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Expression analysis of Igs and mucosal immune responses upon SVCV infection in common carp (*Cyprinus carpio L.*)

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A B S T R A C T

The immunoglobulin (Ig) is a crucial component of adaptive immune system in vertebrates including teleost fish. Here complete cDNA sequence of IgD heavy chain gene from common carp (*Cyprinus carpio*) was cloned and analyzed. The full-length cDNA of IgD heavy chain gene contained an open reading frame (ORF) of 2460 bp encoding 813 amino acids. According to amino acids sequence, multiple alignment and phylogenetic analysis showed that carp Igs are closely related to those of Cyprinidae fish. Transcriptional expression of IgD as well as IgM, IgZ1 and IgZ2 showed similar expression patterns in different organs, this is, high expression level in systemic immune tissues (ie, head kidney, heart and spleen) and low expression in mucosal tissues (ie, gill, skin and gut). Following viral infection with spring viraemia of carp virus (SVCV), obvious pathological changes in skin, gill and gut mucosa and up-regulated expression of antiviral related genes in skin, gill, gut and spleen were observed, indicating that SVCV successfully infected common carp and activated the systemic and mucosal immune system. Interestingly, IgM showed a significant up-regulation only in systemic tissue (spleen), but not in mucosal tissues (gut, gills and skin), while increased expression of IgZ1 and IgZ2 was found in gut. In contrast, the expression of IgD increased significantly in spleen, gills and skin. These strongly suggest that fish Ig isotypes play different roles in mucosal and systemic immunity during viral infection.

Common carp (Cyprinus carpio); Igs; Spring viraemia of carp virus (SVCV)

1. Introduction

In vertebrates, the mucosal immune system acts as the first line of defense against pathogen invasion [1,2]. Immunoglobulins (Igs) are humoral mediators that play the prevailing role in adaptive immunity, and provide obligatory duties to protect the organisms from a wide variety of pathogens [3–6]. Over the course of evolution and water-to-land transition in vertebrates, jawed vertebrates have evolved a very different strategy to generate considerably more diverse repertoires of antigen receptors, depending on the recombination-activation gene (RAG)-mediated rearrangement of the variable (V), diversity (D), and joining (J) segments within the Igs [7–9]. At present, five Ig classes including IgM, IgD, IgY, IgX and IgF have been identified in amphibians [10]. Four IgH isotypes, IgM, IgD, IgY, and IgA, have been found in birds and reptiles to date [11–13]. Most mammals express five IgH isotypes: IgM, IgD, IgA, IgG, and IgE [9]. However, so far, only three Ig isotypes (i.e., IgM, IgD, and IgT/Z) have been identified in teleost, each comprising two identical heavy and two identical light polypeptide chains [14,15]. Specifically, as the most abundant Ig class in plasma, IgM regulates the systemic immune responses of teleost depending on water temperature and quality, as well as fish species, size, stress, stimulation, and immunization [16,17]. Similar to IgA in mammals and IgX in frogs, IgT/IgZ acts as a mucosal-associated Ig in bony fish, which has been described in studied teleost fish except for medaka and catfish [18–21]. Interestingly, two separate IgZ loci encoding IgZ1 and IgZ2 have been identified in zebrafish and common carp [22,23]. IgZ1 represents a typical teleost IgHt/z feature, whereas the Cm 1 and Cz 4 domains constitute a chimeric transcript of IgZ2. IgD is an ancient Ig class that has been found in most jawed vertebrates including yellow catfish (*Pelteobagrus fulvidraco*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus morhua*) [8,24–27]. Similar to IgM and IgT/Z, IgD monomers are composed of two identical light and heavy chains. Human and other primate IgDs have three Cδ domains, however, compared to the limited (i.e., normally two or three) Cd domains in mammals, the number of Cd domains varies widely in different fish species [28–30]. Moreover, the Hinge region (H region) of human IgD

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has a flexible T-shaped structure, like two Fab "long arms" that rotate on either side of the Fc segment, facilitating antigen binding while also acting as a "replacement" for the absence of IgM function. IgD is mainly divided into secreted IgD (sIgD) and membrane-bound IgD (mIgD). Compared with the c-terminus of sIgD, mIgD contains more transmembrane and intracellular regions and can act as a receptor and also involve in the immune response as a ligand [9–13]. In addition, although sIgD has been found coating a small portion of commensal microbiota in mucosal tissues including gut, gill, and skin, the immune function of fish IgD remains unknown [22,31,32].

Common carp (*Cyprinus carpio*), one of the important economic species cultured in China, usually suffer from the infectious diseases caused by bacterial, parasitic and viral pathogens [33,34]. Spring viremia of carp virus (SVCV), as a rhabdovirus [35], could cause spring viremia of carp (SVC) disease in cyprinids, especially in cultured common carp, leading to significant morbidity and mortality in fish industry. By far, there is still a lack of prevention strategy because of limit information of carp Igs response to the viral infection. In order to assess this, the sequences of IgD heavy chain were characterized using rapid amplification of cDNA ends (RACE). In addition, we analyze the expression profiles of IgD as well as IgM, IgZ1 and IgZ2 in different tissues of common carp. More critically, we construct an SVCV infection model in the carp by injecting with a low lethal dose of SVCV. The morphological changes of skin, gill, and gut were detected following SVCV infection. Moreover, the mRNA expressions changes of Igs and antiviral genes in systemic tissues and mucosal tissues were analyzed.

2.Materials. and methods

2.1. Ethics statement

All experimental protocols were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. They were approved by the Scientific Committee of Huazhong Agricultural University (HZAU, permit number HZAUFI-2017–013). All efforts were made to minimize the suffering of the animals.

2.2. Fish husbandry and sampling

Five-month-old common carp (10–15 g) used in this experiment were obtained from a fish farm in Chongqing, China, and maintained in aquarium tanks using a water recirculation system with thermostatic temperature control and extensive biofiltration. The fish were kept at 18 °C for at least two weeks and fed with commercial carp pellets twice per day (9:00 a.m. and 4:00 p.m.) with a rate of 0.5–1% body weight. The feeding was terminated 48 h prior to sacrifice both in control and infected groups. Before infection, the fish were acclimatized to the water temperature by increasing the water temperature from 18 to 12 °C by 2 °C per day for the SVCV infection.

To investigate the expressions of common carp Igs in different tissues, 9 fish were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222) and then tissues including head kidney, heart, spleen, nose, eyes, swim bladder, gill, pharynx, fin rays, skin and gut were collected. All collected samples were immediately frozen in liquid nitrogen, and then stored at −80 °C for further analysis.

2.3. SVCV challenging and sampling

The common carp epithelioma papillosum cyprini (EPC) cell line was maintained in minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution in a 26 °C incubator with 5% CO_2. The SVCV used in this study was gifted from Professor Xue-Qin Liu at Huazhong Agricultural University and propagated in EPC cells until cytopathic effect (CPE) was observed, subsequently adjusted to 1×10^7 pfu ml^{-1} in MEM and stored at -80 °C until use. Fish were anaesthetized with methanesulfonate (MS-222) and intraperitoneally (i.p.) injected with 100 μl of MEM containing SVCV. As the control group, fish were treated similarly and i.p. injected with 100 μl of MEM collected from non-infected cells.

At 1, 7, 14, 28 days post infection (dpi), the common carp were anesthetized with MS-222 for sampling as previous study [36]. For histological and pathological studies, skin, gill and gut of common carp were directly taken from control and infected fish, and then fixed immediately at 4% (v/v) neutral buffer paraformaldehyde for at least 24 h. For RNA extraction and qPCR, tissues including gill, skin and gut were collected in Trizol reagent and stored at -80 °C until use.

2.4. Return infection plaque experiment of SVCV

The skin, gill and gut from control and infected groups were obtained and placed in clean petri dishes to remove muscle tissue. Thereafter, the tissues were cut into small pieces (~ 0.1 cm^2) and treated with cold PBS (pH7.2) for 3 min at 4 °C with continuous agitation. The tissue pieces were mechanically disaggregated on a 100- μm cell strainer, and the cell suspension was collected. All cell suspension were then freeze-thawed three times to break the cells and release the virus after adding 5% FBS, which can prevent virus inactivation. The cell suspension was centrifuged for 5 min at 400 g once and 10,000 g thrice at 4 °C to remove large cell debris, and then the supernatant was filtered with 0.45- μm membrane filter to remove bacteria from the surface of mucosal tissue. The resulting liquid was added to a 6-well cell culture plate covered 90% monolayer EPC cells in the bottom of the plate and maintained in 1.5 mL MEM. The results of plaque experiment were examined under a light microscope (Phenix) and imaged on MShot image analysis system.

2.5. RNA isolation and cDNA synthesis

Total RNA from different tissues was extracted using Trizol Reagent (Invitrogen Life Technologies) following the manufacturer's protocol. A spectrophotometry (NanoPhotometer NP 80 Touch) was used to quantitate the extracted RNA and agarose gel electrophoresis was used to determine the integrity of the RNA. Then approximately 1 μg of RNA was reverse transcribed into cDNA with Hifair III 1st Strand cDNA Synthesis SuperMix for qPCR (YEASEN) following the manufacturer's instructions.

2.6. Common carp IgD cloning

The partial IgD nucleotide sequence was obtained from the common carp transcriptome. The gene-specific primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) [8,37–39] to amplify the internal region of IgD. Then smart 5′-RACE and 3′-RACE were performed using HiScript-TS 5′/3′ RACE kit (Vazyme, China) following manufacturer's instructions. The PCR product was purified with the FastPure® DNA Extraction Mini Kit (Vazyme, China) according to the manufacturer's instructions and then cloned into pCE2 TA/Blunt-Zero vector (Vazyme, China). Positive colonies were selected for sequencing. All primers used were shown in Supplementary Table 1.

2.7. Multiple sequence alignment and phylogenetic analysis

The ORF of the common carp IgD heavy chain gene was found using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) based on the full-length cDNA sequence. The amino acid sequence was predicted using the DNAMAN software and multiple alignment of amino acid sequences were performed with Multalin (http://multalin.toulouse.inra.fr/multalin/) [39]. The signal peptide was predicted using the online SignalP software (http://www.cbs.dtu.dk/services/SignalP/) [40]. Glycosylation sites were predicted using the online software NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/#opennewwindow). The transmembrane domain was predicted using

TMHMM software (http://www.cbs.dtu.dk/services/TMHMM-2.0/). We also predicted the functional domains of IgD through IMGT tool (http://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi).

The phylogenetic analysis of Igs was carried out based on the protein sequence with MEGA7.0 software by the Neighbor-Joining (NJ) method. Reliability of tree nodes was estimated using 1000 bootstrap replicates [41].

2.8. Histological assays

After fixed in 4% neutral formalin buffer, skin, gill and gut were dehydrated in a graded ethanol series, washed with xylene, embedded in paraffin, and then sectioned into 5 μm pieces. The paraffin pieces were stained with classic hematoxylin and eosin (H&E) as described previously [42]. Images were acquired in microscope (Olympus, Japan) using the Axiovision software. The microscopic pathological changes of the skin mucosa were evaluated by measuring the thickness of the epidermis layer. Similarly, the length–width ratios of the lamellae and villi were measured to evaluate microscopic pathological changes in gill and gut, respectively. The parameters of each image were measured by three different researchers and averaged to reduce random errors.

2.9. QPCR and statistical analysis

The qPCR was performed by the qTOWER3G PCR system (Analytik Jena AG, Germany) using the EvaGreen 2 × qPCR Master mix (YEASEN) as following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and at 58 °C for 30 s. Relative fold changes of genes were calculated by the methods of $2^{-\Delta\Delta Ct}$ and 40S was used as control gene for normalization of expression. The results were obtained from three independent experiments and each was performed in triplicate.

All data were expressed as the mean ± SE and checked for normality and homogeneity of variances before statistical analysis. An unpaired Student's *t*-test (Prism version 6.0; GraphPad) was used for gene expression and histology data analysis. Statistical significance was checked by setting the alpha at 0.05 for all analyses.

3. Results

3.1. Characteristics of cDNA sequence of common carp, multiple sequence alignment and phylogenetic analysis

The full-length cDNA of IgD heavy chain is 2813 bp, containing an ORF of 2460 bp, which encodes a predicted protein of 819 amino acids. The IgD protein structure includes one VH, seven Ig-like constant domains (CH1,CH2,CH3,CH4,CH5,CH6,CH7) and a transmembrane region (TM) (Fig. 1). We identified the conserved amino acid residues by multiple sequence alignment with other known teleost sequences. Conserved cysteine residues were labeled, where disulfide bonds could be formed, as shown in Fig. 2.

Multiple alignments analysis showed that the commo carp IgD had high identities to IgD of *Megalobrama amblycephala* (53%) and *Ctenopharyngodon idella* (47%), but low identities to IgD of *Ietalurus Punetaus* (30.18%), *Paralichthysolivaceus* (26.21%), *Epinephelus coioides* (24.15%) and *Takifugu rubripes* (25.02%) (Supplementary Table 2). The NJ phylogenetic tree was constructed based on the deduced amino acid sequences of Igs from common carp and other vertebrates. The phylogenetic analysis suggested that common carp IgM and IgD clustered with their respective protein sequences from other teleost fishes, while common carp IgZ2 clustered with IgM sequences from other teleost fishes. Common carp IgZ1 clustered with IgZ sequence of zebrafish (Fig. 3).

3.2. Igs basal expression analysis in different tissues

The qPCR demonstrated that Igs were ubiquitously expressed in eleven tissues of common carp, but the expression level was different among tissues (Fig. 4). IgM had the highest expression level in the head kidney, followed by the nose, and the lowest level in the gut (Fig. 4A). For IgD, the highest expression level was detected in the heart, followed by the spleen, head kidney and nose, and much lower expression in other detected tissues (Fig. 4B). IgZ1 and IgZ2 of common carp showed similar tissue distribution (Fig. 4C and D), with the highest expression level in the head kidney, followed by the heart, spleen and swim bladder, and the lowest expression in gut.

3.3. Detection of SVCV in common carp post infection

After SVCV infection, obvious pathological changes were observed on the surface of gill, skin, intestine and fins. The pathologic features such as gill whitening, scales shedding, intestinal bleeding and fin erosion were observed (Fig. 5A). Through the Return Infection Plaque Experiment of SVCV, obvious cytopathic effect (CPE) and a certain number of plaques can be found in the infected group, while CPE and plaques were not observed in the control group (Fig. 5B). Finally, the virus load changes in skin, gill and gut were detected by qPCR at 1,7,14,28 dpi. It can be clearly observed from the heat map that the strongest infection was observed at 7 dpi (Fig. 5C). All the above results indicated that the gill, skin and gut of common carp were successfully infected with SVCV.

3.4. SVCV infection induced morphological changes

In order to further observe the pathological changes of common carp tissues caused by SVCV infection, Hematoxylin and eosin (H&E) staining of skin, gill and gut tissues was performed (Fig. 6). The thickness of skin epidermis layer began to decrease significantly at 1 dpi, and gradually increased at 7 dpi, and recovered to the same thickness as that of control group at 14 dpi (Fig. 6A and D). Moreover, significant changes were also observed in gills, as evidenced by wider and shorter secondary lamellae, especially at 7dpi, the changes were particularly obvious (Fig. 6B and E). However, no conspicuous changes were found in the gut of common carp after virus infection compared to control fish (Fig. 6C and F). Overall, these results demonstrated that SVCV successfully invaded common carp and induced significant morphological changes in the external mucosa.

3.5. Immune genes expression in common carp

To gain insights into the kinetics of the immune responses following SVCV infection, the relative expression of 17 antiviral and immune-related genes was quantified at 1,7,14,28 dpi in external (skin, and gill) and internal (gut) mucosal tissues, as well as spleen tissues via qPCR. These gene include antiviral genes (ISG15,Mx1,VIG, Protein Kinase R [PKR], IRF7 and TLR7) and immunoglobulin heavy chain genes (IgZ1,IgZ2,IgM,IgD and PIGR), antimicrobial peptide (Apolipoprotein14 [ApoA14], Hepcidim,Muc2,Muc19), pro-inflammatory cytokines genes (Interleukin 1β (IL1-β) and TNFα) (Fig. 7). Primers for these genes used in this study are shown in Supplementary Table 3. After infection with SVCV, most genes were up-regulated to varying degrees in skin, gill and gut tissues, especially for the antiviral genes, reaching a peak value at 7dpi (Fig. 7). However, most genes' expressions were up-regulated significantly in the spleen at 1dpi and 7dpi.

We also detected the expression changes of Ig genes (IgZ1,IgZ2,IgM, IgD) in four tissues. In the skin and gill tissues, only IgD was up-regulated at 14dpi in the skin, and at 1dpi, 7dpi and 14dpi in the gill. In the gut tissues, the expression level of IgZ1 and IgZ2 increased significantly at all the detected time points. The expression of IgM in gut tissue was up-regulated at 7dpi and 14 dpi, and then decreased to a low level. In the spleen, the expression of IgM and IgD were significantly increased at most time points, reaching a peak value at 7dpi for IgM, and at 14dpi for IgD. IgZ1 was also up-regulated at 14 dpi in spleen (Fig. 8).

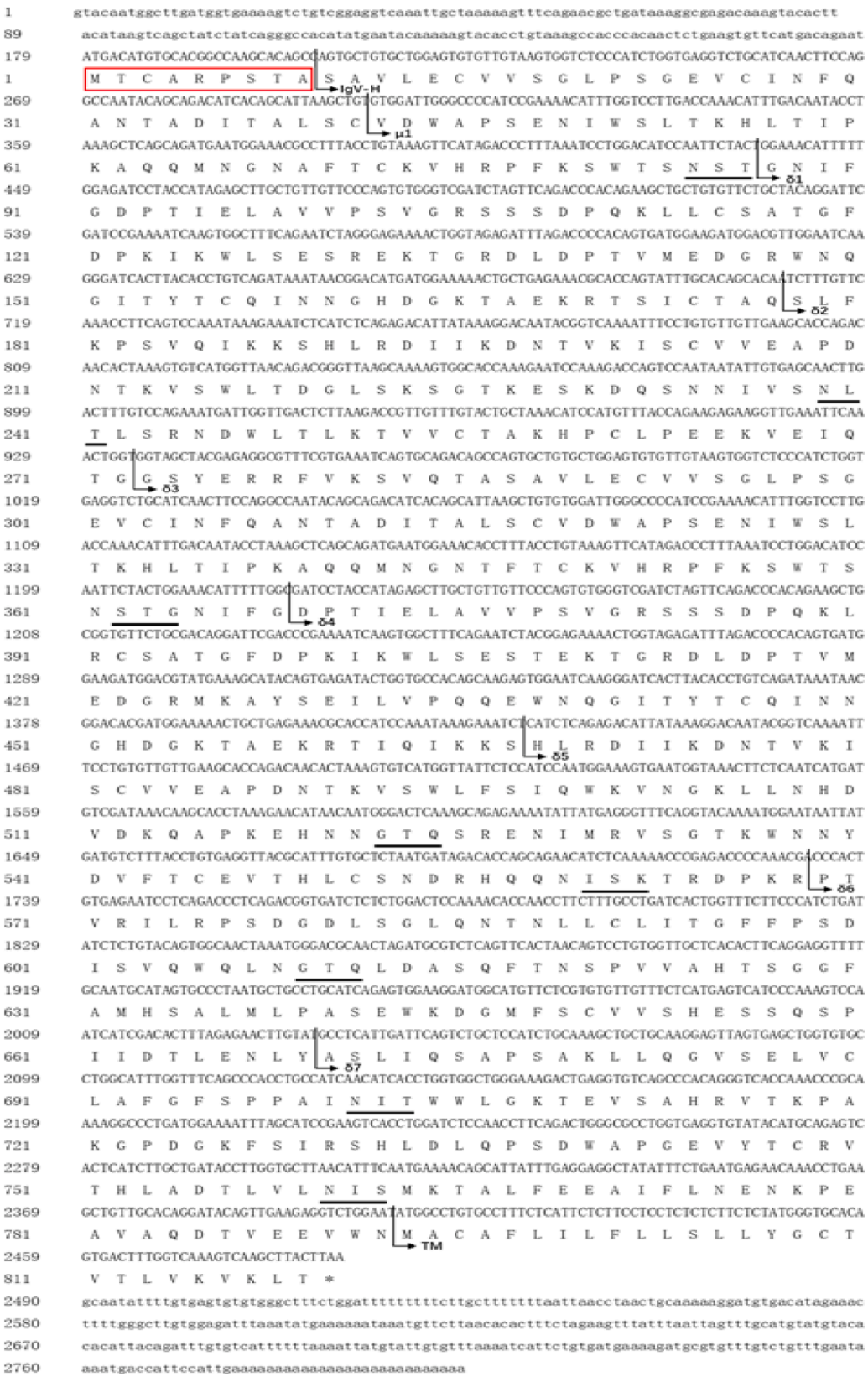

Fig. 1. The nucleotide and deduced amino acid sequences of the heavy chain of IgD in common carp. The numbers on the left show the nucleotide positions. The variable region and constant region are marked with arrows. The signal peptide is marked with a red border. N-linked glycosylation sites are circled. The stop codon is represented with an asterisk (*).

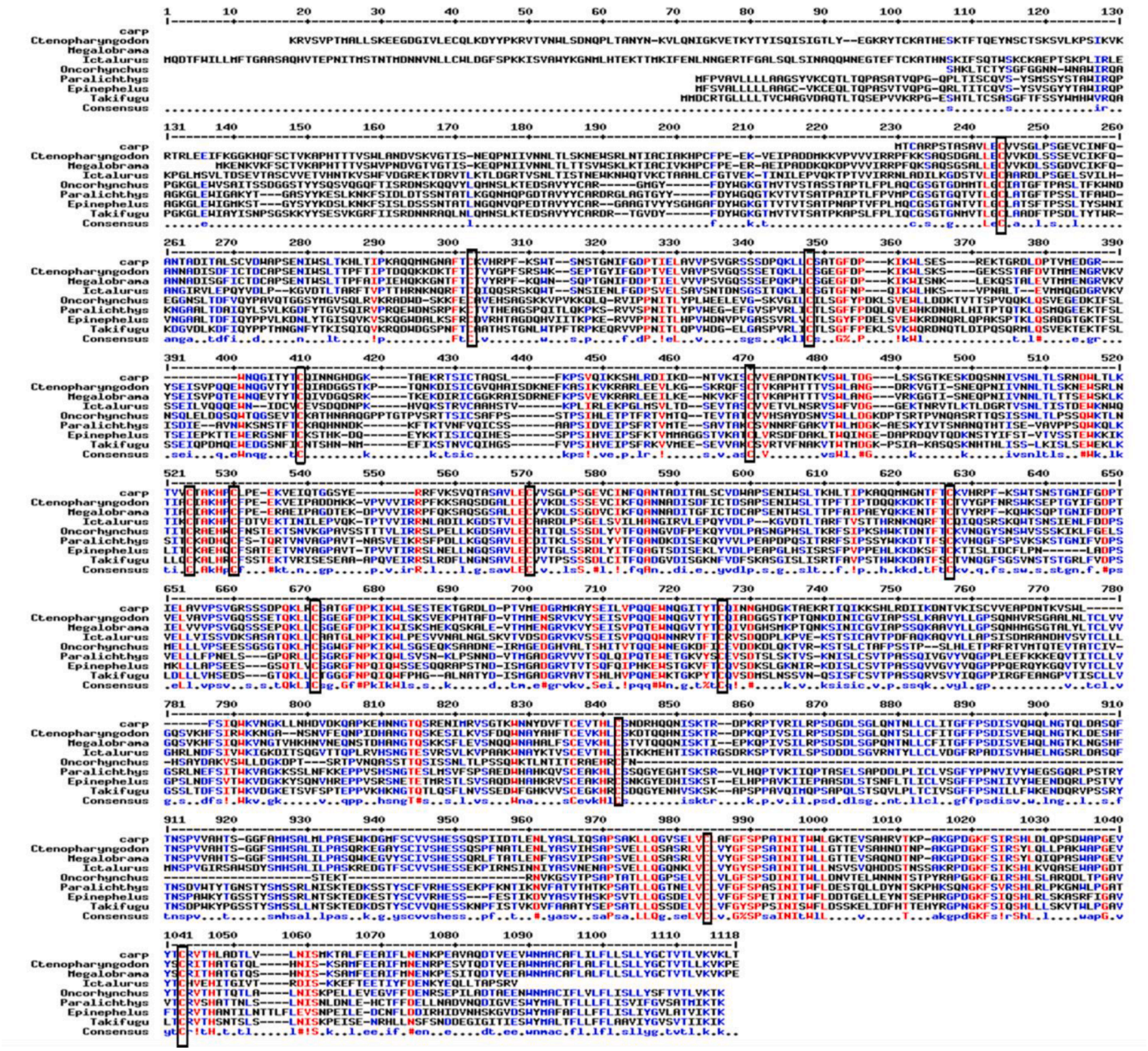

Fig. 2. Multiple sequence alignment of deduced amino acid sequences of common carp IgD with the corresponding sequences of other species, respectively. Cysteine residues are marked with black frame. The GeneBank accession numbers of the sequences used in the alignment are shown as follows: Megalobrama amblycephala IgD (AGR34025.1); Ctenopharyngodon idella (ACV21057.1); Oncorhynchus mykiss (AAW66976.1); Ietalurus Punetaus (ADF56020.1); Paralichthysolivaceus (BAB41204.1); Epinephelus coioides (AFI33218.);Takifugu rubripes (BAD34541).

4. Discussion

Immunoglobulins are highly specialized recognition glycoproteins that can recognize a great variety of antigens from bacteria, viruses, and other disease-causing organisms, and recruit other cells and molecules to exclude these pathogens [19]. Although three types of immunoglobulins IgM, IgD and IgT/IgZ are found in teleost [22], the function of IgD has been controversial and unclear [32]. In addition, there is no report about the gene information and biological function of common carp IgD. Therefore, in the present study, we characterized the sequence of IgD heavy chain gene in common carp and explored the relative mRNA expression changes of Igs and other immune genes during SVCV infection.

Multiple sequence alignments showed that the common carp IgD heavy chain gene had a high identity to that of blunt snout bream. The phylogenetic tree analysis showed that the common carp IgM and IgD heavy chain genes clustered with their respective protein sequences from other teleost fish, while common carp IgZ2 clustered with IgM sequences from other teleost fish. Common carp IgZ1 clustered with IgZ sequence of zebrafish. Gene structure analysis showed that the common carp IgD heavy chain gene consists of a variable region (VH), a Cu1 region, seven constant regions (CHI-CH7) and a transmembrane region (TM), which is similar to that of blunt snout bream [43]. Tissue distribution results showed that IgM, IgZ1 and IgZ2 have the highest expression level in head kidney, and IgD has the highest expression in heart, followed by head kidney. The high expression in the heart may be caused by residual blood cells, and therefore further studies are needed to verify it. All the Immunoglobulin genes of common carp showed expression bias in systemic immune tissues. In addition, IgZ1 and IgZ2 showed similar expression profiles in different tissues, which are similar

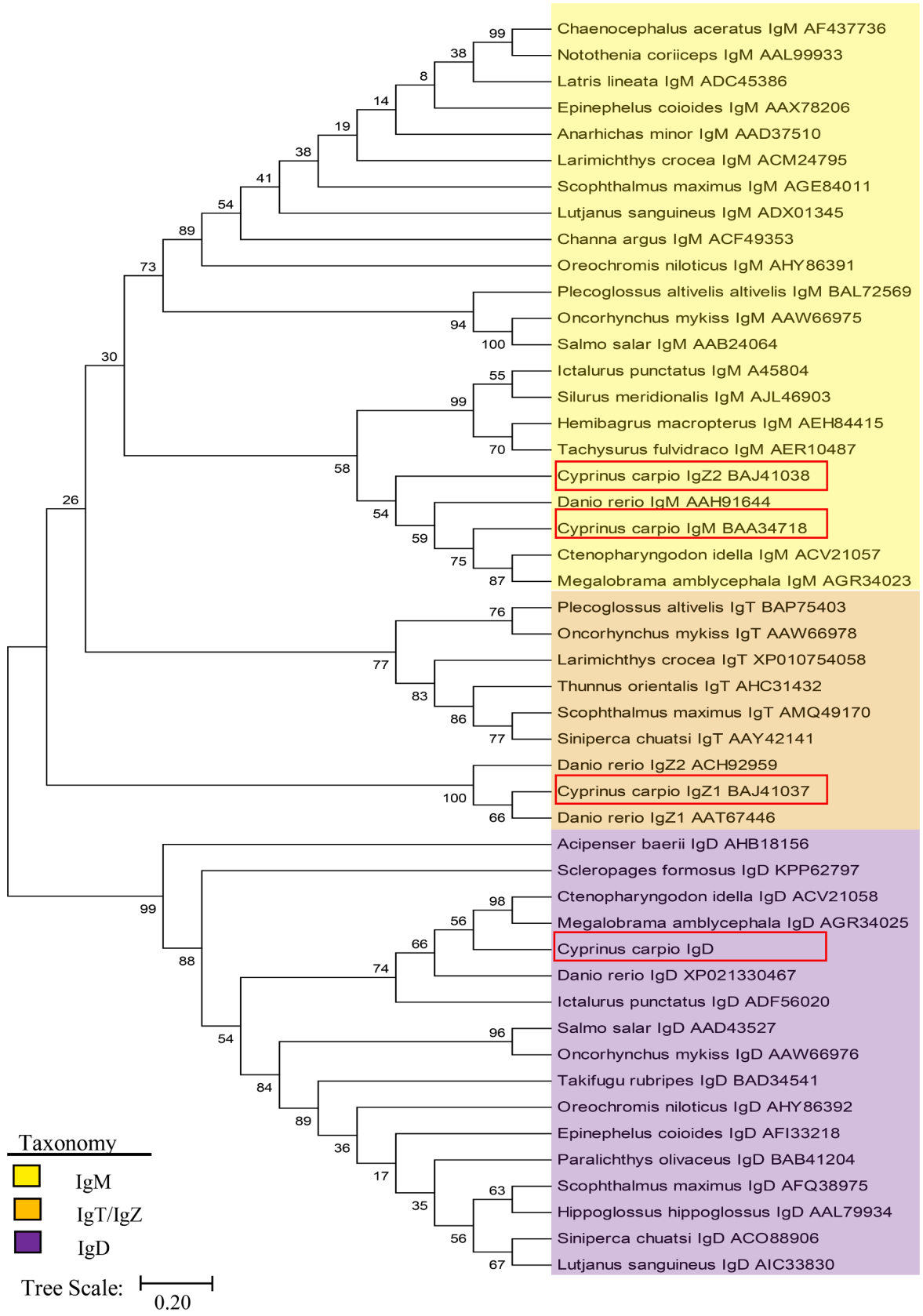

Fig. 3. Phylogenetic analyses of common carp IgZ1, IgZ2, IgM and IgD sequence with other teleost fish by using neighboring-joining (NJ). GenBank accession numbers of all sequences are shown in parentheses behind the name of the sequences. The evolutionary distances are presented as scale bars.

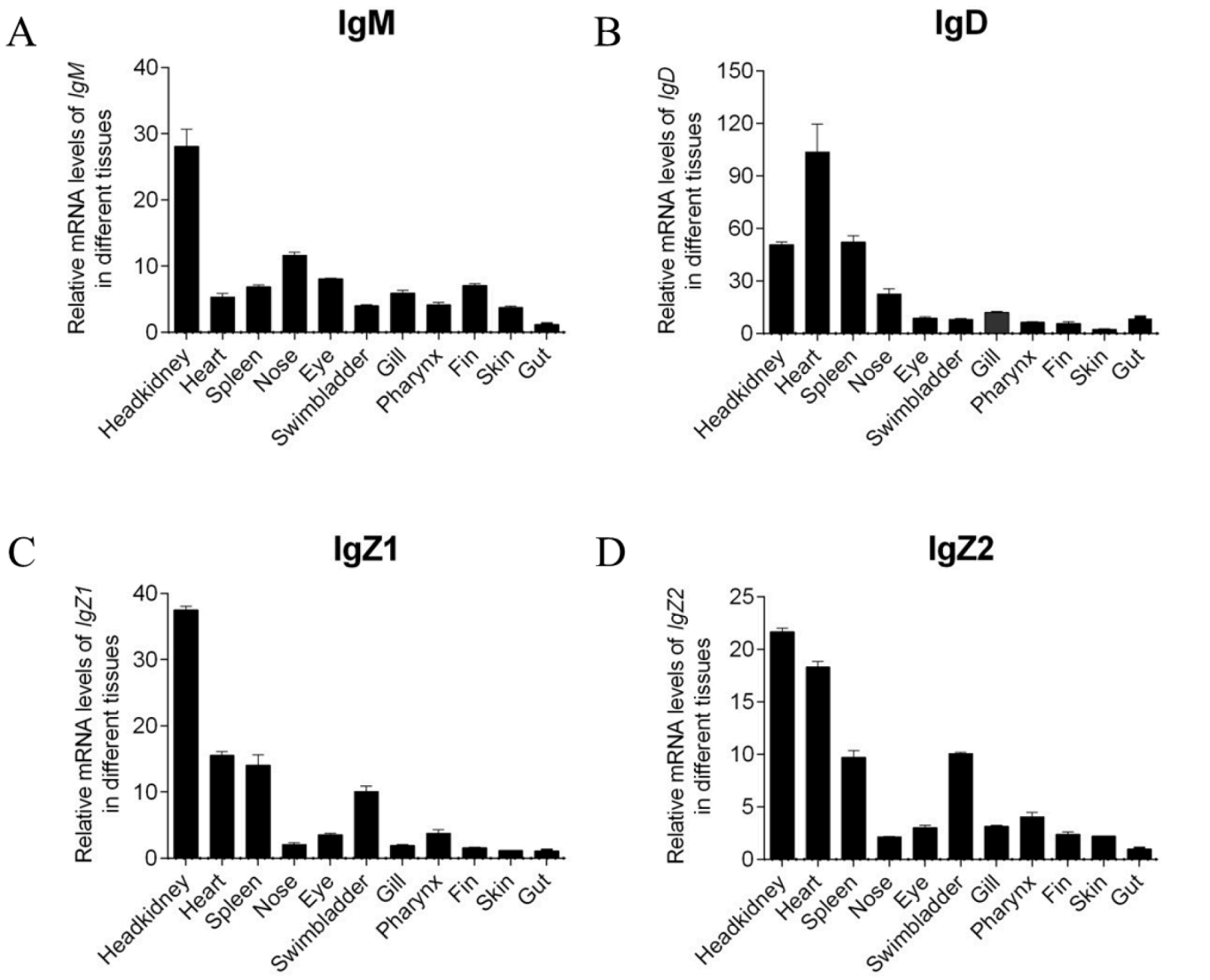

Fig. 4. Basal expression of Igs in different tissues of common carp. Igs mRNA levels were expressed as a ratio relative to 40S levels in the same tissue after qPCR. Relative expression of IgM, IgD, IgZ1 and IgZ2 mRNA were detected in 11 tissues ($n = 9$). The liver was used as calibrator. Data were expressed as the mean ± SEM.

to the results reported by Ryo et al. [23]. It is worth noting that the highest expression of common carp IgD was observed in the heart, followed by head kidney and spleen. The research in *Catla catla* showed that IgD also had a higher expression in gill except the kidney [44]. These results indicated that the IgD showed different tissue distribution patterns from other Igs in common carp, which also implied that the IgD might play different roles in fish immunity. Moreover, IgD has been found in all teleost species examined so far [8,45]. Compared to the limited (i.e., normally two or three) Cd domains in mammals, the number of Cd domains varies widely in different fish species. The distribution of IgD gene transcripts in different tissues was reported in puffer fish, Atlantic cod and rainbow trout [29,46–47]. In puffer fish, IgD gene is mainly expressed in lymphoid tissues, such as peripheral hemolymph, spleen, head kidney and body kidney [46]. In Atlantic cod, IgD producing cells are mainly distributed in hematopoietic organs, such as head kidney and spleen [47], and in rainbow trout, the effector cells secreting IgD mainly exist in head kidney and spleen [29]. In the present study, the mRNA expression level of IgD gene was mainly expressed in head kidney and spleen. These suggest that the head kidney and spleen are the main immune organs of fish. However, recent studies have shown a lack of pathogen-specific IgD titers in trout serum and mucus against parasitic and bacterial infections [47]. Thus far, the immune function of IgD in teleost fish is not very clear [26], although several studies have shown that IgD can be transcriptionally induced during viral, bacterial and parasitic infections [8,48–52].

By far, the research on histological changes of SVCV-infected fish is rare [53,54]. In the present study, we developed an infection model in common carp with SVCV. Upon infection, typical disease symptoms caused by SVCV appeared in infected carp, that is, white gill, bleeding, skin congestion, and fin ray erosion [54]. Moreover, significant morphological changes were detected in external tissues, including lamellae epithelium thickening or mucosal epithelium thinning, which was mainly caused by the loss of epithelial mucous cells while released mucus in response to viral invasion [42]. However, compared to control fish, no pathological changes were detected in the gut, which was consistent with the previous study [55]. We speculated that it may take a long time for the virus to invade the interior of the gut given the abundant mucus coating of the internal tissue, which constitutes a gel-like substance that effectively prevents pathogen entry [33]. The morphological changes caused by SVCV may also lead to changes in the expression of immune genes in the corresponding tissues [42]. Previous studies have shown that viral infection often leads to the activation of innate immune signaling pathways [56]. In our study, a strong immune response was observed both in the internal and external mucosa of

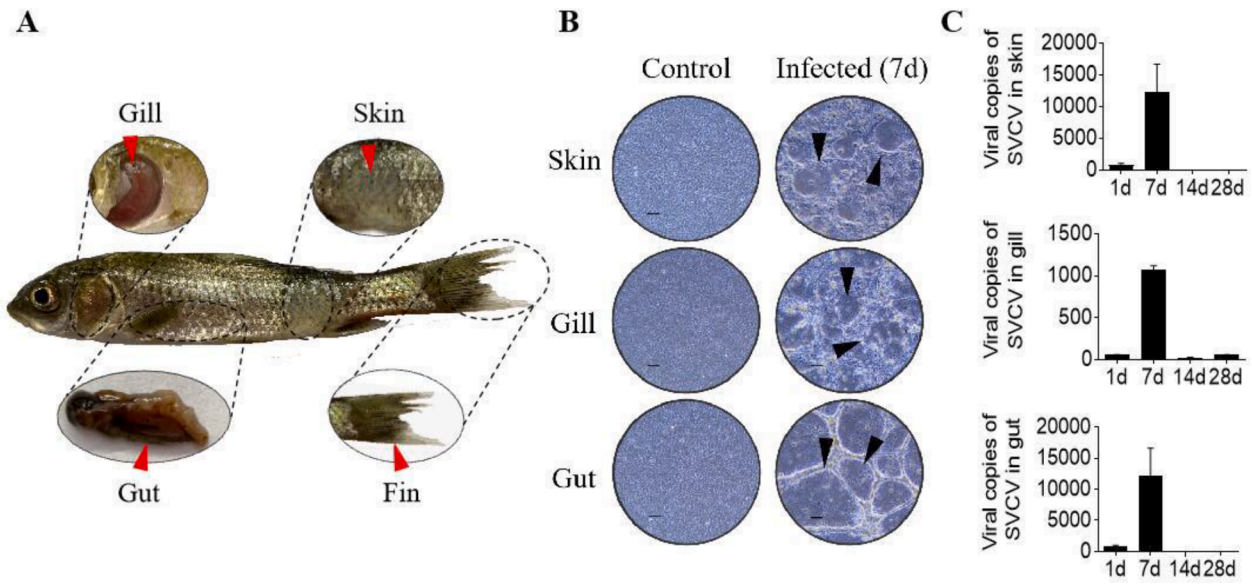

Fig. 5. SVCV infection model of common carp. (A) The phenotype of common carp after SVCV infection. Red arrows indicate the characteristics (gill whitening, scales shedding, intestinal bleeding, and fin erosion) of common carp lesions. (B) The skin, gill and gut tissues of the control and infected carp were re-infection to EPC cells, and the infected group showed significant cytopathic effect (CPE), the black triangles indicate plaque. Scale bar, 100 μm. (C) The load of SVCV in the skin, gill and gut at 1, 7, 14 and 28 dpi ($n = 6$).

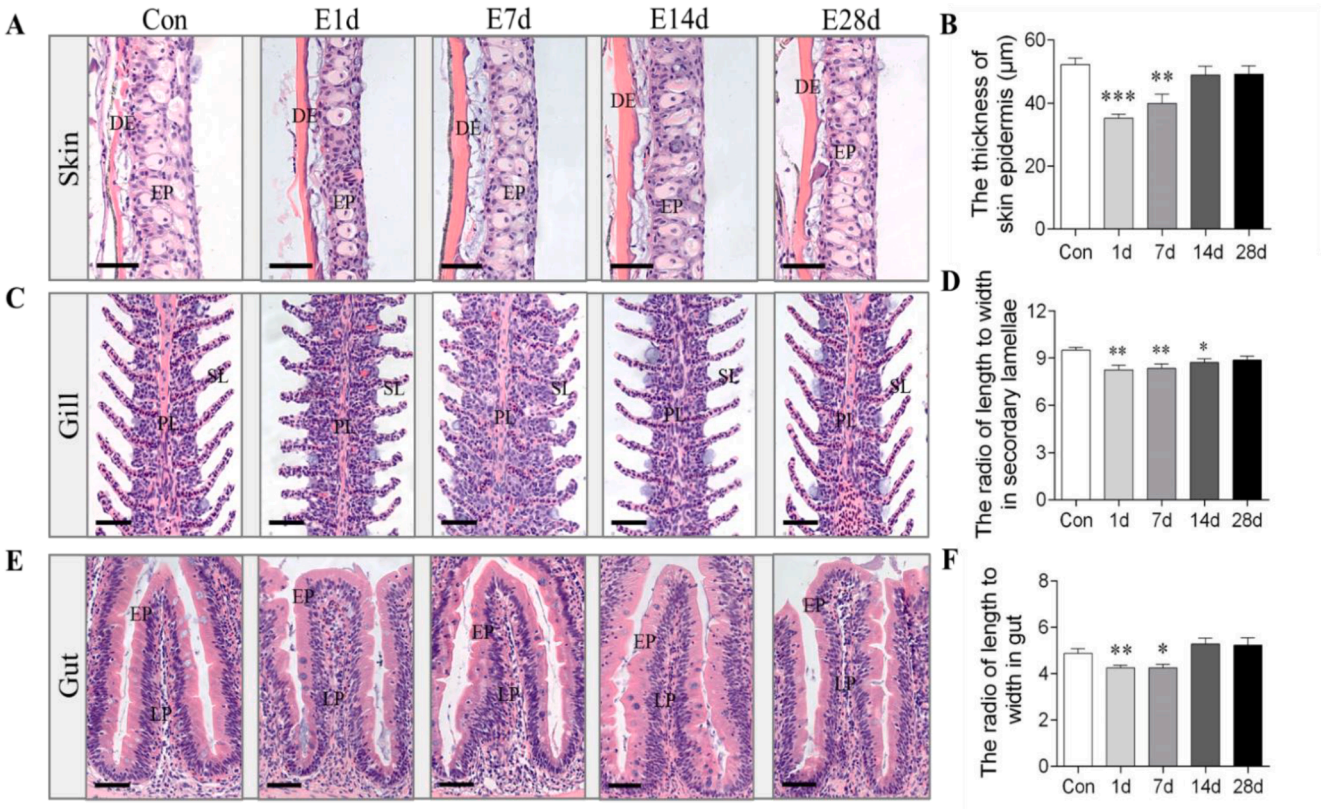

Fig. 6. Morphological changes in skin, gill and gut of common carp. Hematoxylin & eosin staining of common carp skin (A), gill (B) and gut (C) at 1, 7, 14 and 28 dpi, respectively. (D) The thickness of skin epidermis in control and infected fish ($n = 6$ per group). (E) The length ratio of gill SL versus SW of fish in control and infected group ($n = 6$ per group). (F) The length-width ratio of gut LP versus EP of fish in control and infected group ($n = 6$ fish per group). DE, dermis; EP, epidermis; SL, secondary lamellae length; PL, primary lamellae; SW, secondary lamellae width; LP, lamina propria; Scale bars, 20 μm. Control vs. Infected: $^{*}P < 0.05$, $^{***}P < 0.001$, unpaired Student's *t*-test. Data are representative of three different independent experiments (mean± SEM).

infected common carp.

As key components of intercellular signal transmission and regulation, the expression of cytokines increased significantly after viral infection [57,58]. According to previous studies, IFNs play fundamental role in the innate immune response against virus invasion in different vertebrate classes [59]. Our study detected a high expression of

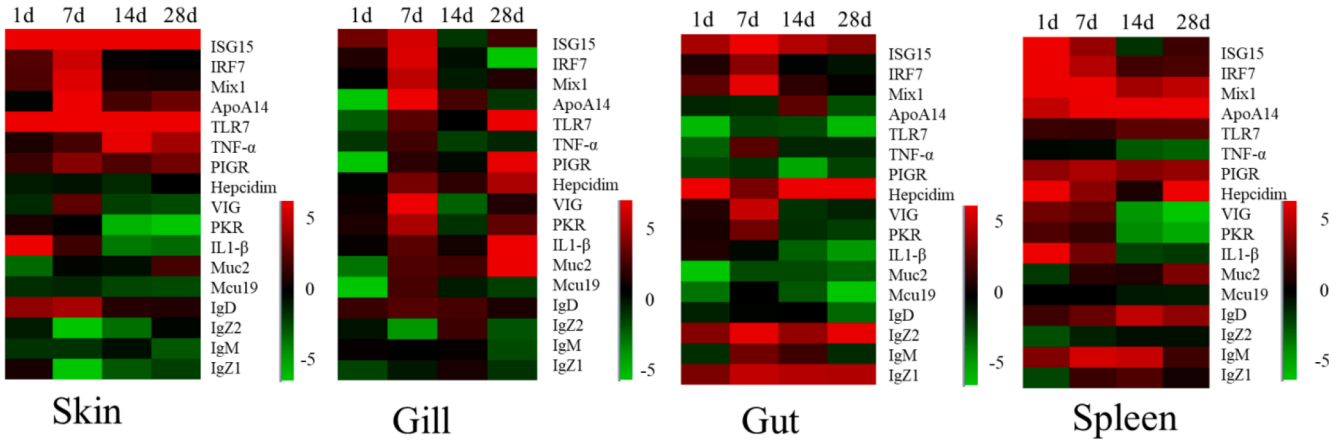

Fig. 7. Kinetics of immune responses in skin, gill, gut and spleen of common carp following SVCV infection. Pheatmap package of R (version 3.4.4) was used to picture heat maps to illustrate the kinetics of immune responses in common carp skin, gill, gut and spleen after infection with SVCV ($n = 6$).

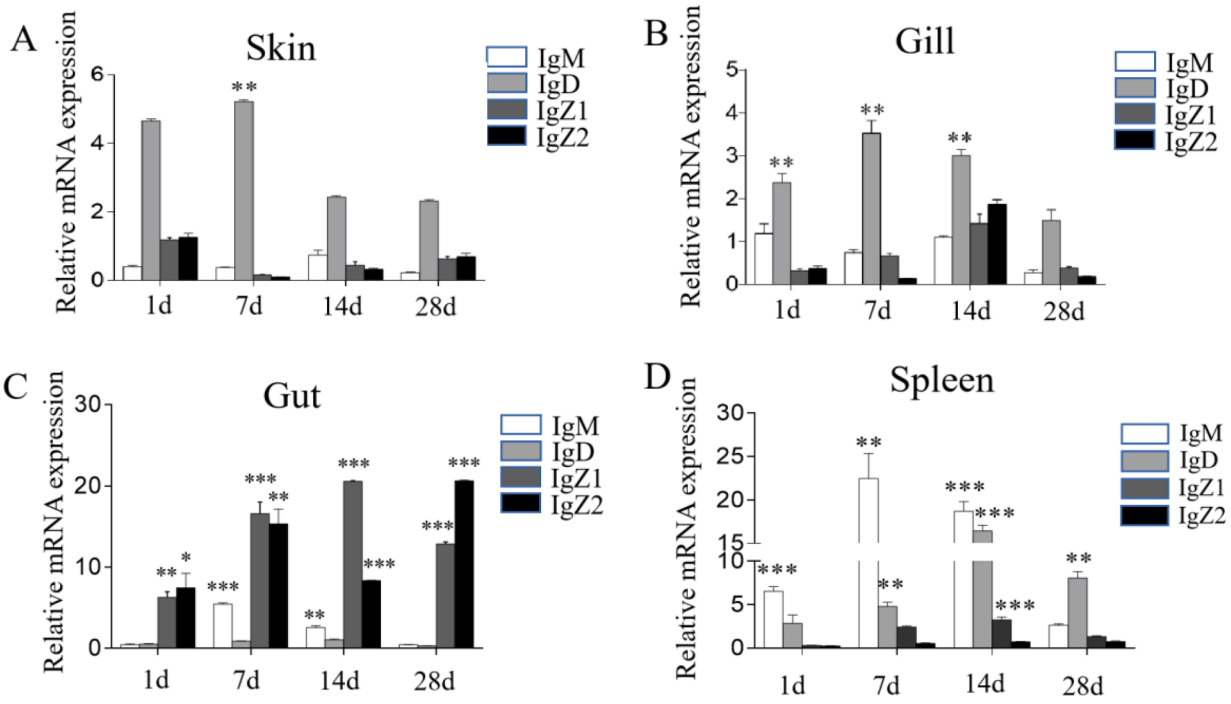

Fig. 8. The expression of Igs in different tissues after infection with SVCV. A-D represents the expression of skin, gill, gut and spleen respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's *t*-test). Data are representative of three different independent experiments (mean ± SEM).

interferon-related genes in mucosal tissues including interferon regulator factors (IRF7). Moreover, innate immune genes such as TNF were also detected in all selected mucosal tissues, further suggesting that innate immunity was involved in the antiviral process [60]. More importantly, the mRNA expression of antiviral genes such as VIG, ISG15, PKR, and Mx1 were up-regulated in infected individuals, suggesting that SVCV activated the antiviral pathway in common carp [57,61,62]. Consistent with previous studies, strong immune responses were detected in the gut after virus infection though no pathological changes were observed in gut [55]. Antimicrobial peptides (AMPs), also known as host defense peptides, are small peptide fragment that have antibacterial activity, which are expressed by the innate immune cells of organism under motivation [63]. Notably, fish skin mucins (MUCs) have been shown to play important roles in environmental stress and immune defense [37,64]. In our study, Muc2 and Muc19 have certain expressions in various tissues after SVCV infection. Hepcidin is a cationic antimicrobial peptide (AMP) rich in cysteine, which plays an important role in the host's innate immune system and iron regulation [65]. Therefore, our data showed that the expression of hepcidin was significantly up-regulated in gut and spleen from 1dpi to 28 dpi, and the similar results were observed in carp after *V. anguillarum* infection, indicating the important role of hepcidin in the whole process of immunity in carp. The higher expression of hepcidin in intestinal tract after infection may be due to the mucosal immune response [65].

In addition, we further analyzed the expression of Igs genes responding to SVCV infection. In this study, no significant expression changes were observed in the skin and gill for all the Igs except IgD, which showed a slight up-regulation in the skin at 14dpi and significant up-regulation in the gill at 1,7, and 14 dpi. IgD was also up-regulated significantly in the spleen after SVCV infection. The expressions of carp IgM were significantly increased in spleen and gut during SVCV infection. Importantly, the expression of IgZ1 and IgZ2 showed similar response to SVCV infection, with significant up-regulation in the gut. These results indicate that IgD functions may not only in systemic tissues

but also in mucosal tissues, while IgM and IgZ1 and/or IgZ2 mainly specialized in systemic and mucosal immune responses, respectively. However, these results were only explored at the transcriptional level, and further studies are needed to clarify the specific roles of these Igs in response of viral infection.

In conclusion, we firstly cloned and characterized IgD heavy chain gene of common carp and detected the mRNA expression levels of four Ig heavy chain genes in different tissues. The present study also constructed infection models of common carp with SVCV. The significant morphological changes were observed in skin and gills. Importantly, after SVCV infection, the mRNA expression of IgM was significantly up-regulated only in spleen, while significantly increased IgZ1/IgZ2 mRNA expression was observed in gut. Critically, the mRNA expression levels of IgD were up-regulated in both mucosal and systemic tissues upon viral infection, suggesting the potential role of IgD in mucosal and systemic immunity.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2021.100048.

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