RNase-free DNase I (Fermentas), followed by reverse transcription into cDNA, using oligo $(dT)_{20}$ primers with MMLV reverse transcriptase (Toyobo, Osaka, Japan) following the instructions provided.

Cloning and sequencing of IRF3 and NCCRP-1 in grass carp

Degenerate primers were designed based on *IRF3* or *NCCRP-1* sequences in fish to amplify the 3' and 5' flanking sequences using the SMART RACE cDNA Amplification Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. These degenerate primers were used to perform polymerase chain reaction (PCR), and the PCR products were purified on 0.8% agarose gels using the DNA Gel Extraction Kit (Axygen, Union City, CA, USA) following the manufacturer's instructions. Purified DNA fragments were ligated into pMD-18T vectors (Takara), transformed into competent *Escherichia coli* DH5α cells, and incubated at 37 °C on LB agar plates. Positive colonies were screened by colony PCR using M13 universal primers and sent to Sangon Biotech (Shanghai, China) for sequencing. To acquire the full-length cDNA sequences of *IRF3* and *NCCRP-1* in grass carp, rapid amplification of cDNA ends (RACE) was performed using the BD SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instruction.

Sequence analysis of IRF3 and NCCRP-1 and phylogenetic analyses

The nucleotide and deduced amino acid sequences of IRF3 and NCCRP-1 from grass carps were analyzed using BioEdit 7.0.1 and Expasy (www. expasy.org/tools). The sequences of *IRF3* and *NCCRP-1* from different species were aligned using DNAstar with ClustalV, and final adjustments were performed manually. The transmembrane domains were predicted with the DAS-TMfilter (http://mendel. imp.ac.at/sat/DAS/ DAS.html), PRED-TMR (http://athina.biol.uoa.gr/PRED-TMR/ inp ut.html), MINNOU (http://mi nnou.cchmc.org/), and TMMOD (http:// liao.cis.udel.edu/website/servers/TMM OD/scripts/frame.php?p=submit).

Signal peptides were predicted using SignalP (http://www.cbs.dtu.dk/

services/SignalP/). Domains were predicted using the SMART server (http://smart.embl-heidelberg.de/).

Neighbor-joining (NJ), maximum parsimony (MP), and minimum evolution trees were constructed using MEGA 4.1, with *Homo sapiens* as the outgroup and bootstrap analysis (Livak and Schmittgen, 2001).

Determination of the tissue-specific expression of IRF3 and NCCRP-1 by qRT-PCR

Real-time quantitative PCR was performed using the Rotor Gene 3000 with SYBR Green Master Mix (Toyobo) to quantify the expression levels of *IRF3* and *NCCRP-1*. Each pair of gene-specific primers (Table 1) for RT-qPCR was designed to amplify 200 bp. *GAPDH* and ß-actin was used as reference genes. The final volume reactions were 20 µl, including 1 µl of 2× SYBR Green Master Mix, and 250 nM each primer. Cycling parameters were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. Three parallel assays were performed for each sample to assure the specificity of the amplification products.

Table 1: Oligonucleotide primers used for PCR.

Primer name	Sequence (5′–3′)	Usage
Oligo(dT)$_{30}$-anchor	AAGCAGTGGTATCAACGCAGAGTACT(30)VN	3' RACE-PCR
NCC F317	TGCCTCAGTACAGGTGGTTCTCT	
NCC R1	GACCGTCTTAGCATCAGCACCC	5' RACE-PCR
NCC R2	CCAGGTGTGTGAGTAGTTCTCC	
NCC R3	CACACTGGATTTGCTGGTCTTG'	
Oligo(dT)-anchor	GACCACGCGTATCGATGTCGACT(16) V	
Anchor primer	GACCACGCGTATCGATGTCGAC	
RV	GAGCGGATAACAATTTCACACAGGA	Identification
M13	CGCCAGGGTTTTCCCAGTCACGAC	
IRF3-31	GACGGCAGGATAAATGGA	PCR
IRF3-32	CGAGTCTTTCCACTTGGTCAC	
R1 IRF3a	CCAGTGAAATGTCCCTCTA	5' RACE-PCR
IRF3-R1b	GGATTAGCAGCATCGTTC	
IRF3-R1c	TAGCAGCATCGTTCTTGTTGTC	
Oligo(dT)$_{16}$	GACCACGCGTATCGATGTCGACT(16) V	
5′ PCR anchor	GACCACGCGTATCGATGTCGAC	
β-actinF	GGCATCACACCTTCTACAACGAG	qRT-PCR
β-actinR	ACACCATCACCAGAGTCCATCAC	
GAPDHF	ATCAAGGAAGCGGTGAAGAAGG	
GAPDHR	CGAAGATGGAGGAGTGGGTGTC	
NCCRP-F	CTCTTGGGTGCTGATGCT	
NCCRP-R	CCTGTGGAGGAAGTGAATG	
IRF3-F	AAGAACGATGCTGCTAAT	
IRF3-R	TTCATAGACTTGTAAGGGACT	

Statistical analysis

Relative *IRF3* and *NCCRP-1* expression levels were normalized against *GAPDH* and β-actin expression using the $2^{-\Delta\Delta Ct}$ method (Kuleshov *et al*., 2016). Statistical analyses of *NCCRP-1* and *IRF3* expression levels were performed using MS Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Groups were compared using unpaired *t*-tests.

Antiviral signaling pathways in grass carp

Based on the 160 antiviral immune-related genes of grass carp identified in GenBank (Supporting information), using the KEGG (Kyto Encyclopedia of Gene and Genomes) database, Wiki (Wikipedia) Pathways database, Reactome database, BioCarta database, HumanCyc database, NCI-Nature database, Panther database, and the Enrichr tool (Evans *et al*., 1999), signaling pathways involved in the immune response of grass carp against GCRV were predicted. The 160 antiviral immune-related genes were listed in the supplementary information.

Results

Characteristics of IRF3

The cDNA of *IRF3* was 1831 bp (GenBank accession number: KC898261), with a 192-bp 5′ UTR (Untranslated regions), a 1377-bp open reading frame (ORF), and a 262-bp 3′ UTR. The predicted gene product was 459 amino acids. The sequence we acquired was identical to the sequence reported by Xu (Xu *et al*., 2015). A phylogenetic tree based on *IRF3* (Fig. 1) showed that grass carp *IRF3* is highly similar to *Carassius auratus IRF3*.

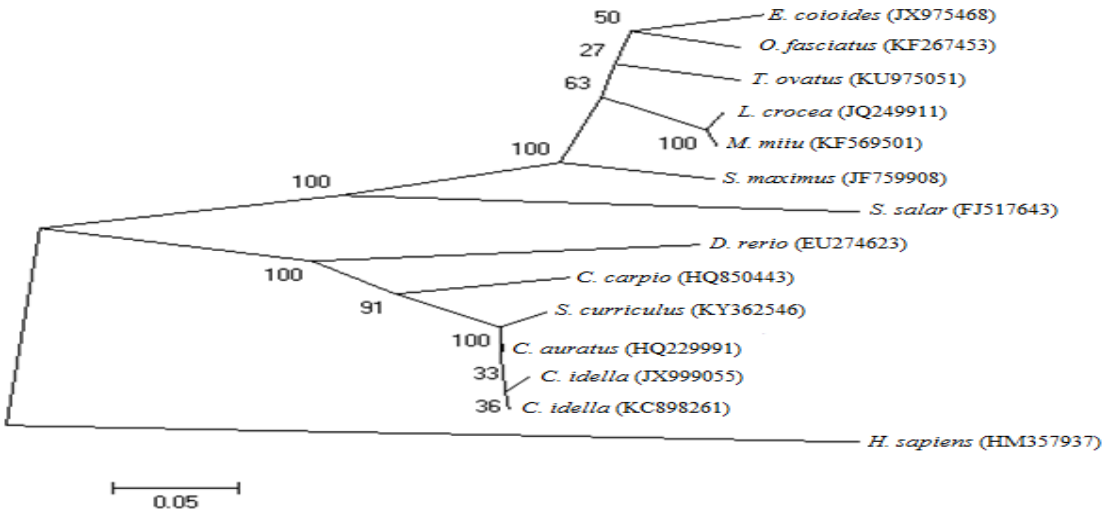


Figure 1: NJ tree based on the *IRF3* gene (numbers indicate the degree of confidence, and numbers inside parentheses are GenBank accession numbers.).

Characteristics of NCCRP-1

The full-length cDNA of *NCCRP-1* was 900 bp (GenBank accession number: HQ388293), with a 3-bp 5′ UTR, a 714-bp ORF, and a 183-bp 3′ UTR. The predicted gene product was 237 amino acids, with a molecular weight of 27.3 kDa. Based on the analyses using SignalP and the TMHMM server, NCCRP-1 in grass carp had no signal peptide or transmembrane region. NCCRP-1 contained Box-1 (PxPxxP), Box-2 (PxP), an FBA domain, and a putative antigen-recognition site (LPxxxWFxxEQxVxLKA) (Jaso-Friedmann et al., 2001; Huang et al., 2014). A phylogenetic tree based on *NCCRP-1* (Fig. 2) showed that grass carp is closely related to *Cyprinus carpio*.

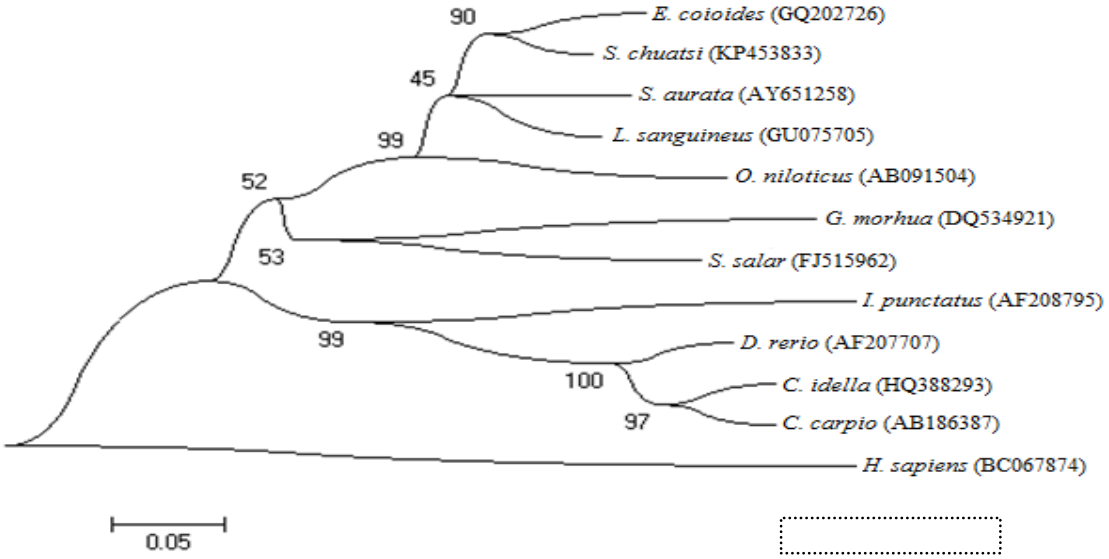
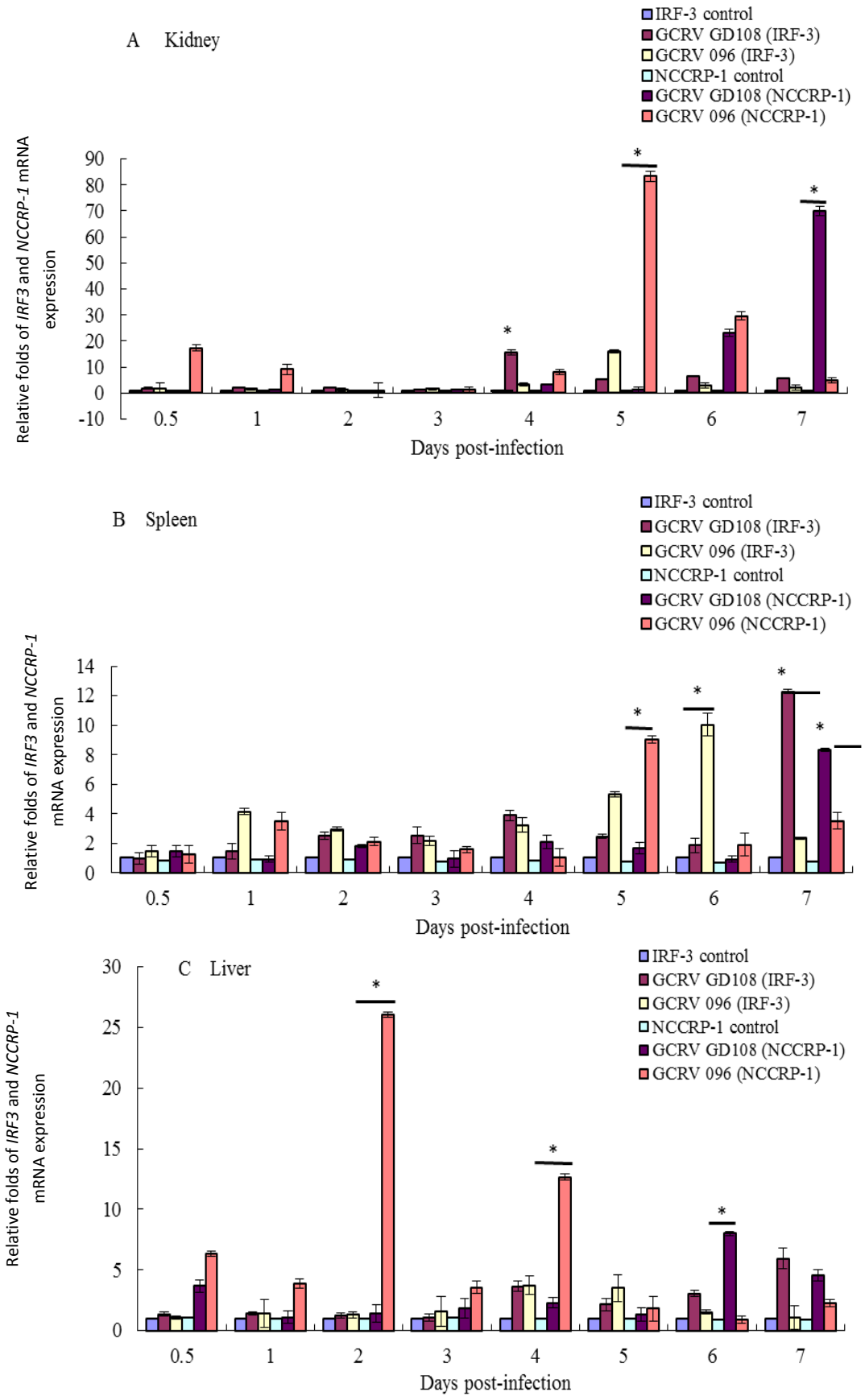
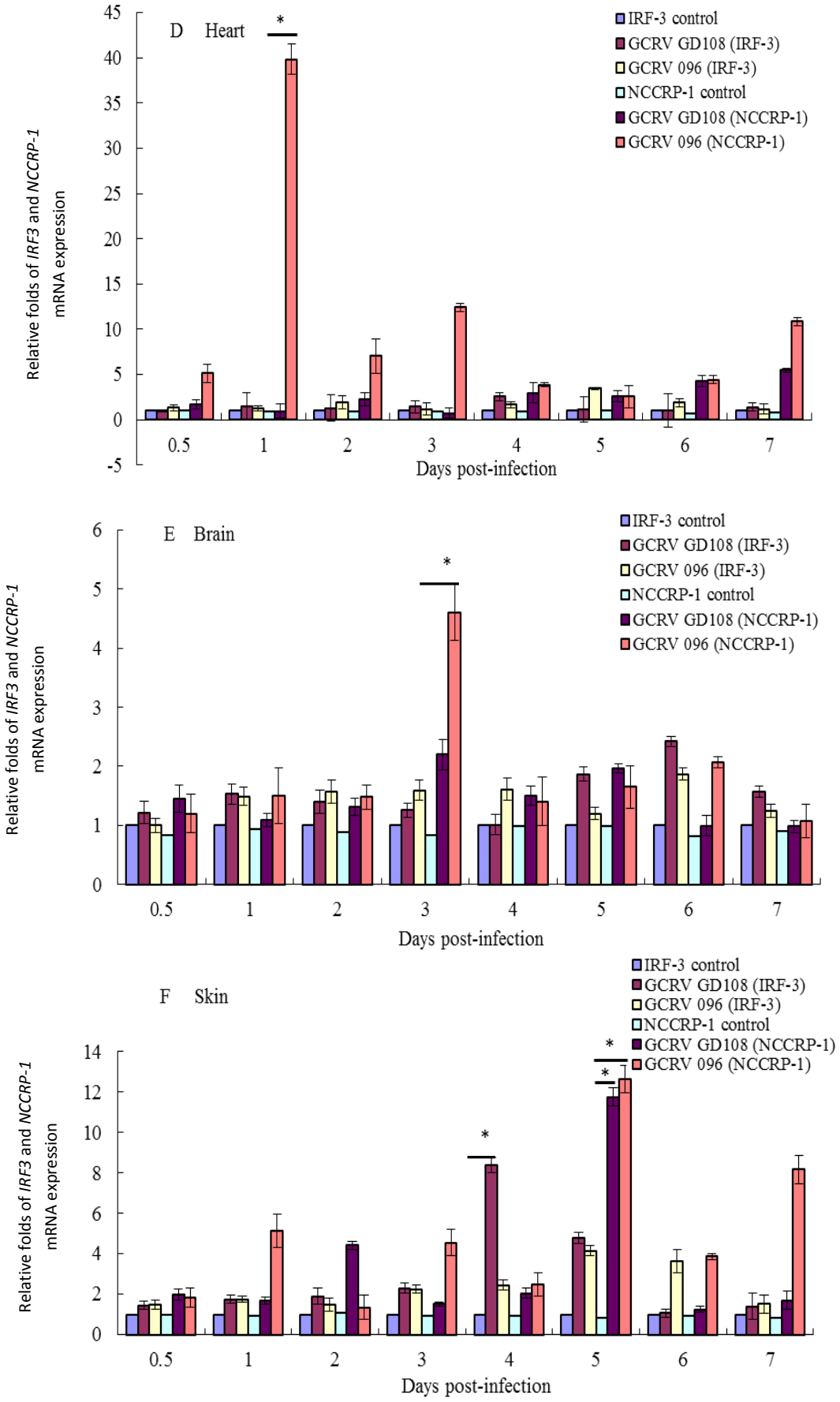


Figure 2: NJ tree based on the *NCCRP-1* gene (numbers indicate the degree of confidence, and numbers inside parentheses are GenBank accession numbers.).

Dynamic expression of IRF3 and NCCRP-1 in grass carp after GCRV challenge

Dynamic expression of *IRF3* and *NCCRP-1* in the kidney, spleen, liver, heart, brain, skin, gill, intestine, and muscle of grass carp during a 7-day period after infection with GCRV GD108 and GCRV 096 was detected by qRT-PCR. *IRF3* was clearly expressed in all tissue types and its expression level differed among tissues (Fig. 3). For *IRF3* in grass carp after challenges with GD108 and GCRV 096, the most pronounced up-regulation was detected in the kidney and spleen (~10–15-fold greater expression than that of the control group), intermediate up-regulation was observed in the liver, skin, gills, and intestine (~5–8-fold increase compared with the control group), and low up-regulation was observed in the heart, brain, and muscle. Dynamic expression of *IRF3* was similar after the challenge with GCRV GD108 and GCRV 096. For *NCCRP-1* in grass carp after challenges with GD108 and GCRV 096, the most pronounced up-regulation was detected in the kidney, skin, gills, intestine, and muscle (~10–80-fold greater expression than that of the control group), intermediate up-regulation was observed in the liver, spleen, and heart (~5–9-fold increase compared with the control group), and low up-regulation was observed in the brain. Especially, expression of *NCCRP-1* in the liver, heart, and intestine was higher and earlier after the challenge of GCRV 096 than GCRV GD108. The results suggested antiviral immune responses of grass carp to GCRV 096 and GCRV GD108 were different to an extent.





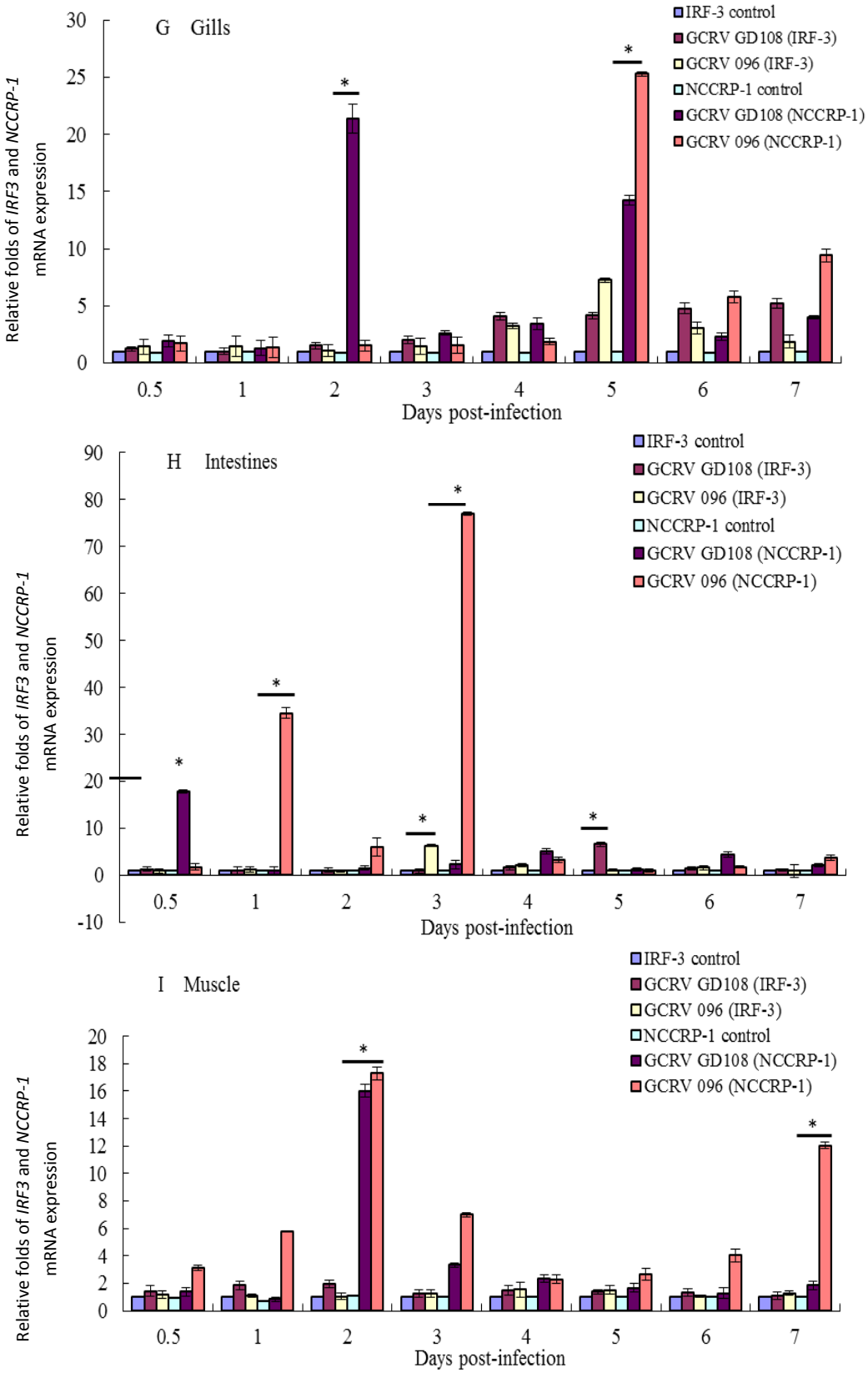


Figure 3: Expression of *IRF3* and *NCCRP-1* in grass carp with the challenge of GCRV GD108 and GCRV 096.

Pathway-enrichment analysis

Based on 160 identified antiviral immune-related genes in grass carp, using the KEGG database, Wiki Pathways database, Reactome database, BioCarta database, HumanCyc database, NCI-Nature database, Panther database, and the Enrichr tool, we predicted that the most enriched pathways in virus-infected grass carp were the toll-like receptor signaling pathway, RIG-I (retinoic acid-inducible gene-I)-like receptor signaling pathway, NOD (nucleotide-binding oligomerization domain)-like receptor signaling pathway, measles-induced pathway, and influenza A-induced pathway (Fig. 4). Using the KEGG database, we determined the potential virus-induced signaling pathways in grass carp (Fig. 4). In addition, homologs of TRAF (tumor necrosis factor receptor-associated factor)3, TANK (TRAF family member associated nuclear factor κB activator), NAP1 (nucleosome assembly protein 1), ERK (extracellular signal-regulated kinase), MKK (Mitogen-activated protein kinase kinase), IP-10 (IFN-γ-inducible protein 10), MIG (monokine induced by IFN-γ), RANTES (chemokine ligand 5), MIP-1 (macrophage infectivity protein 1), FADD, MEKK1 (mitogen-activated protein kinase kinase kinase 1), IRAK (interleukin-1 receptor-associated kinase) 4, and RIP (receptor-interacting protein) 2 should be involved in the immune response in grass carp (Fig. 4), according to KEGG database pathways and the reported immune response relative genes in other fishes (GenBank accession numbers of them in other fishes are respectively KJ789921, KP861886, NM_201318, NM_001124424, NC_031739, AJ417078, NC_024318, NC_019876, BT074182, NC_030425, NC_007121, FN598578 and NC_007113).

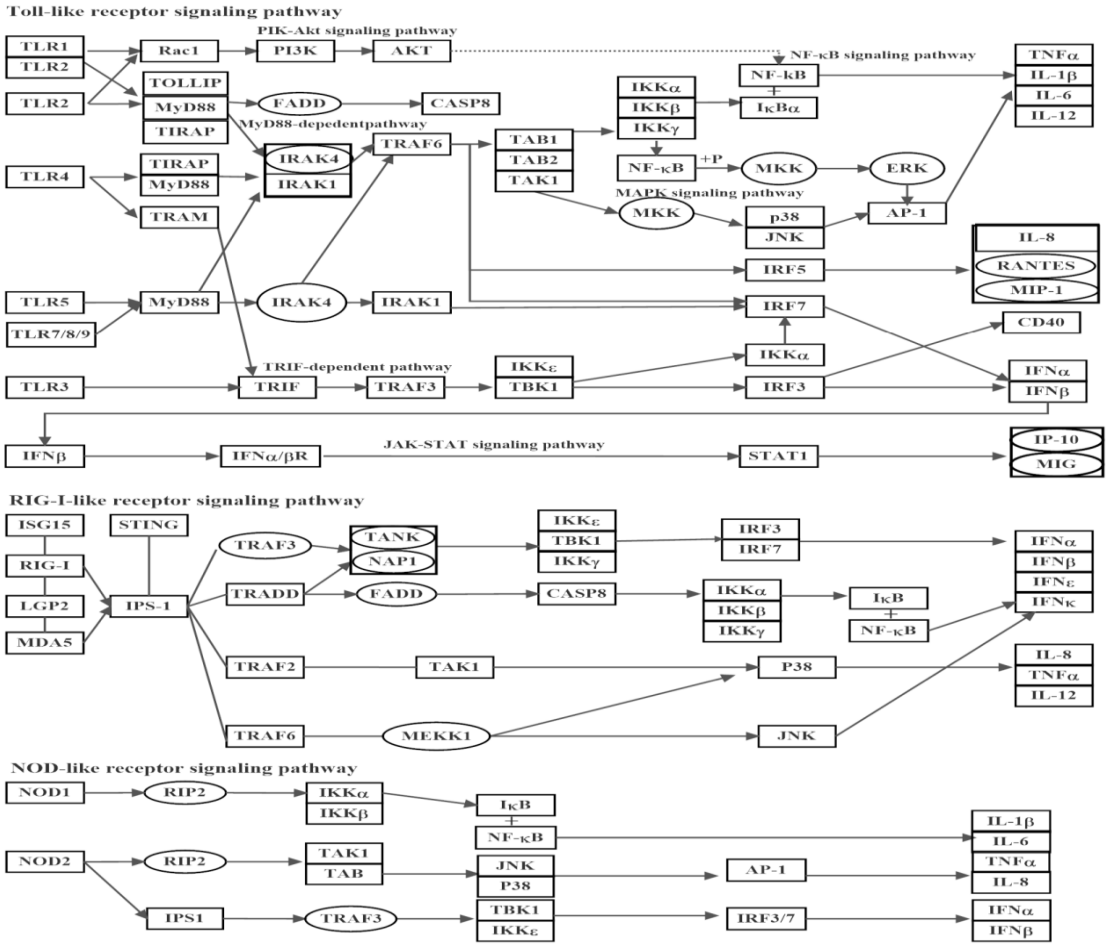


Figure 4: Part speculated GCRV-induced signaling pathway in grass carp.

Discussion

Despite many studies of GCRV and immune responses in grass carp, the mechanisms of grass carp against GCRV are still unclear, and prevention and treatment of grass carp hemorrhage is still a difficult problem. In the present study, we sequenced *IRF3* and *NCCRP-1* of grass carp, sharing conserved motifs with the IRF3 subfamily and the FBA subfamily (Jaso-Friedmann *et al*., 2001; Xu *et al*., 2015). Additionally, NCCRP-1 in grass carp was a soluble protein, similar to NCCRP-1 in the orange-spotted grouper (Panne *et al*., 2007), rather than a type III membrane protein (Jaso-Friedmann *et al*., 2001).

In this study, the dynamic expression of *IRF3* and *NCCRP-1* were qualitatively similar, but quantitatively different after challenges with GCRV GD108 and GCRV 096. Both genes were up-regulated in all tested tissue types, especially the kidney. The kidney might be an important organ for the grass carp defense against GCRV infection.

Our results suggested that IRF3 in grass carp participates in the immune response against GCRV infection and might play an important role in defense against viral infection. After viral infection, fish IRF3 is activated and regulated by phosphorylation, ubiquitination, dimerization, nuclear translocation, etc. Once activated, fish

IRF3 mediates the induction of type I interferons (Kumar *et al*., 2000; Bergstroem *et al*., 2010; Sun *et al*., 2010; Briolat *et al*., 2014; Gu *et al*., 2016; Zhao *et al*., 2016). Moreover, the overexpression of fish IRF3 affects components of the antiviral response in fish (Hiscott, 2007; Bergstroem *et al*., 2010; Sun *et al*., 2010; Zhu *et al*., 2015). IRF3 is expressed in all tested tissues and is up-regulated in response to poly I:C stimulation in various fishes, such as the large yellow croaker, grass carp, miiuy croaker, and tilapia (Yao *et al*., 2012; Xu *et al*., 2015; Gu *et al*., 2016). In this study, *IRF3* in most of the tested tissues in grass carp was up-regulated after challenges with GCRVs, similar to the results obtained in fishes after stimulation with viruses and poly I:C (Yao *et al*., 2012; Xu *et al*., 2015; Gu *et al*., 2016).

NCCRP-1 expression was also up-regulated in various tissues, indicating that NCCs in grass carp are involved in the immune response to GCRV challenge. Expression of *NCCRP-1* is up-regulated following the activation of NCCs by stimuli, resulting in higher killing activity (Chaves-Pozo *et al*., 2012; Huang *et al*., 2014). The increased *NCCRP-1* expression has been linked to the augmentation of killing mechanisms by NCCs (Huang *et al*., 2014). NCCs kill virally transformed cells without prior activation. They collaborate with other effector cells of the immune system, acting as an important component of innate immunity in fish, and they provide innate resistance against virus challenges. In addition, *NCCRP-1* is widely expressed in various organs and is responsible for the recognition of target cells to initiate the lytic cycle. NCCRP-1 may have at least two functions, i.e., target cell binding and the activation of cytokine release (Jaso-Friedmann *et al*., 2001). However, few studies of fish NCC activity have focused on the roles of NCC and NCCRP-1 in defense against viral infections. Our results were similar to those of Chaves-Pozo, who also showed that *NCCRP-1* is up-regulated in sea bream and sea bass infected with nodavirus (Vaziri *et al*., 2012).

Further, our results suggest that the responses of *IRF3* and *NCCRP-1* expression in grass carp differed depending on the GCRV strain. *NCCRP-1* expression increased to a greater extent and more rapidly after challenges with GCRV 096 than GCRV GD108. Immune responses of hosts differ after challenges with different virus strains. For example, differences in immune response are influenced by the virulence and pathogenicity of virus strains (Eldaghayes *et al*., 2006; Adams *et al*., 2009; Ecco *et al*., 2011; Camp *et al*., 2013; Badaoui *et al*., 2014; Kumar *et al*., 2017; Shevtsova *et al*., 2017). The virus-induced host immune response plays an important role in the differences in viral pathogenesis among pathotypes of influenza virus (Cui *et al*., 2014). Little is known about the response of grass carp infected with GCRV and the relationship between the innate immune response and the severity of the clinical disease. Based on the findings of the present study, differences in the host immune response

might explain, in part, differences in pathogenicity between high and low pathogenic GCRV strains in grass carp. The immune response to GCRV in the early stages of infection may be a critical determinant of disease course (Kumar *et al*., 2017).

In addition, the expression of *NCCRP-1* was up-regulated more, earlier, and more broadly than that of *IRF3* after GCRV challenge. For *IRF3*, the earliest and highest expression was observed at 3 days (for GCRV 096, Fig. 3 (H)) or 4 d (for GCRV GD108, Fig. 3 (A, B, C, F)) after infection. *IRF3* was highly up-regulated in the kidney, gill, and intestine (4–15-fold higher expression than that in the control group). For *NCCRP-1*, the earliest and highest expression in different tissues was detected 1 day (for GCRV 096, Fig. 3 (D)) or 0.5 days (for GCRV GD108, Fig. 3 (H)) after infection. *NCCRP-1* was highly up-regulated in the kidney and intestine (40–80-fold higher expression than that in the control group) (Fig. 3). The dynamic expression pattern of *IRF3* was obviously different from that of *NCCRP-1* after the GCRV challenge (Fig. 3). However, the mechanism underlying this difference is not clear. To explore the antiviral immune responses of grass carp and the development of grass carp hemorrhage depending on GCRV genotypes needs more researches.

We also found 13 types of new genes in grass carp that may play roles in the antiviral immune response (Fig. 4). Based on 160 known antiviral immune-related genes in grass carp and the KEGG database, we predicted that the virus-induced signaling pathways in grass carp was highly enriched with toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, and NOD-like receptor signaling pathway (Fig 4). According to the three enriched pathways in KEGG database and reported immune response related genes in other fishes, homologs of TRAF3, TANK, NAP1, ERK, MKK, IP-10, FADD (fas associated via death domain), MEKK1, MIG, RANTES, MIP, IRAK4, and RIP2 in grass carp were expected to be involved in the antiviral immune signaling pathway of grass carp (Fig. 4).

Furthermore, the virus-induced signaling pathways in grass carp was highly enriched with the measles- and influenza A-induced signal pathways (Fig. S1). According to the 173 antiviral immune-related genes in grass carp and the KEGG database, we speculated that the toll-like receptor signaling pathway (Fig. 4), T-cell receptor signaling pathway, NF-κB and MAPK signaling pathway, B-cell receptor signaling pathway, antigen-processing and presentation, complement cascades (e.g. C2, C3, C5, C6), and chemokine signaling pathway should be involved in the immune response of grass carp against virus infection, similar to previous results (Li *et al*., 2017). And that, Measles-induced pathways include T-cell receptor signaling pathway, Toll-like receptor, B cell receptor and RIG-I-like receptor signaling pathway, which were involved in the virus-induced signaling pathways in grass carp. Influenza A-induced pathways include

Toll-like receptor, NOD-like receptor and RIG-I-like receptor signaling pathway, which were also involved in the virus-induced signaling pathways in grass carp. Therefore, this study may explain the enrichment of the virus-induced pathways in grass carp with measles- and influenza A-induced pathways. Maybe measles- and influenza A-induced pathways provide new thoughts for exploring GCRV's pathogenic mechanism.

In conclusion, we found that the expression levels of IRF3 and NCCRP-1 were depended on the GCRV strain. More investigations on the expression differences of *IRF3* and *NCCRP-1* in grass carp infected with different GCRV strains may help to explain the mechanisms underlying grass carp hemorrhage and host immune responses. Bioinformatics speculation of signaling pathway suggested that GCRV-induced signaling pathway was enriched with measles- and influenza A-induced signaling pathways.

Acknowledgments

This work was supported by the National 973 Plan Project of China (2009CB118704) , National Natural Science Foundation (31602199), the Natural Science Foundation of Guangdong Province in China (2015A030313622) and the project of Department of Education of Guangdong Province in China (2012LYM_0075).

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