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Protective immunity induced by DNA vaccine encoding viral membrane protein against SGIV infection in grouper

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ABSTRACT

Singapore grouper iridovirus (SGIV) is the main grouper-infecting virus in southern China that causes serious economic losses. However, there is no effective way to control this viral disease. In this study, SGIV *ORF19R* (*SGIV-19R*) encoding a viral membrane protein was constructed into pcDNA3.1-HA and then was used to evaluate the immune protective effects in grouper *Epinephelus coioides*. Subcellular localization showed that SGIV-19R distributed in the cytoplasm and co-localization analysis indicated the protein partially co-localized with the endoplasmic reticulum (ER). RT-PCR and Western blot analyses confirmed the expression of the vaccine plasmids in grouper muscle tissues. Moreover, the transcription levels of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), myxovirus resistance 1 (Mx1) and immunoglobulin M (IgM) genes were significantly up-regulated in the spleen, liver and kidney of vaccinated groupers. SGIV challenge experiments showed the relative percent survival (RPS) was significantly enhanced in fish with 49.9% at the DNA dose of 45 μg pcDNA3.1–19R, while 75.0% RPS when using 90 μg pcDNA3.1–19R. Meanwhile, vaccination with pcDNA3.1–19R significantly reduced the virus replication, evidenced by a low viral load in the spleen of survivals groupers after SGIV challenge. These results imply that pcDNA3.1–19R could induce protective immunity in grouper, and might be a potential vaccine candidate for controlling SGIV disease.

1. Introduction

Groupers (*Epinephelus* spp.) are valuable fish species in Asian aquaculture and widely distributed in eastern and southern areas of China [1]. However, viral diseases caused by Grouper nervous necrosis virus (GNNV) and Singapore grouper iridovirus (SGIV) or Grouper iridovirus (GIV) are the major threat to grouper *Epinephulus* spp. These pathogens are responsible for tremendous economic losses of grouper farms in southern provinces of China, such as Hainan [2–4]. SGIV or GIV is a large, double-stranded DNA (dsDNA) virus that belongs to genus *Ranavirus*, family *Iridovirdae* [5], and the virus particle is icosahedral that has envelope outside. The SGIV caused massive loss of wild and farmed grouper *Epinephulus* spp. and other marine teleosts in aquaculture [6], however, there is no effective way to control this viral disease in grouper fish farm.

Viral surface antigens are mainly localized to the outer envelope or capsid surface of virions [7,8]. This feature could be a potential vaccine target against viral infection. Vaccination is deemed one of the most effective approaches to control viral diseases in aquatic animals. Several different types of candidate vaccines, including inactivated virus vaccine, virus-like particle vaccine and recombinant subunit vaccine have been developed against ranavirus diseases. These vaccines conferred effective protection to the host against viral challenge in laboratory trials [9,10]. DNA vaccine represents an attractive substitute of traditional vaccines due to its ability to elicit potent humoral and cellular immunity. Moreover, DNA vaccine has various advantages, which include low production cost, high safety, and ease of long-term storage [11]. In aquaculture, DNA vaccines have been well studied and have been proven to be effective against viral pathogens, such as red seabream iridovirus (RSIV) [12], and infectious spleen and kidney necrosis virus (ISKNV) [13].

The SGIV genome contained 162 putative open reading frames (ORFs) which are classified as immediate-early (IE), delayed early (DE) and late (L) genes [14]. SGIV *ORF19R* (*SGIV-19R*), a core gene of iridovirids, has homologues in all sequenced iridovirids. It consists 1029 bp and encodes a protein of 342 aa with a predicted molecular weight

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of 37 kDa. SGIV-19R contains a DUF230 domain (aa 173–253), a proline-rich domain (aa 267–315) and a transmembrane domain (aa 318–342) [15]. Previous study indicated that *SGIV-19R* was a late gene, encoding a putative viral envelope protein [15]. However, the knowledge about protective immunity induced by *SGIV-19R* against SGIV remains unclear. As an economic and marine fish species, groupers have suffered from ranavirus diseases in natural habitats or on farms since 2000 [3]. In this study, we developed a DNA vaccine that expresses SGIV-19R protein, and evaluated its efficacy in protecting groupers from SGIV infection.

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed in accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of China. The protocol was approved by the Institutional Animal Care and Use Committee of the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, and all efforts were made to minimize suffering.

2.2. Fish, cells, and virus

Juveniles of orange-spotted groupers, *Epinephelus coioides* (116.62 ± 18.26 g in weight) were obtained from Wenchang City, Hainan Province, China. The groupers were maintained in aerated seawater at room temperature, and were fed daily with fishing bait. Prior to experiments, the groupers were assured free of SGIV infection by PCR assay. *Epithelioma papulosum* cyprinid (EPC) cells were cultured at 25 °C in medium 199 with 10% fetal bovine serum (FBS). SGIV was originally isolated from diseased groupers in Hainan, China, and was propagated in Fathead minnow (FHM) cells [3].

2.3. Plasmids construction

Based on the complete genome sequences of SGIV (GenBank accession no. AY521625), the full-length sequence of *SGIV-19R* open reading frame (ORF) was amplified from SGIV infected FHM genomic DNA using specific primers SGIV-19R–F1/R1 (Table 1). The amplified fragment was cloned into eukaryotic expression vector pEGFP-N3 (Invitrogen, USA) using *Xho* I and *EcoR* I restriction enzyme sites. The recombinant plasmid, designated as pEGFP-19R, was confirmed by restriction enzyme digestions and DNA sequencing. The pEGFP-19R is used for analysis of the intracellular distribution of SGIV-19R.

Likewise, the designed primers SGIV-19R–F2/R2 (Table 1) were also used to amplify the *SGIV-19R* ORF to introduce restriction enzyme sites. The amplified fragment was cloned into eukaryotic vector pcDNA3.1-HA (Invitrogen, USA) to obtain the plasmid pcDNA3.1–19R, which will be used for a DNA vaccine. The plasmids pEGFP-19R, pEGFP-N3, pcDNA3.1–19R and pcDNA3.1-HA were purified from the Endo-free Plasmid Midi Kit (Omega Bio-Tek, USA).

2.4. Subcellular localization of SGIV-19R in EPC cells

EPC cells were seeded into 6-well culture plates and grown in medium199 supplemented with 5% fetal bovine serum (FBS). The cells were transfected with pEGFP-19R or the empty vector (pEGFP-N3) using Lipofectamine® 3000 (Invitrogen) according to the manufacturer's instruction. At 24 and 48 h post transfection (hpt), the cells were rinsed with 1 × PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min. Subsequently, the cells were permeabilized with 0.2% Triton X-100 for 15 min and stained with hoechst33342 (1 μg/mL) for 10 min. Finally, the cells were mounted with 50% glycerol, and visualized under fluorescence microscope (OLYMPUS DP80).

To find out the relationship between SGIV-19R protein and organelles such as endoplasmic reticulum (ER), mitochondria (Mito) and Golgi apparatus (Golgi), three plasmids of organelle-specific markers pDsRed2-ER, pDsRed2-Mito and pDsRed2-Golgi were used. Each plasmid of organelle-specific marker was co-transfected with pEGFP-19R into EPC cells using Lipofectamine 3000 transfection kit. The cells were fixed at 24 hpt and 48 hpt and were observed by fluorescence microscopy as described above.

2.5. Vaccination and challenge experiments

The plasmid pcDNA3.1–19R was diluted in sterile 1 × PBS buffer to 150 μg/ml and 300 μg/ml, respectively, while pcDNA3.1-HA was diluted to 300 μg/ml. Ninety-nine healthy groupers were randomly divided into three groups (33 animals/group). Each group of animals were intramuscularly injected with 300 μl of pcDNA3.1–19R (150 μg/ml), pcDNA3.1–19R (300 μg/ml), or pcDNA3.1-HA (300 μg/ml), respectively. These groups were designated as pcDNA3.1–19R(L) group, pcDNA3.1–19R(H) group, and pcDNA3.1(C) group. Three groupers of each group were necropsied to collect muscle (of injection sites), spleen, liver and kidney samples at 7 days post vaccination (dpv). The samples were frozen in liquid nitrogen immediately, then stored at −80 °C until used. The rest groupers were challenged with 5×10^6 $TCID_{50}$ SGIV through intraperitoneally injection at 15 dpv. Groupers from same group were randomly distributed into three separated tanks and kept as described above at room temperature (27 ± 1 °C). The dead animals were collected daily and mortality rates were calculated at 21 days post viral challenge. Relative percentage survival (RPS) was calculated according to the following formula: RPS = [1- (death

Table 1
The primers used in this study (enzyme cleavage sites are underlined).

Primers	Primers sequences (5′-3′)	Usage
SGIV-19R–F1	CC<u>CTCGAG</u>AAATGGCATCGTCCACTATAC (*Xho* I)	pEGFP-19R
SGIV-19R-R1	CC<u>GAATTC</u>TTATTTTGTCGCGAGAAAAAATATAAACACC (*EcoR* I)	
SGIV-19R–F2	CC<u>GAATTC</u>AATGGCATCGTCCACTATAC (*EcoR* I)	pcDNA3.1–19R
SGIV-19R-R2	CC<u>CTCGAG</u>TTATTTTGTCGCGAGAAAAAATATAAACACC (*Xho* I)	
β-actin-F	TACGAGCTGCCTGACGGACA	RT-PCR, qRT-PCR
β-actin-R	GGCTGTGATCTCCTTTTGCA	
Mx1-F	CGAAAGTACCGTGGACGAGAA	qRT-PCR
Mx1-R	TGTTTGATCTGCTCCTTGACCAT	
TNFα-F	GTGTCCTGCTGTTTGCTTGGTA	qRT-PCR
TNFα-R	CAGTGTCCGACTTGATTAGTGCTT	
IL-1β-F	AACCTCATCATCGCCACACA	qRT-PCR
IL-1β-R	AGTTGCCTCACAACCGAACAC	
IgM-F	ATACCTGATGCTGTTCCAGTG	qRT-PCR
IgM-R	AAGGTGATGGCTGTGGTTAC	
MCP-F	ACTCGTAAGATCGCCACGGAAGATT	qPCR
MCP-R	ACGTTTCTCAAATGCATGTCTGCCAC	

vaccinated group/total vaccinated group)/(death control group/total control group)] × 100% [10].

2.6. Detection of transcription and expression of vaccine plasmids in groupers

Total RNA of injection site muscle was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Two μg of RNA was reverse transcribed into the first strand cDNA using PrimScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). PCR was carried out to detect the transcription of vaccine plasmid using specific primers SGIV-19R–F2/R2. The detection of *β-actin* mRNA was used as an internal control.

To prepare total protein lysates, frozen muscle tissues were homogenized in 50 mM Tris-HCl buffer (pH 7.5) with protease inhibitor cocktail (Roche, USA). The lysates were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane for Western blot analysis. Mouse anti-HA sera (Vector Laboratories, USA) was used as primary antibody (1: 2000), and peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (Vector Laboratories, USA) was used as the secondary antibody (1: 2000). The signals were detected with chemiluminescent horseradish peroxidase (HRP) substrate (Millopore). Simultaneous internal control was performed by detecting β-actin protein with anti-β-actin antibody (Boster; 1: 2000).

2.7. Quantitative real-time PCR (qPCR) analysis of transcription of immune genes

Total RNAs of spleen, liver and kidney samples were extracted and then subjected to cDNA synthesis as described above. Quantitative reverse transcription PCR (qRT-PCR) were used to measure the relative RNA amounts of immune response related genes, which included tumor necrosis factor alpha (TNF-α, FJ009049), interleukin-1 beta (IL-1β, EF582837), myxovirus resistance 1 (Mx1, DQ440574) and immunoglobulin M (IgM, AY885708) genes. qRT-PCR was carried out by using SYBR green real-time PCR master mix reagents kit (CWBIO, China) in StepOne real-time PCR system (Applied Biosystems, USA) with a cycle condition of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All samples were tested in triplicate. Transcription level of grouper immune genes, which was normalized by internal control *β-actin*, was evaluated based on $2^{-\triangle\triangle Ct}$ method [16]. All data were analyzed with Student's *t*-test. Values of $P < 0.05$ were considered significant.

2.8. Determination of SGIV viral load by qPCR

Three death and three surviving individuals were randomly selected from each group to evaluate SGIV viral load. Total DNA were extracted from the spleen tissues of the sampled animals using TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (Takara) according to the manufacturer's instructions. Viral copy numbers were quantified by qPCR as described previously using specific primers MCP-F/R (Table 1) that anneals to SGIV major capsid protein (MCP) gene [17]. Viral load in samples was measured as the mean of the three replicates, then viral DNA copies per microgram of tissue was calculated.

3. Results

3.1. SGIV-19R localized in cytoplasm of EPC cells

The subcellular localization of SGIV-19R was assessed by detecting the distribution of SGIV-19R-GFP fusion protein in EPC cells. As shown in Fig. 1, SGIV-19R was mainly detected in the cytoplasm at 24 and 48 hpt. Interestingly, the diffused green fluorescence signals at 24 hpt clamped into granular appearance at 48 hpt. While the control, which expressed GFP alone, distributed in whole cells and mainly in the nucleus of fish cells at 48 hpt. These results indicate that SGIV-19R is localized in the cytoplasm.

3.2. SGIV-19R partially co-localized with endoplasmic reticulum (ER)

To determine the precise subcellular localization of SGIV-19R protein, the relationship between SGIV-19R protein and organelles such as ER, Mito and Golgi were analyzed. As shown in Fig. 2, SGIV-19R localized in the cytoplasm which further confirmed the above result, while DsRed2-ER localized in the specific regions in the ER. Further analysis indicated that the signal from EGFP-labelled SGIV-19R partially overlapped with signal from ER-specific RFP, which was manifested by the yellow fluorescence in the merged channel of cells at 24 and 48 hpt. However, such overlapping was not observed in cells that mitochondria or Golgi apparatus were RFP-labelled (data not shown). The results suggested that SGIV-19R protein partially co-localized to the ER in EPC cells.

3.3. DNA vaccine expressed SGIV-19R in vaccinated groupers

RT-PCR was performed to analyze the transcription of pcDNA3.1–19R in the muscle tissues of vaccinated groupers at 7 dpv. The result showed that the transcripts of the *SGIV-19R* genes were detected in both pcDNA3.1–19R(L) group and pcDNA3.1–19R(H) group by a pair of *SGIV-19R* specific primers, while no amplification was obtained in the negative control group (Fig. 3A). Furthermore, the amount of transcripts in pcDNA3.1–19R(L) group was less than that of pcDNA3.1–19R(H) group. This is contrast to the transcript accumulation level of the reference gene *β-actin*, which are maintained at a similar level in pcDNA3.1–19R(L) and pcDNA3.1–19R(H) group.

To analyze the expression of pcDNA3.1–19R in the muscle tissues of vaccinated groupers, Western blot was performed at 7 dpv. The result showed the specific immunoreactive bands of approximately 36 kDa (SGIV-19R) were detected in pcDNA3.1–19R(L) group and pcDNA3.1–19R(H) group, but no specific band was obtained in pcDNA-3.1(C) group (Fig. 3B). Furthermore, the amount of SGIV-19R in pcDNA3.1–19R(L) group was less than that of pcDNA3.1–19R(H) group when the equal amounts of input were loaded. Equal amounts of loading were evident by the similar amounts of β-actin protein in all three groups (Fig. 3B, lower panel). Collectively, these results demonstrated that DNA vaccine pcDNA3.1–19R expressed SGIV-19R in vaccinated groupers and the higher expression level of SGIV-19R correlated with higher dose of vaccination.

3.4. Transcription level of immune related genes

We evaluated the transcription levels of four immune-related genes, namely *TNFα*, *Mx1*, *IL-1β*, and *IgM* genes in spleen, liver, and kidney of groupers at 7 dpv by qRT-PCR. As shown in Fig. 4, all four examined genes were up-regulated to different extent in the tested organs of vaccinated groupers. In detail, the transcription levels of *TNFα* were significantly up-regulated (more than 1.4-fold) in the spleen, liver and kidney of pcDNA3.1–19R(L) group comparing with pcDNA3.1(C) group. The highest up-regulation was found in spleen (1.9-fold), followed by liver (1.6-fold) and kidney (1.4-fold). Meanwhile, the transcription levels of *TNFα* were found more effective in the pcDNA3.1–19R(H) group, which was 2.11-, 2.01- and 1.50-fold in the respective spleen, liver and kidney tissues. The transcription of *Mx1* were significantly increased at least 1.9-fold in the pcDNA3.1–19R(L) group and 1.97-fold in the pcDNA3.1–19R(H) group. Similarly, the mRNA levels of *IL-1β* were also up-regulated in vaccinated groups. However, the up-regulation of this gene varied among organs, with about 2.7-fold increase in the spleen while much less significant increase in the liver and kidney. The mRNA levels of *IgM* was remarkably up-regulated in the spleen and liver more effective than that of in the kidney between the SGIV-19R expressing group and vector expressing

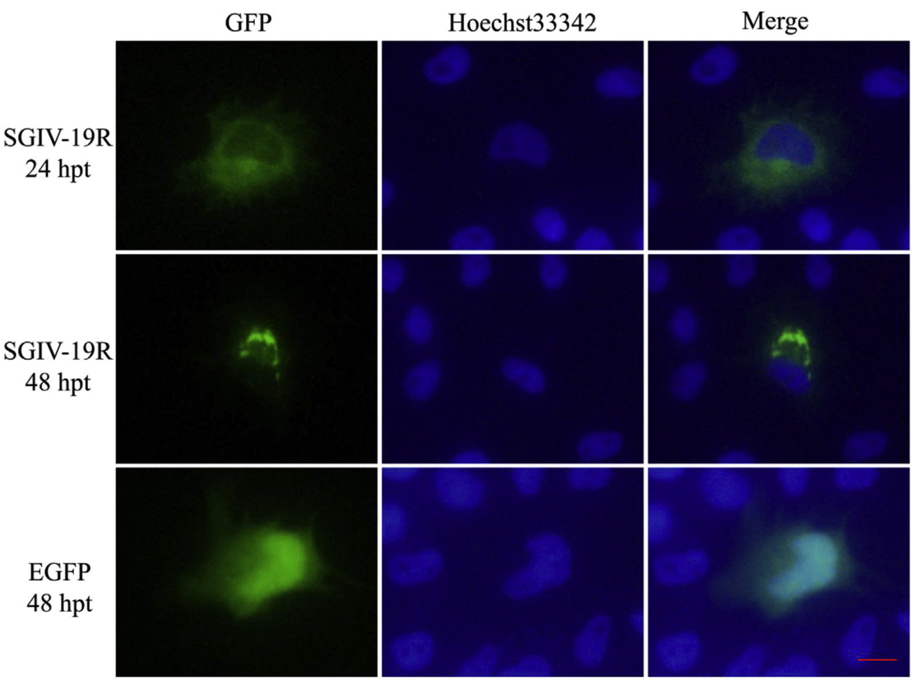

Fig. 1. SGIV-19R localized in cytoplasm of EPC cells. EPC cells were transfected with pEGFP-19R or pEGFP-N3. The fluorescence signals were observed at 24 hpt and 48 hpt. Green fluorescence showed the distribution of EGFP or EGFP-fused SGIV-19R; blue fluorescence showed the nucleus. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

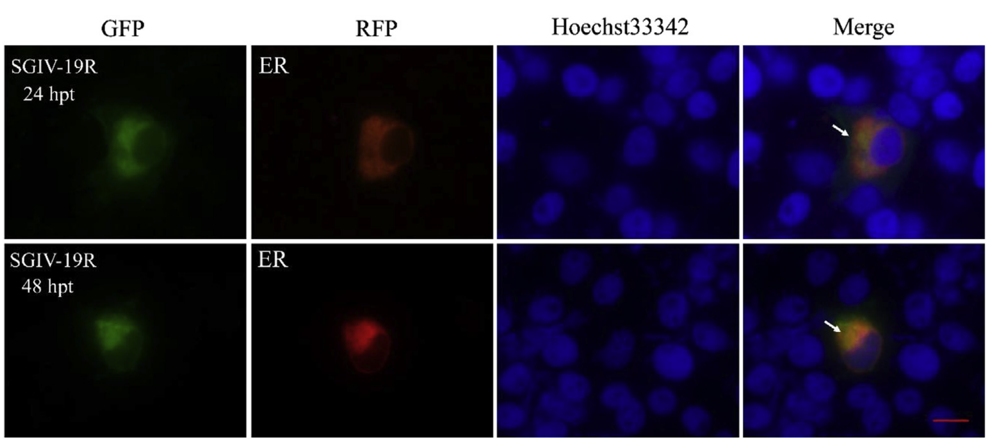

Fig. 2. SGIV-19R partially co-localized with ER. EPC cells were transfected with pEGFP-19R and pDsRed2-ER. The fluorescence signals were observed at 24 hpt and 48 hpt. Green fluorescence showed the distribution of EGFP-fused SGIV-19R; red fluorescence showed the distribution of ER containing RFP; blue fluorescence showed the nucleus; yellow fluorescence (white arrows) showed co-localization of SGIV-19R and ER. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

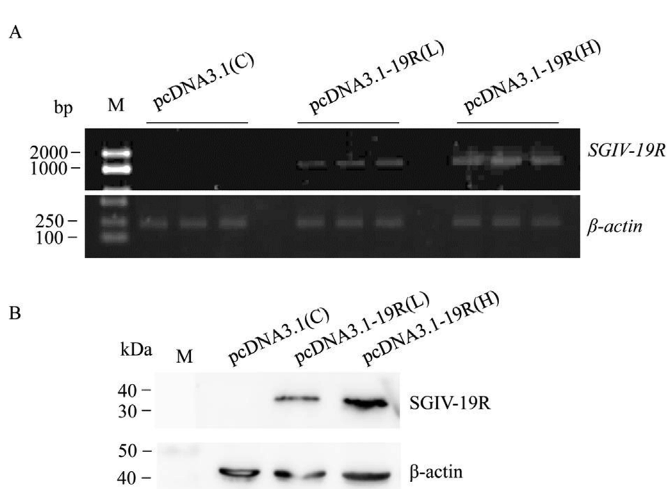

Fig. 3. Transcription and expression of the *SGIV-19R* in vaccinated groupers muscles. RT-PCR (A) and Western blot (B) analysis of transcription and expression of *SGIV-19R* gene in muscle tissues from vaccinated groupers at 7 dpv (n = 3). The *β-actin* mRNA and its protein were used as internal controls.

group.

3.5. Protection of DNA vaccination

To examine the protective effect of the vaccines, the vaccinated groupers were challenged intraperitoneally with SGIV at 15 dpv and the mortality was monitored until to 21 days post-inoculation (dpi). As shown in Fig. 5, the death was firstly recorded at 6 dpi in the pcDNA3.1(C) group, followed at 8 dpi in the pcDNA3.1–19R(L) group and at 10 dpi in the pcDNA3.1–19R(H) group. The cumulative mortalities reached 53.3% in pcDNA3.1(C) group, whereas the cumulative mortalities of the pcDNA3.1–19R(L) group and pcDNA3.1–19R(H) group were 26.7% and 13.3%, respectively (Fig. 5). Compared to the pcDNA3.1(C) group, the relative percent survival (RPS) values of the pcDNA3.1–19R(L) and pcDNA3.1–19R(H) groups were 49.9% and 75.0%, respectively (Table 2). Overall, these data indicate that plasmid pcDNA3.1–19R, at a proper dose, could function as an effective vaccine against SGIV infection in grouper.

3.6. Detection of SGIV viral load

The SGIV viral load in the spleen tissues of death and survivals

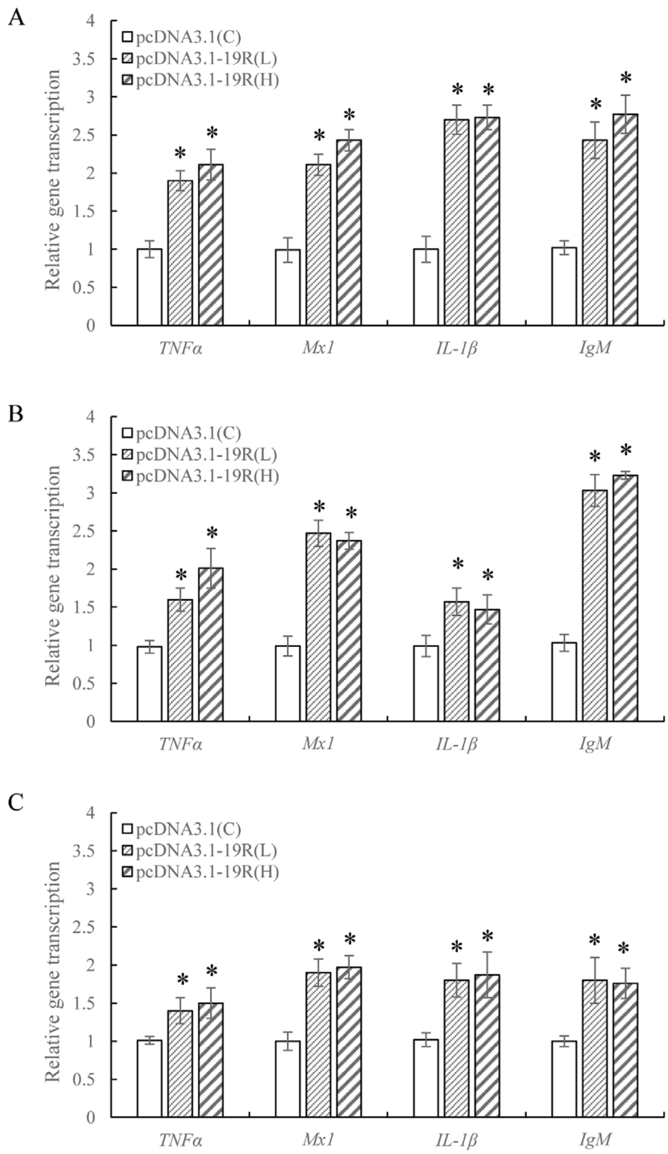

Fig. 4. qRT-PCR analysis of the transcription levels of *TNFα*, *Mx1*, *IL-1β*, and *IgM* genes in spleen (A), liver (B), and kidney (C) tissues from vaccinated groupers. The mRNA level of each gene was normalized to that of *β-actin* mRNA. For each gene, the mRNA level of the pcDNA3.1(C) group was set as 1. Asterisks indicate significant differences from the control group. Data are presented as means ± SE (n = 3). $^{*}P < 0.05$.

groupers in each group were further determined by qPCR. As shown in Table 3, the survival (S) groupers had a low level of viral load ranging from 1.32×10^1 to 1.21×10^2 copies/μg viral DNA, and less of viral load when more DNA vaccine used. In contrast, a high level of viral load with more than 5.33×10^6 copies/μg viral DNA in the spleen tissues of dead groupers was detected both from SGIV-19R expressing group and vector expressing group groups. During challenge trials, the dead groupers showed typical hemorrhage symptoms of SGIV infection, while survival groupers did not show the symptoms.

4. Discussion

In this study, SGIV-19R localized in the cytoplasm and partially co-localized to the ER. RGV 2L, a homologue of SGIV-19R, has been reported as viral membrane protein. It also partially co-localized with ER in the cytoplasm, but it aggregated into viral factories during virus infection [18]. Similarity, SGIV-19R initially localized in the cytoplasm,

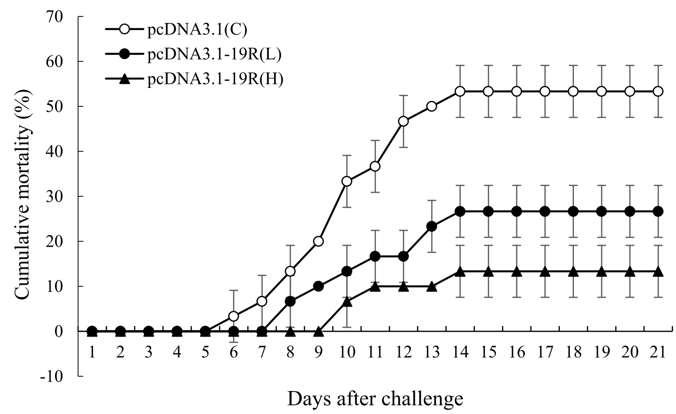

Fig. 5. Cumulative mortality curves of vaccinated groupers upon challenge with SGIV. The vaccinated groupers were intraperitoneally challenged with SGIV and monitored daily for mortality over a 21-days observation period. Data are presented as means ± SE (n = 3).

Table 2
Cumulative mortality and relative percentage survival (RPS) of vaccinated groupers challenged with SGIV.

Vaccinated groups	Cumulative mortality (death/total)	RPS%
pcDNA3.1–19R(H)	13.3% (4/30)	75.0
pcDNA3.1–19R(L)	26.7% (8/30)	49.9
pcDNA3.1(C)	53.3% (16/30)	–

RPS = [1-(death vaccinated group/total vaccinated group)/(death control group/total control group)] × 100%.

Table 3
SGIV viral load determined by qPCR in the spleen tissues from the survival and dead of vaccinated groupers after viral challenge.

Vaccinated groups	Death (D) or survival (S)	Viral load (DNA copies/μg)
pcDNA3.1(C)	S	1.21×10^2
pcDNA3.1(C)	D	9.27×10^6
pcDNA3.1–19R(L)	S	2.44×10^1
pcDNA3.1–19R(L)	D	5.67×10^6
pcDNA3.1–19R(H)	S	1.32×10^1
pcDNA3.1–19R(H)	D	5.33×10^6

and then aggregated into the virus factory during virus infection. Furthermore, *SGIV-19R* was revealed to encode a viral envelope protein, and its homologues in other ranaviruses have been proved to be essential for virus infection and assembly [15]. As a viral membrane protein, it could be a potential vaccine target against virus infection. In addition, the major capsid protein (MCP) has been used to against GIV-Taiwan isolate and the protein effectively protected groupers from the virus infection [19]. Meanwhile, the DNA vaccines of *36L*, *39L*, and *MCP* from SGIV have exhibited effectively protect effects [20]. Although significant progresses have been achieved by vaccines responses against SGIV infection, no report from viral membrane protein as a DNA vaccine against the SGIV is available.

RT-PCR and Western blot analyses indicated that the transcription and expression of *SGIV-19R* were confirmed at muscle tissues of injection site. In addition, the low levels of *SGIV-19R* transcripts in spleen, liver and kidney were detected (data not shown). However, the specific protein bands could be not detected at these tissues by using Western blot analysis (data not shown). Therefore, multi-points booster vaccinations may significantly up-regulate the transcription and expression levels of *SGIV-19R*. Although low expression of *SGIV-19R* in spleen, liver and kidney tissues, the transcription level of *TNFα*, *Mx1*, *IL-1β*, and *IgM* genes were significantly up-regulated. Furthermore, vaccination with pcDNA3.1–19R significantly reduced the virus replication in the spleen of survival groupers after SGIV challenge. Moreover, the groupers vaccinated with more dose of DNA vaccine, the more protective effects against SGIV challenge was obtained.

Active antibody response is an important process for vaccine-mediated protection against viral diseases [21]. In this study, transcriptional profiles of four immune-related genes of vaccinated groupers were analyzed. The transcription of *IgM* genes was significantly up-regulated in spleen, liver and kidney. IgM is mainly distributed in the serum [22]. Similarly, orange-spotted groupers infected with GIV, could active IgM antibody, which provided partial passive protection to groupers [23]. Furthermore, the transcription levels of *TNFα*, *Mx1*, and *IL-1β* were also significantly up-regulated in liver, spleen and kidney in SGIV-19R expressing groupers. The biological activity of TNFα accounts for 70%–95% of the total activity of TNF and it is produced by macrophages [24]. *Mx1* is a broad antiviral host gene that can be induced by viruses [25]. Therefore, it is attempting to speculate that pcDNA3.1–19R triggers the innate antiviral immune response and humoral and cell-mediated immune responses in vaccinated groupers, and induces protective effects against SGIV [11,26].

Previous reports have revealed that the spleen of groupers is the main target organ of SGIV [27,28]. In this study, viral load in the spleen of survival groupers at 21 days after SGIV challenge were investigated. As demonstrated, the survivals of SGIV-19R expressing groupers had a viral load of less than 2.44×10^1 copies/μg viral DNA, which was significantly lower than that of dead groupers and survivals from pcDNA 3.1(C) group. The low viral load in the survivors of SGIV-19R expressing groupers suggested that pcDNA3.1–19R had triggered effective immune responses that resulted in inhibition of viral replication. However, the iridovirids are often carried in the bodies of vertebrates and some of them may be chronic or conditional that are not easily inactivated in host body [29,30]. It is also evidence that ranavirus may exist long-term in the asymptomatic groupers [30]. The fate of SGIV in the survivals of vaccinated groupers after challenge needs further investigation.

In conclusion, our results showed that pcDNA3.1–19R, a DNA vaccine encoding SGIV-19R protein, conferred effective protection upon SGIV challenge in groupers. Moreover, pcDNA3.1–19R expressing could enhance innate immune response and adaptive immune response in grouper, which are essential for combating SGIV infections.

Author contributions

N.T. Yu conceived and designed the experiments; N.T. Yu and X.B. Zheng performed the experiments; N.T. Yu and Z.X. Liu analyzed the data and wrote the paper.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References

[1] T.S. Chen, Y.C. Wu, S.C. Chi, Decreasing salinity of seawater moderates immune response and increases survival rate of giant groupers post betanodavirus infection, Fish Shellfish Immunol. 57 (2016) 325–334.
[2] C. Li, J.X. Liu, X. Zhang, S.N. Wei, X.H. Huang, Y.H. Huang, et al., Fish autophagy protein 5 exerts negative regulation on antiviral immune response against Iridovirus and Nodavirus, Front. Immunol. 10 (2019) 517.
[3] N.T. Yu, Y.L. Zhang, Z. Xiong, L. Qu, Z.X. Liu, A simplified method for the simultaneous detection of nervous necrosis virus and iridovirus in grouper *Epinephelus* spp, Acta Virol. 63 (2019) 80–87.
[4] Q. Yu, M. Liu, H. Xiao, S. Wu, X. Qin, Z. Lu, et al., The inhibitory activities and antiviral mechanism of *Viola philippica* aqueous extracts against grouper iridovirus infection *in vitro* and *in vivo*, J. Fish Dis. 42 (2019) 859–868.
[5] V.G. Chinchar, P. Hick, I.A. Ince, J.K. Jancovich, R. Marschang, Q. Qin, et al., ICTV virus taxonomy profile: *iridoviridae*, J. Gen. Virol. 98 (2017) 890–891.
[6] P.J. Walker, J.R. Winton, Emerging viral diseases of fish and shrimp, Vet. Res. 41 (2010) 51.
[7] L. Liu, S. Gao, W. Luan, J. Zhou, H. Wang, Generation and functional evaluation of a DNA vaccine co-expressing Cyprinid herpesvirus-3 envelope protein and carp interleukin-1 beta, Fish Shellfish Immunol. 80 (2018) 223–231.
[8] H. Zhang, S. Zhou, L. Xia, X. Huang, Y. Huang, J. Cao, et al., Characterization of the VP39 envelope protein from Singapore grouper iridovirus, Can. J. Microbiol. 61 (2015) 924–937.
[9] X. Zhou, X. Zhang, Y. Han, Q. Jia, H. Gao, Vaccination with recombinant Baculovirus expressing Ranavirus major capsid protein induces protective immunity in Chinese giant salamander, *Andrias davidianus*, Viruses 9 (2017) 195.
[10] Z.Y. Chen, T. Li, X.C. Gao, C.F. Wang, Q.Y. Zhang, Protective immunity induced by DNA vaccination against ranavirus infection in Chinese giant salamander *Andrias davidianus*, Viruses 10 (2018) 52.
[11] C. Collins, N. Lorenzen, B. Collet, DNA vaccination for finfish aquaculture, Fish Shellfish Immunol. 85 (2019) 106–125.
[12] C.M. Caipang, T. Takano, I. Hirono, T. Aoki, Genetic vaccines protect red seabream, Pagrus major, upon challenge with red seabream iridovirus (RSIV), Fish Shellfish Immunol. 21 (2006) 130–138.
[13] X. Fu, N. Li, Q. Lin, H. Guo, D. Zhang, L. Liu, et al., Protective immunity against infectious spleen and kidney necrosis virus induced by immunization with DNA plasmid containing mcp gene in Chinese perch *Siniperca chuatsi*, Fish Shellfish Immunol. 40 (2014) 259–266.
[14] V.G. Chinchar, T.B. Waltzek, K. Subramaniam, Ranaviruses and other members of the family *Iridoviridae*: their place in the virosphere, Virology 511 (2017) 259–271.
[15] X. Huang, J. Gong, Y. Huang, Z. Ouyang, S. Wang, X. Chen, et al., Characterization of an envelope gene VP19 from Singapore grouper iridovirus, Virol. J. 10 (2013) 354.
[16] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$, Method 25 (2001) 402–408.
[17] D.V. Koubourli, E.S. Wendel, A. Yaparla, J.R. Ghaul, L. Grayfer, Immune roles of amphibian (*Xenopus laevis*) tadpole granulocytes during Frog Virus 3 ranavirus infections, Dev. Comp. Immunol. 72 (2017) 112–118.
[18] L.B. He, F. Ke, J. Wang, X.C. Gao, Q.Y. Zhang, *Rana grylio* virus (RGV) envelope protein 2L: subcellular localization and essential roles in virus infectivity revealed by conditional lethal mutant, J. Gen. Virol. 95 (2014) 679–690.
[19] H.I. Liu, P.P. Chiou, H.Y. Gong, H.Y. Chou, Cloning of the Major capsid protein (MCP) of Grouper Iridovirus of Taiwan (TGIV) and preliminary evaluation of a recombinant MCP vaccine against TGIV, Int. J. Mol. Sci. 16 (2015) 28647–28656.
[20] Z. Ou-yang, P. Wang, Y. Huang, X. Huang, Q. Wan, S. Zhou, et al., Selection and identification of Singapore grouper iridovirus vaccine candidate antigens using bioinformatics and DNA vaccination, Vet. Immunol. Immunopathol. 149 (2012) 38–45.
[21] D.R. Burton, Antibodies, viruses and vaccines, Nat. Rev. Immunol. 2 (2002) 706–713.
[22] M. Faisal, I.F. Standish, M.A. Vogelbein, E.V. Millard, S.L. Kaattari, Production of a monoclonal antibody against of muskellunge (*Esox masquinongy*) IgM heavy chain and its use in development of an indirect ELISA for titrating circulating antibodies against VHSV-IVB, Fish Shellfish Immunol. 88 (2019) 464–471.
[23] M.S. Wu, C.W. Chen, Y.C. Liu, H.H. Huang, C.H. Lin, C.S. T, et al., Transcriptional analysis of orange-spotted grouper reacting to experimental grouper iridovirus infection, Dev. Comp. Immunol. 37 (2012) 233–242.
[24] J. Alijotas-Reig, E. Esteve-Valverde, R. Ferrer-Oliveras, E. Llurba, J.M. Gris, Tumor necrosis factor-alpha and pregnancy: focus on biologics. An updated and comprehensive review, Clin. Rev. Allergy Immunol. 53 (2017) 40–53.
[25] P. Dam Van, D. Desmecht, M.M. Garigliany, D. Bui Tran Anh, A.S. Van Laere, Anti-influenza a virus activities of type I/III interferons-induced Mx1 GTPases from different mammalian species, J. Interferon Cytokine Res. 39 (2019) 274–282.
[26] R.A. Dalmo, DNA vaccines for fish: review and perspectives on correlates of protection, J. Fish Dis. 41 (2018) 1–9.
[27] X.W. Wang, J.Q. Ao, Q.G. Li, X.H. Chen, Quantitative detection of a marine fish iridovirus isolated from large yellow croaker, *Pseudosciaenacrocea*, using a molecular beacon, J. Virol. Methods 133 (2006) 76–81.
[28] N. Jiang, Y. Fan, Y. Zhou, W. Liu, J. Ma, Y. Meng, et al., Characterization of Chinese giant salamander iridovirus tissue tropism and inflammatory response after infection, Dis. Aquat. Org. 114 (2015) 229–237.
[29] J. Go, R. Whittington, Australian bass *Macquaria novemaculeata* susceptibility to experimental megalocytivirus infection and utility as a model disease vector, Dis. Aquat. Org. 133 (2019) 157–174.
[30] A.L.J. Duffus, T.B. Waltzek, A.C. Stöhr, M.C. Allender, M. Gotesman, R.J. Whittington, et al., Distribution and host range of ranaviruses, in: M.J. Gray, V.G. Chinchar (Eds.), Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates [M], Springer, New York, NY, 2015, pp. 9–59.