Fish and Shellfish Immunology 89 (2019) 337–344

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transcriptome analysis of rare minnow (*Gobiocypris rarus*) infected by the grass carp reovirus

Yusheng Lin[a,b], Bing Wang[a,b], Nenghan Wang[a,b], Gang Ouyang[a], Hong Cao[a,*]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China

ARTICLE INFO

Keywords:
Transcriptome
Rare minnow
GCRV
Immune response
qRT-PCR

ABSTRACT

Grass carp shares the largest portion of the aquaculture production in China, but hemorrhagic disease caused by grass carp reovirus (GCRV) often results in tremendous loss of fingerlings and yearlings, causing significant economic damages. However, it is difficult to study antiviral mechanisms in grass carp *in vivo* due to its large size and long reproductive cycle. Therefore, a small cyprinid species named rare minnow with high sensitivity to GCRV, is regarded as a useful model to study the mechanisms of this disease. In this study, rare minnows were infected with the type-IIGCRV (GCRV-HZ08), and pathogenesis was investigated by BGISEQ-500 transcriptome sequencing of four cDNA libraries, hepatopancreas, gills, head-kidney and spleen, and real time quantitative PCR (qRT-PCR). We obtained 51.22 Gb bases in total, and de novo assembled 107,756 unigenes with an average length of 1,441 bp. GO analysis revealed that the differentially expressed genes (DEGs) involved in the defense mechanisms were the most enriched GO terms in all four tissues. KEGG analysis revealed that the most enriched pathways were “Influenza A”, “Herpes simplex infection”, “Transcriptional misregulation in cancer” and “Metabolic” pathways. We also performed a comparative transcriptomic study between GCRV-infected rare minnow and grass carp data. This revealed that “IL-17 signaling pathway”, “NF-kappa B signaling pathway” and “Influenza A” pathways are conserved (important) in the regulation of anti-GCRV infection in both species, and need to be further investigated. Furthermore, a total of four immune-related DEGs were selected for qRT-PCR validation, and the results confirmed the RNA-seq data. These results enhance our understanding of the antiviral responses of cyprinid fish infected by GCRV.

1. Introduction

Grass carp (*Ctenopharyngodon idella*; Cyprinidae) is an economically important species that accounts for more than 20% of the total freshwater fish production in China [1]. However, the aquaculture industry of grass carp is often hampered by the grass carp hemorrhagic disease, caused by the grass carp reovirus (GCRV), a double-stranded RNA (dsRNA) virus belonging to the genus *Aquareovirus* of the family Reoviridae [2–4]. So far, many measures have been taken to attempt to control this disease but to little effect [4]. This is mainly a consequence of the fact that mechanisms underlying the GCRV infection remain largely unknown. Until recently, most studies on this disease were focused on the mechanisms of viral infection [5,6] and functions of several immune response-associated genes, especially pattern recognition receptors [7–10]. However, the large size and long reproductive cycle of grass carp make it difficult to study the antiviral mechanisms of hemorrhagic disease *in vivo*.

Therefore, it is necessary to find a suitable model to better understand the immune defense mechanisms involved in the response to reovirus infection. It is noteworthy that GCRV also infects rare minnow (*Gobiocypris rarus*), black carp (*Mylopharyngodon piceus*), and topmouth gudgeon (*Pseudorasbora parva*), also causing hemorrhagic symptoms and death [11]. Among them, the rare minnow, a cyprinid species with a relatively small size (5–6 cm) and short reproductive cycle (3–4 months), is regarded as a potentially valuable model to elucidate the molecular pathways of the resistance to GCRV.

Gills and hepatopancreas are the organs particularly susceptible to GCRV; for example, gills often bleed during the GCRV infection [12]. In addition, spleen and head-kidney are important immune system organs in fish: the head-kidney is involved in adaptive immunity and antibody production, while the spleen plays essential roles both in innate and adaptive immunity [13–15]. Therefore, in this study, RNA-seq

* Corresponding author. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences Wuhan, Donghu South Road 7#, China.
E-mail address: regancao@ihb.ac.cn (H. Cao).

https://doi.org/10.1016/j.fsi.2019.04.013
Received 20 October 2018; Received in revised form 31 March 2019; Accepted 5 April 2019
Available online 08 April 2019

sequencing was performed on these four tissues in rare minnow before GCRV infection and 5 days post the viral-challenge. We used the BGISEQ500 platform, which can yield highest quality reads with lowest error rate. The results revealed that there were significant changes of gene expression in these four tissues. Furthermore, the expression levels of four immune response-related genes were confirmed by qRT-PCR.

2. Materials and methods

2.1. Virus

The GCRV strain used in the study was kindly provided by Prof. Yaping Wang (Institute of Hydrobiology, Chinese Academy of Sciences). It was isolated in July 2015 in Huanggan city, Hubei Province, and classified as a type II GCRV due to the high similarity (98.4%) of the S2 segment to the typical type II strain, GCRV-HZ08. The virus was diluted to the titer of 2.97×10^3 RNA copy/μl for use in the experiments.

2.2. Experimental fish

Healthy full-sib adult rare minnow at 3 months of age, weighing 1–1.5 g and with an average length of 5 cm, were used in the study. The fish were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences and maintained in aerated freshwater at 26–28 °C. Experiments involving rare minnow in this study were approved by the Animal Research and Ethics Committee of the Institute of Hydrobiology, Chinese Academy of Sciences, and all experiments were conducted in accordance with the guidelines of the committee.

2.3. Viral challenge experiment, sample collection and histological observation

The adult rare minnow (three-months old) were randomly divided into two groups (approximately 50 per group). Each fish in the group I was infected with 50 μl GCRV-HZ08 (2.97×10^3 RNA copy/μl) by intraperitoneal injection, while fish from the group II were injected with 50 μl PBS as control. At 120 h (hours) post-injection, samples of gill, spleen, hepatopancreas and head-kidney tissues were harvested from both groups (n = 4 specimens from each group as biological replicates). The muscle samples of both GCRV-treated and control groups (n = 4, respectively) were fixed in the formalin, sectioned, stained with hematoxylin and eosin, and analyzed by two pathologists independently using light microscopy.

2.4. RNA extraction and construction of RNA-seq libraries

After grinding each sample in liquid nitrogen, 1 ml of Trizol reagent was added per 100 mg of tissue. Total RNA was purified by beads containing oligo (dT). After addition of fragmentation buffer to generate short mRNA fragments (each of approximately 200 bp), random hexamer-primers were applied to synthesize the first-strand cDNA. Buffer, dNTPs, RNase H and DNA polymerase I were added to synthesize the second strand. Short double-stranded cDNA fragments were purified with a QIAquick PCR extraction kit (Qiagen, Valencia, CA, 28104) and eluted with EB buffer for end repair and single nucleotide adenine addition. Next, short fragments were ligated to Illumina sequencing adaptors. DNA fragments of a selected size were gel-purified and amplified by PCR. The amplified library was sequenced on a BGISEQ500 platform sequencing machine (Qiagen, Valencia, CA). The details of the experiment were as follows: Expected library size: 200 bp; Read length: 90 nt; and Sequencing strategy: paired-end sequencing.

2.5. Sequencing reads filtering and de novo assembly

Low-quality, adaptor-polluted and high content of unknown bases (N)-containing sequencing reads were filtered (the adaptor sequences; unknown bases more than 10%; the percentage of no more than Q 5 bases is over 50% in a read). The Q20 and Q30 of the clean data were calculated, and all downstream analyses were performed using the clean, high-quality data. Trinity (http://trinityrnaseq.sourceforge.net/, version: v2.0.6) [16] was used to perform de novo assembly with clean reads, and Tgicl (http://trinityrnaseq.sourceforge.net/, version: v2.0.6) [17] then used to cluster transcripts to Unigenes.

Expression of genes was quantified using the DESeq package, and DEGs between the two groups (GCRV-infected and control) were identified using the following criteria: fold change ≥ 2, and adjusted p value ≤ 0.05 [18]. The false discovery rate test (adjusted p value) was applied to correct significant levels by eliminating random errors and fluctuations [19].

2.6. GO and KEGG enrichment analyses

After assembly, Unigenes were used for functional annotation against the NR, NT, KOG, SwissProt and InterPro databases. Unigenes aligned to the NR database were annotated in the GO database (GO, http://www.geneontology.org) with Blast2GO software [20]. GO terms with corrected p values less than 0.01 were considered significantly enriched by DEGs.

The Kyoto Encyclopedia of Gene and Genomes (KEGG, http://www.genome.jp/kegg/) database is used for understanding high-level functional information in biological systems, from molecules, cells, organisms and ecosystems, and is particularly powerful for large-scale molecular datasets generated by genome sequencing and other high-throughput experimental approaches [21]. In this study, KOBAS software [22] was employed to test the statistical enrichment of DEGs and NDGs. KEGG terms with corrected p values smaller than 0.01 were considered significant.

2.7. Comparative analysis of rare minnow and grass carp transcriptome data

A comparative data analysis was performed between our rare minnow transcriptome data and a previously published grass carp transcriptome data [12]. Grass carp genome [23] were used as a reference for data mapping of both transcriptome datasets, conducted using Hisat [24]. Read counts was obtained using Htseq [25] For the detection of DEGs, we used EdgeR package [26] with FDR < 0.01 and $\log_2$(TPM fold) > 1 parameter. Blast [27] was used to compare the DEGs between the two species, with the p value < 1E-5. Subsequent GO and KEGG pathway analysis followed the previous description.

2.8. Validation of DEGs by qRT-PCR

To validate the reliability of data obtained by RNA-seq, qRT-PCR was performed on an Applied Biosystems StepOne™ Real-Time PCR System (AB, Foster City, CA). Four DEGs which were significantly differentially expressed at least at two time points in each tissue were selected for the qRT-PCR validation. Total RNA from gill, hepatopancreas, spleen and head-kidney tissues of each individual fish was isolated by means of an SV total RNA isolation system kit (Promega, Madison, WI, Z3100). Then, 4 μg of isolated total RNA was transcribed to cDNA through the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA K1662) with oligo-dT primers. Then, qRT-PCR was performed using the Fast start universal SYBR Green Master Mix (Roche, Penzber, Germany, 11750800). Primers for PCR were designed via an online tool IDT Real Time PCR (http://www.idtdna.com/scitools/Applications/RealTimePCR/). Primer sequences are listed in Supplementary Table 1. Three replicates were included for each sample and *β-actin* gene was used as an internal control for normalization of gene expression. The length of PCR products was about 100–200 bp and Tm ~ 60 °C. Relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$

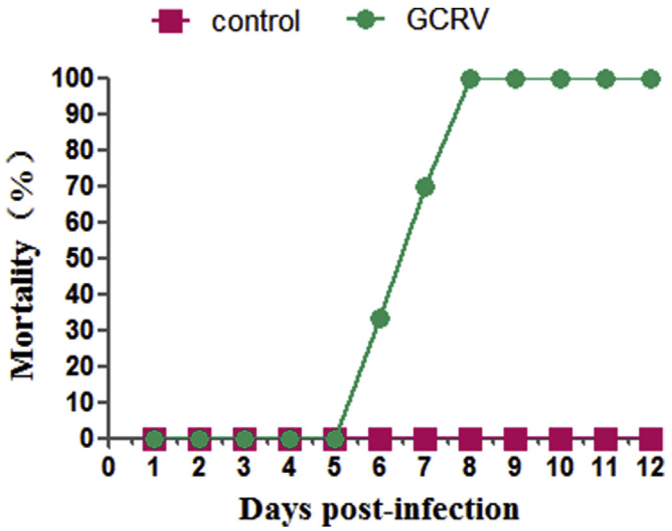

Fig. 1. Cumulative mortality of fish infected with GCRV-II (GCRV-HZ08). The number of dead fish in the GCRV-infected group was counted daily. PBS was the negative control.

method [28].

2.9. Statistical analysis

qRT-PCR data were reported as mean ± SEM of three independent experiments. Statistical analysis (unpaired *t*-test) was performed using GraphPad Prism 5 (GraphPad Software Inc.).

Table 1
Clean reads quality metrics in this study.

Sample	Raw Reads (Mb)	Clean Reads (Mb)	Clean Bases (Gb)	Q20(%)	Q30(%)	Clean Reads Ratio(%)
GCRV-gill	63.61	60.76	6.08	97.97	90.51	95.51
GCRV-kidney	69.68	66.87	6.69	97.95	90.43	95.97
GCRV-liver	69.67	66.1	6.61	97.77	89.84	94.88
GCRV-spleen	69.67	66.23	6.62	97.73	89.75	95.06
Control-gill	69.68	66.03	6.6	97.84	90.02	94.76
Control-kidney	69.68	65.5	6.55	97.47	88.79	94
Control-liver	69.68	65.65	6.56	97.61	89.31	94.22
Control-spleen	58.17	55.11	5.51	97.72	89.62	94.74

3. Results

3.1. Mortality of rare minnow infected with GCRV

The mortality curves of the rare minnow are shown in Fig. 1. In the GCRV-II-infected group, the first death was recorded as early as 5 days post-infection, and the total mortality of 100% was reached at 8 days post-infection. However, in the control group, mortality remained 0%. In addition, fish that died after infection with GCRV-II showed hemorrhagic symptoms, especially in muscles, whereas no hemorrhagic symptoms were observed in the Control group fish (Fig. 2A).

As shown in Fig. 2B, the histological examination showed that the control group had normal muscle tissue appearance: the skeletal muscle fibers are in a long strip shape, with different diameters and lengths, but arranged neatly; muscle fibers contain multiple nuclei; the nuclei are oblate, distributed around the muscle fibers, close to the sarcolemma; and very few blood vessels exist between muscle fibers. In the GCRV-infected muscle samples, the skeletal muscle fibers are disorderly and irregularly arranged; the fibers are distorted and dissolved to varying

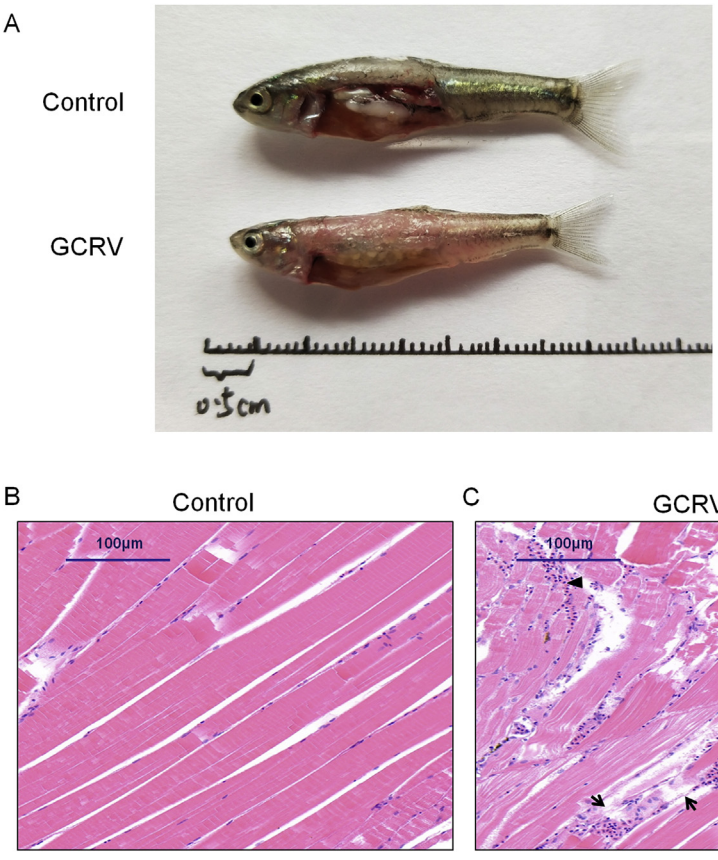

Fig. 2. Hemorrhagic symptoms induced by GCRV-HZ08. (A) Images show representative fish specimens in the Control group and in the GCRV-HZ08 group, the latter exhibiting typical muscular hemorrhagic symptoms. (B) Appearance of the normal skeletal muscle in the control group (H&E staining). (C) Appearance of the skeletal muscle in the GCRV-infected group, exhibiting accumulation and deposition of erythrocytes (arrowhead) and ruptured blood vessels (arrow).

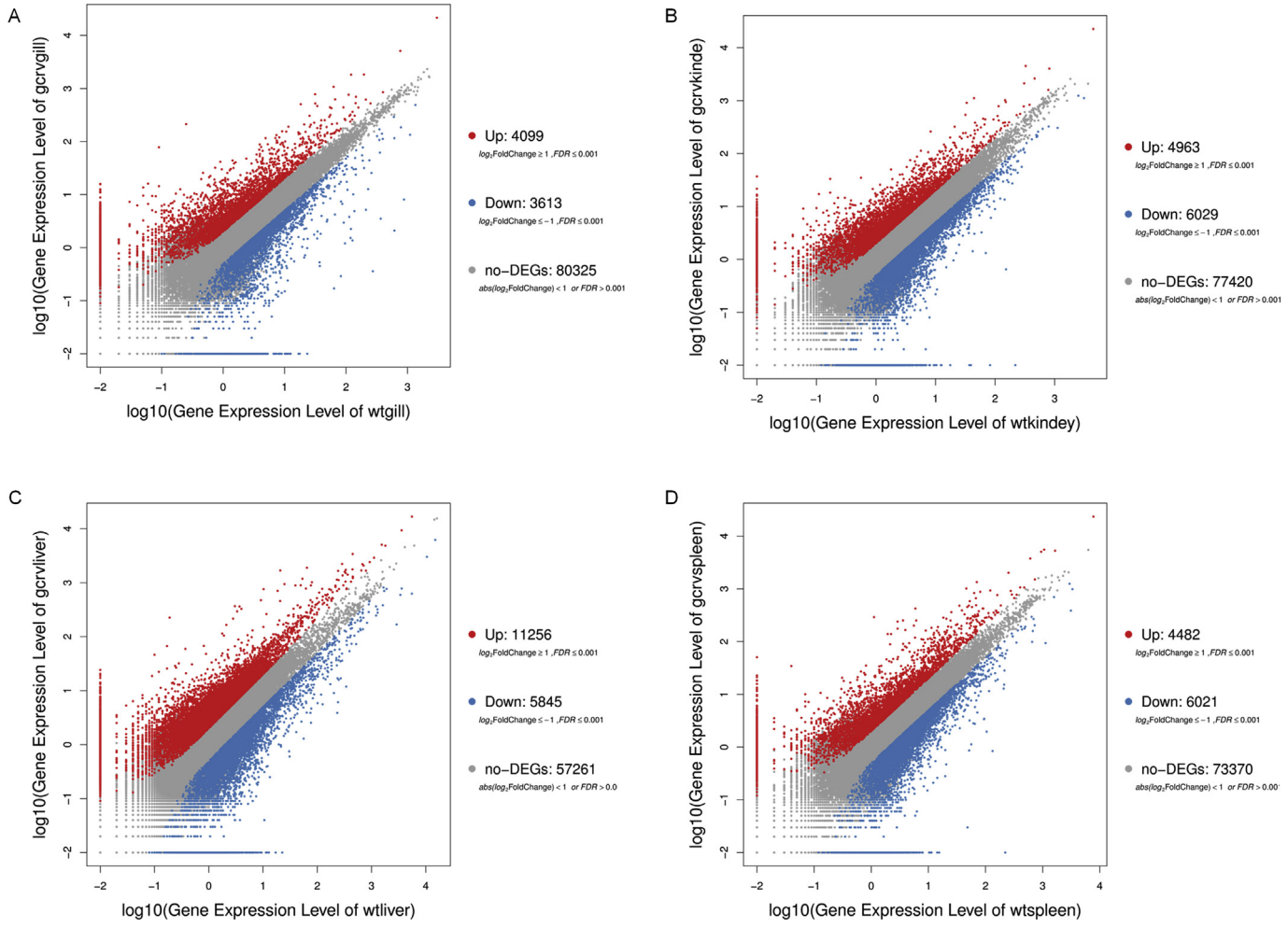

Fig. 3. Scatter plot of DEGs. Image shows the comparative transcriptome profiles of gill (A), kidney (B), hepatopancreas (C) and spleen (D) samples of GCRV-HZ08 vs. control groups. log10-transformed gene expression levels of the two groups are plotted on the two axes: X - control and Y - GCRV-HZ08. Up-regulated genes are shown as red dots, while down-regulated genes are shown as blue dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Top five enriched GO terms of the DEGs in each group.

Groups (Control vs. GCRV-HZ08)	GO term	Number of genes
Gill	Binding	918
	Cellular Process	802
	Cell	699
	Cell part	693
	Catalytic activity	602
Kidney	Binding	1427
	Cellular Process	1321
	Cell	1206
	Cell part	1196
	Catalytic activity	945
Liver	Binding	2406
	Cellular Process	2341
	Cell	2196
	Cell part	2182
	Catalytic activity	1699
Spleen	Binding	1310
	Cellular Process	1131
	Cell	1000
	Cell part	992
	Catalytic activity	831

degrees; the vasodilation between the muscle fibers contains a marked increase in the accumulation and deposition of erythrocytes; some blood vessels exhibit varying degrees of rupture (Fig. 2C).

3.2. Preliminary statistical analysis of RNA-seq data

The expression profiles of gill, spleen, hepatopancreas and head-kidney tissues from rare minnow were obtained at 120 h after the GCRV challenge/PBS injection. Analysis of these 8 RNA-seq datasets generated about 51.22 Gb of bases in total. After filtering of the raw data, we obtained an average of 64.03 M clean reads per RNA-seq that aligned uniquely to the reference genes (Table 1). Furthermore, as shown in Table 1, the raw reads, clean reads, clean bases, clean reads Q20, Q30, and clean reads ratio for each library were recorded. All libraries gave Q20 ratio ≥97%, Q30 ratio ≥88%, and clean reads ratio ≥94%. These results confirmed the high quality of the sequencing data and suitability for further analyses.

3.3. Selection and initial identification of significant DEGs

DEGs among these samples were identified by subjecting the data to a series of paired-comparisons. The expression levels of genes in gill, spleen, hepatopancreas and head-kidney tissues after virus challenge were compared with the control group. The numbers of DEGs identified from the different paired-comparisons are provided in Fig. 3.

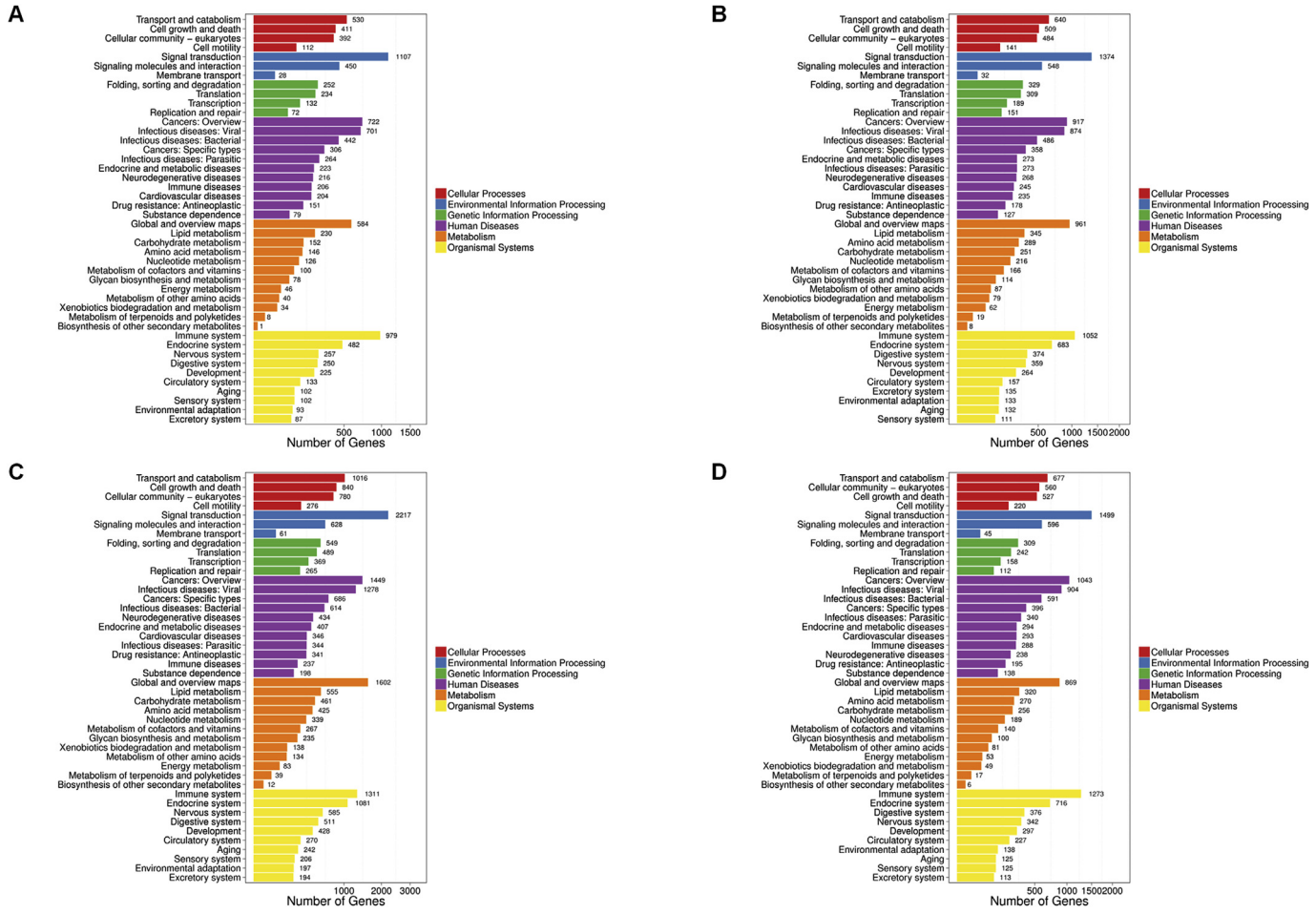

Fig. 4. KEGG pathway classification of DEGs. The functional pathway enrichment of DEGs in gill (A), kidney (B), hepatopancreas (C) and spleen (D) samples of GCRV-HZ08 vs. control groups. The number of DEGs is on the X axis, and functional KEGG classification on the Y axis.

Comparisons with the control group revealed 4099 up-regulated and 3613 down-regulated genes 5 days post-infection in gills, whereas 4963, 11256 and 4482 genes were up-regulated and 6029, 5845 and 6021 genes were down-regulated 5 days post-infection in kidney, hepatopancreas and spleen, respectively.

3.4. GO and KEGG enrichment analysis of the DEGs

GO enrichment analysis was performed to investigate the putative roles of these DEGs. DEGs were categorized into biological process, cellular component and molecular function categories. Some GO terms related to defense mechanisms, such as immune system process and response to stimulus, were significantly enriched, indicating that rare minnow responds strongly to the GCRV infection. The top five enriched GO terms in all groups are listed in Table 2 and details of the GO terms are shown in Supplementary Fig. 1.

In order to identify putative functional biochemical pathways for predicted proteins encoded by DEGs, KEGG pathway enrichment analysis was performed for all the groups and tissues (Fig. 4). KEGG pathway enrichment analysis of DEGs (corrected $p < 0.05$) showed a significantly enrichment of “Influenza A”, “Herpes simplex infection”, “Transcriptional misregulation in cancer” and “Metabolic” pathways in DEGs. In addition, “Herpes simplex infection” was the most enriched pathway in head-kidney, gill and spleen, while “Influenza A” ranked in the second place in gills and spleen. Moreover, the pathway of “Transcriptional misregulation in cancer” was also highly enriched in spleen. Interestingly, the most enriched pathway was “Metabolic” pathway in hepatopancreas, which suggested different defense mechanisms are active in the hepatopancreas, compared with other immune organs.

3.5. Comparative transcriptomic analysis

We performed a comparative study between the transcriptomes of GCRV-infected rare minnow and grass carp. Five days post-infection, in the hepatopancreas, spleen and gill tissues there were 429, 480 and 155 up-regulated, and 218, 494 and 134 down-regulated genes respectively.

GO enrichment analysis revealed that these genes were mainly categorized into “response to external biotic stimulus” and “immune system process” in the gill and hepatopancreas, and “negative regulation of blood coagulation” and “negative regulation of hemostasis” in the spleen (Supplementary Fig. 2). The KEGG enrichment analysis of these genes (corrected $p < 0.05$) showed a significant enrichment of “IL-17 signaling pathway”, “NF-kappa B signaling pathway” and “Influenza A” pathways (Supplementary Fig. 3). This indicates that these genes were conserved in the regulation of anti-GCRV infection in both species, and need to be further investigated.

3.6. Confirmation of DEGs by qRT-PCR

To confirm the RNA-seq data, a total of 4 DEGs which were involved in “immune response” were selected for qRT-PCR validation. These genes included interferon alpha-inducible protein 27-like protein 2A (*Ifi27l2a*), signal transducer and activator of transcription 1a (*STAT1a*), signal transducer and activator of transcription 1b (*STAT1b*), immunoglobulin mu heavy chain (*IgHm*). In general, the expression patterns of all 4 DEGs identified by qRT-PCR were similar to those

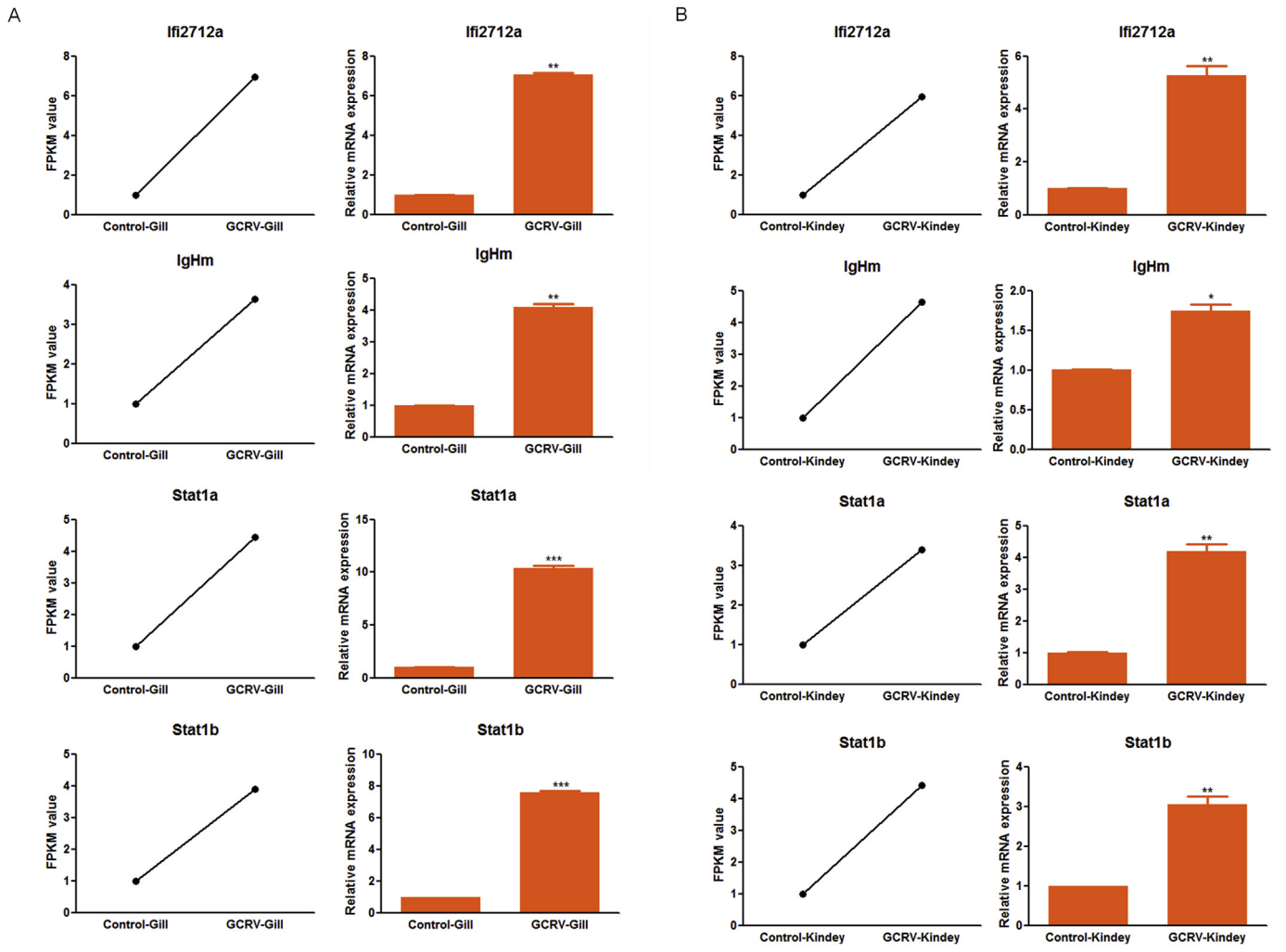

Fig. 5. Transcriptome and quantitative real-time (qRT-PCR) analyses of the expression of four immune response-associated DEGs. For the transcriptome analysis, mRNA expression was measured as fragments per kilobase per million mapped reads (FPKM) and the significance was calculated with a false discovery rate (FDR) < 0.01 (**). The significance of differences between control and GCRV-HZ08 groups was analyzed by one-way analysis of variance (**$p < 0.01$). Vertical bars represent means ± standard deviation (n = 3). HP: hepatopancreas.

obtained in RNA-seq analyses, although the relative expression levels were not completely consistent (Fig. 5). Our results confirmed the reliability and accuracy of the RNA-seq data.

4. Discussion

Globally, aquaculture has been growing rapidly during the last three decades, but its development has been hampered by a diverse range of pathogenic viruses [29]. GCRV can infect most of the organs and cause hemorrhagic disease in many fish species, including grass carp, black carp, rare minnow, topmouth gudgeon, etc. In this study, we used RNA-seq to elucidate the mechanism of hemorrhage symptoms and different immune responses induced in rare minnow infected with the type-II GCRV (GCRV-HZ08). Rare minnow infected with GCRV-HZ08 began to die at 5 days post-infection, reaching a total mortality of 100% at the day 8.

Five days post-GCRV infection, when hemorrhagic symptoms appeared in rare minnow, we collected gills, hepatopancreas, spleen and head-kidney tissues, and analyzed their transcriptome profiles. The KEGG analyses showed that the most enriched pathways in head-kidney, gill and spleen were the “influenza A induced” pathway and the “Herpes simplex infection” pathway. The herpes simplex virus (HSV) is a common human virus, which can persist latently for life in the neurons of infected individuals. Periodically, HSV can restart a lytic-replication cycle, entering, thus, a reactivation process that results in recurrent infection, viral shedding, and transmission to new hosts [30,31]. Influenza A viruses are the causative agents for influenza, and cause respiratory infections that range from asymptomatic to deadly in humans [32]. The host possesses intrinsic antiviral immune system that binds viral components and inhibits viral replication [33]. Both in mammals and in fish, pattern recognition receptors (PRRs) directly sense the presence of pathogen components, so called pathogen-associated molecular patterns (PAMPs) [34,35].

Some previous studies investigated the antiviral mechanisms in rare minnow. For example, Su et [36,37] reported that Toll-like receptors (TLRs), a subfamily of PRRs, regulated Mx expression, and played a crucial role in the anti-GCRV immunity in rare minnow by reducing the mortality and virus yield. Our results, which show that the “Influenza A pathway” plays a critical role in the defense mechanisms against GCRV in rare minnow, are in agreement with results reported in grass carp, where this same pathway was also the most affected pathway after a GCRV infection [38]. Furthermore, we also performed a comparative transcriptomic study between GCRV-infected rare minnow and grass carp data. This revealed that genes enriched in the “Influenza A” pathway are conserved (important) in the regulation of anti-GCRV infection in both species, and need to be further investigated. Therefore,

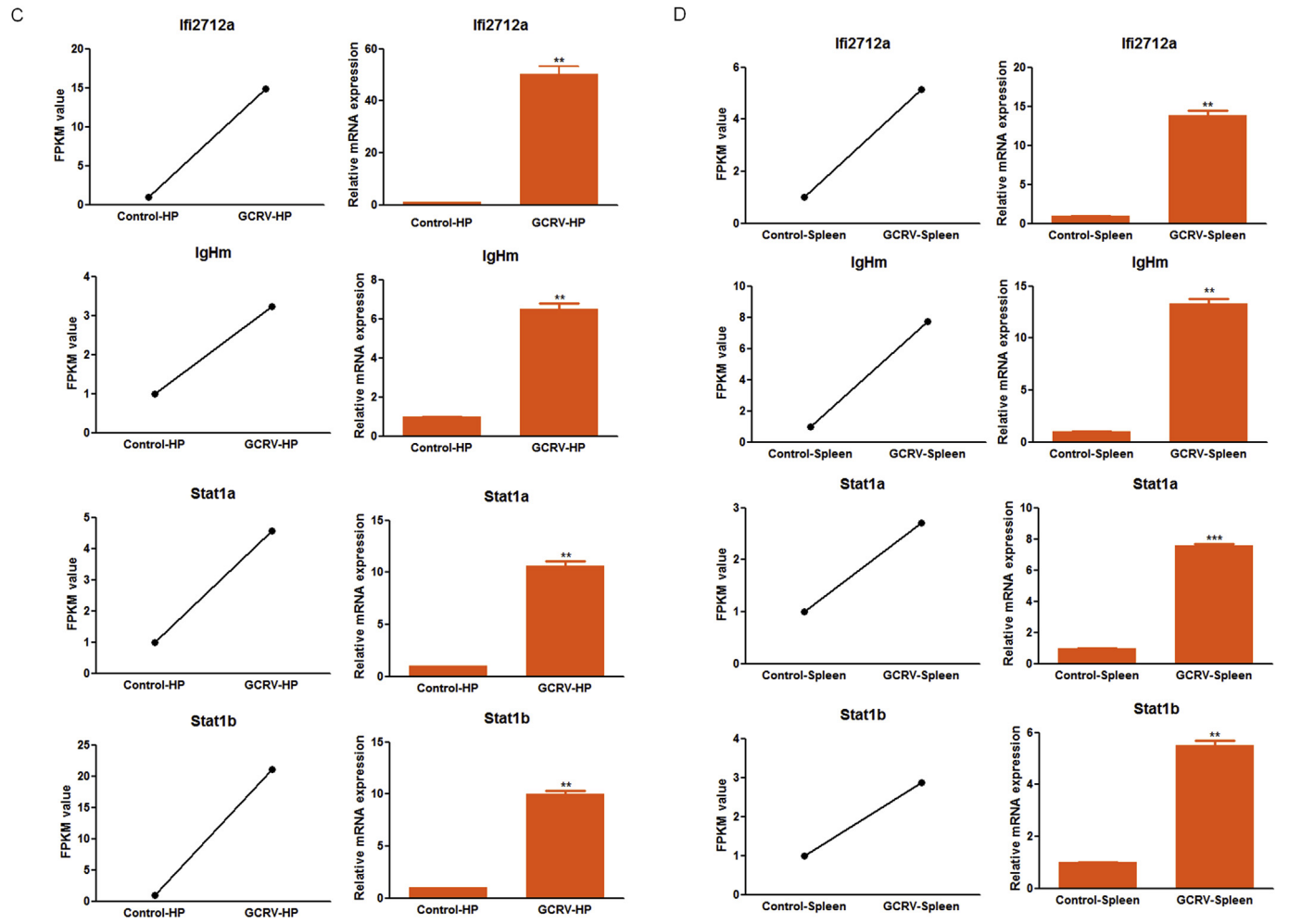

Fig. 5. (*continued*)

this indicates that rare minnow is a suitable model to study the host's defense mechanisms in response to GCRV *in vivo*, not only due to the similarity of immune responses between these two species, but also due to its high susceptibility to GCRV infection [39]. This needs to be further corroborated by further, and more detailed, studies of the antiviral mechanisms in rare minnow.

In conclusion, our study provides a comprehensive understanding of the differences in the pathogenesis and host's immune response to GCRV-HZ08, and the mechanism of hemorrhagic symptoms caused by this virus in rare minnow. Our data will be helpful for future studies aiming to obtain deeper insights into the fish innate immune system and to develop novel vaccines and formulate strategies for disease-resistant breeding in aquaculture.

Acknowledgements

The authors thank Dr. Yaping Wang and Dr. Mijuan Shi (Institute of Hydrobiology, Chinese Academy of Sciences) for assistance with the project design and many advices for manuscript preparation. This work was supported by the State Key Laboratory of Freshwater Ecology and Biotechnology (2018FB02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.013.

References

[1] China Fishery Statistical Yearbook 2016, China Agriculture Press, ISBN 978-7-109-21691-4.
[2] Q. Wang, W. Zeng, C. Liu, C. Zhang, Y. Wang, C. Shi, S. Wu, Complete genome sequence of a reovirus isolated from grass carp, indicating different genotypes of GCRV in China, J. Virol. 86 (22) (2012) 12466.
[3] L. Cheng, Q. Fang, S. Shah, I.C. Atanasov, Z.H. Zhou, Subnanometer-resolution structures of the grass carp reovirus core and virion, J. Mol. Biol. 382 (1) (2008) 213e222.
[4] Y. Rao, J. Su, Insights into the antiviral immunity against grass carp (*Ctenopharyngodon idella*) reovirus (GCRV) in grass carp, J. Immunol. Res. (2015) 670437.
[5] F. Zhang, S. Yan, H. Guo, Q. Chen, Q. Fang, Characterization of viral entry and infection of quantum dot-labeled grass carp reovirus, Virol. Sin. 32 (2) (2017) 163–166.
[6] L. Lu, S. Li, Z. Wang, S. Du, D. Chen, P. Nie, Y. Zhang, Grass carp reovirus VP41 targets fish MITA to abrogate the interferon response, J. Virol. 91 (14) (2017) e00390-17.
[7] L. Chen, J. Su, C. Yang, L. Peng, Q. Wan, L. Wang, Functional characterizations of RIG-I to GCRV and viral/bacterial PAMPs in grass carp *Ctenopharyngodon idella*, PLoS One 7 (7) (2012) e42182.
[8] J. Su, J. Heng, T. Huang, L. Peng, C. Yang, Q. Li, Identification, mRNA expression and genomic structure of TLR22 and its association with GCRV susceptibility/resistance in grass carp (*Ctenopharyngodon idella*), Dev. Comp. Immunol. 36 (2) (2012) 450–462.
[9] C. Yang, J. Su, Q. Li, R. Zhang, Y. Rao, Identification and expression profiles of ADAR1 gene, responsible for RNA editing, in responses to dsRNA and GCRV challenge in grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 33 (4) (2012) 1042–1049.
[10] C. Yang, J. Su, R. Zhang, L. Peng, Q. Li, Identification and expression profiles of grass carp *Ctenopharyngodon idella* tlr7 in responses to double-stranded RNA and virus infection, J. Fish Biol. 80 (7) (2012) 2605–2622.
[11] L. He, A. Zhang, Y. Pei, P. Chu, Y. Li, R. Huang, L. Liao, Z. Zhu, Y. Wang, Differences in responses of grass carp to different types of grass carp reovirus (GCRV) and the mechanism of hemorrhage revealed by transcriptome sequencing, BMC Genomics

18 (2017) 452.
[12] M. Shi, R. Huang, F. Du, Y. Pei, L. Liao, Z. Zhu, Y. Wang, RNA-seq profiles from grass carp tissues after reovirus (GCRV) infection based on singular and modular enrichment analyses, Mol. Immunol. 61 (2014) 44–53.
[13] R. Fänge, S. Nilsson, The fish spleen: structure and function, Experientia 41 (2) (1985) 152–158.
[14] C. Press, Ø. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (4) (1999) 309–318.
[15] P. Rauta, B. Nayak, S. Das, Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms, Immunol. Lett. 148 (1) (2012) 23–33.
[16] M. Grabherr, B. Hass, M. Yassour, J. Levin, D. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (2011) 644–652.
[17] G. Pertea, X. Huang, F. Liang, V. Antonescu, R. Sultana, S. Karamycheva, Y. Lee, J. White, F. Cheung, B. Parvizi, J. Tsai, J. Quackenbush, TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets, Bioinformatics 19 (5) (2003) 651–652.
[18] L. Wang, Z. Feng, X. Wang, X. Wang, X. Zhang, DEGseq: an R package for identifying differentially expressed genes from RNA-seq data, Bioinformatics 26 (1) (2010) 136–138.
[19] J. Ott, Z. Liu, Y. Shen, Challenging false discovery rate: a partition test based on *p* values in human case-control association studies, Hum. Hered. 74 (2012) 45–50.
[20] A. Conesa, S. Götz, J. García-Gómez, J. Terol, M. Talón, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (18) (2005) 3674–3676.
[21] M. Kanehisa, M. Araki, S. Goto, M. Hattori, M. Hirakawa, M. Itoh, T. Katayama, S. Kawashima, S. Okuda, T. Tokimatsu, Y. Yamanishi, KEGG for linking genomes to life and the environment, Nucleic Acids Res. 36 (2008) D480–D484.
[22] C. Xie, X. Mao, J. Huang, Y. Ding, J. Wu, S. Dong, L. Kong, G. Gao, C. Li, L. Wei, KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases, Nucleic Acids Res. 39 (2011) W316–W322.
[23] Y. Wang, Y. Lu, Y. Zhang, Z. Ning, Y. Li, Q. Zhao, H. Lu, R. Huang, X. Xia, Q. Feng, X. Liang, K. Liu, L. Zhang, T. Lu, T. Huang, D. Fan, Q. Weng, C. Zhu, Y. Lu, W. Li, Z. Wen, C. Zhou, Q. Tian, X. Kang, M. Shi, W. Zhang, S. Jang, F. Du, S. He, L. Liao, Y. Li, B. Gui, H. He, Z. Ning, C. Yang, L. He, L. Luo, R. Yang, Q. Luo, X. Liu, S. Li, W. Huang, L. Xiao, H. Lin, B. Han, Z. Zhu, The draft genome of the grass carp (*Ctenopharyngodon idellus*) provides insights into its evolution and vegetarian adaptation, Nat. Genet. 47 (6) (2015) 625–631.
[24] M. Pertea, D. Kim, G. Pertea, J. Leek, S. Salzberg, Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown, Nat. Protoc. 11 (9) (2016) 1650–1667.
[25] J. Dickinson, A. D'Lugos, M. Naymik, A. Siniard, A. Wolfe, D. Curtis, M. Huentelman, C. Carroll, Transcriptome response of human skeletal muscle to divergent exercise stimuli, J. Appl. Physiol. 124 (6) (2018) 1529–1540.
[26] A. Chu, J. Liu, Y. Yuan, Y. Gong, Comprehensive analysis of aberrantly expressed ceRNA network in gastric cancer with and without *H.pylori* infection, J. Cancer 10 (4) (2019) 853–863.
[27] Y. Han, C. Lin, T. Chen, RNA-Seq analysis of differentially expressed genes relevant to innate and adaptive immunity in cecropin P1 transgenic rainbow trout (*Oncorhynchus mykiss*), BMC Genomics 19 (1) (2018) 760.
[28] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T)) Method, Methods 25 (4) (2001) 402–408.
[29] Q. Zhang, J. Gui, Virus genomes and virus-host interactions in aquaculture animals, Sci. China Life Sci. 58 (2) (2015) 156–169.
[30] M. Mancini, S. Vidal, Insights into the pathogenesis of herpes simplex encephalitis from mouse models, Mamm. Genome 29 (7–8) (2018) 425–445.
[31] G. Annunziata, M. Maisto, C. Schisano, R. Ciampaglia, V. Narciso, G. Tenore, E. Novellino, Resveratrol as a novel anti-herpes simplex virus nutraceutical agent: an overview, Viruses 10 (9) (2018) E473.
[32] G. Neumann, Y. Kawaoka, Transmission of influenza A viruses, Virology 479–480 (2015) 234–246.
[33] D. Goubau, S. Deddouche, E. Reis, Cytosolic sensing of viruses, Immunity 38 (5) (2013) 855–869.
[34] J. Liu, S. Liu, X. Cao, Highlights of the advances in basic immunology in 2011, Cell. Mol. Immunol. 9 (3) (2012) 197–207.
[35] J. Liu, X. Cao, Cellular and molecular regulation of innate inflammatory responses, Cell. Mol. Immunol. 13 (6) (2016) 711–721.
[36] J. Su, C. Zhu, Y. Wang, Molecular cloning, characterization and expression analysis of the PKZ gene in rare minnow *Gobiocypris rarus*, Fish Shellfish Immunol. 25 (2008) 106–113.
[37] J. Su, C. Yang, Z. Zhu, Y. Wang, S. Jang, L. Liao, Enhanced grass carp reovirus resistance of Mx-transgenic rare minnow (*Gobiocypris rarus*), Fish Shellfish Immunol. 26 (2009) 828–835.
[38] Z. Dai, J. Li, C. Hu, F. Wang, B. Wang, X. Shi, Q. Hou, W. Huang, G. Lin, Transcriptome data analysis of grass carp (*Ctenopharyngodon idella*) infected by reovirus provides insights into two immune-related genes, Fish Shellfish Immunol. 64 (2017) 68–77.
[39] T. Wang, P. Liu, H. Chen, Preliminary study on the susceptibility of *Gobiocypris rarus* to hemorrhagic virus of grass carp (GCHV), Acta Hydrobiol. Sin. 18 (1994) 144–149 [in Chinese, English abstract].