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Characterization of betanodavirus quasispecies influences on the subcellular localization and expression of tumor necrosis factor (TNF)

Young-Mao Chen[a,b,c,f,g], Chor Siong Tan[b], Ting-Yu Wang[a,b,c,d], Ching-Long Hwong[h,**], Tzong-Yueh Chen[a,b,c,d,e,*]

[a] Laboratory of Molecular Genetics, Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[b] Institute of Biotechnology, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[c] Agriculture Biotechnology Research Center, National Cheng Kung University, Tainan, Taiwan
[d] Translational Center for Marine Biotechnology, National Cheng Kung University, Tainan, Taiwan
[e] University Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[f] Bachelor Degree Program in Marine Biotechnology, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan
[g] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan
[h] Department of Marine Biotechnology, National Kaohsiung University of Science and Technology, Kaohsung, Taiwan

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ABSTRACT

The aim of this study was to investigate the influence of variant coat proteins (CPs) from different quasispecies of betanodavirus on diverse aspects of nodavirus-induced pathogenesis. It is known that variant CPs can acquire either nuclear or cytoplasmic localization, depending on the nodavirus CP genotype, and this variation may arise during viral replication and influence the regulation of host and viral gene transcription. To investigate the role of these variant CPs in pathogenesis, six variant CP expression plasmids were constructed, each containing different quasispecies CP variants from nodavirus genotype red spotted grouper nervous necrosis virus (RGNNV). The CP expression plasmids were transiently transfected into grouper GF-1 cells. At different times, the cell cycle and cell proliferation were assayed using flow cytometry and methyl thiazolyl tetrazolium (MTT) assays, respectively. The proportion of G_2/M-phase GF-1 cells transfected with CP expression plasmids was higher than that of cells transfected with the blank plasmid, especially in regards to quasispecies 2 (QS2). The proliferation ratio of cells transfected with the CP expression plasmids was significantly higher than that of cells transfected with the blank plasmid, with the exception of QS6. We also found that the different quasispecies CPs down-regulated the promoter activity of the tumor necrosis factor (TNF) gene to different degrees. In addition, this is the first report showing the betanodavirus CP derived from different quasispecies of RGNNV provide evidence of a chronically nodavirus-infected grouper. Overall, this study represents the first comprehensive analysis of variant CPs from grouper with persistent nodavirus infections and their effects on different aspects of pathogenesis.

1. Introduction

The orange-spotted grouper, *Epinephelus coioides,* is a commercially important fish that is widely farmed in tropical waters of many countries. Considerable economic losses have been sustained in grouper aquaculture due to the infection of grouper by piscine nodavirus. The virus causes viral nervous necrosis (VNN) on grouper hatchery-reared larvae and juvenilies in Taiwan, Japan, Australia, and Europe, resulting in a high mortality rate (80–100%) [1]. In fact, the pathogen has a world-wide distribution and indeed can infect a very wide range of fish species globally [2–4]. Members of the *Nodaviridae* family are nonenveloped viruses with a genome composed of two single-stranded, positive-sense RNA molecules: RNA1 carries a gene encoding an RNA-dependent RNA polymerase (RdRp) and RNA2 encodes the coat protein (CP). Betanodaviruses usually induce vacuolation and necrosis of the central nervous system in their host. The clinical signs, including

* Corresponding author. Laboratory of Molecular Genetics, Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan.
** Corresponding author.
E-mail addresses: clh001@nkust.edu.tw (C.-L. Hwong), ibcty@mail.ncku.edu.tw (T.-Y. Chen).

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abnormal swimming behavior and noticeable darkening of body color, suggest the involvement of a betanodavirus in the pathology [2]. In addition, RNA virus genomes demonstrate substantial variation, and this can result in different quasispecies of the same genotype. The functional characteristics of different quasispecies of piscine nodavirus are not always identical.

Nodavirus CP is important as a viral structural component in nucleocapsid formation, and it is highly conserved among different piscine nodavirus genotypes [5]. Several studies have established that nodavirus CP is capable of inducing the caspase-dependent apoptosis pathway [6]. Hence, it plays an essential role in the pathogenesis of nodavirus persistent infections, such as viral encephalopathy and retinopathy. However, the mechanism of persistent infection and viral encephalopathy due to nodavirus CP remains unclear. Although several studies have investigated the influence of nodavirus infection on the fish immune response against viral infection [7–9], in the stages of development the adaptive immune system of juvenile groupers are poorly developed, and vaccination of the grouper larva is likely to be difficult. Nevertheless, the presence of nodavirus infection in adult and mature grouper is usually asymptomatic. If there are any remaining fishes, it could become healthy carrier of the nodavirus, and the remaining living fish could develop into chronic nodavirus. No obvious correlations have been found between the parameters describing chronic nodavirus groupers and the course of infection or the outcome of the cultivation process.

In prior studies, fish have been vaccinated with recombinant nodavirus CP for protection against viral challenge [8–12]. Liu et al. [13] demonstrated stimulation of the immune response in grouper following intramuscular injection with recombinant virus-like particles from grouper nodavirus. More importantly, Costa et al. [14] reported identification of B-cell epitopes that were recognized by neutralizing mouse monoclonal antibodies (MAbs) and serum samples obtained from sea bass naturally infected with nodavirus. These studies have found that nodavirus CP may serve as a vaccine component with the objective of inducing a protective immune response. However, several biological features are attributable to the quasispecies nature of viruses, including vaccination failure, persistent infection, resistance to antiviral agents, and alteration in cytopathicity [15]. Therefore, an association between specific quasispecies and the development of disease is also conceivable. As such, comparative genetic analysis of nodavirus CPs from different quasispecies variants may provide important clues for elucidating the mechanism of their influence on pathogenesis. The mechanism that caused nodavirus chronic infection is still unsure, the presence of nodavirus quasispecies implies to be a possible mechanism to cause virus persistence within the host. The main reason is that the virus itself has the ability of escaping the monitoring of the immune system of the host. Nodavirus genome will result in accumulation of mutation when the virus replicates, causing a significant genetic heterogeneity, that is one of the important characteristics of nodavirus [16]. First of all, the reasons for virus infected host to cause viral persistence that the nodavirus itself is able to weaken the virus antigen found on the cell surface in order to reduce the host antibody cognition, thus decrease the damage of the infected virus cell [17]. When the nodavirus antigen influences the host major histocompatibility complex (MHC) directly and interferred the appearance of the MHC during the delivery process of the antigen, the virus will progress and block the antivirus mechanism of the host [18,19]. Mutated virus has the ability of non-lytic phenotypes to allow the virus to persist within the cell, yielding virion which is allowed to replicate wild type viruses causing diseases. It is possible that the feature of nodavirus quasispecies that achieved persistence within the host likely could contribute to escape or relative resistance to host surveillance [20].

The purpose of the present study was to 1) use the direct-sequencing method to analyze amplified cDNA fragments and examining sequence diversity among nodavirus CPs from different genotype RGNNV quasispecies, 2) compare these sequences and develop a basis for the molecular epidemiology of nodavirus infection, 3) clarify the localization of nodavirus CPs from different genotype RGNNV quasispecies, 4) evaluate the influence of CPs on the cell cycle and cell proliferation via flow cytometry and methyl thiazolyl tetrazolium (MTT) assays, respectively, and 5) to analyze the influence of nodavirus CPs from different genotype RGNNV quasispecies on lipopolysaccharide (LPS)-stimulated tumor necrosis factor (TNF) promoter activity by performing transient transfections in grouper GF-1 cells with a grouper TNF-promoter luciferase construct.

2. Materials and methods

2.1. Fish sampling

The first samples of orange-spotted grouper (*E. coioides*) were collected during an acute disease outbreak where mortality rates were as high as 99% in infected juvenile grouper. At this time, the infected juvenile grouper (40–45 days post-hatching) presented with clinical signs that included changes in pigmentation, abnormal swimming behavior, and the inability to follow the rest of the group. Immunohistochemistry (IHC) findings included necrosis and vacuolation in the brain and retina of infected fish. Using the Ct value and the standard curve approach, the copy number of nodavirus *RNA2* was estimated to be approximately 6.04×10^{11}. In the second sampling, orange-spotted grouper, weighing approximately 120 g each, were collected from an indoor fish farm in Linyuan and maintained in 10L containers at 28 ± 5 °C. These fish had survived the high-mortality juvenile stage, where they had been infected with nodavirus. These fish showed no clinical signs and are referred to as healthy carriers. In these fish, IHC results indicted no significant brain or eye vacuolization and the copy number of nodavirus *RNA2* was estimated to be 1.97×10^5. RNA concentrations were determined as follows: the extracted RNA was converted to cDNA and used as a template for PCR amplification. PCR was stopped after a defined number of cycles to avoid plateauing effects. Standard curves were constructed from DNA fragments with known copy numbers [21].

2.2. Total RNA extraction, cDNA synthesis and direct sequencing

Eye samples from the fish (n = 6 per group) were used for total RNA extraction using a MagNALysis homogenizer (Roche, Basel, Switzerland) and TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufactures' recommendations. cDNA was synthesized in a reaction containing 2 μg RNA, 0.1 μM NNVRNA2-A1433 reverse primer (shown in Table 1), 12.5 μM dNTP (Bioman Scientific, Taipei, Taiwan), and 50 units Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 1 h. RNA and cDNA were quantified using an Ultrospec 3300 Pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). The cDNA was then used to amplify the full length *RNA2*, which was then sequenced directly (Mission Biotech, Taipei, Taiwan). The nodavirus-*rna2* region was amplified using a set of primers, NNVRNA2-S1 and NNVRNA2-A1433 (Table 1), specific for nodavirus. The PCR-amplified nodavirus-*rna2* cDNA products were electrophoresed through an agarose gel and visualized under ultraviolet light. The PCR-amplified nodavirus-*rna2* fragment from the healthy carriers was then purified using a gel/PCR fragment extraction kit (Geneaid Biotech, Sijhih City, Taiwan) according to the manufacturer's instructions. The PCR-amplified nodavirus-*rna2* fragment was cloned into pGEM-T vectors (Promega, Madison, WI, USA). For direct sequencing, three sets of forward and reverse primers were designed. The forward primers used in the sequencing reaction were: NNVRNA2-S1, NNVRNA2-S400, and NNVRNA2-S578 (Table 1). The reverse primers used in the sequencing reaction were: NNVRNA2-A1433, NNVRNA2-A1007, and NNVRNA2-A800 (Table 1). The sequencing data were analyzed using Chromas software (Conor McCarthy, Griffith University, Australia).

Table 1
Position and sequence of synthetic oligonucleotide primers.

Name	Location	Sequence 5′ to 3′	Direction
NNVRNA2-A1433	1433/1412	GCCGAGTTGAGAAGCGATCAGC	Reverse
NNVRNA2-S1,	1/21	TAATCCATCACCGCTTTGCAA	Forward
NNVRNA2-S400	400/420	CTGGCTTCCTGCCTGATCCAA	Forward
NNVRNA2-S578	578/598	ACTCCTGTGTGTCGGCAACAA	Forward
NNVRNA2-A800	800/780	CTGGAAGACTGCTCCATCAGG	Reverse
NNVRNA2-A1007	1007/987	AACAGGCAGCAGGATTTGACG	Reverse
NNVRNA2-S22-*Eco*RI	22/53	gcccggaattcTCACAATGGTACGCAAAGGTGAGAAGAAATTG	Forward
NNVRNA2-A1040-*Sal*I	1040/1021	ccgacgcgtcgacgtGTTTTCCGAGTCAACCCTGGTGCAG	Reverse
TNFGWprimary	285/257	CCAAGCAAACAGCAGGACACCTCCTATAC	Reverse
TNFGWnested	230/203	GACACCTTCCATATCTGAACAGCAGAGG	Reverse
AP1	–	GTAATACGACTCACTATAGGGC	Forward
AP2	–	ACTATAGGGCACGCGTGGT	Forward
TNFprodown	−971/–949	GTCTCCAACACTCCGCAACTCAC	Forward
TNFproup	+122/+100	ACTGTGCACAAGTCTGATCTTTA	Reverse
TNFprodown-S-*Nhe*I	−971/–949	ctagctagcGTCTCCAACACTCCGCAACTCAC	Forward
TNFproup-A-*Hin*dIII	+122/+100	actaagcttACTGTGCACAAGTCTGATCTTTA	Reverse

The orientation is indicated as sense (S) and antisense (A). Nucleotide location of the nodavirus *rna2*-specific and *tumor necrosis factor* (TNF) sequence are shown in lowercase letter and the restriction site is underlined. Nucleotide location of the *TNF* primers in DNA is according to Fig. 7A.

2.3. Immunohistochemical studies of healthy nodavirus carrier grouper

The healthy grouper that survived from nodavirus infection, nodavirus outbreak grouper (40–45 days post-hatching grouper) and healthy nodavirus carrier grouper, weighing approximately 120 g, Eye and brain tissues, were fixed in 10% formalin and embedded in paraffin following a routine procedure. Each 5 μm thick section was mounted on a polylysin-coated slide, deparaffinized in xylene, and rehydrate in descending grade (100-70%) of ethanol. Endogenous peroxidase activity was blocked by 10 min incubation at room temperature with Tris-buffered saline and 0.2% Tween 20 (v/v) (TBST) containing 3% hydrogen peroxide (H_2O_2). The sections were sequentially blocked with power block solution (BioGenex, San Ramon, CA, USA), then washed with phosphate-buffered saline (PBS) and incubated with polyclonal rabbit anti-coat protein antibody (1:500) dilution at 37 ˚C for 1 and half hour. The sections were washed twice with PBS, incubated with secondary Ab (Super Sensitive™ Polymer-HRP IHC; Bio Genex) at 37 ˚C for 20 min. The sections were then washed twice with PBS and the peroxidase activity was developed with 3,3′-diaminobenzidine (used as chromogen) for 10 min. After washed twice with PBS, the sections were then counterstained with hematoxylin for 1–2 min for nuclei and washed with water, dehydrated and mounted. Negative controls were performed with preimmune rabbit serum and incubation with PBS instead of the anti-coat protein Ab. The section slides were then observed using the Pannoramic MIDI and analyzed Pannoramic Viewer software (3DHISTECH Kft, Budapest, Hungary).

2.4. Plasmid constructs

Different quasispecies (QSn) nodavirus CP nucleotide sequences were amplified from pGEM-T/nodavirus-QSn-*rna2*, which contained CP sequences from QS1, QS2, QS3, QS4, QS5 and QS6, respectively. The sequences of the six variants isolated at the start of follow-up are designated QSn, where n varies from 1 to 6 (Fig. 1). Six PCR fragments encoding nodavirus CP derived from different QS nodavirus CP genotype RGNNV. The eukaryotic expression plasmids, pQSnCP/CFP and pQSnCP/YFP were generated by subcloning into pAmCyan1-N1 (Clontech) and pZsYellow1-N1 (Clontech), respectively. The primers were designed according to the CP gene sequence (Table 1). PCR products were purified and cleaved with restriction enzyme *Eco*RI and *Sal*I, and cloned into pAmCyan1-N1 and pZsYellow1-N1 to yield CFP or YFP-core fusion protein plasmids. The resulting plasmids, pQSnCP-YFP and pQSnCP-CFP, respectively, contain the nodavirus CP of QS, downstream of the human cytomegalovirus promoter.

2.5. Quantification of nucleolus, nucleus and cytoplasm ratios confocal images

LSM images were exported as Tiff files and fluorescence intensities in the nuclear and cytoplasmic regions were quantified using Image J. Background-corrected nucleolus, nucleus and cytoplasm ratios were calculated from mean fluorescence intensities measured within a small square or circular region of interest placed within the nucleus, cytoplasm, and outside of each cell. Measurements of QSnCP-CFP in the cytoplasm were made in regions of the cell devoid of punctate structures. Note that the absolute nucleolus, nucleus and cytoplasm values varied somewhat between experiments depending on exact imaging conditions and thus comparisons were only made for paired experiments.

2.6. Cell proliferation assay

MTT assays were performed after transfection using a Cell Proliferation Kit I (Roche, Mannheim, Germany) to analyze the effects of QSnCP-YFP overexpression. A total of 2×10^4 *E. coicoides* fin cells [22] (GF-1; BCRC 960094) were seeded in a 24-well plate and grown in a humidified incubator operating at 28 ˚C in antibiotic-free L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% v/v heat-inactivated fetal bovine serum (FBS). After 24 h, when the cells had attached completely, pQSnCP-YFP or pZsYellow1-N1 empty vector (pYFP) was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After removing the medium at 24, 48, 72, and 96 h, 100 μL of fresh medium containing 10 μL of MTT labeling reagent was added to each well and incubated for 4 h at 37 ˚C in the dark. A total of 100 μL of solubilization solution was added to each well, and the plate was then incubated at 37 ˚C overnight. The absorbance was measured at 570 nm [23].

2.7. Flow cytometry analysis

GF-1 cell were transfected with different quasispecies CP in 6-well plate assayed by flow cytometry at 24 h and 48 h 2×10^6 attached GF-1 cells were trypsinized, pelleted by centrifugation and washed with PBS. The cells were then fixed by 70% ethanol and stored overnight at 4 ˚C. After the cells pelleted and washed twice with PBS, cells were incubated in 1 mL PBS with propidium iodine (20 μg mL^{-1}), 0.1% Triton X-100(v/v) and RNase A (0.2 mg mL^{-1}) for 30 min. The GF-1 cells were run with flow cytometry (Beckman Coulter) and data was analyzed by using flow cytometry FlowJo software (Tree Star Inc, Ashland, USA).

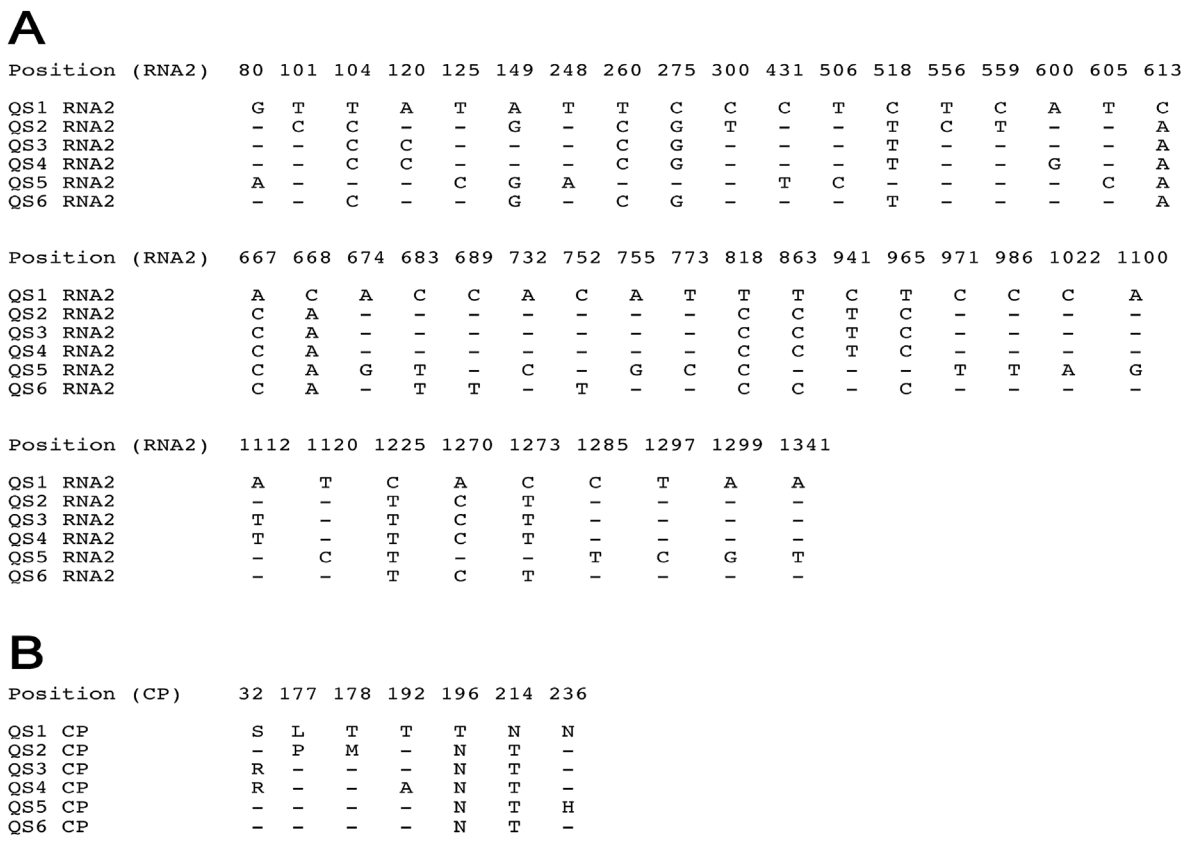

Fig. 1. Alignment of the nucleotide sequences (A) and deduced amino acid sequences (B) of the nodavirus coat proteins (CPs) from the different quasispecies. Dashes represent nucleotide or amino acid identity with the sequence QS1 RNA2 or QS1 CP.

2.8. Cloning of the grouper tumor necrosis factor (TNF) gene promoter

Genomic DNA of grouper was purified from muscle by the Phenol Chloroform method and used to construct a genomic library with the Universal Genome Walker Kit (Clontech, CA, USA) according to the manufacturer's instructions. Two primers, TNFGWprimary and TNFGWnested (Table 1), were designed from the 5′-end of grouper *TNF*, priming upstream amplification through two rounds of PCR, with the Genome Walker primers AP1 and AP2. Thermocycler conditions were set according to the manufacturers instructions. The resulting PCR product was subcloned into a pGEM-T Easy vector (Promega), and then sequenced. Automatic sequencing was performed commercially (Mission Biotech, Taiwan). Two new primers, TNFprodown and TNFproup, were designed to verify the newly amplified sequence (Table 1). PCR was performed using grouper genomic DNA. Thermocycler conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, after which, the purified over a high pure PCR product purification column (Roche) and sequenced as described above.

2.9. Construction of tumor necrosis factor (TNF) gene promoter reporter gene

A promoter region of *TNF*, corresponding to nucleotides −971 to +100 (relative to the transcriptional start site shown in Fig. 7A) was amplified from genomic DNA of grouper by PCR using the following primer: TNFprodown and TNFproup. The PCR product was cloned and sequenced as described above. The MatInspector database was used to search for the potential transcription factor-binding motif in the *TNF* promoter sequence [24]. For *TNF* promoter activity, the pGL3 plasmid (Promega) provided the vector backbone for the construction of the p*tnfp-luc* fusion plasmid, respectively. The −971/+100 5′-region of the grouper *TNF* promoter was amplified from genomic DNA of grouper by PCR using the following primer: TNFprodown-S-*Nhe*I and TNFproup-A-*Hin*dIII. The PCR fragments were subcloned the *Nhe*I- *Hin*dIII double-digested pGL3 vector, upstream of luciferase reporter gene to yield p*tnfp-luc*. Transfection analysis was performed, as described previously [25].

2.10. Reporter plasmid assay

The reporter p*tnfp-luc* vector contained the NF-κB binding elements upstream of *TNF* promoter region driving the luciferase reporter gene. GF-1 cells were transfected with 0.5 μg of p*tnfp-luc* and 1.5 μg of various expression QS CP plasmids with 3 μL of FuGENE 6. At 24 h post-transfection, cells were treated LPS 50 μg mL^{-1} for 6 h as indicated. The luciferase activities in the GF-1 cells were measured by a luminometer with a luciferase assay kit (Promega) as recommended the manufacturer.

3. Results

3.1. Nodavirus sequences encoding variant CPs detected in healthy carriers

We have cloned five nodavirus *rna2* cDNA directly from RNA derived from eyes of healthy carrier using the reverse transcriptase polymerase chain reaction (RT-PCR) method. The nucleotide sequence analyses of the clones isolated from grouper larvae of the acute infection group (QS1) were compared to the nodavirus *rna2* cDNA sequence from the healthy carriers (QS2∼6); Fig. 1A). The nodavirus *rna2* sequences of healthy carrier grouper differed from the acute infection group by a total of 44 nucleotide substitutions. Fig. 1B shows the comparison of the deduced amino acid sequences of the nodavirus CPs. In addition, 10 clones per sample were sequenced from six select

grouper of the acute infection group that had undergone acute infection, and this revealed that nodavirus *rna2* sequences were homogeneous. However, there was apparent genetic variation in the nodavirus *rna2* sequences in asymptomatic healthy carrier (Fig. S1). Phylogenetic analysis of nodavirus *rna2* nucleotide sequences aligned relative to RGNNV genotype (Fig. S2).

3.2. The incidence of nodavirus quasispecies rna2 sequences was compared between healthy carrier and the acute infection group

We directly sequenced PCR products of the nodavirus RNA (CP region) extracted from eye tissues of healthy carriers and the acute infection group. All CP sequences were identical among the six isolates from the acute infection group. Next, the chromatograms from directly sequencing of PCR products obtained from eye tissue of healthy carriers demonstrated a double peak at nucleotide position 506, 667, 668, 752, 794, 818, 863 and 893 in CP region indicating the presence of two different residues (Fig. S1). The results confirm the replicative nodavirus in the eye consisted of quasispecies population that were different between healthy carrier and the acute infection in group. Next, immunohistochemical (IHC) analysis of paraffin embedded sections, healthy (Fig. 2A), acute infection (Fig. 2B) and healthy carrier groups (Fig. 2D) using a rabbit polyclonal anti-CP antibody revealed the presence of different quasispecies of nodavirus CPs in healthy carrier grouper. By contract, no apparent staining observed in the healthy carrier group (Fig. 2C) when assayed using a pre-immune antibody as negative control.

3.3. Subcellular localization of nodavirus CPs derived from different quasispecies of RGNNV

To study the expression and intracellular location of different quasispecies CPs, GF-1 grouper cells were transfected with different quasispecies nodavirus CPs expressing plasmids, followed by analysis with laser scanning confocal microscopy (Fig. 3). Nodavirus CPs have been previously shown to localize to the nucleolus and cytoplasm [26] in cells transfected with a vector expressing the nodavirus CP under the control of a cytomegalovirus (CMV) promoter. To investigate whether localization to both the cytoplasm and nucleolus is a feature common to different quasispecies of nodavirus CPs, we cloned the Cyan Fluorescent protein (CFP) fused with different quasispecies CPs for localization of CP distribution analysis following transient transfection into GF-1 cells. The nucleolus is referred as an important regulator of many cellular functions, such as the regulation of growth, mitosis and stress responses [27]. After transfection with pCFP, control CFP expression was observed in both cytoplasm and nucleolus (Fig. 3A). The ^{23}RRRANNRRR^{31} sequence of nodavirus CP encodes a nucleolus localization signal that functions in both fish and mammalian cells. As can be seen in Fig. 3B, the truncated mutant of pQS1 CP$^{\Delta 23-31}$CFP was distributed throughout the cytoplasm. Assuming that the amount of fluorescence from CFP is proportional to the CP within the cell, variation in CP concentrations among the different quasispecies can be compared. Comparison of cells expressing the different CPs indicated that the subcellular localization of these proteins was consistent with nucleolar localization in the acute infection group. The localization ratio of the QS4 CP was found to be significantly higher in the nucleus compare to the other CPs, but the ratio for QS4 CP was lower than that of QS3 CP (Fig. 4, left panel). To confirm that the QS3 and QS4 CPs had higher localization ratios in the nucleolus region, we co-transfected these expression plasmids with others quasispecies CP plasmids. The cells that were co-transfected with nodavirus QSn CPs were fixed and observed after 24 h of transfection. The nodavirus QS4 CP was conjugated with CFP, while the others nodavirus QS CPs were conjugated with YFP. First, the N-terminal residues of QS1 CP (^{23}RRRANNRRR^{31}), which encoded a nucleolus localization signal, was amplified and fused with YFP (QS1 CP$^{\Delta 23-31}$). The QS1 CP$^{\Delta 23-31}$ expression plasmid was then co-transfected with QS CP4 as a negative control, and the results indicated that nodavirus CPs localized to the nucleus and nucleolus while the QS1 CP$^{\Delta 23-31}$ was distributed in the cytoplasm. Subsequently, QS4 CP was found to localize to a greater extent in the nucleus and nucleolus when compare to QS1, QS2, QS5, and QS6 CPs. However, QS3 CP was found to localize at higher levels in nucleus and nucleolus compare to QS4 CP (Fig. 4, right panel). According to these results, we determined that QS3 CP showed the greatest localization in the nucleus and nucleolus, followed by QS4 CP and the other CPs. The result of this co-localization analysis for the different quasispecies CPs in grouper cells was consistent with previous results which were shown correspond to the intensity of fluorescence.

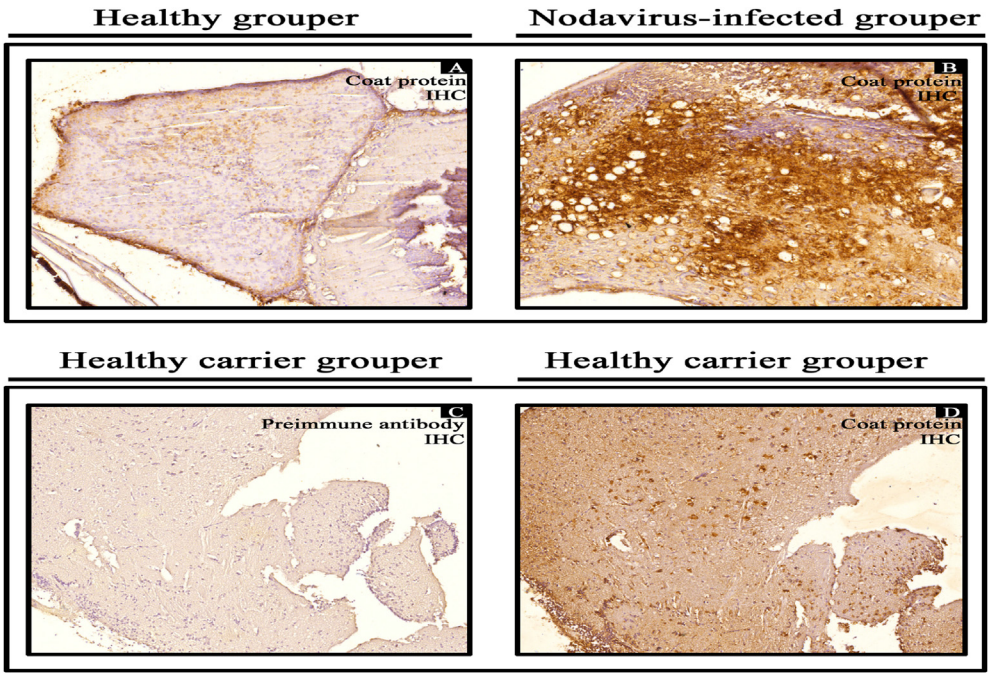

Fig. 2. The expression of nodavirus CP in the acute infection and healthy groups, as observed by immunohistochemistry (IHC) experiments. Histological sections of brain from grouper showing pathological changes attributed to nodavirus infection. Staining for the nodavirus CP in (A) healthy grouper and (B) nodavirus-infected grouper. Reactivity of the pre-immune antibody (C) in healthy carriers and reactivity of the anti-coat protein antibody (D) in healthy carriers.

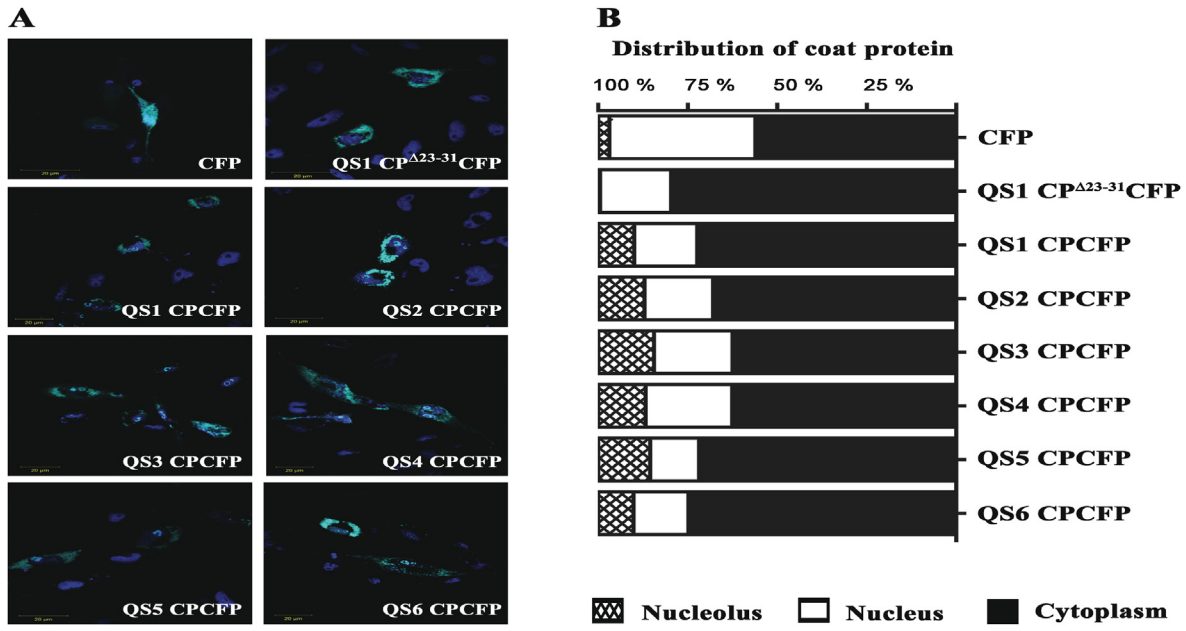

Fig. 3. Subcellular localization of the nodavirus CP. (A) Cells were transfected with 1 μg of each QSn CFP-CP plasmid, and fluorescence images were examined under a fluorescence laser scanning confocal microscope, LSM 780 (B) Cells were scored for localization of QSn CFP-CP. The distribution of the CPs showing each localization pattern expressed as a percentage of the total cell number (n = 10).

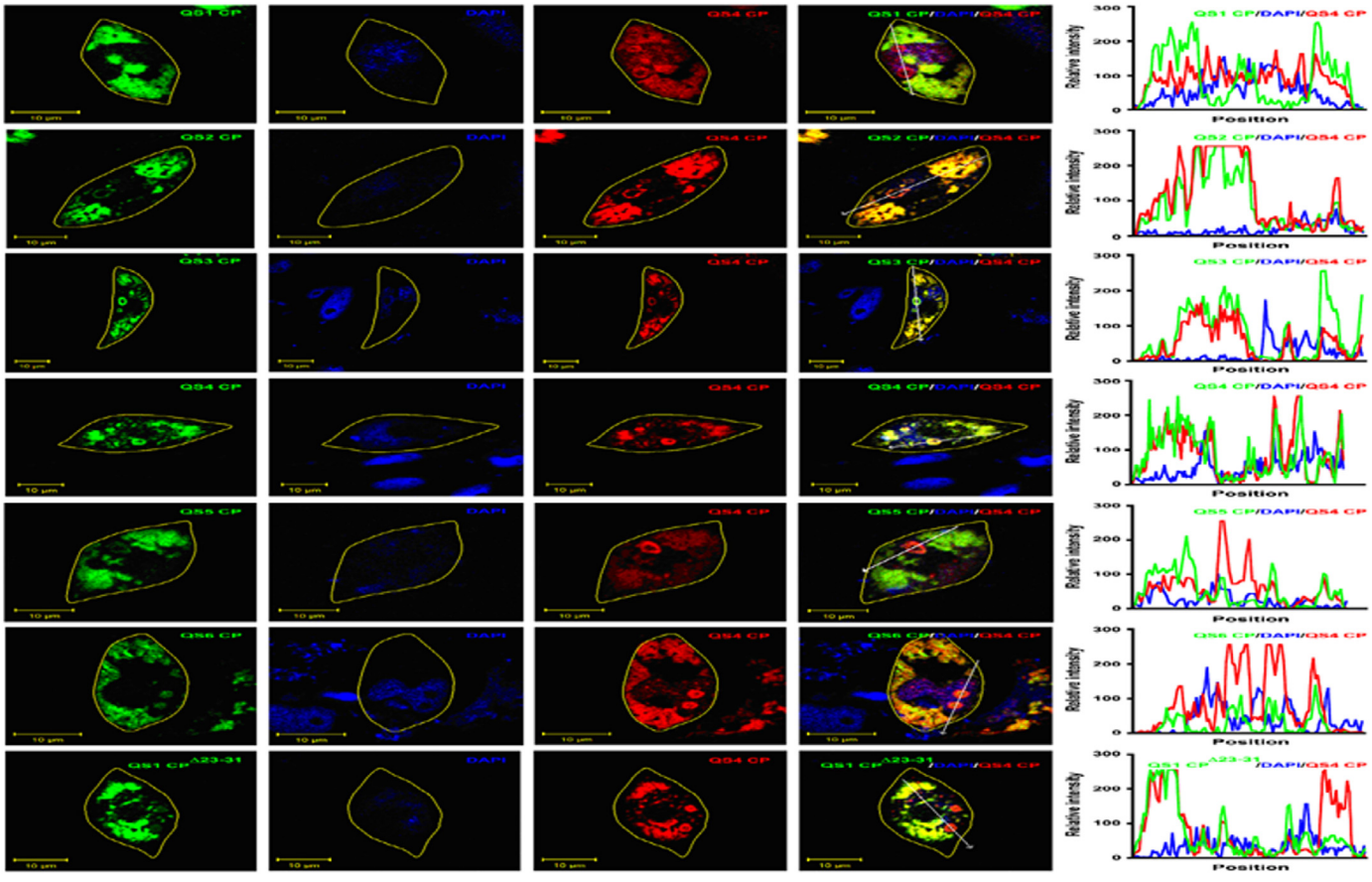

Fig. 4. The co-localization of nodavirus QS4 CP with different QSn CPs was analyzed using the fluorescent intensity profile method. Localization of QSn CP and QS4 CP under steady state conditions in transiently transfected GF-1 cells. Left panel, confocal images showing diffent QSn CPs (green), QS4 CP (red) and nucleus (blue) in grouper cells. Right panel, the fluorescence intensity profiles shown correspond to the quantification of fluorescence (left panel).

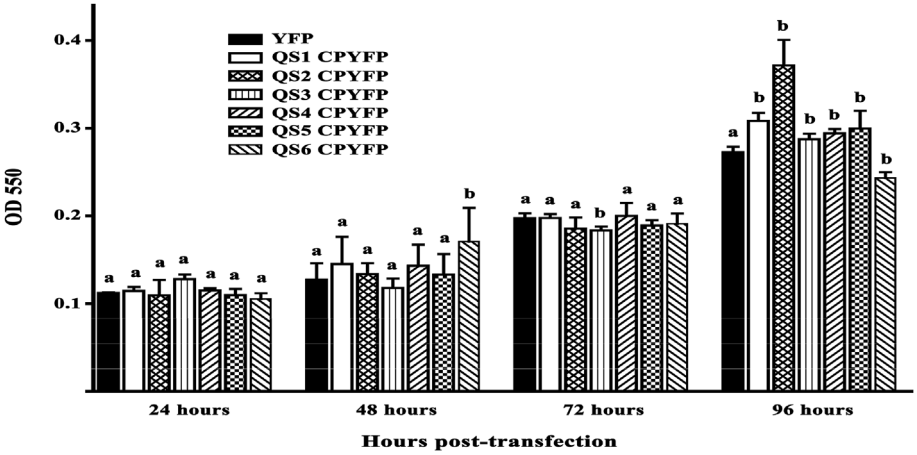

Fig. 5. Influence of CP expression from different nodavirus quasispecies on proliferation of GF-1 cells.

3.4. Different nodavirus quasispecies CPs inhibit proliferation and cell cycle progression

As shown in Fig. 5, the proliferation ratio of GF-1 cells transfected with pQS2CP/YFP was significantly higher than that of cells transfected with control plasmid at 96 h after transfection, but QS6 CP demonstrated lower proliferation than that of cells transfected with control plasmid. In addition, there were no proliferation differences among the GF-1 cells transiently cotransfected with the six different CP plasmids at 72 h. On the other hand, the proportion of G_1/G_0 phase GF-1 cells transfected with different quasispecies CP QS2 and QS5 plasmids were higher than that of cells transfected with control plasmid at 24 h after transfection. Furthermore, GF-1 cells transfected with pQS2CP/YFP plasmid demonstrated inhibition cell-cycle progression at 36 h, resulting from an impaired G_2/M to G_1/G_0 phase transition. In contrast, the quasispecies CPs from QS1, QS3, QS4, QS5 and QS6 demonstrated no significant difference in regard to arrest at the G1/G0 phase. The cell cycle of grouper cells transfected with QS2 CP and QS5 CP was found to arrest at the G2 phase after 36 h. This data was calculated based on the total number of cells arrested at the G2 phase divided by those at the G1 phase (Fig. 6). The results showed that different quasispecies CPs could maintain normal cell growth and influence cell-cycle progression. Moreover, our data indicate that QS2 CP leads to an accumulation of cells in the G_2/M phases of the cell cycle, which is involved in the progression of G_1 into S phase or G_2/M phase transition.

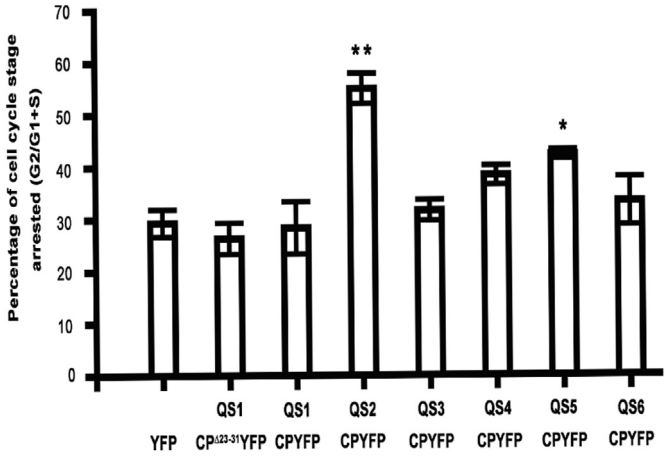

Fig. 6. Influence of CP expression from different nodavirus quasispecies on GF-1 cell cycle. Results indicate an impaired G2/M transition at 36 h after transfection (*$p < 0.05$; **$p < 0.01$).

3.5. Different nodavirus quasispecies of CPs downregulated tumor necrosis factor (TNF) promoter activity in activated GF-1 cells

The promoter-UTR5' region sequence was investigated by genome walking. The PCR product contains a genomic fragment of about 1199 bp (−971 to +229 from the transcription initiation site) of the grouper *TNF* promoter which was homologous to a recently published genomic sequence of the Japanese flounder *TNF* promoter [28]. The sequences obtained for the product was shown in Fig. 7A. Searching the transcription factor database (TRANSFAC) reveals several potential transcription factor binding sites that include a NFκB binding site, position −65 to −57 that is conserved in the promoter of Japanese flounder *TNF*. A potential for basic transcriptional site (position −31 to −25) was also identified in the grouper *TNF* promoter. The *TNF* gene was activated upon lipopolysaccharide (LPS) stimulation in grouper [29]. For functional characterization, the putative 972 bp *TNF* promoter was inserted into the pGL3 reporter vector, generating pTNFp-luc, and this was transiently transfected with different quasispecies CP plasmids into GF-1 for 6 h and then cotreated with 50 μg mL^{-1} LPS for 24 h. The effect on the *TNF* promoter of the different quasispecies CPs were determined based on luciferase activity, as shown in Fig. 7B. Data analysis showed that there was approximately twofold-less luciferase activity in QS2 and QS3 compared to CFP as a control cells.

4. Discussion

The genetic complexity and diversity of *RNA2* nucleotide sequences of nodavirus evolve over the course of nodavirus infection. As such, nodavirus quasispecies diversification is a common event in healthy carriers. As the sequence analysis revealed, the presence of very few nucleotides in analyzed *rna2* genomic regions which the nature nodavirus quasispecies may be far more complex, possibly reflecting basic differences during the course of nodavirus infection. These results are in agreement with the relatively low overall mutation rate and lack of identifiable hypervariable regions within the putative *rna2* genomic region of nodavirus previously reported. This study demonstrated that the nodavirus *RNA2* sequences were relatively conserved among nodavirus quasispecies in host but genetically variants. In the current study, we isolated six different nodavirus *RNA2* variants to investigate the influence of different quasispecies CPs on the host immune response; the results indicated that these mutants may cause strong immunopathogenesis. We found that expression of the nodavirus CPs inhibited cell-cycle progression and cell proliferation. Among the six different CPs, the rates of nucleolar localization and inhibition of cell proliferation were different, which suggested that the different quasispecies CPs may confer differences in pathogenesis during persistent infections.

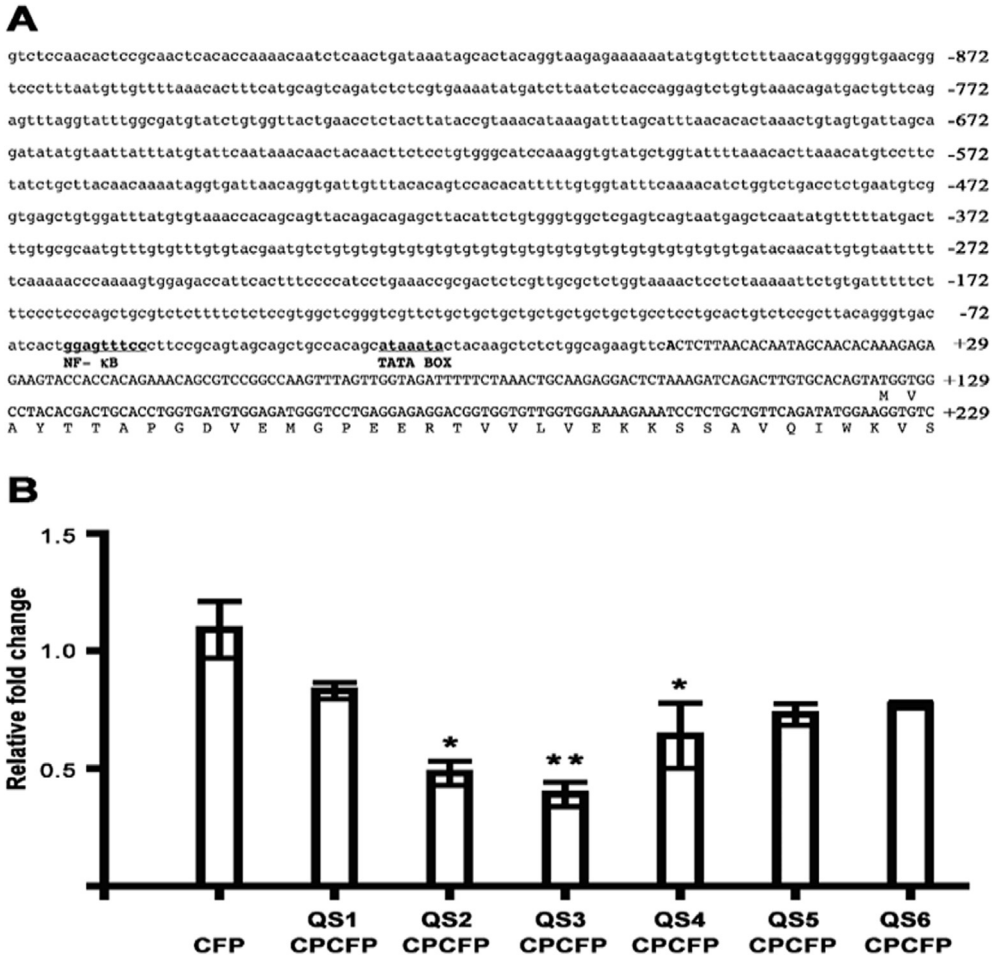

Fig. 7. CPs from different quasispecies of nodavirus inhibited tumor necrosis factor (*TNF*) promoter activity. (A) Transcription-factor binding consensus sequence in the 5′ flanking region of the grouper (*E. coioides*) *TNF* gene. (B) CPs from different quasispecies of nodavirus decreased *TNF* promoter-driven luciferase reporter activity in LPS-stimulated grouper cells. The data are presented as group means ± SEM of three independent flasks of cells. (*$p < 0.05$; **$p < 0.01$).

First, from the total nodavirus population isolated from the healthy carriers, the sequencing results for RNA2 indicated that more than one nodavirus variant was present. This phenomenon could be due to the accumulation of virus variants during the persistence process, resulting in high quasispecies complexity in the total virus population [30]. Based on the alignment of the six isolated nodavirus variants, there were a total of seven amino acid mutations that could result in different nodavirus CP structures. These nodavirus variants had different characteristics, including localization within the host, infectivity, inhibition or stimulation of the cell cycle, and suppression of host immunity. Next, the IHC results from the brains of healthy carriers indicated no obvious vacuolization and low concentrations of nodavirus. This showed that persistent nodavirus infection had occurred in these healthy grouper. The nodavirus populations present in the healthy carriers should have the ability to escape from the host's immune system without causing an outbreak infection in the host. This unique characteristic of nodavirus quasispecies prompted our investigation into the different roles of nodavirus quasispecies variants from healthy carriers and the acute infection group. Although the process of positive-strand RNA virus replication took place in the cytoplasm [30], but nodavirus cp targeting the nucleus is still not obvious. The localization of nodavirus CP in the nucleus and nucleolus of grouper cells from transfected QSn CP may be of great biological significance. This appears to be that QSn CP is able to adjust cellular growth, and a number of noval cellular promoters are involved in the pathogenesis. Other than that, we have gathered from our results that QSn CP can acquire either nuclear or cytoplasmic localization, probably depending on the genotype of QSn CP. The study indicated that different QSn CP forms led to its localization in the nucleus and nucleolus, with a possible involvement in the regulation of transcription of the host. The nuclear localization of QSn CP may be of relevance not only in the nodavirus life cycle but also host cell cycle. From that, a fascinating question popped up that different QSn CP was assembled in the nucleus and CP nuclear transport. Similarly, in the analysis work, using confocal laser scanning microscopy showed that approximately 5% of the nodavirus-infected cell was stained in the nucleus and nucleolus while 95% were stained in the cytoplasm by the anti-CP mAbs (not shown). These studies suggest that while the mechanism of QSn CP migration into the nucleus and nucleolus is not clear and it seems to be regulated, and the reason that nodavirus-infected grouper was infected chronically.

Two important checkpoints of the cell cycle, G_1/S and G_2/M, are responsible for accumulation of detrimental mutations that may result in outcome of disease. Here, we found that transient expression of nodavirus genotype RGNNV CPs could impair progression through the G_1/S in the GF-1 cells. We found that GF-1 cells showed a decreased ability for proliferation compared with control cells. Our results indicated that approximately 70% of GF-1 cells were arrested in the G_1 phase of replication but few apoptotic cells were observed. As such, the reason for slower proliferation of GF-1 cells was likely due to cell-cycle arrest and not to an increase in cell death. However, the exact mechanisms influencing how the different CPs affected the G_1/S checkpoint remain unclear. Nodavirus infected can affect gene expression in the brain of sea bream, as based on subtractive hybridization (SSH) analysis. Specifically, it was found that there was a difference in expression

between infected and control group, including cyclin D and ubiquitin conjugating enzyme 7 interacting protein, which contributed to dysregulation of cell cycle and apoptosis related gene [31]. Research has also indicated that immunoregulatory molecules appear to play an important role in the differences in innate and acquired responses changes between infected and control, and results also indicated that nodavirus-induced protein alterations can affect diverse cellular functions with the host cell [7].

The mechanisms responsible for nodavirus-mediated persistent infection and disease progression remain unclear. Several determinants in higher vertebrates, such as the vigor of the lymphocyte proliferative response to virus antigens in the acute phase of infection, the emergence of viral variants associated with cytotoxic T-lymphocyte antagonists, the genetic factors of the infected hosts, and the genotypes of the virus in association with its quasispecies nature have been suggested to be important factors capable of influencing the outcome of acute virus infections [20]. Teleost fish possess lymphocyte populations analogous in many respects to the T cells and B cells of mammals [32]. It may involve a process of dysregulation in cell-cycle progression and possibly a mechanism that could antagonize the apoptotic response, such as that evoked by the proliferating signal. It has been shown that activation of nuclear factor kappa B (NFκB) inhibits apoptosis and inhibition of enhances antitumor therapy via increased apoptosis [33]. In the current report, the effects of different nodavirus quasispecies CPs on LPS-induced reduction of *TNF*-promoter activity were measured in GF-1 cells transfected with different CPs. One possible explanation for this is that some transcription factor genes might be inhibited. In agreement with Yazawa [28], the *TNF* promoter is known to play an important role in LPS-mediated induction. TNF is a conserved molecule that is involved in cell-mediated cytotoxic reactions and also plays a role during viral infection [34]. TNF manifests a wide range of immune activities, including important functions in the killing of target cells, and is produced mainly by macrophages [35]. It is also one of the cytokines previously detected in the serum of virus-infected fish [36]. Our data showed that the activation of TNF by LPS resulting from the transient expression of nodavirus CPs can inhibit TNF transcriptional activity and may provide another possible mechanism for viral persistence. Understanding these differences in gene expression in nodavirus-infected cells should provide new insights concerning the molecular immune system and also perturbs many signaling pathways.

CRediT authorship contribution statement

Young-Mao Chen: Methodology, Validation, Formal analysis, Data curation, Visualization, Investigation, Writing - original draft, Writing - review & editing, Project administration. **Chor Siong Tan:** Validation, Formal analysis, Data curation. **Ting-Yu Wang:** Validation, Formal analysis, Data curation. **Ching-Long Hwong:** Resources, Supervision, Writing - review & editing. **Tzong-Yueh Chen:** Conceptualization, Methodology, Software, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2020.05.031.

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