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NS38 protein of GCRV 096: Functional analysis and effect on grass carp reovirus replication

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ABSTRACT

Grass carp reovirus (GCRV) seriously affects the grass carp farming, as the causative agent of grass carp hemorrhagic disease. In this study, we found the GCRV 096 NS38 protein contained one conservative PXXP motif similar to σNS of avian reovirus, with very low similarity rates with its homologous GCRV proteins of different genotypes. Furthermore, the soluble GCRV 096 NS38 protein was expressed in *Escherichia coli,* antiserum was raised and characterised. Addition of purified NS38 protein to culture media of *Ctenopharyngodon idella* kidney (CIK) cells clearly inhibited replication of GCRV 096 (genotype I); however, replication of GCRV GD108 (genotype III) was not significantly affected in CIK cells under the same conditions. It was not possible to induce cross-protection among GCRVs of different genotypes. GCRV multi-genotype should be considered and the NS38 protein was a promising candidate for developing therapies against grass carp hemorrhagic disease. Additionally, bioinformatics analysis suggested that the NS38 protein may be involved in viral genome replication.

Keywords: Expression, Functional analysis, Grass carp reovirus 096, NS38 protein, Replication

Introduction

Grass carp hemorrhagic disease restricts the development of the grass carp farming industry in China (Subramanian *et al.,* 1997). Grass carp reovirus (GCRV) is the etiological agent of grass carp hemorrhagic disease (Zou and Fang, 2000). However, pathogenesis of GCRV is not understood completely. Moreover, the genetic diversity of GCRV enhances the difficulty to prevent and treat grass carp hemorrhagic disease (Yan *et al.*, 2014; He *et al.,* 2017). It is imperative that the multiple genotypes of GCRV are researched (Rao and Su, 2015; He *et al.,* 2017).

Mature GCRV virions consist of seven structural proteins and five non-structural proteins. Different GCRV genotypes differ in many ways (Yan *et al.*, 2014; Huang *et al.,* 2016; He *et al.,* 2017). Differences of non-structural proteins of GCRV genotypes are more prominent than that of structural proteins (Huang *et al.,* 2016). Exploring differences and functions of the GCRV non-structural proteins would be helpful for understanding pathogenesis and antiviral strategies. Non-structural proteins play important role in viral infection, viral replication and proliferation, pathogenesis and immune evasion (Schierhorn *et al.,* 2017). Some viral non-structural proteins have been considered as virulence factors (DeDiego *et al.,* 2016), vaccine candidates (Hurtado-Melgoza *et al.,* 2016; Kitagawa *et al.,* 2017; Reyes-Sandoval and Ludert, 2019) and major targets for antiviral therapy (Chu *et al.,* 2017). However, functions of GCRV non-structural proteins are not well understood.

The NS38 protein was shown to trigger the formation of viral inclusions and was expressed only in the process of viral infection of the host (Qiu *et al.,* 2001). After viral proteins are synthesised in the cytoplasm, the NS38 protein may combine with the VP3 protein to form spherical inclusions (Benavente *et al.,* 2007). In the present study, GCRV 096 and GCRV GD108 acted as representatives of GCRV genotypes I and III, respectively (Ye *et al.,* 2012; Yan *et al.*, 2014). GCRV genotype I and III are commonly found in China (Wang *et al.,* 2012; Ye *et al.,* 2012; Yan *et al.*, 2014; Rao and Su, 2015). GCRV genotype I (GCRV 873 is the representative) is associated with low virulence and a long latent period before the induction of a mild host immune response, while GCRV genotype III (GCRV HZ08 is the representative) is associated with high virulence, a short latency and stimulates a strong and extensive host immune response (He *et al.,* 2017). GCRV 096 can induce significant cytopathic effect (CPE) in CIK cells, while GCRV GD108 can replicate in CIK cells without obvious CPE (Yan *et al.*, 2014; Ye *et al.,* 2012). The identity of GCRV 096 with GCRV 873 (genotype I) based on the *vp4*, *vp6* and *vp7* genes is more than 92%. The identity of GCRV GD108 with GCRV HZ08 (genotype III) based on the *vp4*, *vp6* and *vp7* genes is more than 96.3% (Yan *et al.*, 2014). In this study, we investigated the impact of the GCRV 096 NS38 protein on GCRV replication in CIK cells by

real-time quantitative PCR (qRT-PCR) and western blot, and analysed its function.

Materials and methods

Virus, cells, vectors, bacteria and animals

GCRV 096 and GCRV GD108 were isolated from diseased grass carp in Xiaogan, Hubei Province and Zhongshan, Guangdong Province, respectively. Grass carp *Ctenopharyngodon idella* kidney (CIK) cells were procured from Shenzhen Inspection and Quarantine Bureau in China. The vectors pMD18-T, pET-28a (+) and *Escherichia coli* DH5α cells were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Adult New Zealand white rabbits (about 2.5 kg) were procured from the Experimental Animal Centre of Guangdong Medical College (experimental animal quality certification: 0001906), for immunisation experiments and were fed a standard laboratory chow diet.

Virus culture and purification

Viral inoculation in cell culture and propagation were performed as previously described (Fang *et al.,* 1989). GCRV 096 particles were extracted by differential centrifugation at 250-6000 *g* and the supernatant was subsequently ultracentrifuged at 35000 *g* at 4°C for 2.5 h. The purified virus pellet was resuspended in phosphate buffered saline (PBS) at pH 7.4 and stored at -70°C.

RT-PCR amplification and sequence analysis

GCRV 096 genomic RNA was extracted from purified GCRV 096 virions using a viral RNA kit (Takara, China) and cDNA of GCRV 096 genomic RNA was prepared using RT-PCR kit (Takara, China). Primers designed for amplification of the GCRV 096 *ns38* gene were 5′-ATCATGGCACACACAGGCAC-3′ and 5′-TGACCACACCCTT ACATACC-3′. PCR cycling conditions comprised an initial denaturation at 95°C for 3 min, followed by 30 cycles at 94°C for 30 s, 58°C for 60 s and 72°C for 60 s and a final extension step of 30 min at 72°C. The PCR reaction mixture (50 µl) comprised 33 µl dH_2O, 3 µl dNTP (each is 2.5 mmol l^{-1}), 10 pmol l^{-1} primer (2 µl) each, 5 µl of 10×buffer (containing Mg^{++}), 100 ng DNA and 0.25 µl Taq polymerase (5 U μl^{-1}). The target gene was cloned into the pMD18-T vector at 16°C, followed by transformation into competent *E. coli* DH5α cells. The correct recombinant plasmid sequence was verified by sequence analysis at Sangon Biotech (Shanghai, China).

Prediction of the open reading frame (ORF) of the *ns38* gene, identities and similarities of the GCRV 096 *ns38* gene with known *ns38* genes and multiple alignments of the sequences of the NS38 proteins were performed using DNAstar software and BLAST search in NCBI-GenBank.

Expression and purification of recombinant protein and western blot

The primer pair, 5′-CGC*GGATCC*ATGGCACAC ACAGGCAC-3′ and 5′-ACGC *GTCGAC*TTACATACCC CCGAT-3′ was designed to amplify the ORF of the *ns38* gene (*BamH* I and *Sal* I restriction sites were incorporated). The PCR system was the same as the above amplification system, except that the annealing temperature was 65°C. The target ORF of the *ns38* gene was ligated into pET-28a (+) vector using T4 DNA ligase. The resulting recombinant expression plasmid pET28a-ns38 was transformed into *E. coli* BL21 (DE3) competent cells for expression as previously described (Jian *et al.,* 2013). The positive plasmids were verified by restriction analysis, PCR amplification and sequencing at Sangon Biotech, Shanghai, China to confirm that the correct insert was cloned.

The positive colonies were harvested and purification of the recombinant NS38 protein and western blot were performed as previously described (Jian *et al.,* 2013). Antibodies used for western blot were mouse monoclonal anti-His antibody (1:1000; Invitrogen) and goat anti-mouse antibody (1:3000; Takara, Dalian, China).

Preparation of antiserum against the GCRV 096 NS38 protein

Normal serum (prior to immunisation) was collected from the Zealand white rabbits, as the negative control. Equal amounts of the purified NS38 protein (500 µg) and Freund's complete adjuvant (Sigma, America) were mixed and emulsified completely and antiserum against the GCRV 096 NS38 protein was raised as previously described (Yan *et al.,* 2018). The prepared antiserum was separated and stored at -20°C. ELISA and western blot were performed to determine the antibody titre and antigen-binding specificity respectively.

Replication of GCRVs in CIK cells

Three types of experimental media containing 2% fetal bovine serum (FBS) were prepared: medium A, B and C. Medium A was a mixture of 100 µg purified NS38 protein with 5 ml of M199 and media B and C were 1:2 and 2:1 (V:V) mixtures of antiserum with M199, respectively. The control medium was M199 (containing 2% FBS). Experimental media were filtered (0.2 µm) and stored at 4°C.

Trypsinised CIK cells in the logarithmic phase of growth were equally divided between each well of six-well plates (2 ml per well) and incubated at 25°C for 36 h. After incubation, the original M199 medium (containing 2% FBS) was substituted for the experimental media A, B or C (control). Following incubation for 12, 24 and 48 h,

experimental medium was removed and sterile GCRV 096 or GCRV GD108 virus suspension (approximately 2130 μg ml^{-1}) was added at 50 μl per well. After incubation for 1 h, the virus suspension was removed and M199 medium (containing 2% FBS) was added. Cells were collected at 4, 8 and 13 h post-infection (Zou and Fang, 2000). GCRV 096 or GCRV GD108 viral RNA was extracted from equal volume aliquots of cells from each treatment group and reverse transcription was performed. Finally, levels of the viral *ns38* gene and NS38 protein in CIK cells were detected using qRT-PCR and western blot.

qRT-PCR amplification, statistical analysis and western blot

Primers designed for qRT-PCR amplification were 096F (5′-TGTGACGACATCCACGAGA-3′), 096R (5′-TACCGAGTTGGTCCGCTTT-3′), 108F (5′-CC AGACAGCTTGGCGTAA-3′) and 108R (5′-ACTT GTTTGACGTTCACAA-3′), based on sequences of the *ns38* genes in GCRV 096 and GCRV GD108 (the *ns38* gene in GCRV GD108 was also named *NS3*), respectively. Primers based on the inner reference genes *β-actin* and *GAPDH* for qRT-PCR amplification were βF (5′-GTGAATCGGATACTCTCGG-3′), βR (5′-GCA GACATAACGCAAGGCA-3′), GF (5′-GGCTGTGCT GTCCCTGTA-3′) and GR (5′-GGGCATAACCCTCGTA GAT-3′). Primers were synthesised in Sangon Biotech. The qRT-PCR system (25 μl) comprised 9.5 μl sterile water, 0.5 μl of each primer (10 pmol l^{-1}), 12.5 μl SYBR® *Premix Ex Taq*™ and 2 μl cDNA. The PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. qRT-PCR experiments were performed in triplicate. The data of qRT-PCR were analysed using the $2^{-\Delta\Delta CT}$ method. The experimental data were analysed in SPSS software using one-way analysis of variance. Significant differences were also analysed ($p<0.05$).

The infected CIK cells and media were centrifuged at 35000 *g*, at 4°C for 2.5 h. Then, proteins were extracted from the precipitate for western blot (Yan *et al.,* 2018). Antibodies used were mouse monoclonal anti-β-Tubulin antibody (1:1000; Invitrogen), mouse monoclonal anti-NS38 antibody (prepared in Vipotion, China) and goat anti-mouse antibody (1:3000; Takara, China).

Bioinformatics annalysis of the GCRV 096 NS38 protein function

Hydrophobicity of the GCRV 096 NS38 protein was analysed using ProtScale (https://web.expasy.org/protscale). Peptide-binding domains, glycosylation sites, interaction sites and the protein-protein interactions of GCRV 096 NS38 were predicted using NeOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/), InterPro (www.ebi.ac.uk/inter pro), Phobius (http://phobius.sbc.su.se/), METALDETE CTOR (http://metaldetector.dsi.unifi.it/v2.0/), ITASSER (http://zhanglab.ccmb.med.umich. edu) and DIP (http://dip.doe-mbi.ucla.edu).

Results and discussion

Cloning of the GCRV 096 ns38 gene and sequence analysis

The *ns38* gene of GCRV 096 (GenBank Acc. No. HQ407464) contains a 1059 bp ORF (Fig. 1), encoding 352 amino acids with high antigenic index. The predicted molecular weight of the NS38 protein was 37.7 kDa. The NS38 protein was homologous to the σNS protein of avian reovirus and the similarity rate was approximately 14.8%. The non-structural reovirus protein σNS exhibits single-stranded RNA (ssRNA)-binding activity and thought to be involved in assembling the reovirus mRNAs for genome replication and virion morphogenesis (Gillian *et al.,* 2000; Becker *et al.,* 2003). σNS may play an important role in recruiting viral RNAs to inclusions and in controlling or complementing μNS activity (Tourís-Otero *et al.,* 2005). Moreover, the GCRV 096 NS38 protein contains one conservative PXXP motif (319-322 aa) similar to σNS of avian reovirus (Xie *et al.,* 2016).

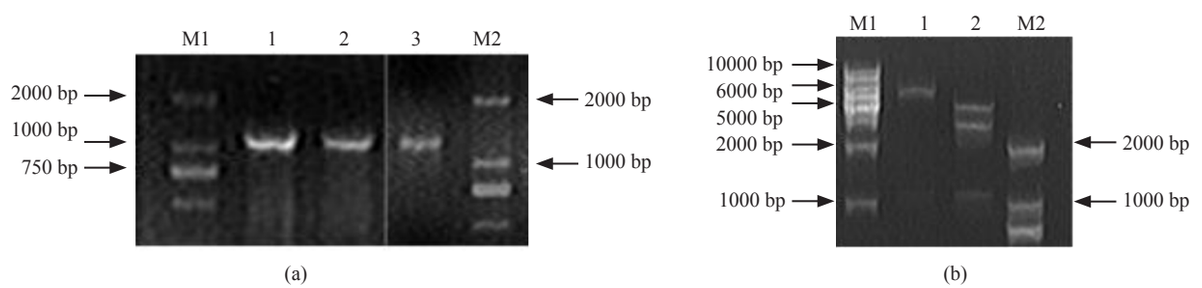

Fig. 1. (a) Amplification of the *ns38* gene in GCRV 096 and the recombinant plasmid pMD18-ns38, (b) double restriction enzyme digestion of the pET28a-ns38 plasmid with *BamH* I and *Sal* I
a. M1, M2: DL2000; 1, 2: PCR products of the *ns38* gene in GCRV 096; 3: PCR products of the *ns38* gene in pMD18-ns38.
b. M1: DL 10000; M2: DL 2000; 1: pET28a-ns38 plasmid; 2: Double restriction enzyme digestion of the pET28a-ns38 plasmid

However, the similarity rates of the GCRV 096 NS38 protein with its homologous GCRV proteins of different genotypes are extremely low, and accordingly named differently (Huang *et al.,* 2016). For example, the similarity rate of the NS38 protein with the homologous protein in GCRV GD108 was 13.3%, with one PXXP motif. By comparative analysis, we found that homology rates of the NS38 protein among GCRVs of the same genotype were more than 99%.

Expression and purification of recombinant GCRV 096 NS38 protein

We successfully expressed the soluble NS38 protein in *E. coli* BL21 cells by constructing the recombinant expression plasmid pET28a-ns38. The optimal conditions for induction were 6 h with 0.1 mM IPTG at 37°C. SDS-PAGE showed that the expressed target protein was soluble and consistent with the theoretical weight value (the fusion tag is 2.77 kDa) (Fig. 2a). SDS-PAGE (Fig. 2b) and western blot (Fig. 2c) with His-Tag monoclonal antibody (Invitrogen) indicated that the GCRV 096 NS38 protein was expressed and purified successfully (150 mM imidazole was the optimum elution buffer).

Indirect plate ELISA and Western blot analyses using antiserum raised against the GCRV 096 NS38 protein

ELISA plates with the expressed NS38 protein were coated with the antiserum raised against GCRV 096 NS38 protein for indirect ELISA assay. The experimental results indicated that the titre of the antiserum was 1:38400, higher than that of the GCRV 096 VP6 protein (Yan *et al.,* 2018). Western blot showed that the antiserum could specifically bind the NS38 protein (Fig. 2).

Replication of GCRVs in CIK cells

The relative levels of the *ns38* gene and NS38 protein in CIK cell samples challenged with GCRV 096 and incubated with NS38 protein or the prepared antiserum, were analysed by qRT-PCR and western blot. The results demonstrated that levels of the *ns38* gene and NS38 protein decreased over time (Fig. 3) and were lower in experimental samples treated with NS38 protein or antiserum than in control samples. The levels of the *ns38* gene and NS38 protein detected was the lowest when CIK cells were incubated with the NS38 protein or antiserum for 48 h; therefore, in subsequent experiments CIK cells were incubated with NS38 protein or antiserum for 48 h.

Notably, in samples infected with GCRV 096, levels of the *ns38* gene and NS38 protein decreased over time post-infection, and replication of GCRV 096 decreased significantly at 8 and 13 h post-infection (Fig. 4). There are two possible explanations for these results. The expressed NS38 protein may have blocked the binding of viral protein with a receptor or protein in the CIK cell membrane, or it might have anchored GCRV 096 to exosome membrane through its hydrophobic regions, thereby preventing cellular entry of GCRV and leading to a decrease in GCRV 096 replication. Many reports have indicated the interaction of virus non-structural proteins with membranes for viral infection (Gonzalez-Magaldi *et al.,* 2014; Klima *et al.,* 2017; Tenorio *et al.,* 2018). For example, foot-and-mouth disease virus non-structural protein 3A and 3AB interact with membranes, which is required for virus replication (Gonzalez-Magaldi *et al.,* 2014); the reovirus σNS triggers the peripheral endoplasmic reticulum to form organelles for viral genome replication and particle morphogenesis

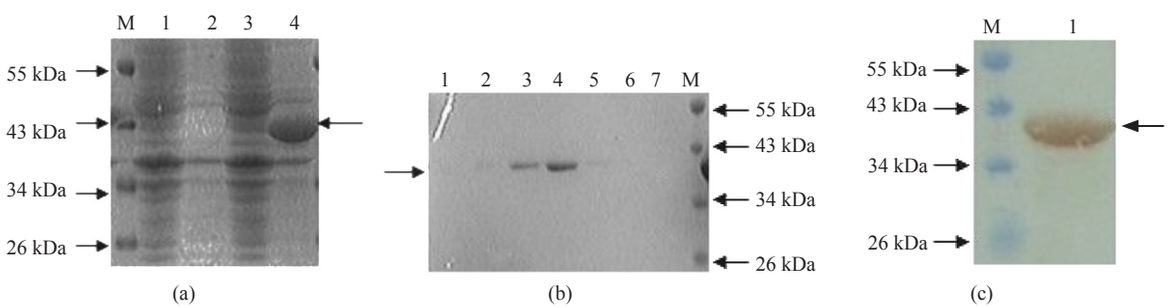

Fig. 2. Expression of the recombinant NS38 protein in *E. coli* BL21(a), purification (b) and western blotting of the expressed recombinant NS38 protein

a. M: Standard protein marker; 1: pET-28a (extract of tansformed pET-28a BL21 without IPTG induction); 2: pET-28a (the supernatant of tansformed pET-28a BL21 with IPTG induction); 3: pET28a-ns38 (extract of tansformed pET-28a BL21 without IPTG induction); 4: pET28a-ns38 (the supernatant of tansformed pET-28a BL21 with IPTG induction).

b. M: Standard protein marker; 1-7: The Purified recombinant NS38 protein with 60, 90, 120, 150, 200, 250, 300 mmol l^{-1} imidazole in the eluent buffer, respectively (indicated by arrows).

c. M: Standard protein marker; 1: The expressed recombinant NS38 protein.

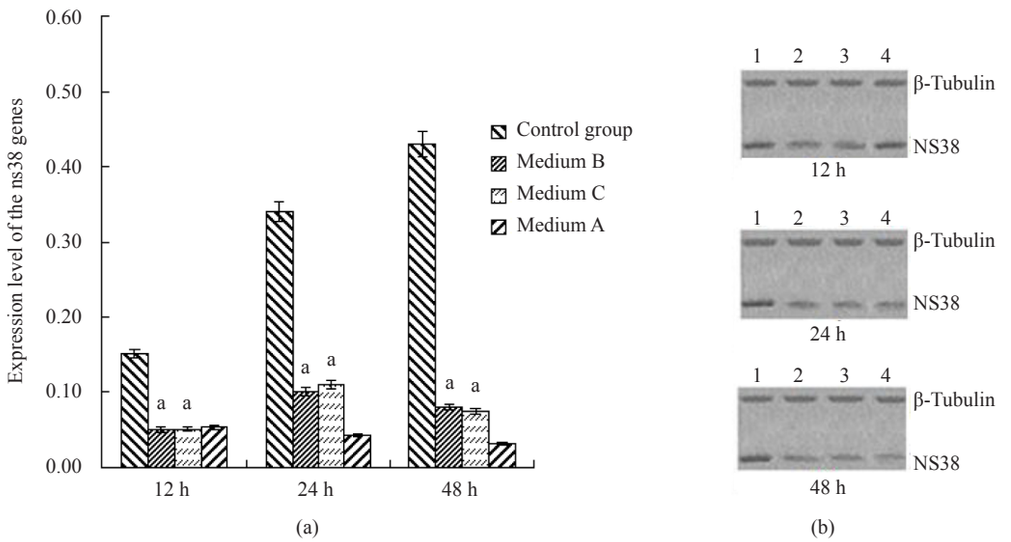

Fig. 3. Expression levels of the *ns38* gene (a) and NS38 protein (b) in CIK cells (infected with GCRV 096) incubated with the NS38 protein and anti-serum

1: Control group; 2: Medium B; 3: Medium C; 4: Medium A.

h: Incubation time of CIK cells with the proteinVP6 or antiserum, a: Indicates significant difference. Medium A: mixture of the purified protein VP6100 μg with 5 ml of M199; Medium B: mixture of antiserum with M199 in volume ratio 1: 2; Medium C: mixture of antiserum with M199 in volume ratio 2: 1

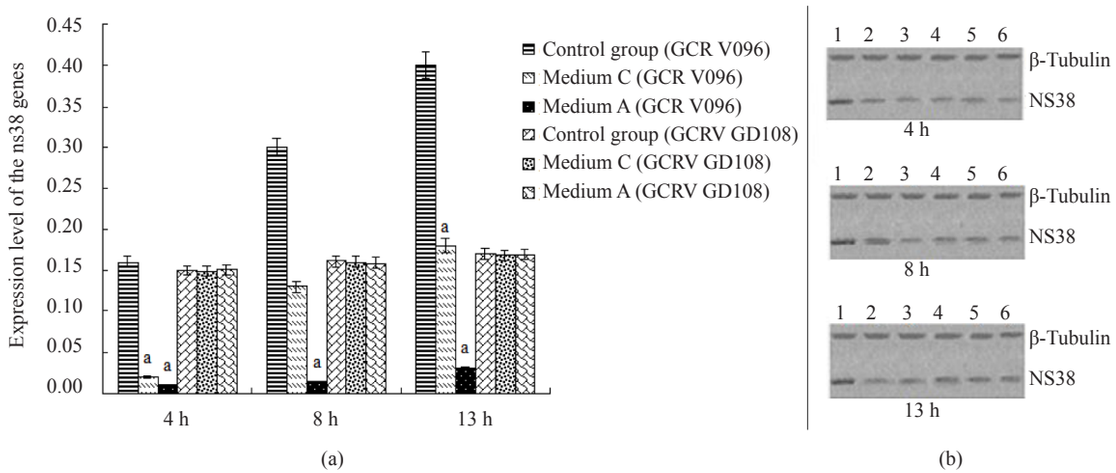

Fig. 4. Expression levels of the *ns38* gene (a) and NS38 protein (b) in CIK cells incubated with the NS38 protein or antiserum and infected GCRV 096 or GCRV GD108

1: Control group (GCRV096); 2: Medium C (GCRV096); 3: Medium A (GCRV096); 4: Control group (GCRV GD108); 5: Medium C (GCRV GD108); 6: Medium A (GCRV GD108).

h: Time after CIK cells infected GCRV 096 or GCEV GD108, a: Indicates significant difference. Medium A: mixture of the purified protein VP6100 μg with 5 ml of M199; Medium C: mixture of antiserum with M199 in volume ratio 2: 1

(Tenorio *et al.,* 2018); the Kobuvirus 3A proteins act as molecular harnesses to enslave the ACBD3 protein leading to its stabilisation at target membranes (Klima *et al.,* 2017). Alternatively, after addition of the antiserum to the cell medium, the combination of antibody with GCRV 096 may affect cellular entry of GCRV 096, also leading to decreased replication of GCRV 096.

Conversely, in samples incubated with the NS38 protein or antiserum and then challenged with GCRV GD108, there was no significant change in the level of the GCRV GD108 *ns38* gene and NS38 protein (Fig. 4). We speculate that the GCRV 096 NS38 protein and its antiserum may not affect cellular entry of GCRV GD108. We deduce that there are some differences in cell entry

and infection of GCRV 096 and that of GCRV GD108. Further, antiserum against the GCRV 096 NS38 protein may not be able to generate immune protection in response to challenge with GCRV GD108.

Therefore, we infer that it may not be possible to induce cross-protection among GCRVs of different genotypes. Comparably, it has been previously reported that there is no serological cross-reaction or cross-protection between GCRV JX01 and JX02 (Wang *et al.,* 2013). GCRV JX01 and GCRV 096 belong to GCRV genotype I, while GCRV JX02 and GD108 are genotype III. Consequently, multi-genotypes of GCRV should be considered for the development of vaccines and other therapies against grass carp hemorrhagic disease.

We consider that the NS38 protein should be a promising candidate for GCRV vaccine development and other therapies against grass carp hemorrhagic disease. Figs. 3 and 4 indicate that the replication of GCRV 096 was inhibited in the samples incubated with antiserum against the GCRV 096 NS38 protein. Moreover, we found that the expressed soluble NS38 protein elicited a higher antibody titre than the GCRV 096 VP6 protein did (Yan *et al.*, 2018). Recent researches have shown viral non-structural proteins as promising vaccine candidates and major targets for antiviral therapy (Hurtado-Melgoza *et al.,* 2016; Chu *et al.*, 2017; Kitagawa *et al.,* 2017; Reyes-Sandoval and Ludert, 2019).

Prediction of the GCRV 096 NS38 protein function

Bioinformatics analysis indicated that the GCRV 096 NS38 protein was non-cytoplasmic protein, with the hydrophobicity sites (174, 175, 241, 242, 243 aa), disulfide-bonded sites (145, 150, 242, 294 aa), glycosylation sites (8, 78, 80, 82, 89, 90, 220, 227 aa) and the ligand binding sites (23, 24, 25, 59, 63, 65, 66, 67, 68, 105, 108, 112, 115, 117, 164, 167, 171, 175, 237, 240 aa). The NS38 protein should have ssRNA binding and RNA-directed 5′-3′ RNA polymerase activity. The predicted interaction proteins of NS38 may be PHD-finger (interacting selectively and non-covalently with zinc ions) and alpha karyopherin-4 (interacting selectively and non-covalently with a nuclear localisation sequence). We deduced that the GCRV 096 NS38 protein probably plays a role in the viral genome replication. Some studies have demonstrated that the σNS protein of reovirus is responsible for reovirus-induced autophagy and benefits virus replication (Gillian *et al.,* 2000; Wu *et al.,* 2017) and it also played a role in translation and was found to connect with both eukaryotic translation initiation factor 3 subunit A (eIF3A) and the ribosomal subunit pS6R (Desmet *et al.,* 2014). Moreover, the σNS protein could induce and activate the PI3K/Akt signaling pathway (Xie *et al.,* 2016), which may be helpful for escaping the host's immunity to GCRV. Results of the present study may help in development of a protective GCRV vaccine and revelation of GCRV's pathogenesis. Functions of the GCRV NS38 protein need to be researched deeply.

Acknowledgements

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