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Article

Molecular cloning, Expression and Functional Analysis of Interferon Regulatory Factor 5 (IRF 5) in Malabar grouper *(Epinephelus malabaricus)*

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Abstract: Interferon regulatory factor 5 (IRF5) is known to be involved in the innate immune response and pro-inflammatory cytokines. However, the roles of IRF5 in immune responses in Malabar grouper (*Epinephelus malabaricus)* have not been extensively explored. In this study, IRF5 gene was identified and characterized from M. grouper. The full-length IRF5 cDNA consisted of a 5′ terminal untranslated region (5′-UTR) of 289 bp and a 3′-UTR of 542 bp, an open reading frame (ORF) of 1500 bp encoding a polypeptide of 499 amino acids with a predicted molecular mass of 56.28 kDa and isoelectric point (pI) of 5.2. The putative MgIRF5 protein consists of four important conserved domains: a helix DNA-binding domain (DBD) at the N-terminus, a middle region, an IRF association domain (IAD) and a virus activated domain (VAD) at the C-terminus. Sequence alignment and phylogenetic analysis showed that highest sequence similarity of IRF5 was observed between the IRF5 genes from *Oplegnathus fasciatus* and *Miichthys miiuy*. The mRNA transcripts of IRF5 were detected in a wide range of tissues types from healthy M. grouper with highest expression in muscle, liver and skin. After treatment with poly (I: C), it was significantly up-regulated in spleen and liver tissues. When infected with NNV, the expression level of MgIRF5 was up-regulated in spleen and head kidney and their transcriptional responses to IRF5 increased in the grouper kidney cells. This approach suggests that MgIRF5 is important in the underlying mechanism of the innate immune responses against antiviral response.

Keywords: IRF5, Transcriptional factors, Immuno-stimulation, Nervous necrosis virus, Malabar grouper *(Epinephelus malabaricus)*

1. Introduction

Interferon regulatory factors (IRFs), a family of transcription factors play a viral role in the regulation of IFN genes and IFN-stimulated genes (ISGs) [1], which mainly acknowledge in regulating many facets of innate and adaptive immune responses [2]. In vertebrates，IRF1 (IRF1, 2

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and IRF11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9, 10) and IRF5 (IRF5 and 6) are the four subfamilies of IRF 11 members [3]. IRFs have the highly conserved region (DBD) and a low homologous region (IRF association domain, IAD) the former with five tyrosines, which are responsible for recognizing IFN-stimulated response element (ISRE) [4]. The expressions of genes are vital to the cellular antiviral response.

IRF5 serves as a direct transducer for viral infection-mediated signaling pathways in type I IFN genes and can induce expression of a gene with both IFN-α and IFN-β, upon viral infection [5]. Interestingly, recent studies have demonstrated that IRF5 plays an important role in RIG-I and toll-like pathways [5-9], and is separated as lymphoid cells and apoptosis [7]. IRF5 is subjected to TRAF6-mediated K63-linked ubiquitination [10] and the signaling connection amongst IRF5 and mitochondrial antiviral signaling protein (MAVS) was distinguished in mice [11]. The receptors mediated signaling pathways for activation of IRF5 dimerizes and translocate to the nucleus, binding to the ISRE motifs in the promoters positively regulating cytokines, for example, IFN1, IL-6, IL-1β, and TNF-α while suppressing IL-10 expression [10, 12]. IRF5 is communicated in dendritic cells, monocytes and B cells, is a transcription factor that controls the transactivations of type I IFN framework related genes and also inflammatory and immune response associated genes [13]. IRF5 is a vital transcription factor that regulates the transcription of type I interferon upon viral disease. IRF5 has been characterized in turbot [14], Paddlefish [15], Zebrafish [16], Grass [17], Japanese flounder [18], Rock bream [19], Tongue sole [20] and Common carp [21]. Precise evidence regarding IRF5 induction by type I IFNs in grouper fish has not been identified.

Viral nervous necrosis (VNN) [22], also called viral encephalopathy and retinopathy [23], is a disease in more than 40 species of freshwater and marine fish around the world. Clinical symptoms of NNV affected fish develop vacuolization in the central nervous system, retina, abnormal swimming behavior, and resulting in a high mortality rate (up to 100%) particularly in larvae and juvenile stages [24]. In this research, the complementary DNA coding for the IRF5 gene of grouper were successfully cloned and characterized. Expression analyses of these IRF5 mRNAs were performed using real-time quantitative PCR in the developmental stages and various tissues of healthy fish and fish injected by IP with Poly (1: C), LPS and NNV. The current study contributes to understanding the immune system of grouper and provides important knowledge that can assist in the creation of tools to control diseases of economic importance to the fish industry.

2. Results

2.1. Characterization of MgIRF5

The full-length sequence of MgIRF5 was identified from the transcriptome database and deposited in Gene bank (accession no. KY428937). The full-length IRF5 cDNA sequence was 2331 bp in length and contained an open reading frame (ORF) of 1500 nucleotides including a 289 bp 5′-UTR and a 542 bp 3′-UTR. The MgIRF5 gene encoded a protein of 499 amino acids with a predicted molecular weight of 56.28 KDa and a predicted isoelectric point (pI) of 5.2. Two mRNA instability motifs ATTTA (1882-1887bp) and (2306-2310 bp) were identified in the 3′-UTR along with polyadenylation signals sequences (AATAAA) located at 2041-2046 and 2280-2285 bp, respectively (Figure 1A). The MgIRF5 protein structural domains were characterized by SMART software tool (Figure 1B).

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-11            tcagtttgaagttttgcgagcagcgggtgtttgtttcggccgccgattg
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290  atgagcgtccagccccgcaggatccgtctgaagccctggttgttggctcaggtgaacagt
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       G  R  Y  P  G  L  Q  W  L  S  P  D  H  R  L  F  Q  I  P  W
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650  gatgctggtgatgatgatgatgaagagatgccgaacctgatggacctcagcatcaacccc
       D  A  G  D  D  D  D  E  E  M  P  N  L  M  D  L  S  I  N  P
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       R  T  S  D  S  A  S  F  N  S  F  P  P  H  S  D  V  D  H  F
770  agtatgtcctccaacgggtcatttggacttccacatgtgatcccagtacctctcctcccc
       S  M  S  S  N  G  S  F  G  L  P  H  V  I  P  V  P  L  L  P
830  gacagaggcctccccatgaactctgacctccccatgacctctgacctccaggctcagagt
       D  R  G  L  P  M  N  S  D  L  P  M  T  S  D  L  Q  A  Q  S
890  ggtatggtggatcaggggagcttcgtccatcctgtaggacaccctaatggactccaggac
       G  M  V  D  Q  G  S  F  V  H  P  V  G  H  P  N  G  L  Q  D
950  ctgagttctctcccccttcagctgcctctggtccccccagccggcttcaatggaggcc
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1070 gacctgctgagcagcgtcccactaacagatctggacctgaagttccagtaccggggccgg
       D  L  L  S  S  V  P  L  T  D  L  D  L  K  F  Q  Y  R  G  R
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       E  P  T  P  E  Q  V  D  L  F  G  P  V  T  L  Q  Q  V  R  F
1250 ccagggacatctgagatccagaaccagaagcaaaggttctacactgaggccctgctggat
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1430 atggagagggagaagaagatcaaagtgttcagcctcaacaacttcctgcaaggtctgatc
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1490 tcgttccagaaaggtgaagctcaaaaccctccacccttcgaggtctatttctgcttcggg
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1610 gtggtggctcggatcctgacagagatgttctcaggagaactcagctggtccaccgacagc
       V  V  A  R  I  L  T  E  M  F  S  G  E  L  S  W  S  T  D  S
1670 atccgtctgcagatctcgaacccggatgtgaaggaccagacggtggagcagttcaaagag
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2210 caaacctgggaaacgatcagtgaccacaaactgctgtagaacatttcagtgactctgttt
2270 ttaggatgtcaataaacaccactgcagtattcattcatttattcatatgtgtgctgtcgt
2330 cc
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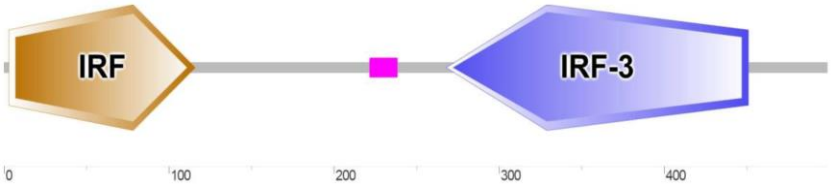

B

Figure 1. (A) Full-length cDNA and detected amino acid sequence of MgIRF5 from M. grouper (*E. malabaricus)*. The detected amino acid sequence is shown under the nucleotide sequence. The 5′-UTR and 3′-UTR are shown in small letters. The bold letters inside box represent the start codon (ATG) and stop codon (AAA). Two mRNA instability motifs ATTTA (1884-1888bp) and (2307-2311bp) were identified in the 3′-UTR along with polydenelation signals sequences (AATAAA) are located at 2042-2047 and 2281-2286 bp, respectively. (B) The putative protein structure of IRF5 from E. malabaricus was generated using SMART-MODEL program.

2.2. Homology Sequence Comparison and Multiple Amino Acid Alignment

A pairwise alignment of the MgIRF5 protein sequence with other known IRF5 species revealed that MgIRF5 shared 94% similarity and 92% identity with the *O. fasciatus* IRF5 homolog. The teleost IRF5 homologs demonstrated 94 to 53% identities between *O. fasciatus* and *H. sapiens* (Table 2). Multiple sequence alignments showed four conserved domains in both human and fish IRF5 (Figure 2).

Table 2. The amino acid identities (%) and similarities (%) and gap of Malabar grouper IRF5 to reprehension in other species.

Common name	Species	Accession no.	AA	Identity (%)	Similarity (%)	Gap
Malabar grouper	*Epinephelus malabaricus*	ARI70656.1	500	100	100	0
Sea bream	*Oplegnathus fasciatus*	AFZ93894.1	498	92	94	0
Large yellow croaker	*Larimichthys crocea*	KKF19013.1	492	89	92	1
Sciaenidae drum	*Miichthys miiuy*	AHB59743.1	492	89	92	1
Turbot fish	*Scophthalmus maximus*	AEG76957.1	487	84	88	2
Nile tilapia	*Oreochromis niloticus*	XP_003448868.1	474	82	87	5
Japanese flounder	*Paralichthya olivaceus*	ADZ96215.1	472	79	84	7
Atlantic salmon	*Salmo salar*	NP_001133324.1	532	70	78	6
Crass carp	*Ctenopharyngodon idella*	ACT83674.1	579	67	78	4
Zebrafish	*Danio rerio*	NP_001314746.1	516	66	77	4
Paddlefish	*Polyodon spathula*	AEW27153.1	496	63	74	5
Green sea turtle	*Chelonia mydas*	EMP28595.1	472	58	69	10
African clawed frog	*Xenopus laevis*	NP_001088065.1	517	54	67	6
Mouse	*Mus musculus*	NP_001239311.1	506	54	63	8
Human	*Homo sapiens*	XP_011514463.1	504	53	64	6

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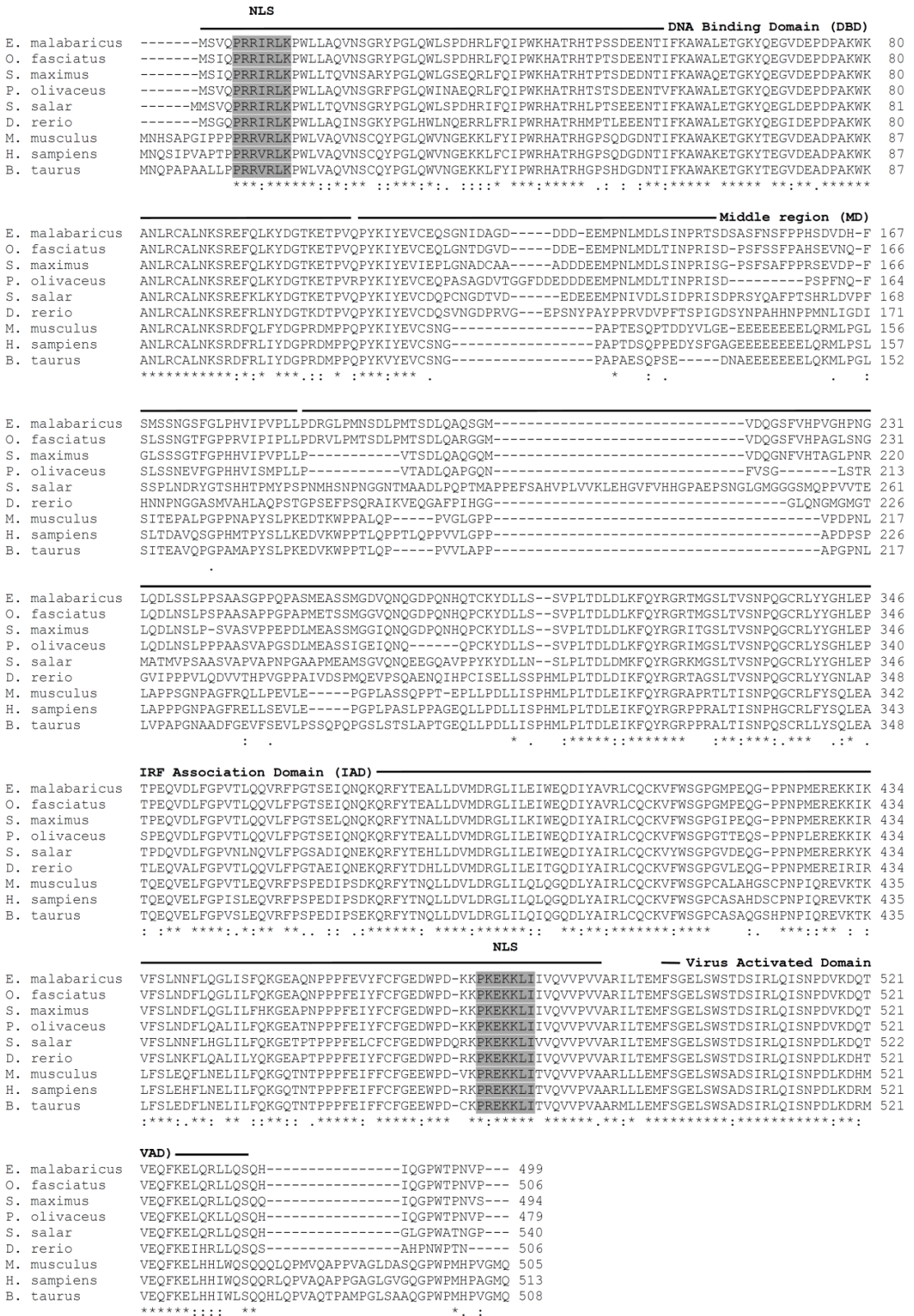

Figure 2. Multiple sequences alignment of the deducted MgIRF5 with their counterparts from several species. The proposed signal peptides, helix DNA-binding domain (DBD, an IRF association domain (IAD) and a virus activated domain (VAD) and two nuclear localization signals (NLSs) are labeled above the alignment. Identical and similar sites are indicated with asterisks (*) and dots (. or :), respectively.

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2.3. Phylogenetic Tree Analysis

Phylogenetic analysis of amino acid sequences of IRF members were conducted based on cluster W alignment by using the neighbor-joining method with bootstrap values of 1000. The results revealed that the MgIRF5 sequences are more closely related to *O. fasciatus* IRF5, 93%, than to *M. miiuy*, 64 %. This cluster shows that this is a sister group to the clad comprising IRF5 belonging to *Homo sapiens* to *Gallus gallus* and *Xenopus laevis*, though it has a low bootstrap value of 27 (Figure 3).

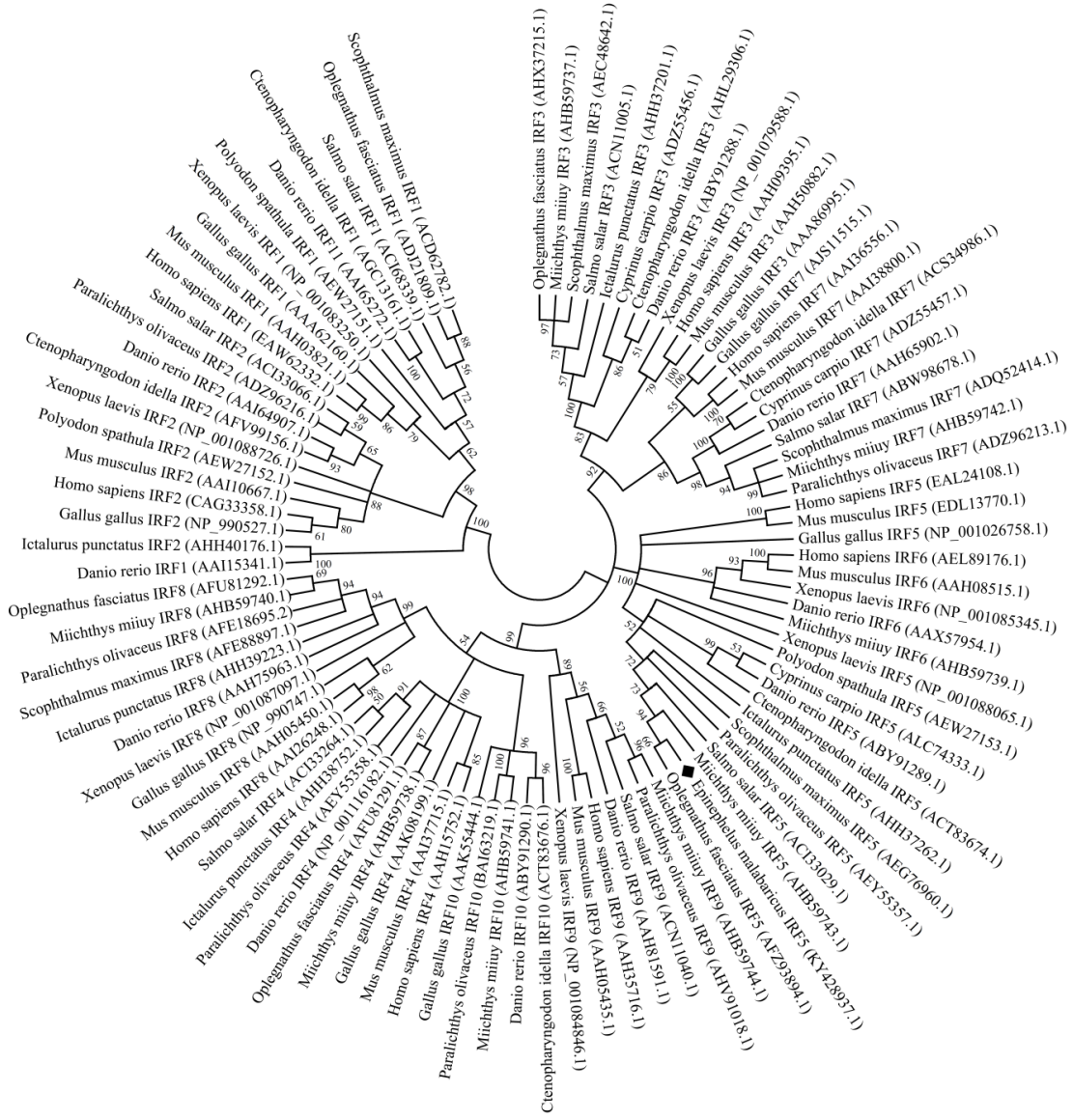

Figure 3. Phylogenetic tree constructed based on the amino acid sequences of IRF5 from different species. The amino acid sequences were aligned using CLUSTAL W and the tree constructed by the Neighbor-Joining method supported with 1000 bootstrap replication using MEGA 7 software.

2.4. Embryo Developmental Stages and Tissues Distribution of the MgIRF5

The expression pattern of MgIRF5 gene was determined in embryonic developmental stages and larvae differential tissues in M. grouper fish by a quantification real-time PCR method (Figure 4). The expression of IRF5 from embryonic stages was initially low and gradually increased from

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0.005-fold at 0 h to 0.023-fold at 24 h, after that expression level decreased to 0.012-fold at 72 h post fertilization (Figure 4A). The broad expression in the differential tissues tested showed highest expression in the muscle (0.012-fold) and liver (0.01-fold) and lowest expression in head kidney (Figure 4B).

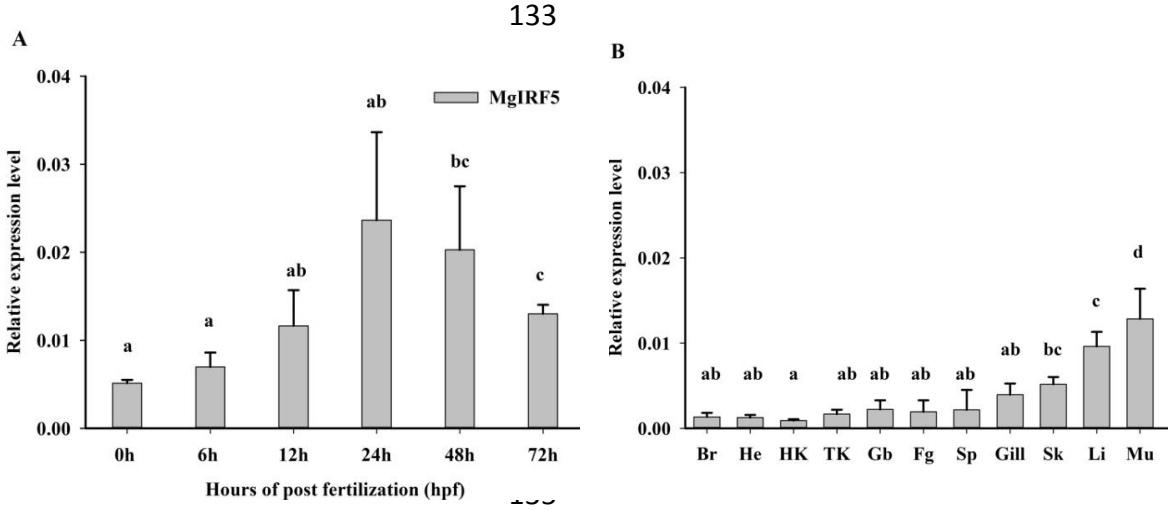

Figure 4. Relative mRNA expression levels of MgIRF5 transcripts in developmental stages and healthy grouper various tissues were performed by quantitative real-time PCR analysis. A. MgIRF5 mRNA expression in developmental stages at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h post fertilization. MgIRF5 mRNA expression in various tissues: eye (Ey) brain (Br), heart (He), head kidney (Hk), trunk kidney (Tk), spleen (Sp), gas bladder (Gb), intestinal (In), gill (Gill), skin (Sk), liver (Li) and muscle (Mu). The mRNA expression levels were normalized to the expression of the β-actin. Vertical bars represented mean ± SD (n=3). The statistical analysis was conducted with Student's t-test. Significant differences at the experimental group are indicated by asterisks * ($p<0.05$); ** ($p< 0.01$).

2.5. Expression Pattern of MgIRF5 during Immune Stimulation

Poly (I: C) and LPS was used to investigate the expression pattern of MgIRF5, in spleen, head kidney and liver using the quantitative real-time PCR (Figure 5). The results showed that MgIRF5 expression was significantly up-regulated in spleen (1.84-fold), liver (5.51 fold) under Poly (I: C) stimulations (Figure 5A). The Mx gene expression showed significantly up-regulated in spleen (0.97 fold) and liver (1.48 fold) at 24h post infection of poly (I: C) with compared to the experimental control group, respectively (Figure 5B). In the LPS treatment, the immune genes expression was observed to not be significant on any of the organs (Figure 5).

2.6. Expression Pattern of MgIRF5 during NNV Challenge

To understand the expression of MgIRF5 during NNV challenge, the spleen, head kidney, and liver tissues of infected and non-infected fish were examined by real-time PCR analysis (Figure 6). The expression of NNV in the brain was detected at 48 and 72 h post infection by the RT-PCR method, and CP was highest at 72 h post infection (Data not shown). In vivo mRNA expression levels of IRF5 gene were significantly up-regulated in the spleen, head kidney and liver (Figure 6A-C). Spleen had the highest expression (16.51 and 12.65-fold) obtained at 12 h, and 72 hpi, respectively (Figure 6A). MgIRF5, mRNA levels analyzed in the head kidney and liver tissues

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(Figure B, C) showed significantly up-regulation with the treatment of NNV in all time courses. The highest fold expression (1.26 and 8.23-fold) occurring at the 12 h and 72 hpi, respectively.

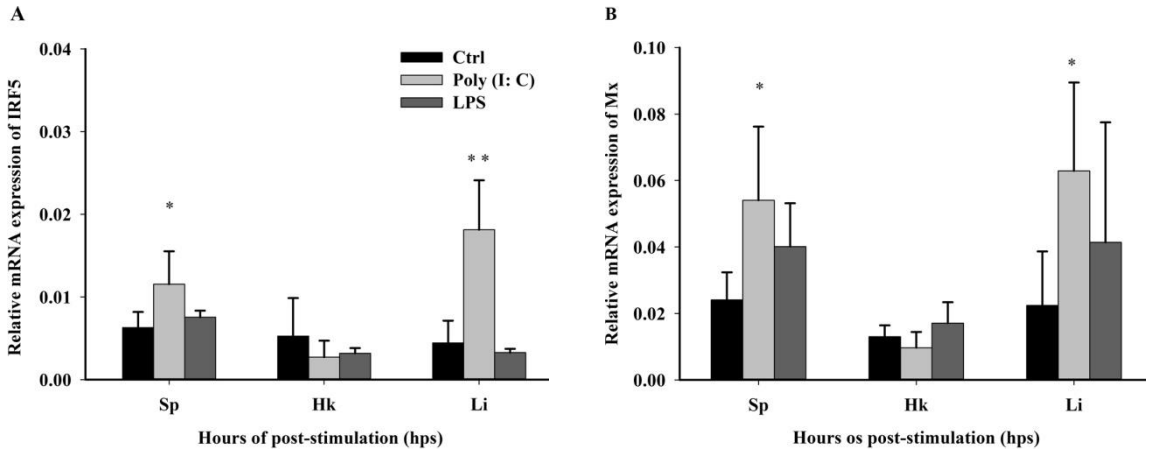

Figure 5. Relative mRNA expression level of IRF5, Mx mRNA in spleen, kidney and liver by (A) poly (I: C) and (B) LPS injected fish at 24 h samples were performed by quantitative real-time PCR analysis. The β-actin gene was used as an internal control. All data were expressed as the mean ± standard deviation (n=3). Significant differences between the experimental and the control group are indicated by asterisks * ($p<0.05$); ** ($p< 0.01$).

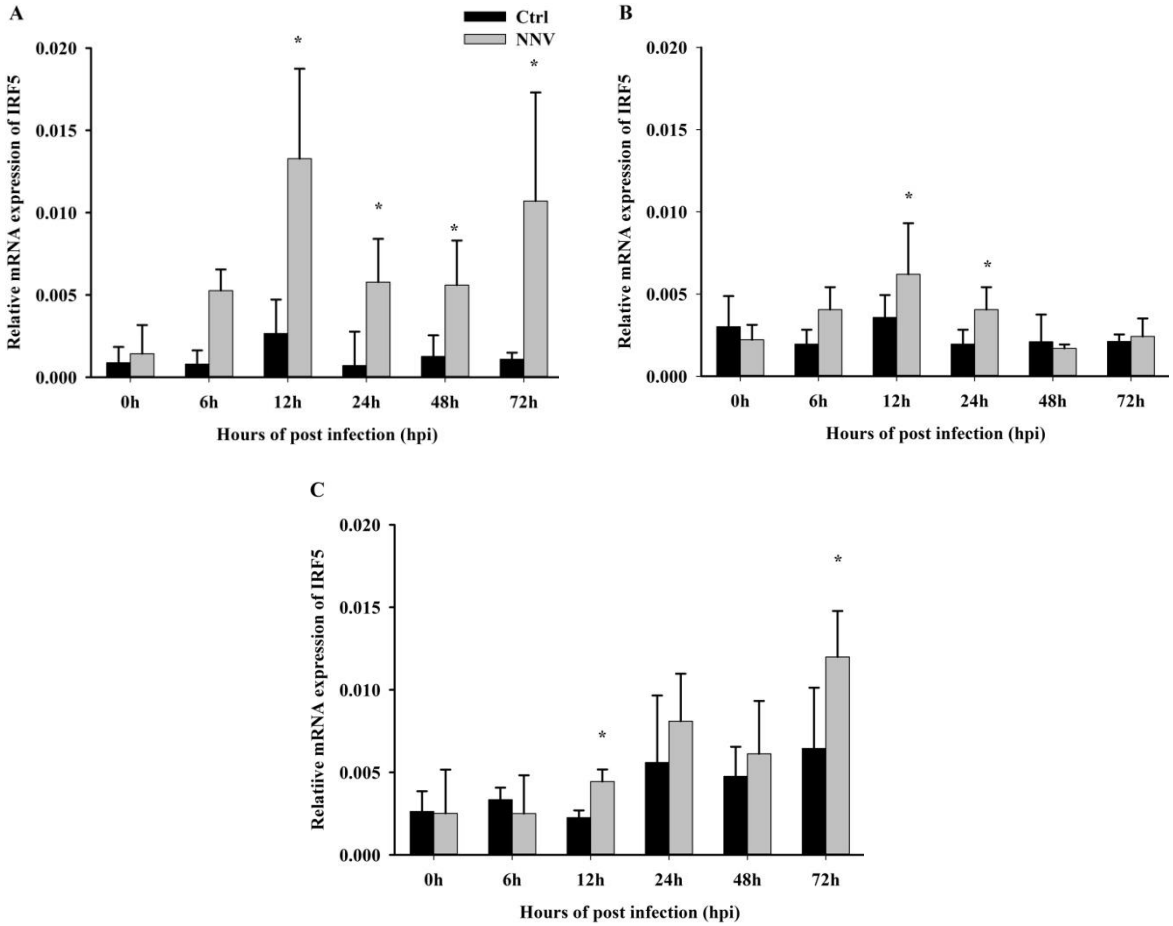

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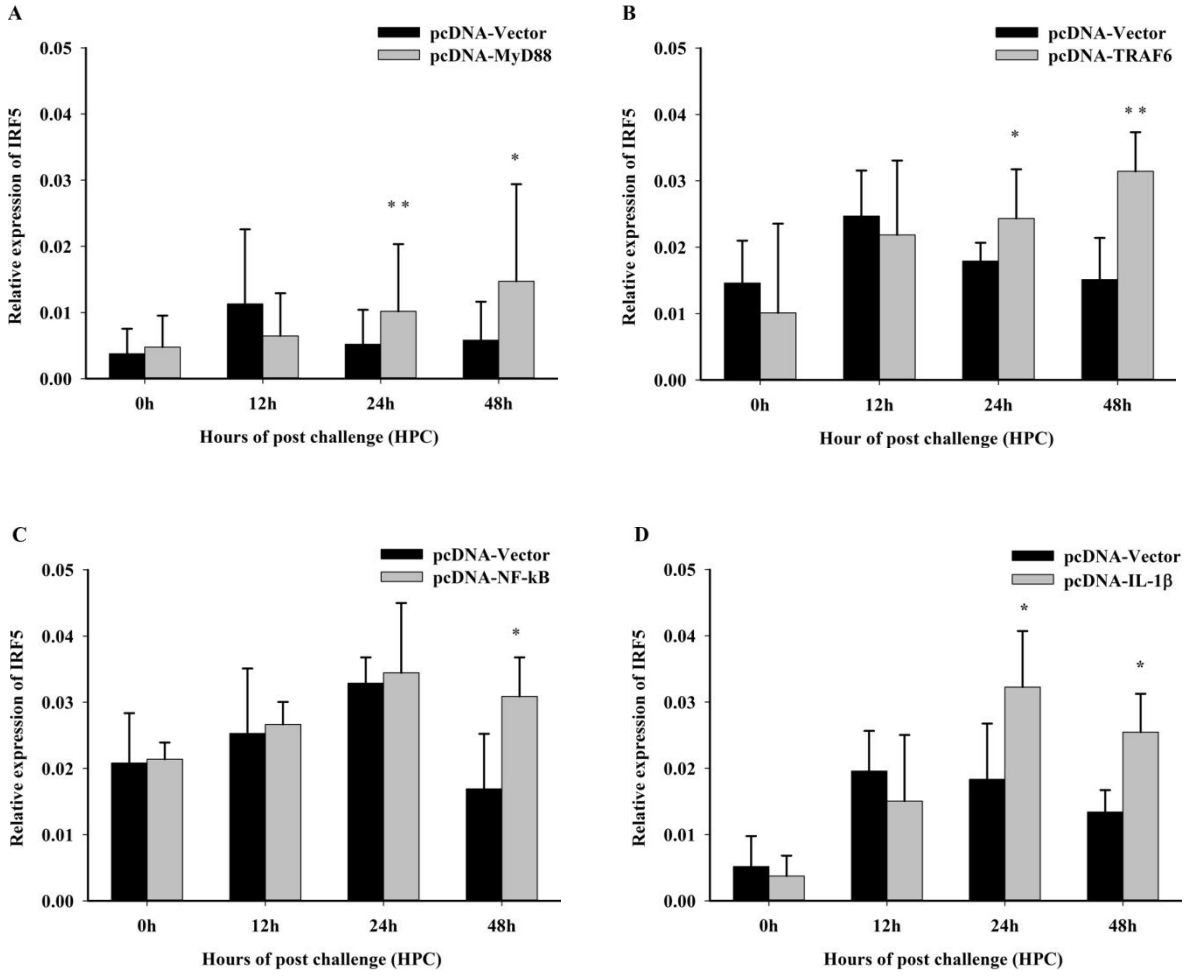
Figure 6. Relative mRNA expression level of the immune gene in different tissues from M. grouper challenged with intraperitoneally injected NNV (10^7 $TCID_{50}$ ml^{-1}). The tissues were harvested at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h post infection. The relative mRNA expression levels in (A) Spleen, (B) head kidney and (C) liver after NNV challenge and control group received an equal volume of BPS. The expressions of IRF5 mRNA were used by quantitative real-time PCR analysis. All data were expressed as the mean ± standard deviation (n=3). Significant differences between the experimental and the control group are indicated by asterisks * ($p<0.05$); ** ($p< 0.01$).

2.7. Overexpression of MgIRF5 Altered the Transcriptional Modulation

To determine the role of IRF5 inducted signal transduction, the pcDNA3.1-IRF5 and pcDNA3.1vector were transfected in the grouper kidney cells. The transcript was determined at 0 h, 12 h, 24 h and 48 h post treatment by quantitative real-time PCR method (Figure 7). The significant increase the transcriptional modulation by the expression of MgIRF5 expressing cells was only observed in the TNF-α (2.55-fold), IL-1b (2.16-fold) and IFNI (fold 2.21-fold), with were observed to be highest at 24 h. At 48 h, the expression of plasmid-IRF5 resulted in MyD88 (2.70-fold) and NF-kB (2.13-fold) significantly increased. The expression of Mx (1.10-fold) gene was lowest at 24 h post infection.



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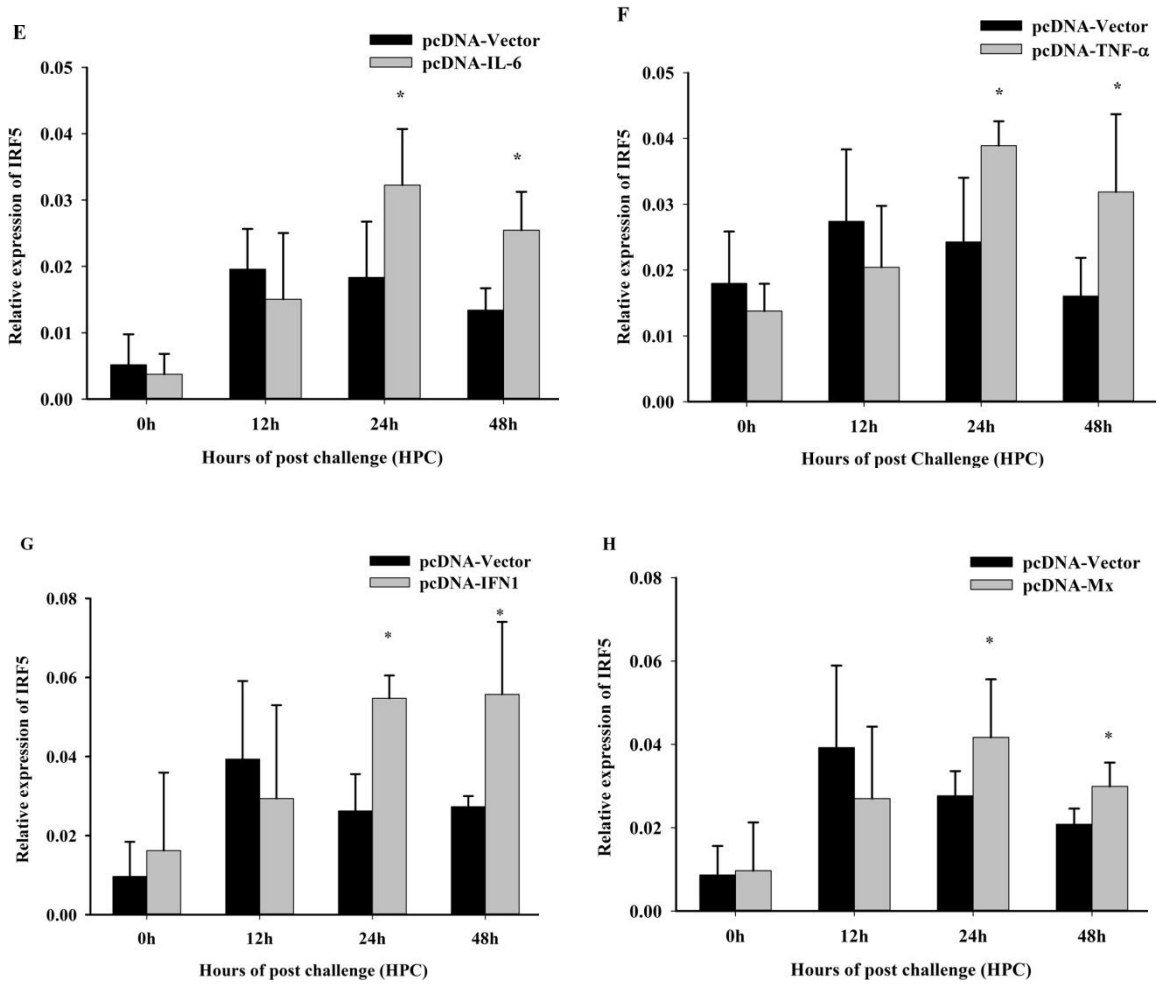

Figure 7. Relative mRNA expression level of transcriptional modulation in kidney cells after MgIRF5 transfection. The experimental samples were harvested at 0 h, 12 h, 24 h and 48 h post infection. The Amount of mRNA expression level of transcriptional modulator genes was normalized to β-actin. Results are presented as the mean ± SD (n=3). Data with asterisks are significantly different at (p<005); ** (p< 0.01).

2.8. Overexpression of MgIRF5 Inhibited the Viral Replication

In order to determine the antiviral activity of MgIRF5 against NNV infection, the NNV-CP and NNV-RdRp genes were examined by real-time PCR in pcDNA3.1-vector and pcDNA3.1-IRF5 overexpressing cells (Figure 8). The NNV gene expression was down-regulated compared to the pcDNA3.1-vector treated control cells at 24 h, indicating that overexpressed MgIRF5 affect viral replication.

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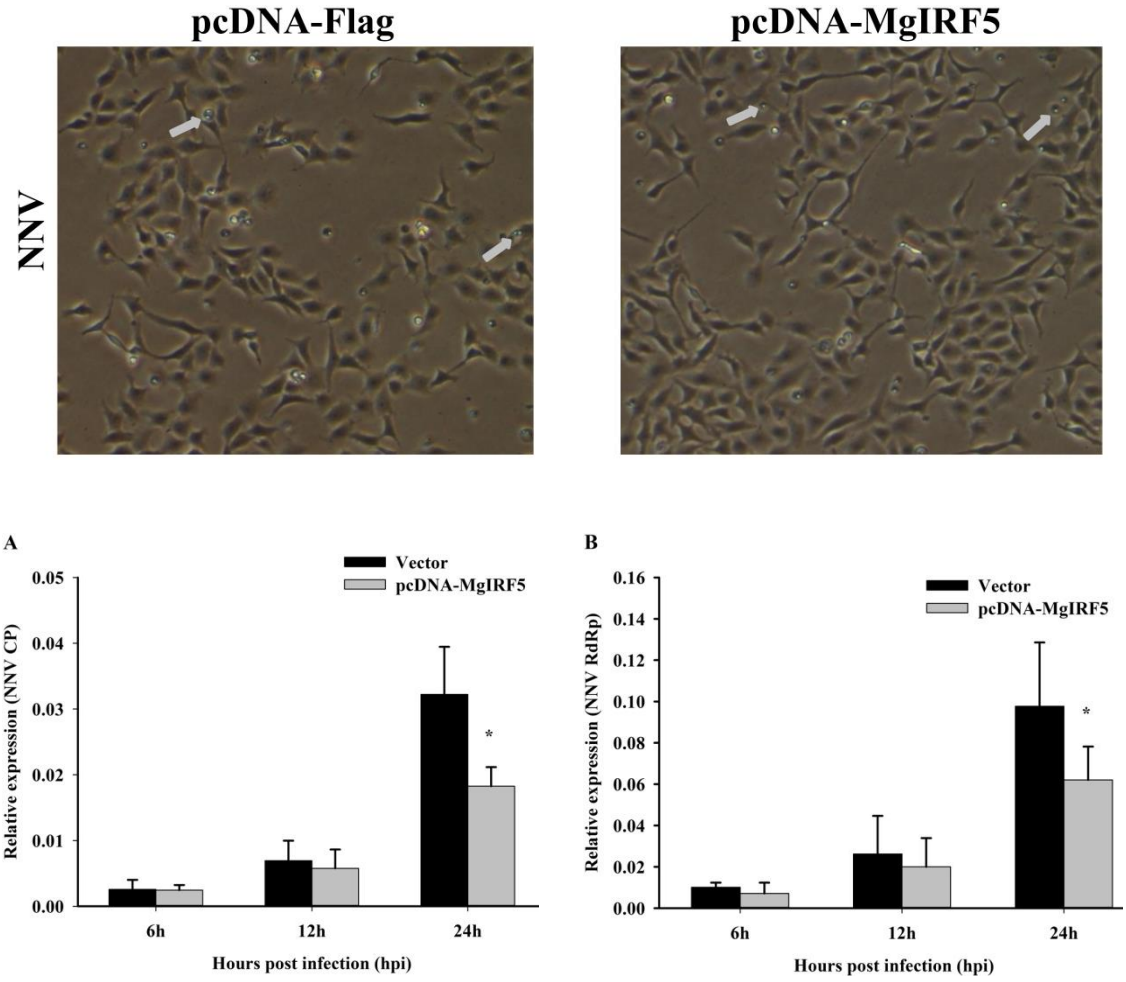

Figure 8. Effects of MgIRF5 overexpression on viral gene transcription. The expression of MgIRF5 was conformed in vector or MgIRF5 transected cells using Assay. After transfection, cells were infected with grouper NNV for 6 h, 12 h and 24 h, respectively. Total RNA of infected cells or control cells were extracted were determined NNV gene by quantitative PCR, respectively. The expressed data was normalized to β-actin. Results are presented as the mean ± SD (n=3). Data with asterisks are significantly different at ($p<005$); ** ($p< 0.01$).

3. Discussion

Numerous IRFs family members have been identified and characterized from different species. However, there is no prior information about the identification and characterization of IRF5 in Malabar grouper. In the present study, we obtained the M. grouper IRF5 cDNA sequence; this gene is a conserved sequence and contains a DBD, an MR, an IAD and a VAD (Figure 2). The highly conserved five tryptophans residues are present in the DBD, and are essential bind to ISRE/IRF-E in the promoter region of target genes [25]. IRF5 also shares a conserved interferon associated domain (IAD) domain with other IRF family members, with the exception of IRF1 and IRF2. IRF5 is responsible for the formation of homo/hetero-dimers and association with other transcription factors [26]. IRF5 contains a protein-rich domain in the MR and shares less homology with different IRFs. This is common between mammals and fish. IRF5 also contains a VAD which possesses all the

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conserved serine residues which function as virus-induced phophorylation sites. The putative IRF5 protein possesses two NLS conserved domains found in the N- and C-terminal regions, which are necessary for nuclear translocation and retention in virus-infected cells [5] (Figure 2). Phylogenetic analysis indicated that *E. malabaricus* IRF5 was well clustered and was closely related to *O. fasciaus* IRF5 (Figure 3). Collectively, this evidence suggests that MgIRF5 belongs to the IRF5 family member.

The IRF5 expression profiling has been reported in many fish, and is significantly expressed in different tissues under healthy condition [14-17, 19-20]. In this study we found that MgIRF5 was predominantly expressed in muscle and liver and moderately expressed in skin and gill (Figure 4B), which is different to the other fish species. In contrast, the differential tissue distribution of IRF5 expression in vertebrate species may be due to the diverse potential immune systems of fishes. At the embryonic developmental stages, IRF5 transcription in the fertilized egg suffices for early development stages and this transcript plays an important role up to the segmentation period in *D. rerio* [16]. In the present study, grouper fertilized embryo and larvae had higher expression of IRF5 at 24hpf (Figure 4A). These results suggest that the profiles of gene expression in different species in development stages may imply its function involved in specific stages.

In fish, the transcript expression of IRF5 gene was detected after Poly (I: C) and LPS stimulation in previous studies [14-16, 18-19, 21]. The results demonstrated that Poly (I: C) could induce MgIRF5 expression significantly in spleen and liver, respectively (Figure 5). The results are consistent with previous expression studies in fish [15, 18-19, 21], they also suggest that the gene might play an important role in fish defense against viral infection. After incubation with LPS for 24h, IRF5 gene expression in all immune tissues did not significantly induce increased expression when compared to the control. Similar research demonstrated that the in-vivo response of zebrafish to LPS stimulation is not mediated via a TLR-4 and MyD88-dependent signaling pathway [27-28], suggesting that the immune response to LPS challenge in fish might be different from mammals.

The expression of the MgIRF5 gene was found to be induced after virus infection in previous studies [14, 17-20]. In our study IRF5 expressions were significantly up-regulated by experimentally challenging with NNV and detected by real-time PCR. NNV replication was observed 48h post infection in the brain by a RT-PCR method and the NNV capsid protein was highly expressed at 72 h (Data not shown). The results indicate that NNV induces MgIRF5 expression significantly in lymphoid organs or tissues such as in spleen, head kidney and liver, suggesting that the gene might play an important role in fish defense against the viral pathogen (Figure 6 A-C). This was confirmed by studies in other fish species, which also showed up-regulation of IRF5 gene when infected with pathogens [14, 17-20]. This suggests IRF5 plays a pivotal role in preventing viral infection in vertebrates.

A number of signaling pathway studies show that IRF5 leads to activation and nuclear translocation and results in the production of type IFNs and proinflammatory cytokines [12, 29-33]. In fish, the potential roles of IRF5 in IFN, MX and TNF-α response were confirmed by the evidence of over-expressed IRF5 in Rock bream when transfected [19]. In this study, we found that the over-expression of IRF5 transcripts was inducing downstream signaling which translocate to the nucleus, the target genes such as MyD88, TRAF6, NF-kB, IL-1β, IL-6, TNF-α, IFN and Mx (Figure 7). This phenomenon can be explained by the IRF5 depending upon the molecular mechanisms

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underlying fish immune response. These findings described herein further confirm MgIRF5 acted as a transcription activator in immune response to viral infection.

Previous papers indicated that the potential role of IRF5 in mediating transcription activating type 1 IFN for defense against viral infection [1]. Our results showed that the transcription of IFN-ISGs was mediated by MgIRF5 transfection during NNV infection (Figure 8). Our data indicated that the over-expression of IRF5 inhibited the replication of NNV-CP and RdRp gene transcription of NNV in grouper kidney cells. It may activate a host's antiviral function similar to that of interferon-stimulated gene 15 (ISG15), Vig and MDA5 in RGNNV infection [34-36]. Collectively, the M.gIRF5 might participate in host antiviral responses and may inhibit NNV replication and transcription inducing type I IFN expression as well as activating the JAK-STAT signaling pathway.

The full-length cDNA sequence of IRF5 was predicted from transcriptome data bank in the M. grouper. The IRF5 gene in M. grouper is structurally and phylogenetically closely related to *Oplegnathus fasciatus* and *Miichthys miiuy* IRF5. The gene was expressed in immune related tissues with highest expression levels in muscle, liver and spleen. IRF5 expresses at a higher level in response to NNV and Poly (I: C) stimulation as compared with LPS, suggesting that IRF5 has an important role in antiviral immune response of fish. The overexpression of MgIRF5 enhanced the expression of transcriptional factors and significantly inhibited NNV replication. This study of IRF5 may help better understand immune response in grouper, which would be beneficial for prevention of diseases in grouper larvae culture and provide valuable information for the study of origin and evolution of immunity.

4. Materials and Methods

4.1. Cell Culture and Viral Pathogens

The grouper kidney cells (GK) [37] and GF-1 cells [38] were maintained in L-15 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen), 10 I.U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen) at 28 ℃. Malabar grouper nervous necrosis viruses (MgNNV) was isolated from infected M. grouper brain and were proliferated in GF-1 cells [31], then cultured supernatant was used to determine by the virus tittered to be 10^7TCID50 ml^{-1}, as described by Reed and Muench [39].

4.2. Experimental Animals and Immune Challenge

The healthy M. grouper (4-5 cm) were purchased from a commercial farm (Kaohsiung, Taiwan) and acclimatized to the laboratory conditions in a circulating water system for one week before use in experiments. Experimental animals were acclimated with the institutional IACUC guidelines of the National Taiwan Ocean University (Keelung, Taiwan). Fish samples were randomly divided into four groups. The experimental group of grouper were injected intraperitoneally with 0.1 ml of 5mg/ml conc. in polyinosinic:polycytidylic acid (poly (I: C), Sigma Aldrich) per fish, 10mg/ml conc. of lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8, sigma Aldrich) per fish, and NNV at the dose of 10^5 $TCID_{50}$ ml^{-1}. The control group was administrated with the same volume of BPS and L-15 medium. At 0 h, 6 h, 12 h, 24 h, 48 h and 72 h post injection (phi), three fish samples from each

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group were anesthetized in 0.05% 2- phenoxyelthanol, and samples of spleen, head kidney, liver, gill were collected and immediately stored in TRIzol® reagent (Ambion, USA) at -80 ℃.

4.3. M. grouper De novo Transcriptome Database Search and Cloning

Malabar grouper IRF5 transcripts were distinguished by the de novo transcriptome sequencing approach [40]. Total RNA was extracted from the spleen of healthy Malabar grouper utilizing TRIzol® reagent (Ambion, USA) and reverse transcribed into first strand cDNA using a PrimeScript 1st strand cDNA synthesis kit (*BIONOVAS*, USA) according to the manufacturer's instructions. PCR amplification primers were designed based on the IRF5 Unigene from the Malabar grouper transcriptome database (Table 1). PCR amplification was performed under the following conditions: 1 cycle of 95 ℃ for 5 mins, 35 cycles of 95 ℃ for 30 sec, 53 ℃ for 30 sec, and 72 ℃ for 60 sec, followed by a final extension at 72 ℃ for 10 mins. The PCR products were gel-extracted and cloned into yT&A-vector and pcDNA3.1-vector. Following transformation into competent *E. coli* (DH5α) cells, positive clones were screened utilizing ampicillin selection and colony PCR and after that sequenced.

Table 1. Oligonucleotide primers used to amplify the genes in the M. grouper.

Primer name	Orientation	Nucleotide Sequences (5′ – 3′)	Primers Usage
IRF5	Sense	ATGAGCGTCCAGCCCCGC	RT-PCR
IRF5	Antisense	TCAGGGGACGTTAGGGGT	RT-PCR
IRF5	Sense	AAGGTTCTACACTGAGGCCC	RT-PCR detection
IRF5	Antisense	TTGCAGGAAGTTGTTGAGGC	RT-PCR detection
IRF5	Sense	ATATCAGGAAGGCGTGGAC	Real-time RCR
IRF5	Antisense	TCTATGTTCCCGCTCTGCTCA	Real-time RCR
MyD88	Sense	GCGACGCCTGTGACTTTCA	Real-time RCR
MyD88	Antisense	TTCGAGCTCCGGGACAGA	Real-time RCR
TRAF6	Sense	TACAGGACAGAGGTGCCC	Real-time RCR
TRAF6	Antisense	AGCGGACAGTTAGCGAG	Real-time RCR
NF-kB	Sense	CAGGACGGCAACGGAGA	Real-time RCR
NF-kB	Antisense	TGCTGCTGACTGCTGAG	Real-time RCR
IL-1β	Sense	CCAGCGTTGAGGGCAGAA	Real-time RCR
IL-1β	Antisense	ATCGTCTCCAGATGTAAGGTT	Real-time RCR
IL-6	Sense	GGAGAGGCTCAGAGGAAG	Real-time RCR
IL-6	Antisense	ACACCTGAGTGTGAGAACAGTAA	Real-time RCR
TNF-α	Sense	GCAAAGCCTCGCTGATG	Real-time RCR
TNF-α	Antisense	GCCCAGATAAATGGCGTTGT	Real-time RCR
Mx	Sense	AGAAGGTGCGTCCCTGCAT	Real-time RCR
Mx	Antisense	CTGACAGCGCCTCCAACAC	Real-time RCR
IFN1	Sense	GGGTTACAGCTCTGCATCA	Real-time RCR
IFN1	Antisense	CTCTTGCTCTCCCTGTGG	Real-time RCR
β-actin	Sense	CACAGTGCCCATCTACGAG	Real-time RCR
β-actin	Antisense	CCATCTCCTGCTCGAAGTC	Real-time RCR

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4.4. Bioinformatics Analysis

The cDNA sequence containing the complete open reading frame (ORF) of MgIRF5 was obtained from transcriptome data of M. grouper spleen by using BLAST at the NCBI web servers (http://www.ncbi.nlm.nih.gov/blast). The PROSITE database was searched to detect conserved domain structure within the deducted amino acid sequences (http://us.expasy.org/tools/scanprosite). The amino acid conserved domains were analyzed by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de). Multiple alignments of the amino acid sequence were built using ClustalW2 (http://www.ebi.ac.uk) with default settings. Molecular evolutionary analyses of the polygenetic trees based on multiple protein alignments were constructed using the Bootstrap neighbor-joining (NJ) method from the MEGA software (http://www.megasoftware.net) and bootstrapped 1000 times.

4.5. Developmental Stage and Tissue Expression Analysis of MgIRF5

To determine IRF5 gene expression in different tissues, eyes, brain, heart, head kidney, trunk kidney, liver, spleen, intestine, gas bladder, gill, muscle, and skin were taken aseptically from healthy M. grouper. Fertile eggs and fry were collected during developmental stages, approximately 0.4 ml of fertilized eggs, or 20 fry at 0 h - 72 h post fertilization [16]. At each time interval three tissue samples were freshly collected and immediately stored in TRIzol® reagent (Ambion, USA) at -80 ℃.

4.6. Transfection of pcDNA3.1-MgIRF5 into Grouper Kidney (GK) cells

The grouper kidney cells were seeded in 24 well plates with a density of $1.5x10^5$ cells/well in 0.5 mL/well L-15 medium (Gibco, USA) and incubated at 28 ℃ overnight. Cells were washed with 1X PBS and then replaced with L-15 medium. For each well 1ug of IRF5-pcDNA recombinant vector construct or an empty vector (Mock control) was transfected with grouper kidney cells by using Maestrofectin (Omics Bio, Taiwan), according to the manufacturer's protocol. At 2 days post infection, the cells were infected with NNV. At 2h post infection, GK cells were washed and refreshed with L-15 medium. At 6 h, 12 h and 24 h post infection (PI), the cells were harvested and immediately stored in TRIzol® reagent (Ambion, USA) at -80 ℃.

4.7. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from experimental samples by TRIzol® reagent (Ambion, USA) according to the manufacturer's instructions. The quality of the extracted RNA was determined with 1% agarose gel by electrophoresis and the concentration was determined by spectrophotometerically absorbance at 260nm (Thermo Scientific, USA). The total RNA was used to synthesize the first-strand cDNA with HiScript I Reverse Transcriptase (BIONOVAS, CA) in accordance with the manufacturer's instructions. The cDNA was stored at -80 ℃ until use.

4.8. Quantitative real-time PCR (qRCR)

Evaluation of the mRNA expression was carried out via quantitative real-time PCR (qPCR) using a ABI Applied Biosystems® 7500 Real-Time PCR System V2.0.6 (Applied Biosystems, USA). The qPCR mixture consisted of 1μl of cDNA samples, 7μl of nuclease-free water, 10μl of KAPA SYBR® FAST qPCR kit (Kapa Biosystems, USA), and 0.5μl of forward and reverse primers (10μM)

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(Table 1). The threshold cycle (CT) value was determined using the default setting on the ABI 7500HD system and exported into a Microsoft excel sheet for subsequent data analyses where the relative expression ratios of target genes were normalized to β-actin and calculated by $2^{-\Delta\Delta Ct}$ method.

4.9. Statistical Analysis

Quantitative data from three independent experiments were expressed as mean ± standard deviation and the experimental groups were compared using Student t-test for paired samples. The critical level of statistic significant differences was $p<0.05$. High significance was $p<0.001$.

5. Conclusions

In the present study, the full-length cDNA sequences and expression level of M. grouper IRF5 were identified and characterized. The evolution relationship was analysis and closely related to *Oplegnathus fasciatus* and *Miichthys miiuy* IRF5. Our data suggest that IRF5 expression in M. grouper immune tissues significantly expressed by immuno-stimulation and NNV infection.

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Author Contributions: Thirunavukkarasu Periasamy designed the experiment, conducted, analyzed the results and drafted the manuscript. Ming-wei Lu monitored throughout the experimental process and read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

VER	Viral encephalopathy and retinopathy
NNV	Nervous necrosis virus
IRF 5	Interferon regulatory factor 5
Poly (I: C)	polyinosinic: polycytidylic acid
LPS	lipopolysaccharide
Mg	Malabar grouper *(Epinephelus malabaricus)*

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