Structure and function of S9 segment of grass carp reovirus Anhui strain

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Abstract A highly virulent grass carp reovirus (GCRV) strain, named GCRV-AH528, was recently purified from a diseased grass carp with hemorrhage disease in Anhui, China. GCRV-AH528 S9 segment was 1320 nucleotides in length and encoded a 418 amino acid VP6 protein. BLAST search showed that the VP6 protein owned a conserved domain belonging to the reoviral $\sigma 2$ family. Phylogenetic analysis of VP6 presented that GCRV-AH528 belonged to GCRV genotype II, which was more closely related to *Orthoreovirus* than GCRV genotype I and genotype III. Further analysis revealed that GCRV-AH528 S9 and mammalian orthoreovirus S8 might have evolved from a common ancestral precursor and have identical mechanism in virus assembly. The expression level of *vp6* gene was detected by quantitative real-time PCR (qRT-PCR). Over time, the expression level of *vp6* gradually increased in *Ctenopharyngodon idellus* kidney cells. However, the level of *vp6* expression in blood sharply increased at 4–6 days, and then decreased to a low level after GCRV-AH528 challenge ($P < 0.05$). The *vp6* gene was detected in all tissues examined, whereas at relatively higher levels in blood, kidney, and liver ($P < 0.05$). The yeast two-hybrid (Y2H) system was used to identify VP6 self-interaction, while no interaction was detected in VP6–VP6. This study not only revealed the S9 segment structure and expression

pattern but also analyzed the VP6 mechanism by yeast hybridization method. The present study provides valuable informations for further experimental design and investigation of VP6 functions.

Keywords Grass carp reovirus (GCRV) · VP6 · Grass carp · Yeast two-hybrid (Y2H)

Introduction

Grass carp (*Ctenopharyngodon idellus*) is a vital freshwater aquaculture species that is extensively cultured in Asian countries, particularly in China. The production of grass carp is about 600 million t annually which is the highest amount yield in all aquatic animals [12]. In pond farming, the species is susceptible to hemorrhage disease, gill-rot disease, red-skin disease, and bacterial enteritis of grass carp. Thereinto, the hemorrhage disease is the most pathogenic disease that damages the grass carp breeding industry and leads to more than 80% mortality rate in fingerlings and yearlings grass carp. Epidemiological studies have confirmed that the hemorrhage disease is caused by grass carp reovirus (GCRV), resulting in at least 148 million dollars in economic losses each year [17]. In addition, GCRV is also fatal to various aquatic animals including rare minnow (*Gobiocypris rarus*), black carp (*Mylopharyngodon piceus*), and topmouth gudgeon (*Pseudorasbora parva*) [26]. It has been suggested that globalization of aquaculture operations has apparently accelerated the spread of GCRV [24].

GCRV is composed of 11 double-stranded RNA (dsRNA) segments and belongs to genus *Aquareovirus* (AQRV), family *Reoviridae*. The segmented genome results in high complexity and variability among different

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strains of GCRV [28, 38]. Currently, nine GCRV strains have been completely sequenced and are divided into three groups (genotype I, II, and III) based on genomic and biological characteristics [31]. Recent studies have shown that GCRV VP6 protein plays a critical role in the immune response and thus can be used as an antigen [11]. To further study the role of VP6 protein in viral pathogenesis and immune response, researchers have already constructed VP6 protein expression vectors used for expression *in vivo* or *in vitro* and the products show good immunogenicity [19, 36, 37, 41]. These results suggest the VP6 is a good candidate for the design of GCRV DNA vaccines. A better understanding of the viral dynamic and distribution of GCRV strain in tissues may facilitate in controlling further spread of the disease. However, information on the distribution and dynamic change of GCRV *vp6* gene in tissues or organs is lacking after infection by GCRV.

A highly virulent GCRV strain named GCRV-AH528 was recently isolated from cultured grass carp with severe hemorrhage disease in Anhui province [33]. To obtain further information on GCRV-AH528 genome and use available data on AQRV, GCRV-AH528 S9 segment (GenBank Accession Number KR180376) that encoded VP6 protein was cloned, and the characteristics and functions of this sequence were analyzed by the methods comprised bioinformatic and experimental approaches.

Materials and methods

Experimental sample collection

The original strain of GCRV-AH528, which was isolated and maintained in our laboratory, was used in the present study [33]. The stored GCRV-AH528 cell suspension was thawed and diluted to a ratio of 1:100, and then 1 ml diluted solution was added into *Ctenopharyngodon idellus* kidney (CIK) cells that were seeded in six-well plates. After incubating for 1 h, the supernatant was removed and replaced with 2 ml of Medium199 supplemented with 2% fetal bovine serum. The CIK cells were further cultured at 28 °C. The virus-containing cell suspension was harvested every two days for *vp6* gene expression analysis. Three wells cells were sampled at each time point.

Grass carp (100–150 g) were used in the artificial infection experiments. Fish were cultured in three tanks (50 fish in each tank) with cycling water at 28 ± 1 °C. They were challenged by intraperitoneal injection with 0.35 ml of GCRV-AH528 cell suspension at a concentration of 10^5 TCID₅₀/0.1 ml. The blood was drawn from the caudal vein of each grass carp at 2, 4, 6, 8, 10, 12, and 14 days post-injection. Three grass carps in each tank were sampled

at each time point. In addition, the intestine, kidney, liver, spleen, heart, gill, muscle, and blood of the infected and moribund grass carp were collected.

RNA extraction

Total RNA was extracted from the collected samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. All RNAs were quantified by using a spectrophotometer (Eppendorf BioPhotometer D30, Germany) and freezed at −80 °C. The isolated RNAs were used as template for quantitative real-time PCR (qRT-PCR) analysis.

Amplification and analysis of the S9 segment

Amplification of the entire S9 segment was conducted using the full-length amplification of cDNA (FLAC) technique [21, 25]. Online services (<http://blast.ncbi.nlm.nih.gov>, <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>, and <http://web.expasy.org/protparam/>) were used for open reading frame (ORF), conserved domain, and physical and chemical properties analyses. Multiple sequence alignments were performed using MegAlign. The secondary structure of the VP6 protein was predicted by Antheprot software [8]. A phylogenetic tree was built with the neighbor-joining method using 1000 bootstrap replications [27].

One step qRT-PCR

The SYBR Green PCR was performed on the Bio-Rad CFX96 Sequence Detection System (BIO-RAD, USA). The reaction mixture contained following components: 2 µl RNA template, 12.5 µl 2× One Step SYBR Green PCR buffer (Takara, China), 0.5 µl Ex Taq HS (5U/µl), 0.5 µl PrimeScript RT Enzyme Mix, 0.5 µl each primer (10 µM, listed in Table 1), and ddH₂O in a total volume of 25 µl. The reactions were firstly executed for 5 min at 42 °C and 10 s at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. To test the amplification specificity, a melting curve was generated by gradually increasing the temperature to 95 °C after qRT-PCR. All reactions were performed in triplicate, and the negative controls with no template were run at the same. Threshold cycle (C_T) values were automatically determined by the instrument. Target gene expression was calculated by using the $2^{-\Delta\Delta C_T}$ method.

Statistical analysis

The experimental data was analyzed by one-way ANOVA analysis and student's *t* tests using the SPSS 13.0 statistical

Table 1 Primers used in this study

Primers	Nucleotide sequences (5′–3′)	Note
qRT-PCR- <i>vp6</i> -F	TGCACACGCATAGCTTCAAC	Used for qRT-PCR
qRT-PCR- <i>vp6</i> -R	TAATCACTCGCCACCTGTCT	
pGBKT7-VP6-F	<i>ATGGCCATGGAGGCC</i> ATGGAGCGATCCACTTACAATATCT	Used for Y2H
pGBKT7-VP6-R	<i>CCGCTGCAGGTCGAC</i> AGGGAATAAGCGATCCAACACC	
pGADT7-VP6-F	<i>GCCATGGAGGCCAGT</i> ATGGAGCGATCCACTTACAATATCT	
pGADT7-VP6-R	<i>CAGCTCGAGCTCGAT</i> AGGGAATAAGCGATCCAACACC	

Letters in bold and italics are homologous with Y2H carriers

software. A value of $P < 0.05$ was considered statistically significant.

Yeast two-hybrid (Y2H) identification VP6 self-interaction

pGBKT7-VP6 and pGADT7-VP6 vectors were constructed by ligation independent cloning technology. Primers used for expression vector construction are listed in Table 1. After testing the bait and prey constructs for autoactivation and toxicity, a total of three yeast co-transformations were performed to identify interactions of VP6–VP6: pGBKT7-Lam + pGADT7-T (negative control), pGBKT7-VP6 + pGADT7-VP6, and pGBKT7-53 + pGADT7-T (positive control). For each transformation, 100 ng of bait recombinant plasmid and 100 ng of prey recombinant plasmid were mixed and transformed into *Saccharomyces cerevisiae* Y2HGold in small scale reactions. The transformants were then grown at 30 °C on synthetically defined (SD) medium. Each yeast transformation was tested on both plates of SD-Trp/-Leu/X- α -Gal/AbA (Double Dropout, DDO) and SD-Trp/-Leu/-His/-Ade/X- α -Gal/AbA (Quadruple Dropout, QDO).

Results

The structure of S9 segment

GCRV-AH528 S9 genome segment was 1320 bp in size and contained a 1254 bp ORF starting with the first initiation codon (AUG) at bases 23–25 and ending with a termination codon (UAG) at bases 1277–1279. The ORF encoded a VP6 protein consisting of 418 amino acids. The calculated molecular mass of VP6 protein was 47.95 kDa with an estimated isoelectric point of 8.13. Conserved terminal sequences (5′-GUAACU—UUCAUC-3′) and a short specific inverted repeat sequences (5′-GAGUC-3′, 5′-GACUC-3′) were found at the end of S9 segment (Supplementary Fig. S1).

Phylogenetic relationship analysis

A phylogenetic tree was constructed based on VP6 protein (Supplementary Fig. S2). The tree showed that the homologues of GCRV-AH528 VP6 only existed in *Spinareovirinae*, and not in *Sedoreovirinae*. On the basis of previous studies [3, 10, 39], GCRV-AH528 was classified as AQRV belonging to *Spinareovirinae* and had the genetic relatedness with *Orthoreovirus* (ORV). According to the protein sequence, GCRV-AH528 was designated as GCRV genotype II.

Relative expression level of *vp6* gene in CIK cells

In the present study, GCRV-AH528 could be propagated in CIK cells and *vp6* expression was detected at different time points after infection. qRT-PCR analysis demonstrated that the expression level of *vp6* gradually increased with the extending of incubation time (Fig. 1A). At the first test point (2 days), *vp6* expression level was only about 17.9% of the final test point (14 days) ($P < 0.05$). Statistical analysis showed no significant differences between adjacent checkpoints ($P > 0.05$).

Relative expression level of *vp6* gene in blood

In blood, *vp6* expression level initially increased, and then dropped after infection (Fig. 1B). Further analysis showed that *vp6* had a higher expression level from the fourth to tenth day, peaking on the sixth day. The expression quantity of *vp6* at 4 and 6 days had statistically significant differences with all other days of infection ($P < 0.05$). The peak level at 6 days was 3.4-fold higher than that of the last checkpoint (14 days).

Tissue distribution of *vp6* gene in moribund grass carp

The study showed *vp6* distributed in all tissue samples collected from diseased grass carp, particularly in kidney,

Fig. 1 Relative expression level of *vp6* gene detected by qRT-PCR. Bars represent relative expression levels from three biological replicates. Error bars indicate standard deviation. **A** *vp6* expression in CIK cells; **B** *vp6* expression in blood; **C** *vp6* expression in moribund grass carp

intestine, and blood (Fig. 1C). Furthermore, the above three tissues existed significant differences with other tissues ($P < 0.05$). The lowest expression level was observed in heart, which was only 21.8% of the highest level detected in blood ($P < 0.05$).

Identification of VP6 protein self-interaction by Y2H assay

GCRV-AH528 VP6 encoding gene was cloned into the Clontech Y2H bait plasmid pGBKT7 and prey plasmid pGADT7, then the validities of these recombinant plasmids were confirmed by sequencing. For yeast Y2HGold co-transformation assay, bait-prey protein pairs of VP6–VP6 and Lam-T (negative control) showed growth on DDO plates but not on QDO plates, however, 53-T (positive control) underwent growth on both DDO and QDO plates. Further experiments showed that VP6–VP6 and Lam-T (negative control) could not grow on DDO/X- α -Gal/AbA and QDO/X- α -Gal/AbA plates, but 53-T (positive control) could grow on DDO/X- α -gal/AbA and QDO/X- α -gal/AbA plates (Supplementary Fig. S3). Based on these

observations, it was inferred that GCRV-AH528 VP6 protein had no interact with itself.

Discussion

Analysis of the terminal nucleotide sequences contributes to understanding mechanisms of transcription and replication. All members of *Reoviridae* family exhibit conserved terminal sequences which are similar among different species and have been used in reovirus classification [3, 13]. Analysis of the 5' and 3' terminal sequences demonstrated that non-coding regions (NCRs) existed in S9 segment of GCRV-AH528, whose length was 22 nucleotides at the 5' end and 41 nucleotides at the 3' end. The S9 NCRs contained a 5'-GUAACU terminal motif in 5' end and a UUCAUC-3' terminal motif in 3' end that were highly conserved in GCRV-AH528 11 segments (Accession Numbers KR180368–KR180378) [33]. In addition, a domain of inverted repeat adjacent to the 5' and 3' terminus always plays an important role in distinguishing this genome segment from other segments [2]. In GCRV-AH528

S9 segment, the 5' end sequences, GAGUC at position 9–13, were complementary to its 3' end inverted repeat, GACUC at position 1306–1310. The conserved terminal sequences are broadly reported in members of *Reoviridae* family and are essential in sorting and packing functions of the virus [2].

The ORF was considered to encode a protein of 418 amino acids in length, with a deduced molecular weight of approximately 47.95 kDa. The AUG start codon of the ORF was located at nucleotides 23–25 and the UAG stop codon was situated at nucleotides 1277–1279. No additional ORFs of significant length are detected in either the plus or minus RNA strand. Although the first AUG of S9 segment appears to be not in a very favorable context for initiation of translation (UGUAUGG) according to the consensus sequence (A/G)NNAUGG (N = any nucleoside), in most cases, the AUG nearest the 5'-terminal cap is exclusively used for initiation for protein synthesis in a “scanning model” [15, 16]. The putative methionine start codon (nucleotides 23–25) is thus the first triplet rather than the others.

A search made against the protein databases has revealed that the deduced VP6 protein shares some homology with the $\sigma 2$ protein encoded by S8 segment of mammalian reovirus (MRV), a member of genus ORV in the family *Reoviridae*. Further analysis, more similarities between GCRV-AH528 S9 and MRV S8 are observed. They not only have similar segment lengths and molecular weights, but also own the same 3'-terminal sequences (UUCAUC-3') and the amino acids numbers (418 amino acids). Secondary structure predictions reveal that GCRV-AH528 VP6 comprises 16.27% α -Helix, 31.34% β -Sheet, 28.47% Turn, and 23.92% Coil, which are similar to the characteristics of MRV $\sigma 2$ protein (Fig. 2). In addition,

Fig. 2 The secondary structure comparison between GCRV-AH528 VP6 and MRV $\sigma 2$

most of the amino acid residues in the carboxy-terminal regions of VP6 and $\sigma 2$ proteins are hydrophilic. These imply relatively closer relations between them. From an evolutionary standpoint, GCRV-AH528 S9 and MRV S8 may have evolved from a common ancestral precursor and thus utilize identical mechanisms in virus assembly. Previous studies on the core structure of MRV have shown that $\sigma 2$ is located primarily on the inner surface of the core and plays a role in stabilizing the $\lambda 1$ [14, 23]. $\sigma 2$ is also a potent dsRNA-binding protein that binds to the base of $\mu 1$ for outer capsid assembly [18]. Therefore, It has also been hypothesized that GCRV-AH528 VP6 functions as a mediator bridging the inner core with the outer capsid [32].

In the present study, VP6 was used to elucidate the evolutionary relationships among members of the *Reoviridae* family. There are two subfamilies in the *Reoviridae*: *Spinareovirinae* and *Sedoreovirinae*. The subfamily *Spinareovirinae* includes viruses that have relatively large spikes or turrets situated on the surface of the core particle, whereas the subfamily *Sedoreovirinae* comprises viruses without large surface projections on their virions or core particles, thereby rendering them an almost spherical or “smooth” appearance [29]. However, protein sequence analysis showed that GCRV-AH528 VP6 homologues only found in *Spinareovirinae*, not in *Sedoreovirinae*. Although GCRV-AH528 is grouped into ORV cluster in the present phylogenetic tree, GCRV-AH528 defined as AQRV is more scientific and reasonable according to the source of the virus and complete genetic characteristics [3, 10, 39]. At present, there are three GCRV genotypes represented by isolates GCRV-873 (genotype I), GCRV-HZ08 (genotype II), and GCRV-104 (genotype III) [31]. Here, GCRV-AH528 is classified to be of GCRV genotype II on the basis of the genomic sequences and taxonomy guidelines of the International Committee on Taxonomy of Viruses (ICTV) [1]. Compared with GCRV genotype I and genotype III, GCRV-AH528 is closely related to ORV. It means that GCRV-AH528 has a higher evolutionary rank than other GCRV genotype strains and is likely to have originated from a common ancestor with ORV.

In CIK cells, the number of *vp6* gene escalated with the increase of cell culture duration. Statistical analysis shows no significant differences between two successive time points, thus implying that GCRV-AH528 strain proliferation is a progressive process in CIK cells (Fig. 1A). Majority of attenuated virus strains replicate quickly and express large amounts of antigen proteins, thereby eliciting strong adaptive immune responses that result in rapid virus clearance. However, pathogenic virus strains replicate at a lower rate than attenuated strains and they are easier to reach the target tissues [9, 40]. In this research, GCRV-AH528 is deduced as a pathogenic strain that enters the

internal organs to wreak havoc, thereby resulting in grass carp visceral necrosis and bleeding.

In farming, grass carp presents three stages after GCRV infection: incubation (1–3 days), outbreak (4–7 days), and recovery (from 8 days onwards). Intraperitoneal injection of the isolated virus could lead to hemorrhage similar to actual clinical symptoms. Grass carp infected with GCRV-AH528 showed symptoms of blackened dorsum and hemorrhage around the mouth cavity, gill cover, belly, as well as the base of fin ray. Infected grass carp presented a higher *vp6* expression level in blood in the fourth and sixth day of infection, which corresponded to the outbreak period. These findings indicate that this stage is the virus proliferation stage and that the adaptive immune responses of grass carp do not effectively eliminate virus. After the outbreak period, the immune mechanism of grass carp is gradually enhanced, leading to clearance of GCRV-AH528 infection and a sharp drop in *vp6* expression quantity ($P < 0.05$). Although *vp6* can be detected at the fourteenth day of infection, it is relatively lower than the initial level, possibly due to the gradual clearance of GCRV-AH528 from the body. The expression profile of *vp6* is convex-shaped (Fig. 1B), indicating that this gene may be utilized as a molecular marker for GCRV infection.

GCRV-AH528 *vp6* gene could replicate in most splanchnic tissue cells and its expression model was consistent with tissues tropism of GCRV-AH528 resulting in hemorrhage symptoms in the corresponding tissues. The expression level of *vp6* was much higher in some obvious pathological organizations, such as blood, kidney, and intestine. Further analysis showed the three organizations had statistical significances with other checked tissues ($P < 0.05$) (Fig. 1C). In diseased grass carp, the kidney may be a region of virus replication, serving as a factory to produce a large number of GCRV that are subsequently distributed to other parts of the body [17]. Blood circulates among different diseased tissues, and infected visceral tissues release viral particles into blood, thereby leading to a high expression of *vp6* in blood. In clinical symptoms, it is generally difficult to detect the pathological change of heart, which is consistent with the low *vp6* expression quantity. Thus the blood, kidney, and intestine are the main organs that are more easily attacked by GCRV.

Members of *Reoviridae* family have a segmented dsRNA genome that is enclosed by single, double or triple proteinaceous capsid shells. To evade the antiviral defense mechanisms inside the cytoplasm of the host cell, these viruses conduct the entire viral RNA transcriptional process within the inner capsid in a characteristic process called endogenous RNA transcription [35]. The capsid shells are built by virus protein–protein interaction and vital to impede host cell immune clearance mechanism. The Y2H system has been become a powerful genetic

approach to identify protein–protein interaction in *Reoviridae* [4, 7, 30, 34]. For application in GCRV protein–protein interaction identification, GCRV873 (GCRV genotype I) NS80 is verified to interact with proteins NS38, VP4, and VP6 as well as itself, whereas no interactions involving the four protein pairs NS38–VP4, NS38–VP6, VP4–VP4, and VP4–VP6 are observed [6]. Strong self-interaction is evidenced in Rice black-streaked dwarf virus (RBSDV) P10 protein, Mal de Río Cuarto virus (MRCV) P9-1 protein, and avian orthoreovirus (ARV) μ NS protein through Y2H assays [5, 20, 22]. To elucidate the basic role of VP6 protein in GCRV-AH528 (GCRV genotype II) replication and particle assembly, Y2H system was used to identify VP6 self-interaction. Expression of interacting proteins in co-transformed Y2HGold strain must have the ability to activate all reported genes and be cultured on both DDO and QDO plates. However, in this study, protein pairs of VP6–VP6 could only grow on DDO, not on QDO, DDO/X- α -Gal/AbA, and QDO/X- α -Gal/AbA plates. According to the phenotype assessment on plates, the VP6 protein can not combine with itself, suggesting no self-interactions happen in VP6 during the viral life cycle.

In conclusion, this study defined the GCRV-AH528 S9 genomic characteristics and expression mechanisms, providing evidence for the pathogenesis of GCRV. The results contribute to better understanding of the tropism and transmission of GCRV-AH528. However, to dissect the pathogenicity of GCRV-AH528, further work including biochemical and cytological analyses will be required.

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