Comparative analysis of differential gene expression in two species of crucian carps in response to Cyprinid herpesvirus 2 (CyHV-2) infection

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Keywords: Carp; Cyprinid herpesvirus 2; Gene expression

Abstract

We assessed the expressions of MHC, LYZC, keratin8, MPO, DUSP1, IkBa, Rab21, and Rac2 between two species of carps (Erqisi river crucian carp and allogynogenetic crucian carp) after Cyprinid herpesvirus 2 (CyHV-2) infection. The relative expressions of MHC, LYZC, and keratin8 in the virus-challenged groups were significantly higher than control groups. Moreover, the expression of IkBa in the virus-challenged groups was significantly lower than in the control groups. Compared with the virus-challenged ERO group, the expression of IkBa in the virus-challenged ZHO group decreased. The expression of Rab21 in the virus-challenged groups gradually increased and was significantly higher than in the control groups, and then its expression began to decrease after 24 h. At 72 h, the expression of IkBa in both virus-challenged groups was significantly lower than in the control groups. In addition, the expression of Rab21 in the virus-challenged ZHO group was significantly higher than the virus-challenged ERO group at all time points except for 72 h. Before 24 h, the expression of Rac2 remained unchanged in these four groups, and its expression in the virus-challenged ZHO group was significantly higher than in the other three groups. Nevertheless, its expression began to decrease after 24 h but was still slightly higher than the control group at 72 h. MPO showed a similar expression pattern as Rac2. The expression of DUSP1 in the four groups was the same at 0 h. However, its expression in the virus-challenged ZHO group was significantly higher than in the other three groups at other time points.

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Introduction

Erqisi river crucian carp (Carassius auratus gibelio) and allogynogenetic crucian carp (Carassius auratus gibelio) are important freshwater fish species in China. Erqisi river crucian carp is mainly distributed in Xinjiang Uygur Autonomous Region, which is a fast-growth specie with a favorable taste. Allogynogenetic crucian carp is a widely cultured fish species in China with over one million tons of production every year. However, they are susceptible to some viral infections. For instance, when fish are infected with Cyprinid herpesvirus 2 (CyHV-2), they will die within 2 days after some clinical signs appear (Xu et al., 2014).

CyHV-2 is one of the most serious viruses in crucian carp, and it is the second herpesvirus we found in Cyprinid. This virus is widely distributed all over the world, including Japan, America and China, and it is reported firstly in Japan (Jung and Miyazaki, 2010). Moreover, it can cause death of carp, such as the high mortality of goldfish in Taiwan (Chang et al., 1999). This virus always appears in summer and autumn, especially when the water temperature is between 15 to 25 °C (Kong et al., 2017). There are some similar symptoms in infected fish, such as bleeding, pale gills, ascites, abnormal spleen and kidneys (Podok et al., 2014).

It is convinced that MHCI exists in each vertebrate (Bensaid et al., 1991), and the molecular structure of MHCI in fish is similar to that in mammals. MHCI is found firstly in the carp (Hashimoto et al., 1990), and it has been detected in several other fish pieces (Xu et al., 2011; Pinto et al., 2013). As the critical immune gene of fish, it plays an important role in the immune response regulation and antigen processing. Lysozyme is a non-specific immune protein factor that cures the disease directly by hydrolyzing the peptides in the cell walls of bacteria. Lysozyme can be divided into six types, and LYZC is one of them. LYZC accounts for the largest proportion in nature and exists in both vertebrates and invertebrates, and it is firstly reported in Oncorhynchus mykiss (Dautigny et al., 1991). It is an important non-specific immune factor that can protect fish from pathogens. Keratin8 exists mainly in gastrointestinal tract and liver, and it plays an important role in inflammatory response, cell growth and prophylaxis of tumor (Majumdar et al., 2012). MPO is expressed in cytoplasmic granules of myeloid cells, and it is critical in the microbial infections (Kettle et al., 1993). DUSP1 is the negative regulatory gene of MAPK family protein, and it participates in a large number of biological processes, including cell signaling, chondrocyte growth and cell metabolism. Recently, it has been found that DUSP1 can inhibit the growth of hepatocellular carcinoma cells by regulating EKR (Calvisi et al., 2008). In eukaryotic cells, IkBa/NF-Kb is widespread, which can be enhanced with a variety of cellular genes or sequences. IkBa/NF-Kb can promote the transcription and expression of related genes, and they are closely related to the important pathophysiological processes, such as stress response, immune regulation, inflammation, growth control, embryonic development, cell hyperplasia, transformation and apoptosis (Chakraborty and Mann, 2010). Rab21 is involved in the transportation of endocytic vesicles from early endocytic compartment to late endocytic compartment. Moreover, it can interact with integrin within the cell membrane, mediating cell movement and adhesion (Pellinen et al., 2006; Simpson et al., 2004). Rac2 is an important regulatory factor in the cell signal transduction and formation of actin cytoskeleton. It mainly exists at phagosome membrane and can participate in the formation of hematopoietic cells as well as regulation of cell signal transduction and actin cytoskeleton (Pradip et al., 2003; De et al., 2009).

In the present study, we aimed to examine the expressions of some innate immune-related genes (MHCI, LYZC, keratin8, MPO, DUSP1, IkBa, Rab21 and Rac2) between two species, and previous studies have confirmed that these genes are differentially expressed in crucian carp in response to viral infection (Xu et al., 2016). The purpose is to determine which species has the superiority on anti-virus and help to accumulate fundamental data in crucian carp breeding.
Materials and Methods

Experimental animal

The fishes used in this study were Erqisi river crucian carp and allogynogenetic crucian carp with the similar specifications, which were assigned as ERO and ZHO, respectively. The average body weight of the fishes used in the experiment were (200±10) g. Erqisi river crucian carp were cultured in Erqisi River, Xinjiang Uygur Autonomous Region (133 thousand individuals/mu in farming density), and allogynogenetic crucian carp were fed in Dafeng City, Jiangsu Province (444 thousand individuals/mu in farming density).

For the infection group, fish were injected with 1 mL of 10⁶ viral particles mL of CyHV-2 in the abdominal cavity according to the previous method (Xia et al., 2016). Fishes were injected with 1 mL of PBS as the control group. All fishes were maintained in water at a controlled temperature between 20 to 23 °C which was suitable for their living.

Sample collection

After VLPs infection, the expressions of immune-related genes were examined within 72 h. A total of 18 fishes from the infection group were collected for each species, and the same number of fishes were collected from the control group. The kidney tissue was collected at 0, 6, 12, 24, 48 and 72 h post-injection.

PCR was used to confirm viral infection. In each group, two extra replicates were gathered, and all samples were refrigerated at -20° C prior to real-time PCR.

RNA extraction and cDNA synthesis

Total RNA was extracted from kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The kidney tissue for each individual was set as one sample. The purity of the isolated RNA was determined based on the OD₂₆₀/OD₂₈₀ nm ratio, with expected values between 1.8 and 2.0. RNase free-DNase I (Takara, Japan) was used to remove residual genomic DNA, and then the purified RNA was reversely transcribed into cDNA using random hexamer primers and MMLV Reverse Transcriptase (Takara) according to the manufacturer’s instructions.

Primer design and source

For designing primers for amplification of the eight genes, different set of MHCI, LYZC, keratin8, MPO, DUSP1, IxBa, Rab21 and Rac2 sequences of heterologous fish species such as Cyprinus carpio (AB018581.1), Carassius auratus (KJ703112.1), Ctenopharyngodon idella (KY081642.1), Cyprinus carpio (HE584636.1), Danio rerio (AF201451.1 and AY057094.1) and Megalobrama amblycephala (JQ905614.1) were obtained from NCBI database. The sequences of primers are listed as follows:

**MHCI**:
Fw: CTCATCTCCAGTCGTGTC/A: AAAGGTCCCGCTCATCATTAG

**LYZC**:
Fw: ACTTGATGGCTTTGAGGGATT/Rv: TTACACTGGTCTTTCCCACCTG

**keratin8**:
Fw: GTTGAGAGGGAGGTCAAGGAAT/Rv: CAAGGATGCGAGGTGTTGTC

**MPO**:
Fw: CGGCACCTCTCTATGCAGCA/Rv: GTATCTCCCAGCCCAAGGT

**DUSP1**:
Fw: TTTCACCTCTCCATCTCCA/Rv: CATTTTACCCCAACCGAGACAC

**IxBa**:
Fw: AATCTCTGTCGCAACACTGG/Rv: GGGTGACCTCCTCCTCAG

**Rab21**:
Fw: GAAATACCCCGTGAAGTTAGGA/Rv: GGACGTTGGAATTTTGTC

**Rac2**:
Fw: CAGAGATTACAGATGCGATG/Rv: GTCCAGCCAGGAGGTGTT

Real-time RT-PCR analysis

Real-time PCR was performed in a 20 µL reaction system consisting of 1 µL cDNA, 7 µL nuclease-free water, 10 µL 2× SYBR Green Supermix (Bio-Rad) and 1 µL of each specific primer (10 µM) on a CFX96™ Real-time PCR Detection System (Bio-Rad). ß-actin was selected as the housekeeping gene. The relative expression levels of target genes were determined using the 2(-△△CT) method (Livak and Schmittgen, 2012). Single-factor analysis of variance (t-test) was used to compare the differences between groups. P less than 0.05 was considered as statistically significant.
Results

Variations in the expression of eight genes in two species of crucian carp

In the present study, we examined the expressions of eight immune-related genes, including MHC I, LYZC, keratin8, MPO, DUSP1, IκBα, Rab21 and Rac2, by real-time RT-PCR. From investigation, we can see the expression of Rac2 (a) remained unchanged in these four groups, while its expression in the virus-challenged ZHO group was significantly higher compared the other three groups (P < 0.05) before 24 h. Nevertheless, the expression of Rac2 was decreased at 72 h compared with other time points, but it was still slightly higher compared with the control group. The expression pattern of MPO (b) was similar to that of Rac2, except that its expression in the virus-challenged ZHO group was the highest at 48 h. The relative expression levels of keratin8 (c) in the virus-challenged groups were significantly higher compared with the control groups (P < 0.05), and its expression in the virus-challenged ZHO group was significantly higher than that in the virus-challenged ERO group especially at 12 h (P < 0.05). The expression of DUSP1 (d) in the four groups was the same at 0 h. However, its expression in the virus-challenged ZHO group was significantly higher compared the other three groups at other time points (P < 0.05, Figure 1).

As results show, the relative expression levels of MHC I (e) and LYZC (f) in the virus-challenged groups were significantly higher compared with the control groups (P < 0.05). Besides, there was a slight difference between the two virus-challenged groups. However, the expression of IκBα (g) in the CyHV-2-challenged groups was significantly lower compared with the control groups (P < 0.05). Moreover, the expression of IκBα in virus-challenged ZHO group was lower compared with the virus-challenged ERO group. From 0 h to 24 h, the expression of Rab21 (h) in the virus-challenged groups gradually increased, which was significantly higher compared with the control groups (P < 0.05). However, its expression in the virus-challenged groups began to decrease after 24 h, which was significantly lower compared with the control groups at 72 h (P < 0.05). Moreover, the expression of Rab21 in the virus-challenged ZHO group was significantly higher compared with the virus-challenged ERO group at all time points (P < 0.05) except for 72 h (Figure 1).
Relative expression level of Rac2 in ERO and ZHO from 0 h to 72 h.

Note: different letters denote significant differences ($P<0.05$)

Figure 1 Relative expression levels of Rac2, MPO, keratin8, DUSP1, MHC1, LYZC, IkBo and Rab21 in ERO and ZHO from 0 h to 72 h.
Discussion

In the present study, we assessed the expressions of eight immune-related genes in two different species of crucian carp through artificial infection of CyHV-2.

During 72-h infection, the expression of MHC-I in both species of fish was significantly up-regulated, which was consistent with previous reports (Luo et al., 2014; Sever et al., 2014). Moreover, MHC-I exists in each vertebrate (Bensaid et al., 1991), and it is required to deliver the antigen in the initial specific cellular immunity. We found that the expression of MHC-I in the virus-challenged ZHO group was significantly higher compared with the virus-challenged ERO group, and its expression in both virus-challenged groups was significantly up-regulated compared with their own control group, reflecting a better initial specific cellular immunity in ZHO.

It has been confirmed that the expression of LYZC is high in many fish (Jiménezcantizano et al., 2008; Ye et al., 2010; Fernández-Trujillo et al., 2008), and its expression is up-regulated after bacterial infection (Minagawa et al., 2001; Wang et al., 2013). In our current study, we detected a similar expression pattern of LYZC compared with previous reports. Before 48 h, the expression of LYZC in the two virus-challenged groups was up-regulated, and then its expression began to decrease. In a previous report, the author found that the expression of LYZC in grass carp was up-regulated, and then its expression began to decrease, it was the same with ours (Ye et al., 2010).

The expression of keratin8 in both virus-challenged groups was significantly up-regulated, and this finding was consistent with some previous reports (Ku et al., 2007; Podok et al., 2014). This up-regulation could be attributed that keratin protects the cell by falling off from host cells during the late stage in the apoptotic process (Schutte et al., 2004). Moreover, keratin is involved in liver disease by modulating disease progression upon mutation, and its expression is associated with chronic hepatitis C virus (Strnad et al., 2006). Besides, in previous experiments, keratin8 plays a crucial role in protecting hepatocytes when mice are challenged by mechanical and toxic stresses (Fortier et al., 2010).

We found that the expression of DUSP1 was different after 72h post-injection. Some previous studies have reported a similar expression pattern of DUSP1 after infection with the vaccinia virus (Cáceres et al., 2013). The levels of pro-inflammatory and anti-inflammatory cytokines depend on DUSP1 (Dickinson and Keyse, 2006). When fish undergo some stimulations, the expression of DUSP-1 is significantly up-regulated (Lee et al., 2005; Liu et al., 2008; Shields et al., 2011). Similarly, the expression of Rac2 in the virus-challenged ZHO group was significantly up-regulated. The expression of Rac2 reflects the ability of this gene to trigger an inflammatory response, and Rac2 plays an important role in the formation of reactive oxygen and nitrogen types (RONS)-dependent web-like structures (NET) in mice (Lim et al., 2011). However, its expression in the virus-challenged ERO group remained unchanged compared with the control group. The different expression patterns of Rac2 between the two virus-challenged groups might be attributed to the amount of virus injection, which needs to be verified in the following experiment using gradient virus injection.

After CyHV-2 challenge, IkBa was the only down-regulated gene in two virus-challenged groups. In a previous study, when mandarin fish are infected with the spleen and kidney necrosis virus, the expression of IkBa is decreased (Wang et al., 2009). However, in other report, IkBa is up-regulated after being challenged with spring viremia of carp virus (Levraud et al., 2007). Such discrepancy could be explained by different infections (acute or chronic state).

The expression of Rab21 in the virus-challenged ZHO group was significantly higher compared with the virus-challenged ERO group. Rab21 is widely expressed and distributed (Ali et al., 2014), and it is involved in the transportation of endocytic vesicles. The expression of Rab21 affects the endo/exocytic transportation of interns (Pellinen et al., 2006). Moreover, its over-expression can suppress EGF-mediated mitogen-activated protein kinase signaling pathway, suggesting that Rab21 plays a negative role in this signaling pathway (Xi et al., 2014).
2012). According to above-mentioned findings, we believed that the difference in Rab21 up-regulation between the two species of crucian carp could be attributed to that ZHO had a stronger ability to regulate the early endocytic pathway. After 72 h, there was no difference in the Rab21 expression between the two species.

We found that the expression pattern of MPO was similar to that of Rab21. Previous studies have shown that the expression of MPO is significantly increased after CyHV-2 infection (Lau et al., 2005; Phung et al., 2012), which is consistent with our findings. Following trauma in zebrafish, MPO- and peroxidase-expressing cells are localized at the site of acute inflammation within several hours, leading to inflammation at the tip of the embryo’s tail (Lieschke et al., 2001), and the expression of MPO is decreased after phagocytosis of bacteria (Bradley et al., 1982). These data are also consistent with our findings. The expression of MPO in both two species of challenged carp was significantly decreased at 72 h. The difference in its expression between two species of challenged carp at 24 h and 48 h might be attributed to the difference in anti-inflammatory path.

Acknowledgments

This work was supported by a grant from Central Public-interest Scientific Institution Basal Research Fund (No.2014T12).

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The Israeli Journal of Aquaculture – Bamidgeh • ISSN 0792-156X • IJA.74.2022.1774523


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