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Cloning and characterisation of type I interferon receptor 1 in orange-spotted grouper (*Epinephelus coioides*) for response to nodavirus infection

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ABSTRACT

Grouper is known as a highly economical teleost species in the Asian aquaculture industry; however, intensive culture activities easily cause disease outbreak, especially viral disease. For the prevention of viral outbreaks, interferon (IFN) is among the major defence systems being studied in different species. Fish type I IFNs are known to possess antiviral properties similar to mammalian type I IFNs. In order to stimulate antiviral function, IFN will bind to its cognate receptor, the type I interferon receptor (IFNAR), composed of heterodimeric receptor subunits known as IFNAR1 and IFNAR2. The binding of type I interferon to receptors assists in the transduction of signals from the external to internal environments of cells to activate biological responses. In order to study the function of IFN, we first need to understand IFN receptors. In this study, we cloned and identified IFNAR1 in orange-spotted grouper (osgIFNAR1) and noted the up-regulated mRNA expression of the receptor and downstream effectors in the head kidney cells with cytokine treatment. The transcriptional expression of osgIFNAR1, which is characterised using polyinosinic-polycytidylic acid (poly[I:C]) and lipopolysaccharide (LPS) treatments, indicated the involvement of osgIFNAR1 in the immune response of grouper. The subcellular localisation of osgIFNAR1 demonstrated scattering across the grouper cell. Viral infection showed the negative feedback regulation of osgIFNAR1 in grouper larvae. Further loss of function of IFNAR1 showed a decreased expression of the virus. This study reported the identification of osgIFNAR1 and characterisation of receptor sensitivity towards immunostimulants, cytokine response, and viral challenge in the interferon pathway of orange-spotted grouper and possible different role of the receptor in viral production. Together, these results provide a frontline report of the potential function of osgIFNAR1 in the innate immunity of teleost.

1. Introduction

Groupers comprise some of the important economical aquaculture species in Taiwan; however, mortality rates of 80%–100% in grouper larvae and juveniles have been reported to be related to nervous necrosis virus (NNV) in Taiwan [1]. This resulted in huge economical losses in major industry profits. NNV belongs to the genus *Betanodavirus* in the Nodaviridae family, with a non-enveloped T = 3 capsid and virus genome encoding two positive-sense single-stranded RNAs (RNA1 and RNA2) [2,3]. As of now, there is no effective vaccine or solution to eradicate this viral disease in the larval stages of aquaculture species. Different studies have been carried out to investigate interactions between the virus and innate immunity of teleosts to prevent viral disease outbreaks; in a previous study, the grouper Mx protein interacted with the viral protein and could be regulated by the interferon (IFN)-dependent pathway against NNV [4,5].

The IFN system is one of the important innate mechanisms for antiviral defence in the host. IFN has been described as secreted proteins or cytokines capable of inducing an antiviral state in cells [6]. IFNs are categorised into types I, II, and III based on the distinct receptor

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interactions, characteristics, and types of immune response they initiated. Type I and III IFNs belong to specific signalling pathways that activate innate immune defences against viral infection, while type II IFNs are mainly involved in defence against pathogens and host allergic response [7]. Thus far, there are nine identified type I IFN subtypes in mammals; these subtypes transduce signals downstream through type I interferon receptor (IFNAR) complexes [8]. Type I IFN signals are transduced via binding to the IFNAR; the presence of receptors is crucial for delivering IFN antiviral signals [9]. Functions of the IFN receptors have been related to interactions with a variety of ligands, signal transduction, and biological responses [10]. Upon binding of type I IFN to IFNAR complexes, the antiviral state is stimulated through the activation of the JAK-STAT pathway [11]. IFNAR complexes are composed of two receptor subunits, termed interferon receptor 1 and 2 (IFNAR1/IFNAR2), which form a heterodimer complex [10]. Phosphorylation of the IFNAR1 subunit stimulates the recruitment of signal transducer and activator of transcription 1 (STAT1) and STAT2 molecules to its intracellular domain [12]. While IFNAR2 subunit interact with an additional tyrosine kinase to the receptor complex, it does not appear to decide the specificity of signalling [13]. This binding leads to the transcription of dsRNA-dependent protein kinase R (PKR) and GTPase Mx protein antiviral genes [9]. The biological response of interferon was found to be dependent on IFNAR1, suggesting that main response of IFNAR1 in type I IFN interaction is to allow ligand binding and direct tyrosine kinase 2 (Tyk2) recruitment to the receptor complex [14]. The distribution of IFNAR is tightly regulated to control duration and intensity of different IFN signalling processes [7]; the presence of receptors could determine the widespread or cell-specific functions upon interactions with different IFNs. The mechanism behind regulating presence of IFNAR on the surface of the cell and how they recognise the diversity of IFNs and mediate signalling for their respective functions is still unknown.

Type I IFNAR belongs to the class II cytokine receptor family (CRFB) [10]. In CRFBs, type I IFNAR1/2 is a single transmembrane protein comprising fibronectin type III (FN-III) motifs connected to form their extracellular domain [15]. Thus far, teleosts have 17 known CRFB members, containing more CRFB members than humans [16–18]. Based on gene synteny report from previously reported species, zebrafish CRFB1/2 with specificity to IFNs might be the equivalent homologs to human IFNAR2, while CRFB5 could serve as IFNAR1 [19]. The gain and loss of function of IFN and its receptors in zebrafish CRFB1 and CRFB5 proved to be responsible for the antiviral properties that belong to group I type I IFNs containing two cysteines, while CRFB2 and CRFB5, which have similar antiviral results, belong to four cysteine group II type I IFNs [6]. The antiviral immune role is reported when mice lacking interferon receptors showed increasing susceptibility to infection with different kinds of viruses [20]. Using purified recombinant IFN protein to induce the transient expression of downstream ISG or viperin, expression would be down-regulated significantly without the presence of IFN receptors [21]. Recently, more studies on the immune activity of receptors have reported their diverse roles in the immune pathway of aquaculture species [19,22,23]; in Atlantic salmon and Dabry's sturgeon, an increase of cytokine receptor expression was shown in response to polyinosinic-polycytidylic acid (poly[I:C]) and lipopolysaccharide (LPS) treatments [24,25], indicating the sensitivity of the receptor to immunostimulants.

In order to contribute a possible virus prevention strategy involving the innate immune system, grouper type I IFN [26] and IFN-induced antiviral protein Mx [4,5] have been previously reported. To induce downstream effectors, IFN would bind to their receptor; however, the IFN receptor in orange-spotted grouper (*Epinephelus coioides*) is unclear; thus, the purpose of this study were to (i) identify type I interferon receptor 1 in orange-spotted grouper (osgIFNAR1), (ii) assess bioinformatic analysis for osgIFNAR1, (iii) evaluate expression of osgIFNAR1 under LPS and poly(I:C) treatments, (iv) determine localisation of osgIFNAR1 in GF-1 cell, (v) induce activation of Mx promoter reacting to ligands in presence of osgIFNAR1, (vi) evaluate expression of osgIFNAR1 and osgMx1 under ligand stimulation, (vii) evaluate expression of osgIFN and osgIFNAR1 under viral challenge, (viii) determine mRNA expression of NNV in loss of function test. These results may give new insights on the molecular transduction of IFNAR1 in the type I interferon pathway on the innate immunity of fish.

2. Materials and methods

2.1. Fish and challenge experiments

Orange-spotted groupers were obtained from the Core Facility of Grouper Bio-resources in the Translational Center for Marine Biotechnology, An-Nan Campus, National Cheng Kung University (Tainan, Taiwan). For naïve tissue sampling, juvenile fish (body weight: 3.5 inch, 90-dph) were sacrificed and total of 12 different organs were collected for tested NNV free before proceed for naïve tissue sampling. For the poly(I:C) and LPS challenge experiments, juvenile groupers (body weight: 5.0 ± 0.2 g, 60 days post hatching [dph]) reared at 28 °C were given intraperitoneal injections with either 50 μL of poly (I:C) (1 mg/mL in phosphate buffered saline [PBS]; Sigma-Aldrich, St. Louis, MO, USA), 50 μL of LPS (0.4 mg/mL in PBS, Sigma-Aldrich), or 50 μL of PBS to serve as a control. For the NNV challenge experiment, grouper larvae (body weight: 0.3 ± 0.1 g, 30 dph) were divided into two groups (40 fish/group). To confirm that the fish were initially free of NNV, six randomly selected fish were tested for NNV RNA2 primers (Table 1) by real-time PCR (qPCR). Each group was immersed in 1 L of fish-rearing seawater with moderate aeration, which contained either 50 mL of a viral solution (10^6 $TCID_{50}$/0.1 mL) or saline, for 3 h at 28 °C. The fish were then transferred to a virus-free aquarium and reared at 28 °C. The fish were sacrificed and collected at 0, 12, 24, 48, and 72 h after immersion for gene expression measurements. The NNV used in this study was isolated and purified as reported previously [27]. Harvested tissues were placed in TRIzol™ reagent (Ambion, Carlsbad, CA, USA) and stored at −80 °C until processing. All animal experiments were performed in strict accordance with the recommendations in the guide for the Institutional Animal Care and Use Committee (IACUC) under reference number 103084, National Cheng Kung University.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from fish samples homogenised in TRIzol™ reagent (Ambion, Carlsbad, USA) using a MagNA Lyser homogeniser (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo, USA), and the first-strand cDNA was synthesised with 2 μg of total RNA, 0.25 mM dNTP, 4 μM random hexamer, and 200 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) at 37 °C for 60 min.

2.3. Cloning and sequencing of osgIFNAR1

To obtain the full length of osgIFNAR1, rapid amplification of the 5′ and 3′ cDNA ends (5′/3′ RACE) was performed with the 5′/3′ RACE Kit, 2nd Generation (Roche, Basel, Switzerland). The design of gene-specific primers (IFNAR1-5′-SP1, IFNAR1-5′-SP2, and IFNAR1-5′-SP3; IFNAR1-3′-F1 and IFNAR1-3′-F2) for 5′ and 3′ RACE was based on the partial osgIFNAR1 sequence assembled from zebrafish (ABJ97310.1) and rainbow trout (ADU04482.1) derived from NCBI database and laboratory genome database. For 5′ RACE, cDNA was transcribed from total RNA using IFNAR1-5′-SP1 and purified using the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland). The 5′ end was polyadenylated with terminal deoxynucleotidyl transferase (TdT) (Promega, Madison, WI, USA), and PCR was performed with the IFNAR1-5′-SP2 and an oligo-dT adaptor primer. Nested PCR was performed with the IFNAR1-5′-SP3 and an adaptor primer. For 3′ RACE,

Table 1
Primers used in this study.

Primers	Primer sequence (5' - 3')*	Application	Annealing temperature	PCR fragment size
IFNAR-F1	GGCAGATCTTGGGGTACTTCC	Gene cloning	58 °C	
IFNAR-R1	GTACTCCACAGGGGGCAGCTCC		58 °C	
IFNAR-F2	TGAACAACATAGGTGTCATTCC		54 °C	
IFNAR-R2	GAGGGCATTGTAGACATGTGTG		54 °C	
IFNAR1-3′-F1	TGAACAACATAGGTGTCATTCC		60 °C	
IFNAR1-3′-F2	GGCAGATCTTGGGGTACTTCC		60 °C	
IFNAR1-5′-SP1	GAGGGCATTGTAGACATGTGTG		60 °C	
IFNAR1-5′-SP2	AAGGTGTCATTCCATGGTGGC		60 °C	
IFNAR1-5′-SP3	TGCCCTGATAAAGATGCTGCT		60 °C	
Oligo-dT Anchor Primer	GACCACGCGTATCGATGTCGAC TTTTTTTTTTTTTTT		60 °C	
Anchor Primer	GACCACGCGTATCGATGTCGAC		60 °C	
IFNAR1-FL-F1	GGCACATCGACCGGTGGAGAC		58 °C	
IFNAR1-FL-R1	GAGGGGAAAGCATCAGATCTGTA		58 °C	
IFNAR1-FL-F2	GACAGGAGAACGAGTCTGGAC		55 °C	
IFNAR1-FL-R2	AGCTGTCACTGTGAAACCCAGA		55 °C	
IFNAR1_pcDNA_V5_F	GCGGGTACCATGAGAGCATATGA	Plasmid construction	56 °C	
IFNAR1_pcDNA_V5_R	TAGACATGTGTGTCTCTAGACGG	Plasmid construction	56 °C	
IFNAR1-QF	CCACACTTCCAGTATCTTTATGAC	Real Time PCR	60 °C	197 bp
IFNAR1-QR	CTGTGTCTGCCACTGCTGTCTGG			
β-actin QF	CCAGGCATCAGGGAGTGATGG	Real-Time PCR	60 °C	258 bp
β-actin QR	CCAGACAGCAGTGGCAGACACAG			
Mx1-QF	TTCATACAGCTGGCCCACAGT	Real-Time PCR	60 °C	100bp
Mx1-QR	GCAGTGGACTCTTTTTCTTGCTTAA			
NNV RNA2-QF	GACGCGCTTCAAGCAACTC	Real-Time PCR	60 °C	203bp
NNV RNA2-QR	CGAACACTCCAGCGACACAGCA			

cDNA was transcribed using an oligo-dT adaptor primer and PCR was performed with IFNAR1-3′-F1, IFNAR1-3′-F2, and the adaptor primer. The full-length of osgIFNAR1 PCR products were amplified with IFNAR1-FL-F1/R1 and IFNAR1-FL-F2/R2, followed by cloning and sequencing as described as above. All primers (Table 1) were synthesised by Genomics BioSci & Tech (Taipei, Taiwan).

2.4. Bioinformatic analysis

The cDNA of osgIFNAR1 was translated into protein using the Translate Tool (http://web.expasy.org/translate/) and analysed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The homologous conserved domains were identified using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The putative protein structure was determined using the SWISS-MODEL program with the automatic modelling mode (http://swissmodel.expasy.org/). Alignment of multiple sequences was performed by the MegAlign program (LaserGene v7.1, DNASTAR Inc.). The overall similarity of the sequences was determined using the neighbour-joining algorithm from MEGA 5. Bootstrap values were calculated with 10,000 replications to estimate the robustness of internal branches.

2.5. Quantitative real-time PCR

The synthesised cDNA was further diluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a ratio of 1:4 and stored at −20 °C until use. The gene specific primers for qPCR were designed to cross the intron/exon boundaries (Table 1). The qPCR analysis was performed with GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's protocol. Amplification was performed in the StepOnePlus™ Real-Time PCR system (Applied Biosystems, California, USA) with the following parameters: an initial step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, with melting curve analysis. The expression of each target gene was normalised to that of β-actin, and expression relative to the control was calculated using the 2-ΔΔCT method. The quantification method of the virus copy number used in this study was as reported previously [27].

2.6. Plasmid construction

The coding sequences of osgIFNAR1 were amplified by PCR with the primer IFNAR1_pcDNA_V5_F/R. The fragments were separated on a 0.8% agarose gel, purified with a gel extraction kit (Qiagen, Hilden, Germany), and digested with restriction enzymes (KpnI and XbaI). The digested fragments were subcloned into the pcDNA3.1/V5-His A expression vector (Clontech, California, USA), and the resultant plasmids were verified by sequencing.

2.7. Cell culture, transfection, and virus infection

Grouper fin cells (GF-1, BCRC 960094) were obtained from the Bioresources Collection and Research Center (BCRC), Taiwan. GF-1 cells were seeded in 6-well plates (3.0×10^5 cells/well) and cultured in Leibovitz's L-15 medium (Gibco, Massachusetts, USA) with 5% foetal bovine serum (FBS) (Gibco, Massachusetts, USA) at 28 °C [28]. Wells in triplicate were transfected with 4.0 μg of pcDNA3.1-osgIFNAR1-V5-His or pcDNA3.1-V5-His using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. After 24 h, cells were infected with NNV (10^4 $TCID_{50}$/0.1 mL) by incubation in the medium without FBS for 1 h, and washing three times with PBS, and then adding fresh L-15 medium with 1% FBS. At 24 h post NNV infection, total RNAs were extracted for qPCR analysis of NNV RNA2 sequences.

2.8. Loss of function of IFNAR1 and virus infection

For IFNAR1 knockdown experiments, all siRNAs were predicted and synthesised by MDbio Inc. (MDbio, Taiwan) as duplexes: siRNA-IFNAR1, 5′-GCAGACUGAAGGUGUCAUUTT-3′ and siRNA-Control, 5′-UUCUCCGAACGUGUCACGUTT-3′. GF-1 cells (3×10^5) in 6-well plates were transfected with designed siRNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After transfection with designed siRNAs for 24 h, cells were collected to evaluate the effect of siRNA on total IFNAR1 mRNA levels by qPCR using the primers IFNAR1-QF and IFNAR1-QR (Table 1). Other cells were further infected with NNV and cell lysates were used to evaluate the NNV copy numbers through qPCR at different time points post infection.

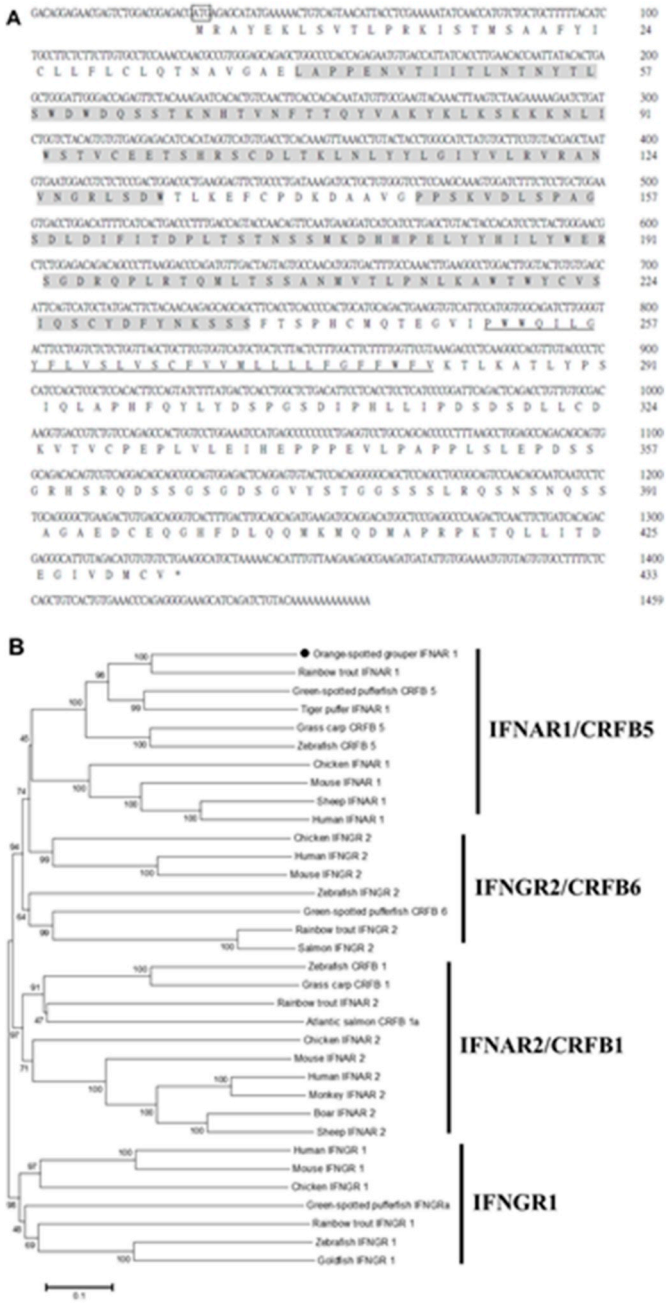

Fig. 1. Nucleotide and deduced amino acid sequences of type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) (A) and phylogenetic tree (B) analysis of osgIFNAR1 with other IFNAR1/class II cytokine receptor family member 5 (CRFB5) family homologs.
(A) Start codon (ATG) is bolded in white, deduced signal peptide is boxed black, conserved fibronectin-III and transmembrane (TM) domains are indicated with grey boxes and underlined, respectively. Deduced intracellular cytoplasmic domain are boxed. (B) A phylogenetic tree was constructed using the neighbour-joining method with MEGA5.0 software, the amino acid sequences were from selected teleosts and mammals, aligned using CLUSTALW with a bootstrap value of 10,000 replicates. The accession numbers of selected sequences are as follows: Chicken IFNAR1 (NP_990190.1), Mouse IFNAR1 (NP_034638.2), Sheep IFNAR1 (CAA65183.1), Human IFNAR1 (NP_000620.2), Grass carp CRFB5 (AGZ04472.1), Zebrafish CRFB5 (ABJ97310.1), Rainbow trout IFNAR1 (ADU04482.1), Green-spotted puffer CRFB5 (CAD67766.1), Tiger puffer IFNAR1 (XP_003976720.1), Zebrafish CRFB1 (ABJ97307.1), Grass carp CRFB1 (AGW21650.1), Rainbow trout IFNAR2 (AGO14284.1), Chicken IFNAR2 (AAD13677.1), Mouse IFNAR2 (CAA70943.1), Human IFNAR2 (CAG46616.1), Monkey IFNAR2 (ABZ80248.1), Boar IFNAR2 (NP_001191704.2), Sheep IFNAR2 (NP_001009342.1), Chicken IFNGR1 (NP_001123859.1), Mouse IFNGR1 (NP_034641.1), Human IFNGR1 (NP_000407.1), Pufferfish IFNGRa (AEI70477.1), Zebrafish IFNGR1 (AAI63407.1), Goldfish IFNGR1 (ACV41808.1), Rainbow trout IFNGR1 (ABY87188.1), Human IFNGR2 (NP_005525.2), Mouse IFNGR2 (NP_032364.1), and Chicken IFNGR2 (NP_001008676.1), Zebrafish IFNGR2 (NP_001071095.2), Green-spotted pufferfish IFNGR2 (Q7ZT07), Rainbow trout IFNGR2 (ABY87189.1), Atlantic salmon IFNGR2 (AIN50150.1). IFNAR1 from orange-spotted grouper is shown with a black circle and different receptor families are indicated on the right.

2.9. Immunofluorescence staining

GF-1 cells were seeded on coverslips in 6-well plates (3.0×10^5 cells/well) and grown at 28 °C. For viral infection, cells were infected with NNV (10^2 $TCID_{50}$/0.1 mL) in the L-15 medium without FBS for 1 h, washed three times with PBS, and then incubated in fresh L-15 medium with 1% FBS for 24 h. Cells were fixed for 15 min at 28 °C with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Cells were washed twice with PBS and then permeabilised with PBST (0.1% Triton X-100 [w/v] in PBS) for 5 min. Cells were washed with PBST and blocked for 30 min at room temperature with 5% BSA (w/v) in PBST. Coverslips were incubated at 4 °C overnight with a mouse monoclonal anti-6 × His tag antibody (Abcam, Cambridge, UK) at 1:1000 dilution. After incubation, cells were washed three times with PBST and incubated for 1 h at room temperature with Alexa Fluor 594 goat anti-rabbit IgG at 1:1000 dilution (Invitrogen Life Technologies, Carlsbad, CA USA). Nuclei were stained with 10 μg/mL of Hoechst 33,342 (Invitrogen Life Technologies, Carlsbad, USA), after which the cells were washed three times with PBST and mounted in Fluoromount-G® (Southern Biotechnology Associates, Birmingham, USA). Images from an LSM 780 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) were recorded and analysed with the manufacturer's software.

2.10. Primary cell culture

The protocol was followed according to Wang (2017, unpublished). Orange-spotted grouper with an average weight of 25 g were anaesthetised with 2-phenoxyethanol (Sigma-Aldrich, MO, USA) and then sacrificed. Head kidneys were collected and transferred through a 100-μm nylon mesh with cold Leibovitz's L-15 medium containing 0.1% foetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD, USA) plus penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich, MO, USA). The cell suspension was centrifuged at 400 × *g* for 5 min and then resuspended with L-15 medium containing 2% FBS and penicillin/streptomycin. The viability of cells was calculated using a haemocytometer with trypan blue staining (Invitrogen, CA, USA). The total viable cells were coordinated to 1.5×10^6 cells/mL in L-15 medium with 2% FBS and penicillin/streptomycin and seeded in 6-well plates. Cells were incubated at 28 °C and prepared for treatment.

2.11. Recombinant type I IFN stimulation on primary head kidney cells

The protocol was modified from that of Wang (2017, unpublished). Head kidney cells were stimulated with an optimal dose of type I IFN (10 ng/mL) diluted in L-15 medium and incubated at 28 °C for 4, 12, and 24 h [26]. After stimulation, suspended cells were collected and centrifuged at 400 × *g* for 10 min to collect adherent and non-adherent cells in head kidney, followed by careful decanting of the supernatant. Then, 1 mL of TRIzol® reagent (Invitrogen, CA, USA) was used to lyse by pipetting the cell lysate several times for RNA extraction, as described earlier.

2.12. Promoter transfection and luciferase assay

The GF-1 cell was co-transfected using Lipofectamine 2000 (Invitrogen). Firstly, cells from the treatment group (30 min post

transfection) were treated with 50 ng/mL of recombinant grouper type I IFN and 50 ng/mL poly(I:C). Another treatment group was transfected with only basic vector and treated with the same inducer. At 0.5 h post transfection, the luciferase activities of GF-1 cells were measured using a luminometer and luciferase assay kit (Promega, Madison, WI, USA). Luciferase reporter was designed according to Ref. [26], Mx promoter luciferase vector (with the luciferase reporter gene) contained an NF-kB-binding element and interferon stimulated response elements (ISREs) that were upstream of the Mx promoter region, pGL3 was the basic vector, which contained the luciferase reporter gene only.

2.13. Statistical analysis

Statistical analyses were carried out with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were analysed using one-way analysis of variance (ANOVA) and Tukey's test, and a p-value less than 0.05 was considered significant. A paired-samples t-test was used to compare expression in the treatment and control groups. All presented values are means and their standard errors.

3. Results

3.1. Sequence analysis of grouper IFNAR1

The full length of osgIFNAR1 consisted of 1459 base pairs nucleotides inclusive of 5′ UTR, 3′ UTR, and open reading frame (ORF). The open reading frame sequence encoded 433 amino acids (a.a), with two conserved fibronectin-III domains encoding 40–132 a.a and 147–237 a.a, and the transmembrane domain encoding 251–281 a.a (Fig. 1A). The IFNAR1 transcript contained a polyadenylation site (CA) along the poly-A tail. The evolutionary relationship of IFNAR was measured using the neighbour-joining method and known IFNAR/CRFB sequences were measured with the interferon gamma receptor (IFNGR) family, and together formed four major clusters in the phylogenetic tree analysis, with the IFNAR1/CRFB5 cluster shown to be closer to IFNGR2. osgIFNAR1 is clustered in a group with the teleost IFNAR1/CRFB5 family (Fig. 1B). Mammalian IFNAR1 formed a separate cluster with teleost species as an evolutionary divergence effect. IFNAR1 and IFNAR2/CRFB1 further formed different clusters, maintaining a high bootstrap value. Amino acid identity was compared to trout (saltwater), pufferfish (saltwater), carp (freshwater), zebrafish (freshwater), and human (mammalian) (Table 2). osgIFNAR1 shared a higher identity to trout IFNAR1 (56.1%), while it shared 37.2%, 46.4%, 39.2% amino acid identities with carp, pufferfish, and zebrafish, respectively. Multiple sequence alignment (Fig. 2A) indicated the presence of conserved fibronectin-III in osgIFNAR1 in relation to related teleosts from the phylogenetic tree analysis. Prediction of the osgIFNAR1 protein domains using SMART software compared the fibronectin-III domain and transmembrane region to selected teleosts and human (Fig. 2B). The putative protein structure of osgIFNAR1 was constructed using SWISS-MODEL (Fig. 2C) and was compared to human and trout IFNAR1. Bioinformatic analysis indicated the presence of IFNAR1 in orange-spotted grouper.

Table 2
Amino acid similarities between orange-spotted grouper and other known members of the type I interferon receptor (IFNAR)/class II cytokine receptor (CRFB) family. LaserGene v7.1 (DNASTAR Inc.) was used for analysis.

Common name	Species name	Identity (%)
Orange-spotted grouper	*Epinephelus coioides*	100.0
Rainbow trout	*Oncorhynchus mykiss*	56.1
Green-spotted pufferfish	*Tetraodon nigroviridis*	46.4
Grass carp	*Ctenopharyngodon idella*	37.2
Zebrafish	*Danio rerio*	39.2
Human	*Homo sapiens*	18.1

3.2. Expression analysis of osgIFNAR1 at the basal level

We used qPCR analysis to measure the transcript expression of IFNAR1 in 12 healthy tissue samples of 3.5-inch orange-spotted grouper (approx. 90 dph) (Fig. 3). At the basal level, higher expression was found in the gill and brain, and lower expression was found in the spleen and liver. Other tissues had fairly similar expression levels. osgIFNAR1 was generally expressed in all tissues, but mucosal- and nervous system-related tissues were found to have higher expression levels.

3.3. Subcellular localisation of osgIFNAR1

Immunofluorescence staining was used to observe the localisation of IFNAR1 in the GF-1 cells over a period of time. Using confocal microscopy image, the localisation of IFNAR1 did not showed co-localise movement with nucleus of cells after 12 h. It was found to be scattered across GF-1 cells with a dotted pattern at 12 h after overexpression; the presence of the dotted pattern was not observed at 6 h (Fig. 4). This result showed that the IFNAR1 protein could be expressed after 12 h.

3.4. Overexpression of grouper IFNAR1 induced downstream antiviral protein promoter

To prove the transduction ability of osgIFNAR1, cells were co-transfected with IFNAR1 and downstream Mx promoter [26]. The luciferase activities of cells were analysed, and treatment with recombinant type I IFN shown an increased fold change compared to the untreated group, while treatment with the viral mimic poly(I:C) did not show a significantly increased fold change (Fig. 5A). These results indicated that type I IFN could stimulate IFNAR1 to induce the downstream Mx promoter compared to viral mimics.

3.5. Expression analysis of osgIFNAR1 with cytokine treatment

We used qPCR to measure the expression of osgIFNAR1 and osgMx1 in response to type I IFN treatment in the primary cell culture (Fig. 5B). Using purified recombinant type I IFN protein as indicated in Ref. [26], the result showed an increased expression level of osgIFNAR1 at 4 h post stimulation and returned to a similar level as the control group (PBS) at 12 h osgMx1 was used as marker to verify the activity of type I IFN treatment, and it was significantly up-regulated (up to 10-fold change) after treatment at 4 h post stimulation.

3.6. Expression analysis of osgIFNAR1 with immunostimulants LPS and poly(I:C)

We used qPCR to measure the effect of LPS and poly(I:C) as immunostimulants on the transcript expression of osgIFNAR1 in various tissues. The results indicated an increased expression level of osgIFNAR1 in the brain, eye, head kidney, and spleen, particularly in the poly(I:C) treatment as a virus mimic (Fig. 6). At 6 h post stimulation, there was high expression of osgIFNAR1 in the head kidney and spleen respectively: expression was almost 6-fold and 2-fold increased compared to that in the control group (PBS). The brain, eye, and spleen showed slightly increased expression level during the early hours of stimulation. For the LPS treatment, a high expression level was observed at 12 h post stimulation in the head kidney and spleen, while the eye showed a slightly increased expression level at 6 h post stimulation. The expression of osgIFNAR1 towards microbial mimics showed a lower and slower response compared to that towards viral mimics.

3.7. Expression analysis of osgIFN and osgIFNAR1 with virus challenge

We infected grouper larvae and measured the expression level of osgIFN and osgIFNAR1 with qPCR analysis (Fig. 7). We also measured the number of viral copies. The viral copy number reached the highest

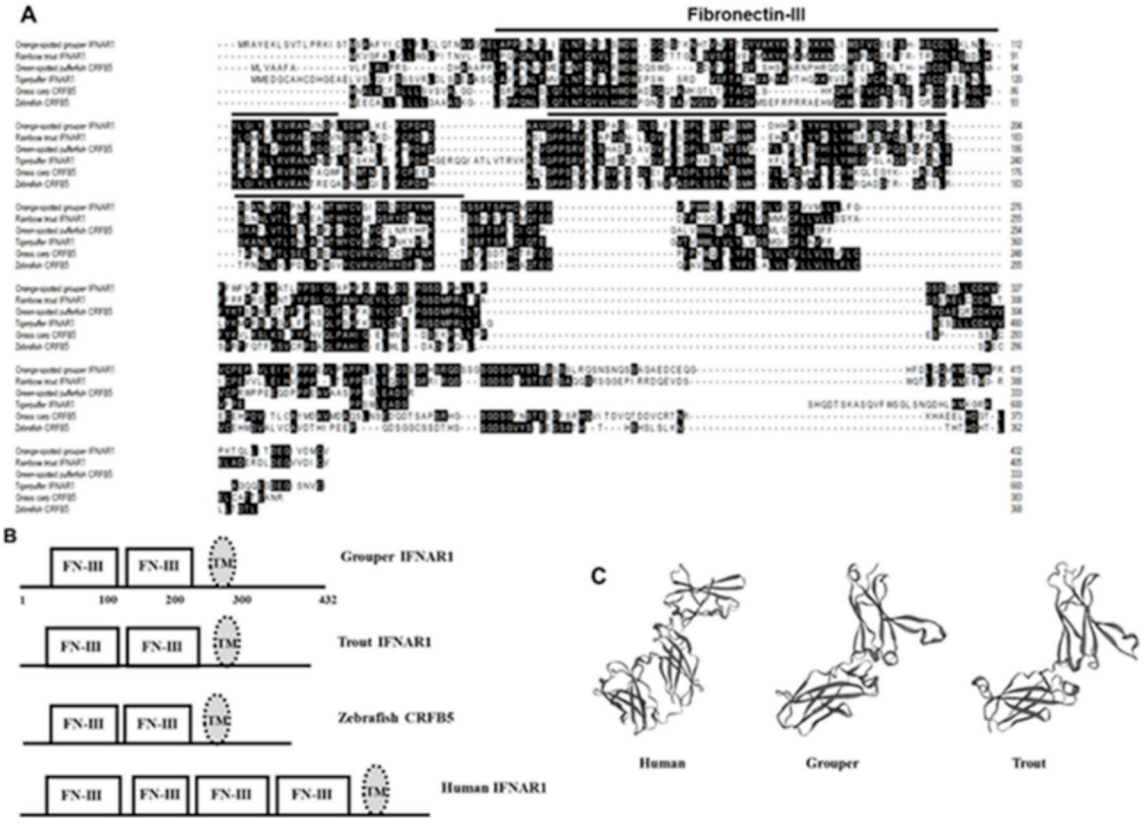

Fig. 2. Amino acid multiple alignment (A) of type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) with other IFNAR1/class II cytokine receptor family member 5 (CRFB5) homologs and schematic functional domain of osgIFNAR1 (B) with other homologs and a homology model (C) of IFNAR1. (A) Multiple deduced amino acid alignment of osgIFNAR1 with other IFNAR1 homologs was produced using ClustalW, and consensus amino acids are shaded using BOXSHADE (version 3.21), conserved fibronectin-III and transmembrane domains are indicated above the sequence with straight and dashed lines, respectively. Homologs, comprising rainbow trout (ADU04482.1), zebrafish (ABJ97310.1), carp (AGZ04472.1), green-spotted pufferfish (CAD67766.1), tiger puffer IFNAR1 (XP_003976720.1). (B) Illustration of conserved functional domains of osgIFNAR1 using SMART software prediction. FN-III denotes the fibronectin type-III domain, TM denotes the transmembrane domain. The accession numbers are zebrafish (ABJ97310.1), trout (ADU04482.1), and human (NP_000620.2). (C) Homology model of amino acids of grouper IFNAR1 to trout and human IFNAR1.

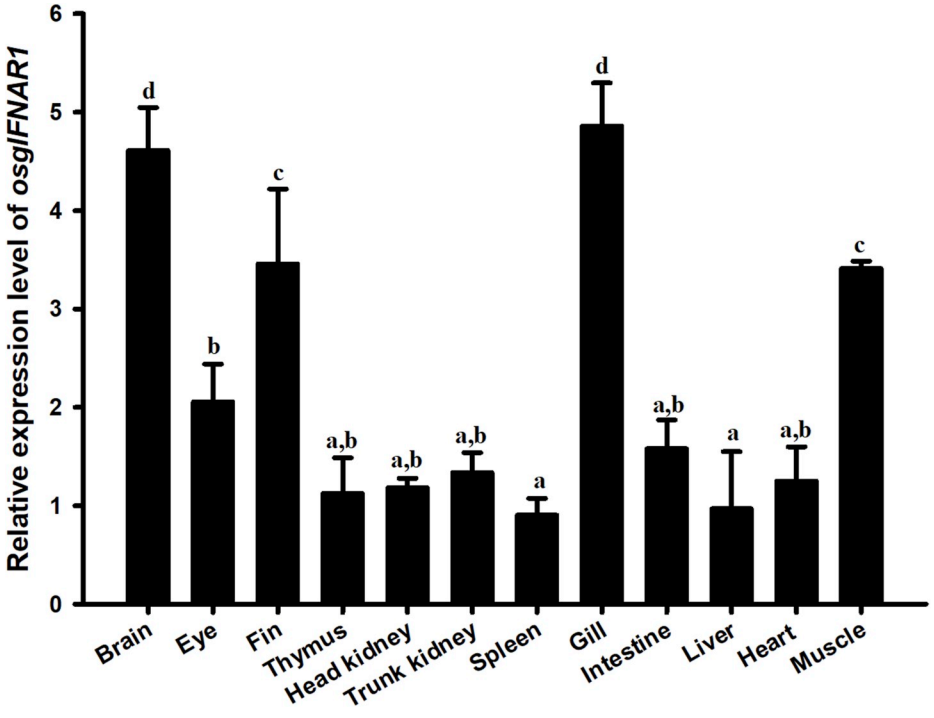

Fig. 3. Expression of type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) in grouper juvenile tissues. Expression was measured by real-time polymerase chain reaction (qPCR) and normalised to β-actin. Results represent the means and standard errors of five fish. Values were compared using one-way analysis of variance (ANOVA), different letters above the bar denote significant differences ($p < 0.05$) and identical letters indicate no significant differences.

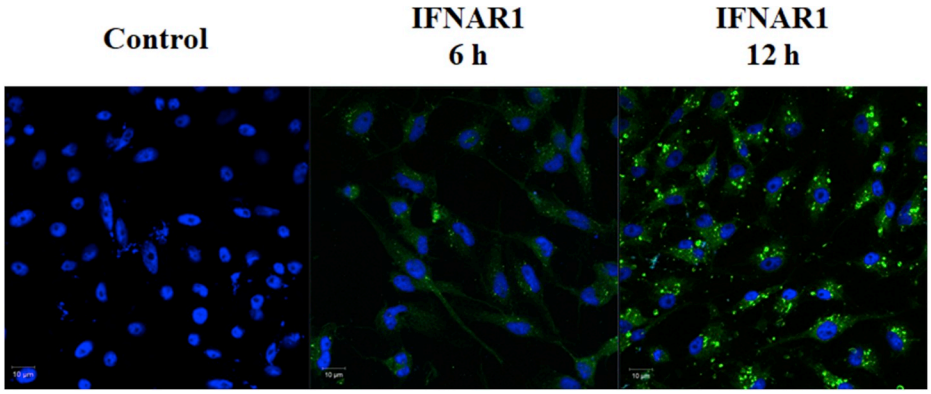

Fig. 4. Subcellular localisation of type I interferon receptor subunit 1 (IFNAR1) in grouper fin (GF-1) cells. Immunofluorescence images of IFNAR1 protein (green) and nuclei (blue; Hoechst 33,342) were recorded at 6 and 12 h after overexpression.

level at 48 h post challenge, confirming the replication activity of the virus in larvae. The transcript expression of osgIFN showed a fluctuating pattern, with increased expression at 24 h and maximum expression at 72 h, while osgIFNAR1 showed a slow increasing trend from 0 to 24 h, followed by a decline to 48 h and maximum expression at 72 h. This result showed that the expression of osgIFNAR1 would be regulated.

3.8. Knockdown of IFNAR1 using siRNA on NNV infection in GF-1 cells

To verify the regulation of IFNAR and viral replication, we further knocked down the expression of IFNAR1 in grouper cells and measured the amount of NNV in cells. It showed that reduced expression of IFNAR1 show a decrease in the NNV amount (Fig. 8A) from 0 to 24 h, meanwhile the overexpression of IFNAR1 in cells with NNV infection showed the lowest expression at 0 h and fairly increased NNV replication up to 24 h (Fig. 8B). This result showed that in virus-infected cell, IFNAR1 could play a role in virus production.

4. Discussion

Type I IFN has been widely reported since the discovering of IFN in

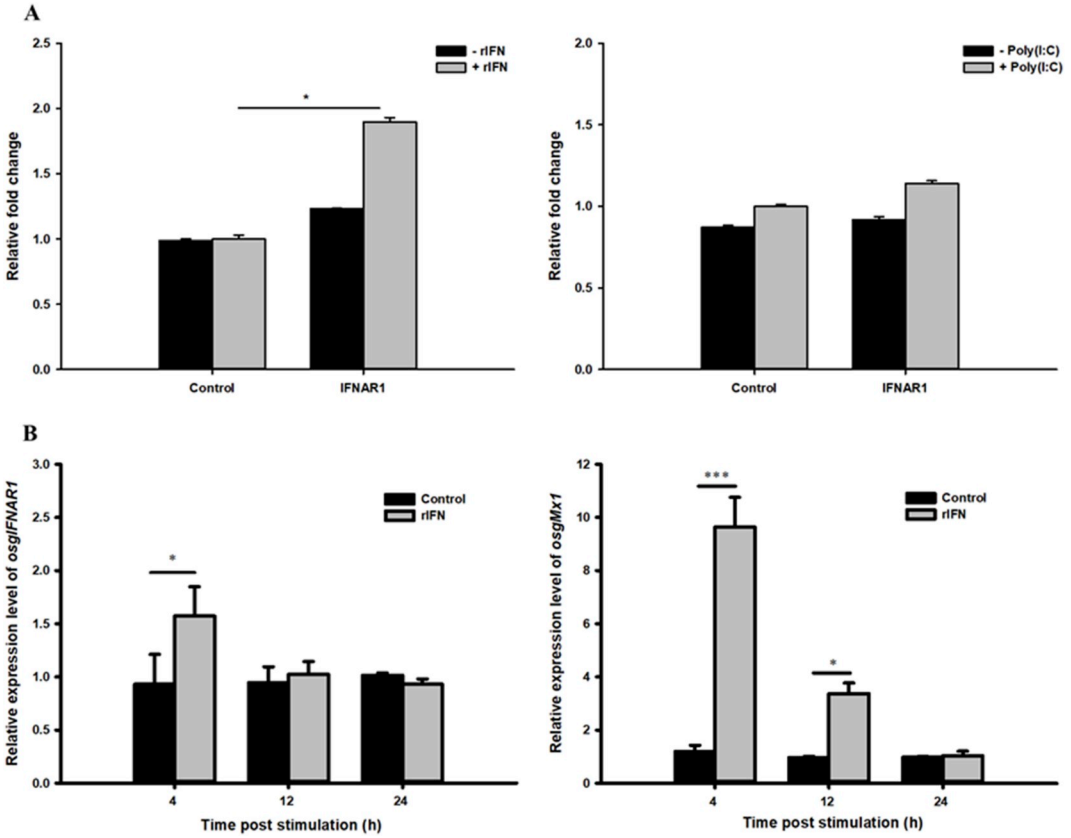

Fig. 5. Effect of recombinant type I interferon (IFN) on the induction of the Mx promoter (A). Grouper fin (GF-1) cells were treated with 50 ng/mL of recombinant type I IFN. The cells were lysed, and luciferase activities were measured 24 h after transfection. Expressions were indicated from triplicate experiments and asterisks represent significant differences between control cells and cells transfected with grouper Mx promoter. Effect of recombinant type I IFN on the expression of type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) and osgMx (B) in the head kidney cells at three different time points (4, 12, and 24 h). Expression was measured by qPCR and levels were normalised to β–actin. The expression of each group was normalised to the control (PBS). All results represent the means and standard errors of triplicates. Values were compared using the *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

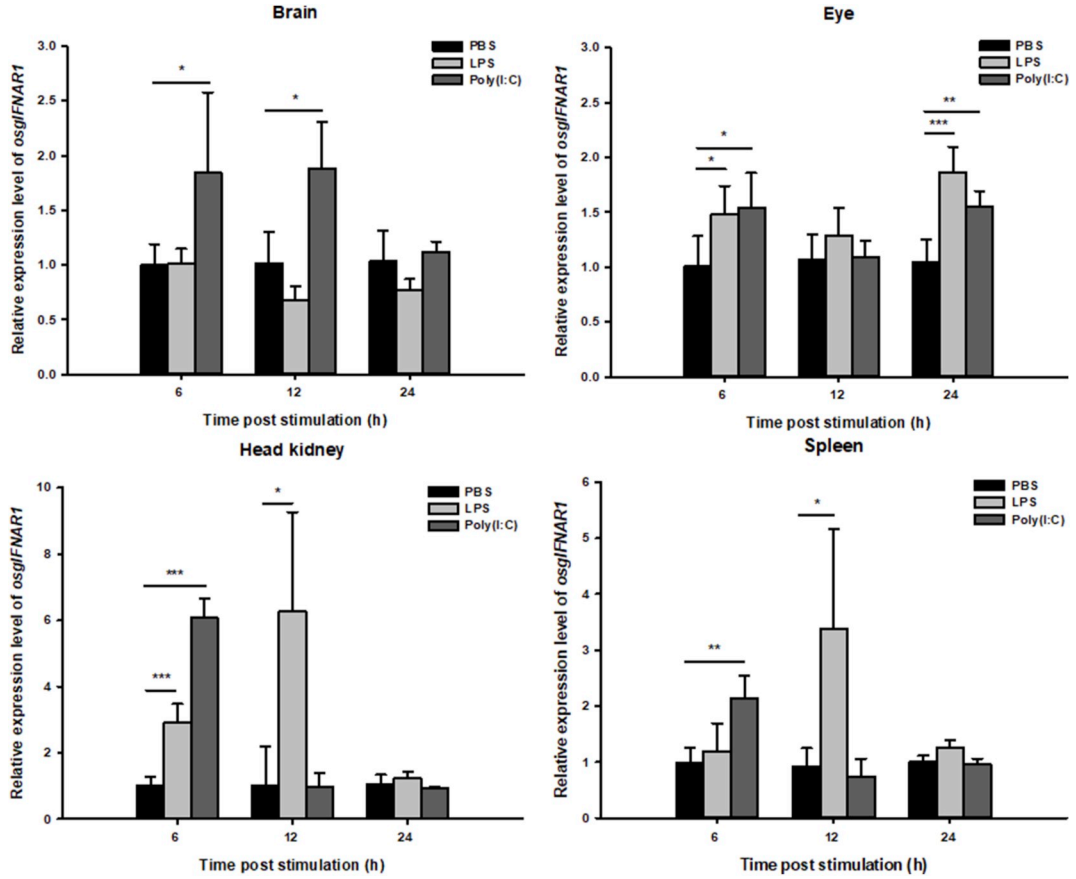

Fig. 6. Effect of LPS and poly(I:C) on the expression of type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) in juvenile grouper tissues (brain, eye, head kidney, and spleen) at three different time points (6, 12, and 24 h). Expression was measured by qPCR and levels were normalised to β-actin. The expression of each group was normalised to the control (PBS). All results represent the means and standard errors of six fish. Values were compared using the *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

1957 [29]. IFNs as ligands would bind to their receptor [30]. However, there are less studies on the receptors, especially the teleost interferon receptor—the first interferon receptor discovered in teleosts was reported just a decade ago [6,16]. Up to now, interferon receptor studies are showing slow progress. In the present study, we reported on the cloning and identification of the type I interferon receptor 1 (IFNAR1) in orange-spotted grouper. Several results supported the characterisation of the IFNAR1 sequence. Phylogenetic tree analysis displayed the

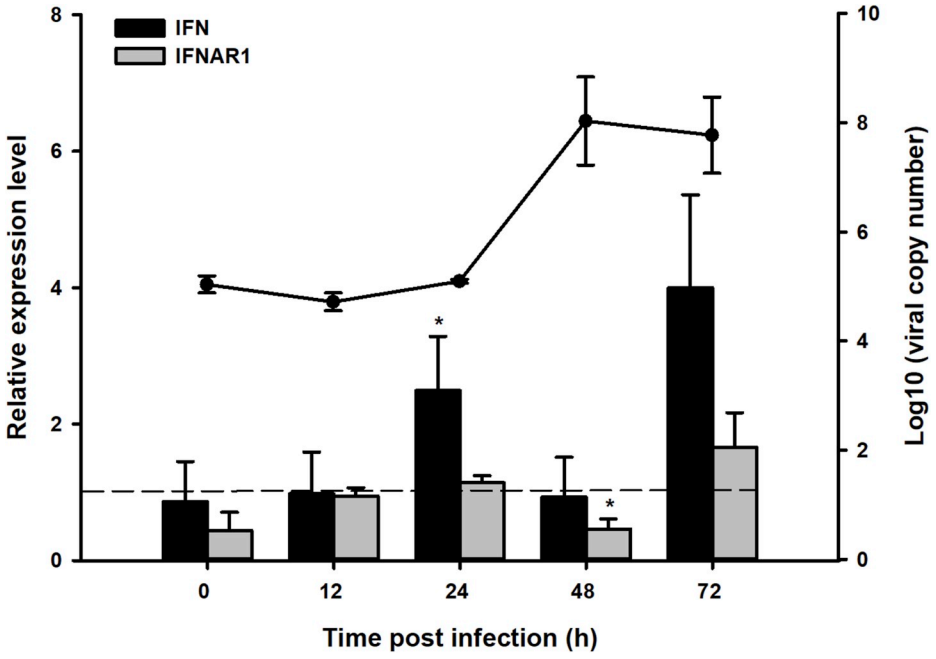

Fig. 7. Effect of nervous necrosis virus (NNV) challenge on the expression of orange-spotted grouper interferon (osgIFN) and type I interferon receptor subunit 1 (IFNAR1) of orange-spotted grouper (osgIFNAR1) in grouper larvae. The expression of osgIFN and osgIFNAR1 at different time points was measured and the expression was normalised to the control (PBS) at each time point. Lines above the bar indicate the virus copy numbers in larvae at different times after the challenge. All results represent the means and standard errors of four fish. All other analysis is described in Fig. 6.

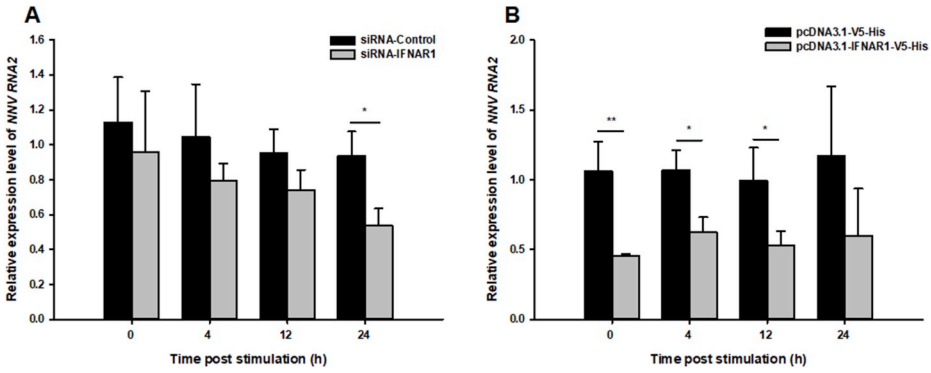

Fig. 8. Effects of type I interferon receptor subunit 1 (IFNAR1) silencing and overexpression on nervous necrosis virus (NNV) infection in grouper fin (GF-1) cells. Upon knockdown of IFNAR1 by siRNA, the relative NNV RNA2 levels (A) were detected after viral infection at 0, 4, 12, and 24 h. The relative NNV RNA2 levels (B) were detected in GF-1 cells transfected with pcDNA3.1-IFNAR1-V5-His or pcDNA3.1-V5-His at 0, 4, 12, and 24 h after viral infection. The analysis is as described previously, and all experiments were performed in triplicated cultures.

clustering of osgIFNAR1 to the IFNAR1 subunit; osgIFNAR1 has a closer ancestor relationship with trout and pufferfish, as they also have higher amino acid identity. Prediction of the functional domain shown conserved fibronectin (FN)-III domains across different known teleost and mammalian species; however, the presence of four tandem functional repeat domains of FN-III in human was found to only contain two repeat domains in teleosts. This could be due to presence of the lineage-specific expansion (LSE) factor in evolution, as explained in pufferfish [16].

The grouper tissue distribution result indicated the expression of IFNAR1 was widely distributed in different organs, with higher expression in the brain and gill; a previous report indicated that the type I interferon receptor protects against viruses within the central nervous system [31]. Our previous study noted that type I interferon could response to mucosal immunity [26], which could explain the higher expression in the gill tissue. Different IFNAR1 expression pattern also noted in salmon and goose [24,32]. However, under different immunostimulant treatments, the expression rate towards poly(I:C) was earlier compared to that of LPS treatment in the head kidney and spleen; IFNAR1 expression showed upregulation at 6 h post stimulation, suggesting that the response towards viral response could be quicker than that towards microbial infection [22]. This is in contrast to the mouse model of IFNAR1-IFNβ signalling LPS treatment [33]. The expression level of IFNAR1 returned to the basal level at 24 h post stimulation; this is similar to the study of Luo et al. [25], in which immunostimulant treatment did not show constitutive IFNAR1 expression. From the different expression levels indicated, we further observed the subcellular localisation of IFNAR1 in the GF-1 cell line. Upon saturation of the surface of cells, it did not express with complete coverage; IFNAR is known to have a generally wide distribution on the surface of cells [34–36]. Scattered pattern of IFNAR1 was observed throughout the cell at 12 h on the overexpression scale. Convincingly, the localisation of IFNAR1 in the cell showed similar patterns to the dotted scattered pattern reported by Kumar et al. [37]. This indicates that the localisation of IFNAR1 has a similar spatial distribution to that seen in mammalian species.

Receptors have been often correlated with signal transduction; however, the affinity of interaction and transduction of signal relying on assembling of receptors, different subunits of cytokine receptor engaged in interactions with different affinities [38]. One study reported that in the presence of ligand, cytokine receptors formed homodimers in carp instead of heterodimers [23], indicating that the presence of homodimers could be activating ligand/receptor interaction. Previously, grouper Mx promoter was induced using grouper type I IFN [26]; our result supported the suggestion that the overexpression of type I IFNAR1 induced the expression of Mx promoter. This result indicated that the transduction of signals from ligands was limited to specific responses; using viral mimics, there is no significant up-regulation level, indicating specificity in IFNAR1 interactions with ligands. We further tested type I IFN and characterised the expression of IFNAR1, and there was up-regulation of the IFNAR1 expression level in the primary cell culture at 4 h post stimulation. The regulation of IFNAR responses is said to be in a fine-tune manner to shape the appropriate immune responses for host defence and survival [39].

As the interferon system accounted for activating the innate immune response, a viral challenge was used to test the expression of IFNAR1 in grouper larvae. The results showed an increasing trend from 0 to 24 h and down-regulation at 48 h; these results are in accordance with those reported by Chen et al. [23]. In a previous study, Chen et al. also reported down-regulation of the osgIFN expression level after four days [26]. In IFNAR1 expression, the downregulation of IFNAR1 at 48 h could be because there was significant increase in IFN at 24 h post infection; in nature of IFNAR regulation, the downstream response has to be limited or constrained to prevent excessive cellular response that could lead to autoimmunity, uncontrolled inflammation, and even death [10]. It is well-known that IFNARs interact with negative regulators (SOCS, UBP43, and SHP) [40–42] to limit the signalling extent and maintain the cellular response balance. Further gain and loss of function in IFNAR1 is carried out, and small-interference RNA was used to silence the expression of IFNAR1 in cells. The result showed a decreasing amount of NNV replication, while in gain of function, the overexpression of IFNAR1 showed a low expression of NNV at 0 h and fairly increased at 4–24 h. From previous understanding, the complete null mutation of IFNAR1 in mice showed the importance of IFNAR presentation on the surface of cell for survival against viral infections and the delivering of IFN responses [20,43]. Here, we would like to propose an alternative mechanism regarding IFNAR1 response towards virus production. NNV is known to utilise the hosts own mechanism to facilitate their entry and replication [44–46]; when the IFNAR1 number is increased, it could be associated with a virus production strategy or delivering responses that facilitate or increase virus production. Thus, when the IFNAR1 number is reduced or knocked down below a certain threshold, viral production is decreased; this decrease could result from low virus entry and replication numbers. IFNAR1 might have different role played in interferon system, apart from known role in antiviral mechanism.

Our previous study noted the inhibition of viral protein expression within 24 h in grouper cells [5]. However, this requires further study to verify the new role of IFNAR1. In summary, we identified and characterised IFNAR1 in orange-spotted grouper. The expression of IFNAR1 can be regulated with type I IFN, poly(I:C), and LPS. IFNAR1 showed a negative regulation feedback when IFN was up-regulated to avoid lethality. From the loss of function experiment, as IFNAR1 is reduced, the amount of NNV is decreased; the IFNAR1 amount could use to increase viral production in early viral infection. Our findings here could provide new insights into the innate immunity of aquaculture species.

CRediT authorship contribution statement

Zhi Zhuang Tang: Validation, Formal analysis, Data curation, Visualization, Writing - original draft. **Ting-Yu Wang:** Formal analysis, Data curation, Visualization, Investigation. **Young-Mao Chen:** Visualization, Investigation, Writing - review & editing, Project administration. **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 to this article can be found online at https://doi.org/10.1016/j.fsi.2020.04.044.

References

[1] B.L. Munday, J. Kwang, N. Moody, Betanodavirus infections of teleost fish: a review, J. Fish. Dis. 25 (2002) 127–142.
[2] K. Mori, T. Nakai, K. Muroga, M. Arimoto, K. Mushiake, I. Furusawa, Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis, Virology 187 (1992) 368–371.
[3] N.C. Chen, M. Yoshimura, H.H. Guan, T.Y. Wang, Y. Misumi, C.C. Lin, P. Chuankhayan, A. Nakagawa, S.I. Chan, T. Tsukihara, T.Y. Chen, C. J Chen, Crystal structures of a piscine betanodavirus: mechanisms of capsid assembly and viral infection, PLoS Pathog. 11 (2015) e1005203.
[4] Y.M. Chen, Y.L. Su, J.H. Lin, H.L. Yang, T.Y. Chen, Cloning of an orange-spotted grouper (*Epinephelus coioides*) Mx cDNA and characterisation of its expression in response to nodavirus, Fish Shellfish Immunol. 20 (2006) 58–71.
[5] Y.M. Chen, Y.L. Su, P.S. Shie, S.L. Huang, H.L. Yang, T.Y. Chen, Grouper Mx confers resistance to nodavirus and interacts with coat protein, Dev. Comp. Immunol. 32 (2008) 825–836.
[6] J.P. Levraud, P. Boudinot, I. Colin, A. Benmansour, N. Peyrieras, P. Herbomel, G. Lutfalla, Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system, J. Immunol. 178 (2007) 4385–4394.
[7] N.A. de Weerd, N. Thao, The interferons and their receptors—distribution and regulation, Immunol. Cell Biol. 90 (2012) 483–491.
[8] S. Pestka, C.D. Krause, M.R. Walter, Interferons, interferon-like cytokines, and their receptors, Immunol. Rev. 202 (2004) 8–32.
[9] S.V. Kotenko, G. Gallagher, V.V. Baurin, A. Lewis-Antes, M. Shen, N.K. Shah, J.A. Langer, F. Sheikh, H. Dickensheets, R.P. Donnelly, IFN-λs mediate antiviral protection through a distinct class II cytokine receptor complex, Nat. Immunol. 4 (2003) 69–77.
[10] P.J. Hertzog, N.A. de Weerd, S.A. Samarajiwa, Type I interferon receptors: biochemistry and biological functions, J. Biol. Chem. 282 (2007) 20053–20057.
[11] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (2001) 778–809.
[12] L.C. Platanias, Mechanisms of type-I- and type-II-interferon-mediated signalling, Nat. Rev. Immunol. 5 (2005) 375–386.
[13] J.A. Langer, E.C. Cutrone, S. Kotenko, The class II cytokine receptor (CRF2) family: overview and patterns of receptor-ligand interactions, Cytokine Growth Factor Rev. 15 (2004) 33–48.
[14] G. Uzé, G. Schreiber, J. Piehler, S. Pellegrini, The receptor of the type I interferon family, Curr. Top. Microbiol. Immunol. 316 (2007) 71–95.
[15] Q. Gao, P. Nie, K.D. Thompson, A. Adams, T. Wang, C.J. Secombes, The search for the IFN-γ receptor in fish: functional and expression analysis of putative binding and signalling chains in rainbow trout *Oncorhynchus mykiss*, Dev. Comp. Immunol. 33 (2009) 920–931.
[16] G. Lutfalla, H.R. Crollius, N. Stange-thomann, O. Jaillon, K. Mogensen, D. Monneron, Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: the class II cytokine receptors and their ligands in mammals and fish, BMC Genom. 4 (2003) 1–15.
[17] C. Stein, M. Caccamo, G. Laird, M. Leptin, Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish, Genome Biol. 8 (2007) R251.
[18] D. Aggad, C. Stein, D. Sieger, M. Mazel, P. Boudinot, P. Herbomel, J.-P. Levraud, G. Lutfalla, M. Leptin, Vivo analysis of Ifn-γ1 and Ifn-γ2 signaling in zebrafish, J. Immunol. 185 (2009) 6774–6782.
[19] J. Zou, C.J. Secombes, Teleost fish interferons and their role in immunity, Dev. Comp. Immunol. 35 (2011) 1376–1387.
[20] U. Muller, U. Steinhoff, L.F.L. Reis, S. Hemmi, J. Paviovic, R.M. Zinkernagel, M. Aguet, Functional role of type I and type II interferons in antiviral defense, Science 264 (1994) 1918–1921.
[21] P. Boudinot, C. Langevin, C.J. Secombes, J. P Levraud, The peculiar characteristics of fish type I interferons, Viruses 8 (2016) E298.
[22] M.M. Monte, T.H. Wang, B. Collet, J. Zou, C.J. Secombes, Molecular characterisation of four class 2 cytokine receptor family members in rainbow trout, *Oncorhynchus mykiss*, Dev. Comp. Immunol. 48 (2015) 43–54.
[23] H.R. Chen, W.Q. Liu, B.H. Wang, H.L. Mao, Z.C. Sun, Q.H. Hou, Y.C. Mi, L.H. Fan, C.Y. Hu, Cloning, identification of the two cytokine receptor family B subunits CRFB1 and CRFB5 from grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 45 (2015) 211–220.
[24] B.J. Sun, L. Greiner-Tollersrud, B.F. Koop, B. Robertsen, Atlantic salmon possesses two clusters of type I interferon receptor genes on different chromosomes, which allows for a larger repertoire of interferon receptors than in zebrafish and mammals, Dev. Comp. Immunol. 47 (2014) 275–286.
[25] K. Luo, S.H. Zhang, D.D. Tang, L.H. Xia, W.H. Gao, G.M. Tian, Z.T. Qi, Q.Q. Xu, W.B. Zhang, Analysis of the expression patterns of the cytokine receptor family B (CRFB) and interferon gamma receptor (IFNGR) in Dabry's sturgeon (*Acipenser dabryanus*), Dev. Comp. Immunol. 84 (2018) 420–426.
[26] Y.M. Chen, C.E. Kuo, G.R. Chen, Y.T. Kao, J. Zou, C.J. Secombes, T.Y. Chen, Functional analysis of an orange-spotted grouper (*Epinephelus coioides*) interferon gene and characterisation of its expression in response to nodavirus infection, Dev. Comp. Immunol. 46 (2014) 117–128.
[27] H.C. Kuo, T.Y. Wang, P.P. Chen, Y.M. Chen, H.C. Chuang, T.Y. Chen, Real-time quantitative PCR assay for monitoring of nervous necrosis virus infection in grouper aquaculture, J. Clin. Microbiol. 49 (2011) 1090–1096.
[28] S.C. Chi, W.W. Hu, B.J. Lo, Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV), J. Fish. Dis. 22 (1999) 173–182.
[29] A. Isaacs, J. Lindenmann, Virus interference. I. The interferon, Proc. R. Soc. London, Ser. A or B 147 (1957) 258–267.
[30] C. Piehler, C. Thomas, K.C. Garcia, G. Schreiber, Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation, Immunol. Rev. 250 (2012) 317–334.
[31] C.N. Detje, T. Meyer, H. Schmidt, D. Kreuz, J.K. Rose, I. Bechmann, M. Prinz, U. Kalinke, Local type I IFN receptor signaling protects against virus spread within the central nervous system, J. Immunol. 182 (2009) 2297–2304.
[32] H. Zhou, S. Chen, Y.L. Qi, Q. Zhou, M.S. Wang, R.Y. Jia, D.K. Zhu, M.F. Liu, K.F. Sun, F. Liu, X.Y. Chen, A.C. Cheng, Type I interferon receptors in goose: molecular cloning, structural identification, evolutionary analysis and age-related tissue expression profile, Gene 561 (2015) 35–44.
[33] N.A. de Weerd, J.P. Vivian, T.K. Nguyen, N.E. Mangan, J.A. Gould, S.-J. Braniff, L. Zaker-Tabrizi, K.Y. Fung, S.C. Forster, T. Beddoe, H.H. Reid, J. Rossjohn, P.J. Hertzog, Structural basis of a unique interferon-β signalling axis mediated via the receptor IFNAR1, Nat. Immunol. 14 (2013) 901–907.
[34] M.P. Hardy, C.M. Owczarek, S. Trajanovska, X. Liu, I. Kola, P.J. Hertzog, The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties, Blood 97 (2001) 473–482.
[35] M.P. Hardy, E.P. Sanij, P.J. Hertzog, C.M. Owczarek, Characterization and transcriptional analysis of the mouse Chromosome 16 cytokine receptor gene cluster, Mamm, Genome 14 (2003) 105–118.
[36] M.P. Hardy, P.J. Hertzog, C.M. Owczarek, Multiple regions within the promoter of the murine Ifnar-2 gene confer basal and inducible expression, Biochem. J. 365 (2002) 355–367.
[37] S.K.G. Kumar, H. Barriere, C.J. Carbone, J.H. Liu, G. Swaminathan, P. Xu, Y. Li, D.P. Baker, J.M. Peng, G.L. Lukacs, S.Y. Fuchs, Site-specific ubiquitination exposes a linear motif to promote interferon-α receptor endocytosis, J. Cell Biol. 179 (2007) 935–950.
[38] J. Kumaran, L. Wei, L.P. Kotra, E.N. Fish, A structural basis for interferon-alpha-receptor interactions, Faseb. J. 21 (2007) 3288–3296.
[39] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat. Rev. Immunol. 14 (2014) 36–49.
[40] M. You, D.H. Yu, G.S. Feng, Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway, Mol. Cell Biol. 19 (1999) 2416–2424.
[41] O.A. Malakhova, K.I. Kim, J.K. Luo, W. Zou, K.G. Kumar, S.Y. Fuchs, K. Shuai, D.E. Zhang, UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity, EMBO J. 25 (2006) 2358–2367.
[42] J.E. Fenner, R. Starr, A.L. Cornish, J.G. Zhang, D. Metcalf, R.D. Schreiber, K. Sheehan, D. J Hilton, W.S. Alexander, P.J. Hertzog, Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity, Nat. Immunol. 7 (2006) 33–39.
[43] S.Y. Hwang, P.J. Hertzog, K.A. Holland, S.H. Sumarsono, M.J. Tymms, J.A. Hamilton, G. Whitty, I. Bertoncello, I. Kola, A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons alpha and beta and alters macrophage responses, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 11284–11288.
[44] J.S. Chang, S.C. Chi, GHSC70 is involved in the cellular entry of nervous necrosis virus, J. Virol. 89 (2015) 61–70.
[45] K.M. Kampmueller, D.J. Miller, The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells, J. Virol. 79 (2005) 6827–6837.
[46] Y.M. Chen, C.E. Kuo, T.Y. Wang, P.S. Shie, W.C. Wang, S.L. Huang, T.J. Tsai, P.P. Chen, J.C. Chen, T.Y. Chen, Cloning of an orange-spotted grouper *Epinephelus coioides* heat shock protein 90AB (HSP90AB) and characterization of its expression in response to nodavirus, Fish Shellfish Immunol. 28 (2010) 895–904.