



Full length article

## Inhibition of Cyclophilin A on the replication of red spotted grouper nervous necrosis virus associates with multiple pro-inflammatory factors

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## ABSTRACT

Cyclophilin A (CypA) is a ubiquitously expressed cellular protein and involves in diverse pathological conditions, including infection and inflammation. CypA acts as a key factor in the replication of several viruses. However, little is known about the role of CypA in the replication of the red-spotted grouper nervous necrosis virus (RGNNV). In the present report, grouper CypA (GF-CypA) was cloned from the grouper fin cell line (GF-1) derived from orange-spotted grouper (*Epinephelus coioides*). Sequence analysis found that GF-CypA open reading frame (ORF) of 495 bp encodes a polypeptide of 164 amino acids residues with a molecular weight of 17.4 kDa. The deduced amino acid sequence shared highly conserved regions with CypA of other animal species, showing that GF-CypA is a new member of Cyclophilin A family. We observed that GF-CypA was up-regulated in the GF-1 cells infected with RGNNV. Additionally, overexpression of CypA could significantly inhibit the replication of RGNNV in GF-1 cells. By contrast, when the GF-CypA was knock-downed by siRNA in GF-1 cells, the replication of RGNNV was enhanced. Furthermore, the expressions of pro-inflammatory factors, such as TNF-2, TNF- $\alpha$ , IL-1b, and ISG-15, were increased in GF-CypA transfected GF-1 cells challenged with RGNNV, indicating that GF-CypA might be involved in the regulation of the host pro-inflammatory factors. Altogether, we conclude that GF-CypA plays a vital role in the inhibitory effect of RGNNV replication that might be modulating the cytokines secretion in GF-1 cells during RGNNV infection. These results will shed new light on the function of CypA in the replication of RGNNV and will pave a new way for the prevention of the infection of RGNNV in fish.

### 1. Introduction

Cyclophilin A (CypA), is one of the most important members of the immunophilin family. It belongs to one of the three superfamilies of peptidyl-prolyl *cis-trans* isomerases (PPIase) that binds to the immunosuppressive agent cyclosporine A with high affinity [1]. The member of Cyp was first identified in mammals, where first human Cyp was identified as a specific cytosolic binding protein for the immunosuppressive cyclosporin A (CsA) drug [2]. Both Cyclosporine and Cyclophilin A make a complex (CypA-Cs) that bind to and inhibit the calcineurin and protein phosphatase activity, which further lead to the prevention of T-cell activation in mammals [3,4]. Cyps are much

conserved, ubiquitously distributed and is one of the most abundant proteins (about 0.1–0.4%) in the cytoplasm [5–7]. It plays an important role in diverse biological activities, i.e., transcriptional regulation, immunomodulation, cell signaling, protein folding, and trafficking [8,9].

Cyps have been isolated from many species that including, bacteria, yeast, plants, and animals [10–12]. CypA was among the first identified cyclophilins, discovered independently by two researchers in 1984 [2,13]. Later in 1989, amino acid sequence analysis confirmed that these proteins were the same [14,15]. CypA increased the proliferation of human pancreatic and lung cancer cells by the activation of p38-MAPK and Extracellular signal-Regulated Kinase 1/2 (ERK 1/2) pathways [16]. The CypA has also acted against immune-mediated injuries,

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e.g., Acetaminophen liver toxicity [17]. It also induced the release of Monocyte Chemoattractant Protein-1 (MCP-1), inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-8, MMP-2, and MMP-9), and cartilage destruction during rheumatoid arthritis [18,19]. Likewise, in hepatocellular carcinoma (SK-Hep-1) cell line, Cyp A upregulated the expression of many cytokine-related genes like IL-1 $\beta$ , IL-6, IL-8, CXCL1, CXCL2, and CXCL3 [20].

Nervous necrosis virus (Betanodavirus) is a small non-enveloped virus having a coat of icosahedral shape (T = 3). It causes viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) which primarily affect the brain and eyes of juveniles and hatchery-reared larvae of several marine fish species [21]. Its genome composes of two segmented positive single-stranded RNAs i.e., RNA1 & RNA2, which encodes RdRP (RNA dependent RNA polymerase) and Capsid genes, respectively [22]. In recent years, these viruses have attracted much attention because of their ecological and economic impacts on the aquaculture industry [23–25]. To date, numerous organisms Cyps are reported with its specific roles in regulating the life cycle and replication of several viruses such as in facilitating the virus replication that including HCV [26], HCMV [27], HIV-1 [28], HBV [29], Corona virus [30], Vaccinia virus [31], Vesicular stomatitis virus [32] etc. Conversely, there are viruses reported to be inhibited by CypA comprising of Influenza virus [33] and Rota virus [34,35]. Thus, it is clear that CypA can able to regulate the stability between the host and a pathogen in either beneficial or destructive manner through different pathways. Since, its role in pathogenesis CypA is considered as a biomarker for disease and been an appealing target for preparation of therapeutic agents. Hence, it is important to reveal the critical role of Grouper fish CypA in the regulation of infectivity and replication of Red-spotted grouper nervous necrosis virus (RGNNV), and these results might offer insight into the complexity of the virus-host interactions also worth of proposing CypA as a target for anti-viral therapy for future.

In the current study, the full-length cDNAs of CypA was cloned from GF-1 cells for the first time, and the expression profiles of GF-CypA and pro inflammatory factors were investigated at different time points on RGNNV replication. Hopefully, the present study will be shedding new light on the immune response of CypA and as a potential therapeutic target for VNN vulnerable fish species.

## 2. Material and methods

### 2.1. Virus and cell line

RGNNV was isolated from diseased red-spotted grouper fish (*Epinephelus akaara*) [36], and stored at  $-80^{\circ}\text{C}$  in our laboratory. Grouper Fin-1 (GF-1) cell line was originally derived from the grouper (*E. coioides*) fin tissue [23] that was purchased from Bioresource collection and research center, Taiwan, China. The GF-1 cells were cultured and maintained at  $28^{\circ}\text{C}$  using Leibovitz's L-15 supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin.

### 2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the GF-1 cells using Trizol (Invitrogen, Japan) reagent following the manufacturer instructions. The total RNAs concentration was determined using NanoDrop (Thermo Scientific), and the RNA integrity was checked using an Agilent 2100 Bioanalyzer (Agilent). The cDNAs were synthesized from total RNA by using the PrimeScript<sup>™</sup> RT reagent Kit with gDNA Eraser (Takara, Dalian, China) as per the manufacturers protocol, and stored at  $-80^{\circ}\text{C}$  until further analysis.

### 2.3. Cloning and sequencing of full-length cDNA of GF-1 CypA

To obtain the core sequence of GF-CypA (GF-1, CypA gene), mRNA

**Table 1**  
Primers used for this study.

S. No.	Primers	Sequence (5'–3')	Application
1	P-GF-CypA-F	AGATTGTCATGGAGCTGAGA	RT-PCR
	P-GF-CypA-R	AAGATCGTGATCACAGACTGC	RT-PCR
2	$\beta$ -actin-F	AGCCAACAGGGAGAAGATGACC	RT-PCR
	$\beta$ -actin-R	TGATCCACATCTGTGGAAG	RT-PCR
3	RGNNV-T4-F	CGTGTCCAGTCACTGTGTCGCT	qRT-PCR
	RGNNV-T4-R	CGAGTCAACACGGGTGAAGA	qRT-PCR
4	Capsid-F	TTGCTGGCTTCTCTGCCTGATC	qRT-PCR
	Capsid-R	ACTGAGGTCGGACTGTTCTGC	qRT-PCR
5	$\beta$ -actin-F	TCTGGTCGTACCCTGGTATTG	qRT-PCR
	$\beta$ -actin-R	GCAGCAGTGCCCATCTCCTGCT	qRT-PCR
6	ISG-15-F	ATGGATATAACCATCGTTAT	qRT-PCR
	ISG-15-R	TTACGTCAATTGCTCAGGTGA	qRT-PCR
7	IL-1b-F	ATGCCTGAGGGACTGGAAC	qRT-PCR
	IL-1b-R	TGGAGTTGCCTCACAACCGA	qRT-PCR
8	TNF-2-F	ATGGAAGGTGAATGCAAAGT	qRT-PCR
	TNF-2-R	TGCCTGTTGTAGATGAGGAC	qRT-PCR
9	TNF- $\alpha$ -F	ATCCTCTGCTGTTTCTGATAT	qRT-PCR
	TNF- $\alpha$ -R	TCTCTGTTTCTCTGACAGA	qRT-PCR
10	GF-CYPA-164	GCGUCAUUCGGUUUCAUTT	siRNA, qRT-PCR
		AUGAAACCGGAAUGACGCTT	siRNA, qRT-PCR
11	GF-CYPA-287	GCACUCUCUCUAUGGCAATT	siRNA, qRT-PCR
		UUGGCCAUAGAGAGUGGCTT	siRNA, qRT-PCR
12	non-silencing (NS-siRNA)	UUCUCCGAACGUGUCACGUTT	siRNA, qRT-PCR
		ACGUGACACGUUCGGAGAATT	siRNA, qRT-PCR

**RT-PCR** = Reverse transcription polymerase chain reaction; **qRT-PCR** = quantitative real time polymerase chain reaction.

from GF-1 cells were used as a template for PCR amplification with degenerated primers. The successful primers used in the present study were listed in Table 1, gene-specific primers and other primer sequences for complete Open Reading Frame (ORF) of P-GF-CypA were designed based on conserved regions of CypA sequences from closely related species shown in Table 2 with their accession numbers. PCR reactions were performed for 35 cycles following the profiles of  $94^{\circ}\text{C}$ , 5 min;  $94^{\circ}\text{C}$ , 45 s;  $60^{\circ}\text{C}$ , 30 s;  $72^{\circ}\text{C}$ , 30 s;  $72^{\circ}\text{C}$ , 10 min. Specificity of the amplified products was confirmed by 1.0% agarose gel electrophoresis, purified by gel extraction kit (CW Biotech Company, China) and ligated into the pMD18-T simple vector (Takara, Dalian, China). The plasmids containing the positive clones of an expected size were purified (Promega, USA) and sequenced at Tsingke Biological Company, Wuhan, China.

### 2.4. Sequence characterization and phylogenetic analysis of GF-CypA gene

The nucleotide and deduced amino acid sequences of GF-CypA cDNA were analyzed and classified using the BLAST search programs (<http://www.ncbi.nlm.nih.gov/blast>) and Expert Protein Analysis System (<http://prosite.expasy.org/>), respectively. The secondary and three-dimensional (3-D) structure of GF-CypA was predicted by using the online Phyre<sup>2</sup> software (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>). Multiple sequence alignment of GF-CypA was performed by using DNAMAN (9.0.1) and Clone Manager Professional (Ver 9.51). An unrooted phylogenetic tree was constructed by the Neighbor-Joining (NJ) algorithm embedded in the MEGA 6.0 program with its reliability assessed by 1000 bootstrap repetitions.

**Table 2**  
Summary of species homology analysis of GF-CypA Amino acids.

S. No.	Species Name	Identity %	Similarity %	E-Value	GenBank Accession Number
1	<i>Azumapecten farreri</i>	82	81	2e-106	AAR11779.1
2	<i>Tachysurus fulvidraco</i>	80	79	6e-104	AKD95367.1
3	<i>Scylla paramamosain</i>	77	77	1e-100	AEN94575.1
4	<i>Gallus gallus</i>	73	72	4e-92	ACX31829.1
5	<i>Penaeus monodon</i>	74	74	1e-96	ABV90639.1
6	<i>Xenopus laevis</i>	76	75	1e-97	NP_001082659.1
7	<i>Siniperca chuatsi</i>	81	83	1e-106	ANN12593.1
8	<i>Homo sapiens</i>	73	72	5e-93	P62937.2
9	<i>Ictalurus punctatus</i>	81	81	1e-102	NP_001187167.1

### 2.5. Recombinant construction of GF-CypA plasmid

Total RNA from GF-1 cells were extracted cells using Trizol (Invitrogen, Japan) and transcribed into cDNAs using SuperScript II reverse transcriptase kit (Invitrogen, USA) according to the manufacturer's instructions. PCR reactions were performed using primer sets given in Table 1, detected by 1.0% agarose gel electrophoresis, purified and ligated into a pMD18-T simple vector (Takara, Dalian, China), as described earlier. The full-length cDNA encoding GF-CypA was PCR amplified using the modified primers with restriction enzymes (*Bam*H1 and *Eco*RI; bold and underlined) 5'-GAATTCAGATTGTCATGGAGCTGAGA-3' and 5'-GGATCCGAGTCTGTGATCAGATCTT-3', were purified to clone into eukaryotic expression plasmid pcDNA3.1 (Invitrogen). Purified PCR fragments and the pcDNA3.1 plasmid were digested with the corresponding restriction enzymes then ligated together. The recombinant plasmid was transformed into *Escherichia coli* BL21 (TransGen, Beijing, China). Plasmid pcDNA3.1-CypA was purified using Endo-free Plasmid DNA Mini Kit (Omega bio-tek, Norcross, Georgia), quantified and stored in  $-20^{\circ}\text{C}$ , until further analysis.

### 2.6. Titration and expression analysis of GF-CypA during RGNNV infection

GF-1 cells was transfected separately with pcDNA-CypA and RGNNV at a Multiplicity of infections (MOI) of 1. After 2 h of adsorption at  $28^{\circ}\text{C}$ , the inoculum was removed. GF-1 cells were washed twice with PBS followed by adding L-15 with 4% FBS. After 12 and 24 h, supernatants were collected for virus titration assay by TCID50. Quantitative real-time PCR (qRT-PCR) assay was carried out to study the regulation pattern analysis of CypA or expression of viral genes or immune-related genes in GF-1 cells during RGNNV infection. The RNA samples of GF-1 cells post infected with RGNNV at an MOI of 1 were collected at 0, 3, 6, 12, 24, and 48 h post infection (hpi) by using the RNAiso Plus (Takara, Dalian, China) following manufacturer's instructions. Reverse transcription was carried out using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Beta-actin served as an internal control for the detection of viral mRNA in the cells, and all the primers used for qRT-PCR are listed in Table 1. The qRT-PCR assay was performed using a Light Cycler® 480 (Roche, USA), triplicate reactions were carried out in a volume of 20  $\mu\text{l}$  containing 10  $\mu\text{l}$  of SYBR Green I Master (Roche), 0.8  $\mu\text{l}$  of 10 mM of each forward and reverse primer, 7.4  $\mu\text{l}$  of nuclease-free water, and 1  $\mu\text{l}$  of cDNA. Cycling parameters were  $95^{\circ}\text{C}$  for 5 min followed by 40 cycles of  $95^{\circ}\text{C}$  for 10 s,  $58^{\circ}\text{C}$  for 10 s,  $72^{\circ}\text{C}$  for 10 s, finally at  $4^{\circ}\text{C}$  for 5 min. Dissociation curve analysis was performed to determine target specificity. The relative expression of the target genes versus  $\beta$ -actin gene was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method and all data were given in terms of relative mRNA expression.

### 2.7. CypA and RGNNV protein expression analysis during RGNNV infection in GF-1 cells

As described above 60–80% of confluent GF-1 cells were infected with RGNNV at MOI of 1 for 2 h in serum-free L-15 at  $28^{\circ}\text{C}$ . RGNNV

infected GF-1 cells were tested for the CypA and viral capsid protein expression by Western blot assay [37], with minor modifications. Briefly, for CypA and capsid protein, cellular lysates at different time points (as mentioned in Section 2.6) were resolved by sodium dodecyl sulfate 15% and 12% polyacrylamide gel electrophoresis (SDS-PAGE), respectively, and transferred onto nitrocellulose membranes (Bio-Rad). Subsequently, the membranes were blocked for nonspecific binding with Odyssey blocking buffer (Li-Cor Biosciences) for 1 h at room temperature. The specific polyclonal antibodies anti-RGNNV (Capsid) and anti-CypA were raised in rabbits and stored at  $-20^{\circ}\text{C}$  in our lab. The anti-RGNNV and anti-CypA primary antibodies were diluted at 1:500 dilutions, also anti- $\beta$ -actin at 1:10,000 with Odyssey blocking buffer then incubated to the respective membranes overnight at  $4^{\circ}\text{C}$ . After washing 3 times with TBST (Tris-buffered saline, 50 mM Tris/HCl, 150 mM NaCl, 0.1% Tween 20), infrared dye-linked donkey anti-rabbit IgG antibody (Gene Co., LTD., Shanghai, China) at 1:15,000 dilution was then added, and membranes were incubated at room temperature for 1 h. The immunoblots were visualized using an Odyssey infrared imaging system (Li-Cor Biosciences). Proteins were visualized, and differences in band intensity were quantified using the Odyssey® CLX Infrared Imaging System (Li-Cor Biosciences).

### 2.8. Transfection and siRNA knockdown

Short interfering RNAs (siRNAs) sequences synthesized by GenePharma Company (Shanghai, China) were utilized to knock down CypA in GF-1 cells. Transfection of siRNA sequences GF-CYP A-164, GF-CYP A-287, and a non-silencing siRNA (NS-siRNA) (Table 1) was performed according to manufacturers' protocol using Lipofectamine® RNAiMAX (Invitrogen, Life Technologies). Briefly, 60–80% of confluent GF-1 cells were transfected with above siRNA sequences in a 6-well plate. Before the transfection, all siRNAs of 10  $\mu\text{M}$  and 20  $\mu\text{L}/\text{mL}$  of Lipofectamine RNAiMAX reagent were suspended in 150  $\mu\text{l}$  of an Opti-MEM (Invitrogen) medium individually in separate tubes and incubated at room temperature for 5 min. Both the suspension (Lipofectamine + siRNA) was mixed together and allowed to stand for 15 min at room temperature to form siRNA-Lipofectamine complexes for all the three samples. Similar protocols were followed to prepare pcDNA3.1-CypA - Lipofectamine complexes. Pre-seeded GF-1 cells were allowed to incubate with 0.3 ml of siRNA or pcDNA3.1-CypA complexes or without pcDNA3.1-CypA for 12 h before infecting with RGNNV (MOI of 1.0). After 12 h of infection, total RNAs of differently treated cells were extracted to analyze for the target genes by qRT-PCR analysis.

### 2.9. Statistical analysis

Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by LSD test using SPSS 17.0. *p* value of  $< 0.05$  was considered significant and is indicated by an asterisk in the figures while *p* value of  $< 0.01$  is indicated by a double asterisk in the figures.



CypA, we used computational tools developed for protein structure prediction. The Phyre<sup>2</sup> online software predicted that GF-CypA contained three  $\alpha$  helical regions (14%) and 10  $\beta$  strands (36%) spread across N to C terminus, respectively (Fig. 1B). The figure highlights the secondary structure along with its confidence rate where 9 different color bands in the figure highlights the specific intensity of the confidence rate. The 3D structure of GF-CypA as shown in Fig. 1C was also predicted through Phyre<sup>2</sup> online software. To further evaluate the molecular evolution of GF-CypA, a phylogenetic tree was constructed based on multiple sequence alignments species as mentioned earlier. As shown in Fig. 1D, the phylogenetic analysis clearly showed that CypA gene obtained from GF-1 cells was formed from the teleost, amphibian, arthropod, avian, and mammalian clade. To get information about the conserved domains of GF-CypA protein, amino acid sequence of 8 different species, including mammal (*Homo sapiens*), avian (*Gallus gallus*), amphibian (*Xenopus laevis*), arthropods (*Penaeus monodon*, *Scylla paramamosain*), mollusks (*Azumapecten farreri*) and Pisces (*Ictalurus punctatus*, *Tachysurus fulvidraco*) were aligned. The results showed that the deduced amino acids of a single Cyp PPIase domain (YKGSFGFHRVIP-GFMCQGG) at site 48–65. This domain was almost conserved from invertebrates to vertebrates (Fig. 1E).

### 3.2. Homology analysis

The deduced amino acid sequence of GF-CypA showed a high homology with the sequence of other animals in GeneBank database, such as *H. sapiens* (72%), *G. gallus* (72%), *P. monodon* (74%), *X. laevis* (75%), *S. paramamosain* (77%), *T. fulvidraco* (79%), *I. punctatus* (81%), and *A. farreri* (81%). Furthermore, the percentage of identity, multiple amino acid alignments, and their representative genbank accession number were presented in Table 2.

### 3.3. Expression analysis of GF-CypA after RGNNV infection

To investigate the GF-CypA response to RGNNV infection in GF-1 cells, mRNAs and the proteins from the GF-1 cells post infected with RGNNV at several time points were analyzed by qRT-PCR and Western blot. The GF-CypA transcript levels gradually increased from 3 h after transfection and showed significantly elevated peaks at 12 hpi. At 24 hpi, the mRNA levels were decreased, while at 48 hpi the expression

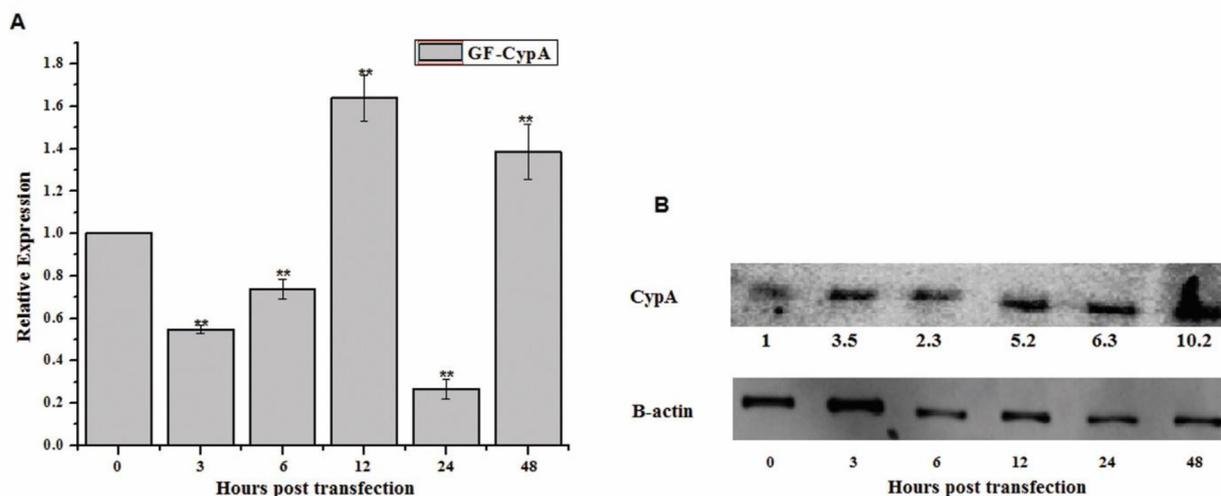
was again increased significantly (Fig. 2A). Similarly the protein expression pattern of GF-CypA results showed lowest band intensity stated as 1 at 0 hpi and gradually improved throughout the experiment up to 48 h after infection (Fig. 2B). Together, these results indicated that CypA expression could be induced in GF-1 cells by RGNNV infection.

### 3.4. Effect of GF-CypA on RGNNV expression

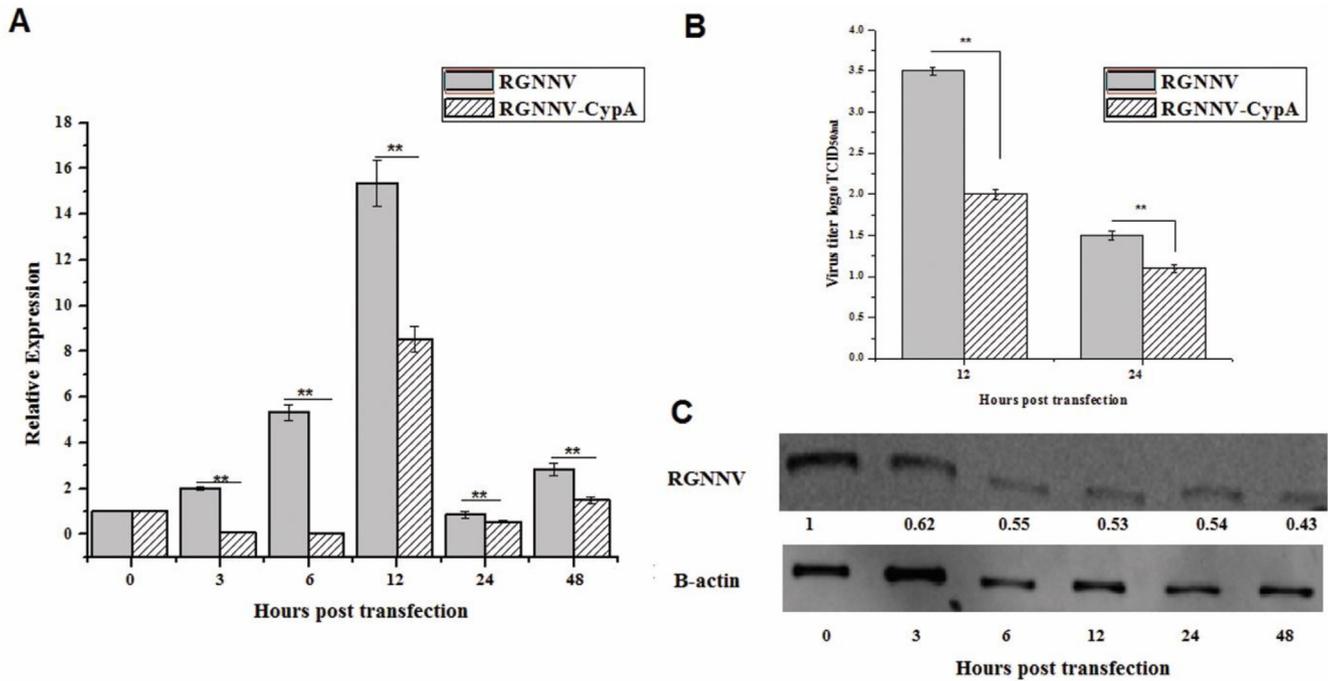
As described earlier, CypA has been reported that it can affect the viral replication. Consequently, it is essential to elucidate that if any RGNNV gene might be influenced during the induction of CypA genes in GF-1 cells. To study this, the mRNA levels of RGNNV capsid gene, expression were examined at different time points in GF-1 cells pre-incubated with or without pcDNA3.1-CypA. As shown in Fig. 3, mRNA transcript levels of RGNNV were gradually raised from 0 to 6 h that reached its highest significant level (approx. 16 points) at 12 hpi incubated without pcDNA3.1-CypA (Fig. 3A). Whereas the mRNA transcript of RGNNV from the pcDNA3.1-CypA pretreated cells were comparatively lower in all-time points and its highest significant level was only 9 points at 12 hpi. Fig. 3B demonstrates that CypA influenced the titration of RGNNV at both 12 and 24 h after transfection. Similarly, the expression pattern of RGNNV capsid protein showed highest band intensity stated as 1 at 0 hpi and gradually reduced throughout the experiment up to 48 h after infection (Fig. 3C).

### 3.5. Knockdown of GF-CypA upregulated the RGNNV level in GF-1 cells

In order to examine the impact of GF-CypA on RGNNV infection, CypA gene knocked down GF-1 cells was developed using siRNA technology and generated NS-siRNA, Cyp-164-siRNA, and Cyp-287-siRNA cell populations. Knockdown of specific genes for 12 h and expression at the transcriptional level was measured by qRT-PCR analysis. Notably, 10  $\mu$ M siRNA concentrations had significantly down-regulated the expression of Cyp-164-siRNA and Cyp-287-siRNA genes to about 80 and 70%, respectively, whereas no altered expression was seen in the control (NS-siRNA) cells (Fig. 4A). Conversely, the relative expression levels of RGNNV after 12 hpi in both 164-siRNA and 287-siRNA knockdown cells showed a remarkable comeback, compared with the cells transfected with NS-siRNA. Western blot results further confirmed



**Fig. 2.** The GF-CypA expression level after RGNNV infection. **A.** The samples were analyzed for mRNA expression pattern at 0, 3, 6, 12, 24, and 48 hpi in triplicates by qRT-PCR method.  $\beta$ -actin gene was used as the internal control to calibrate the cDNA template for all the samples. Data were shown as mean  $\pm$  SE (N = 3). The asterisk indicated a statistically significant difference ( $P < 0.05^*$ ,  $P < 0.01^{**}$ ) compared with that in control (0 h). **B.** Expression levels of CypA protein was determined by Western Blot analysis.  $\beta$ -actin was used as control. Bands were visualized using the Odyssey<sup>®</sup> CLx Infrared Imaging System and bands intensity was measured using Quantity One-4.6.2.



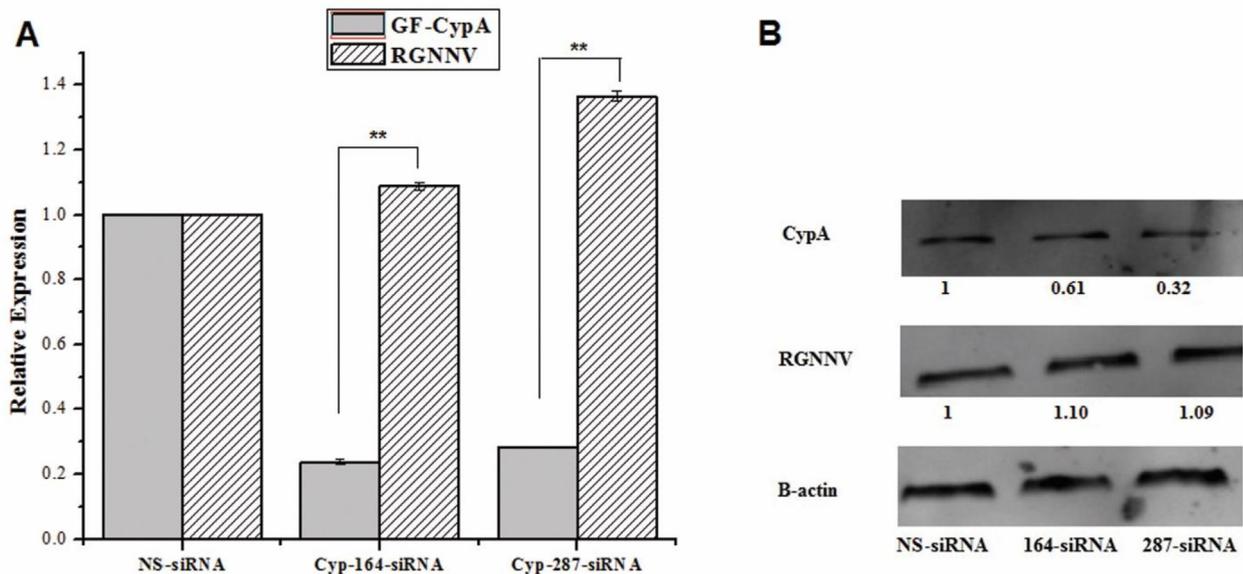
**Fig. 3.** The effect of GF-CypA on RGNNV expression. Expression patterns of genes were determined at 0, 3, 6, 12, 24, and 48 hpi by qRT-PCR method. **A.** RGNNV expression level with or without pcDNA3.1-CypA.  $\beta$ -actin was used as internal control. Data were shown as mean  $\pm$  SE (N = 3). The asterisk indicated a statistically significant difference ( $p < 0.05^*$ ,  $p < 0.01^{**}$ ) compared with that in control (0 h). **B.** The RGNNV titers in the supernatant were measured by using TCID<sub>50</sub>. **C.** Expression levels of RGNNV Capsid protein were analyzed by Western Blot.  $\beta$ -actin was used as control. Bands were visualized using the Odyssey<sup>®</sup> CLx Infrared Imaging System and bands intensity was measured using Quantity One-4.6.2.

the knockdown of cells and the down-regulation of CypA specific proteins, also, significant overcome of RGNNV protein expression levels compared with the NS-siRNA knockdown cells (Fig. 4B).

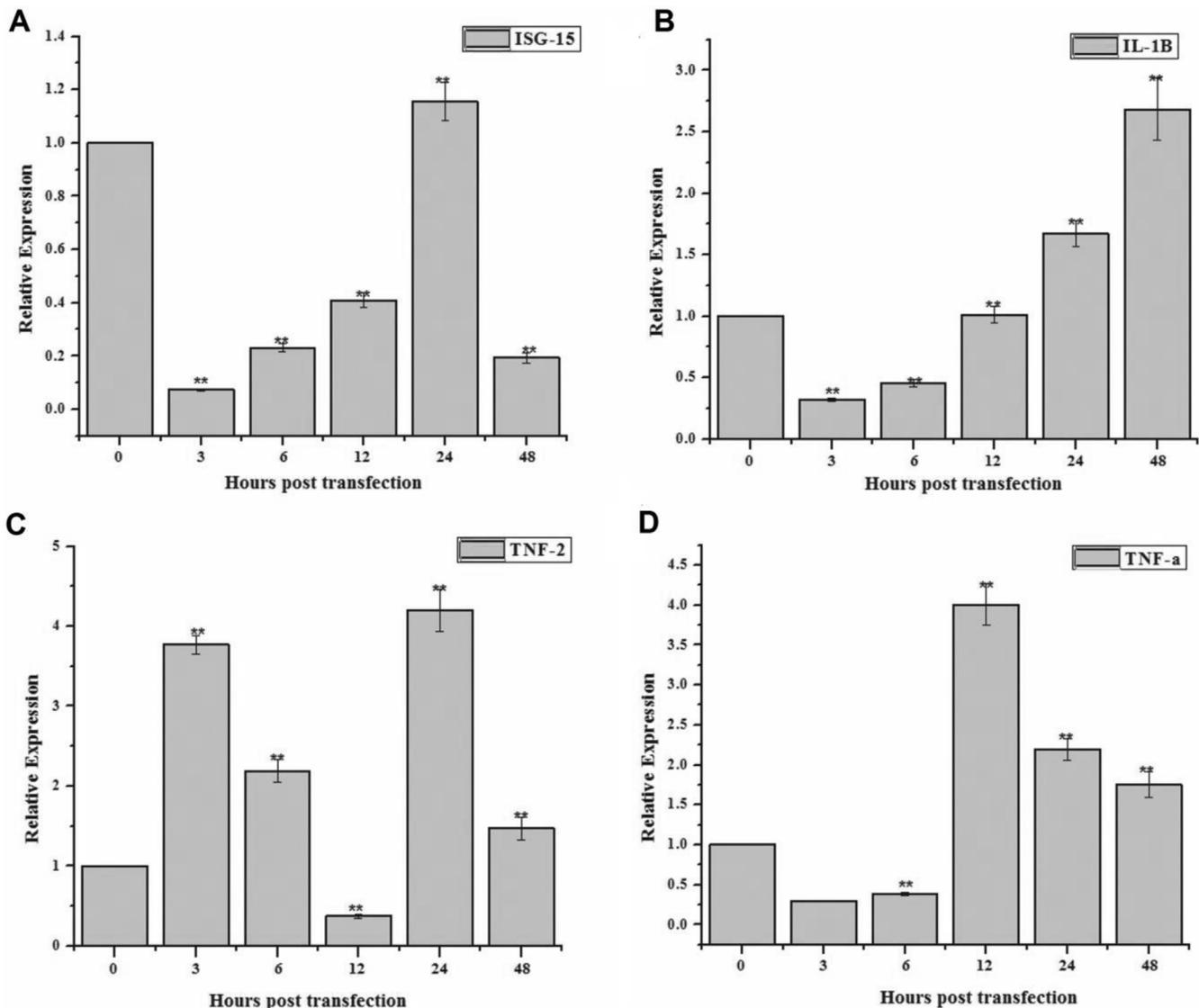
### 3.6. Effect of CypA on cytokines expression during RGNNV infection

It has been reported that CypA can be secreted by cells in response

to viral infection, resulting in overexpression of various cytokines. In the current study, we measured the mRNA levels of several cytokines that including ISG-15, TNF-2, IL-1B, and TNF- $\alpha$  at different time periods in GF-1 cells infected with RGNNV by qRT-PCR. The transcript levels in GF-1 cells before infection were set as 1. Fig. 5A shows the expression levels of ISG-15 that upregulated significantly at 3, 6, and 12 h and reached a peak at 24 hpi. However, we also observed a



**Fig. 4.** siRNA down regulation of GF-CypA gene expression and its effect on NNV replication. GF-1 cells were separately transfected with CypA-164-siRNA and Cyp-287-siRNA for regulation analysis. Non-Silencing (NS) siRNA was used as a negative control. After 12 h of siRNA transfection, GF-1 cells were infected with NNV at MOI of 1.0 and examined at 12 hpi. **A.** The gene expression level of CypA and RGNNV. **B.** Expression levels of CypA and RGNNV Capsid protein were analyzed by Western Blot.  $\beta$ -actin was used as control. Bands were visualized using the Odyssey<sup>®</sup> CLx Infrared Imaging System and bands intensity was measured using Quantity One-4.6.2.



**Fig. 5.** Cytokines expression analysis after NNV infection. Expression pattern analyses of cytokines were determined by qRT-PCR. The samples for relative expression analysis were analyzed at 0, 3, 6, 12, 24, and 48 hpi. **A.** ISG-15. **B.** IL-1B. **C.** TNF-2. **D.** TNF- $\alpha$ .  $\beta$ -actin used as internal control. Data were shown as mean  $\pm$  SE (N = 3). The asterisk indicated a statistically significant difference ( $P < 0.05^*$ ,  $P < 0.01^{**}$ ) compared with that in the control (0 h).

significant decrease in the expression of genes at 48 hpi. Similar gradual and significant increasing expression pattern was observed in IL-1B, and the expression was continued up to 48 hpi with RGNNV infection (Fig. 5B). While mRNA for TNF-2 analysis revealed a significant increase in 3 hpi and steadily decreased up to 12 hpi, still the second peak of expression could be observed at 24 hpi and again decreased at 48 hpi (Fig. 5C). Though, the TNF- $\alpha$  gene expression was not significantly increased (0.25) at 3, where at 6 hpi significantly increased and a sudden increase was stalled at 12 hpi (4.0) then progressively dropped until 48 hpi with RGNNV (Fig. 5D).

#### 4. Discussion

Although the Cyclophilin A has been reported previously as a modulator in several viral replications, little is known about their effects in RGNNV and its regulation, especially in the immune system of red spotted grouper fish. In this study, we discovered the grouper Cyclophilin A and named as GF-CypA after red spotted grouper fish. This is the first ever report of cloning and regulation study of Grouper fish Cyclophilin A. The full-length cDNA of GF-CypA was 743 bp, including an ORF of 495 bp encoding a polypeptide of 164 amino acids.

These results are consistent with the sequence analysis of other known CypAs such as *Pelteobagrus fulvidraco* [37] and *Penaeus monodon* [38]. Multiple sequence alignment and phylogenetic analysis of the amino acid sequences were compared with 10 animals in different taxa for molecular evolutionary studies of grouper CypA. The tree imprints classical taxonomy and phylogenetic transition from invertebrates to vertebrates, from aquatic to terrestrial, indicating that all Cyclophilins were evolved from a single clade, might have related biological functions and shared the same ancestral gene. Numerous reports have indicated that CypA was involved in various pathological conditions, including inflammation and infection [39,40]. Further to investigate the role of GF-CypA in defending infections, the expression of Cyclophilin A in GF-1 cells during RGNNV infection was measured by qRT-PCR. Interestingly, the expression of RGNNV and CypA were gradual and significantly increased in GF-1 cells till 12 hpi. However, the substantial decrease at 24 hpi remains unclear, further transcriptomic and gene expression investigations might reveal the exact immune mechanisms of GF-1 cells related to RGNNV infection, as observed by Pozo and colleagues in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) head-kidney leucocytes (HKLs) infected with NNV [41]. The expression pattern of GF-CypA was added to the

universality and the importance of the role-played by Cyclophilin A family. These results were in line with Liu and co-workers [42], they suggested that CypA expression level was up-regulated in response to avian H9N2 virus infection in human cells.

Nevertheless, for the first time, our study demonstrates that the Cyclophilin A gene has an inhibitory effect against the RGNNV replication in GF-1 *in vitro* model. In the current study, overexpression of Cyclophilin A down-regulated the RGNNV replication, while knocking down of CypA in GF-1 cells led to the overexpression of RGNNV. These results suggested that GF-CypA has a host inhibitory factor during RGNNV infection. In the previous study, overexpression of CypA was observed during the influenza virus infection in human gastric carcinoma (AGS) cell line [43]. It could be due to host antiviral response and CypA may play an essential role in the immune system of red spotted grouper. On the other hand, some researchers have reported that CypA was necessary for replication of HCV [44,45]. It is noteworthy that the CypA protein can play distinctive roles in different viruses. The peculiar contrivance of CypA during RGNNV infection may need to be elucidated in future studies.

Previously, it was believed that CypA functions primarily as an intracellular protein. Subsequent studies have proven that the potential role of CypA secretion has induced the production of pro-inflammatory cytokines [46]. Our results indicated that up-regulation of IL-1 $\beta$  gene expression during RGNNV infection in GF-1 cells transfected with pcDNA3.1-CypA at either time points, suggesting a close molecular association between CypA and cytokines in GF-1 cells. Overexpression studies of CypA performed in rheumatoid arthritis induced expression of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  [18]. Among fish, the upregulation of ISG-15 in black sea bream [47] and IL-1 $\beta$  & TNF- $\alpha$  gene expression in the brains of gilthead sea bream, sea bass [48,49], and turbot [50] after NNV infection has been reported. These CypA regulated cytokines could follow a pathway that is dependent on the NF- $\kappa$ B activation. Even so, the mechanism by which CypA facilitates signaling to activate the NF- $\kappa$ B pathway remains to be explored.

These results implied that GF-CypA plays a vital role in the immune response of grouper fish to NNV infection. Studies on the precise role of these proteins are still in the beginning stage, and the exact mechanism of anti-RGNNV activity remained to be explored in the future. Further investigations of the molecular mechanism of how GF-CypA inhibits RGNNV replication may help us better understand host-virus interactions from anti-infection perspective. These findings will have a major impact on scientific areas comprising genetic improvement to fight against NNV infection, development of viral vectors for gene therapy and discovery of new anti-viral drug targets.

#### Authors contribution

Li Lin, Hongyan Kou and Jiagang Tu designed the research. Muhammad Asim, Li Lin, Jianguo Su, Jun Li and Sarath Babu finalized the manuscript. Muhammad Asim, Zhendong Qin, and Lijuan Zhao, performed the experiments, contributed to the data collection and statistical analysis.

#### Conflicts of interest

The authors declare no conflict of interest.

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