



## Full length article

## Grouper viperin acts as a crucial antiviral molecule against iridovirus

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## ABSTRACT

Virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (viperin), is an antiviral protein, induced by interferon (IFN), poly(I:C) and viral infection to exert antiviral function. To investigate the roles of viperin during fish virus infection, a viperin homolog from orange spotted grouper (*Epinephelus coioides*) (Ecviperin) was cloned and characterized in this study. Ecviperin encoded a 361-aa protein which shared 87% and 69% identity with *Siniperca undulata* and *Homo sapiens*, respectively. Amino acid alignment analysis showed that Ecviperin contained a conserved radical-SAM domain (aa73-281). Phylogenetic analysis indicated that Ecviperin showed the nearest relationship with *S. undulata*. In healthy grouper, Ecviperin was distributed in all tissues, and the expression of Ecviperin was the highest in kidney and spleen. *In vitro*, the mRNA expression of Ecviperin was significantly up-regulated in response to Singaporean grouper iridovirus (SGIV) infection. Subcellular localization analysis showed that Ecviperin was distributed in the cytoplasm and co-localized with endoplasmic reticulum (ER). The ectopic expression of Ecviperin significantly inhibited the replication of SGIV. Furthermore, overexpression of Ecviperin positively regulated the interferon related molecules, including interferon regulatory factor 3 (IRF3), IRF7, interferon stimulated gene 15 (ISG15), myxovirus resistance gene 1 (MX1), interferon-induced 35-kDa protein (IFP35), and TNF receptor-associated factor 6 (TRAF6). In addition, the expression of pro-inflammation cytokines was differently regulated by Ecviperin overexpression. Furthermore, reporter gene analysis showed that the overexpression of Ecviperin enhanced the activity of nuclear factor of kappa B (NF-κB), IFN-1 and interferon-stimulated response element (ISRE) promoter, suggesting that Ecviperin might restrict SGIV replication by the positive regulation of interferon and inflammatory response. Taken together, our results demonstrated that Ecviperin encoded an ER-localized protein, and exerted antiviral function against fish DNA virus by up-regulating interferon and pro-inflammatory response.

## 1. Introduction

During virus infection, virus nucleic acids and proteins stimulate the innate immune system, in which pattern recognition receptors (PRR) recognize pathogen, triggering the IFN-mediated innate immune response and inducing the transcription of hundreds of IFN-stimulated genes (ISGs) through signaling cascades [1,2]. Many ISGs restrict virus infection, such as interferon-induced transmembrane proteins (IFITMs) [3,4], protein kinase R (PKR) [5], MX dynamin-like GTPase 2 (Mx2) [6], tetherin [7], tripartite motif containing protein (TRIM) [8–10] and viperin [11]. Viperin, as an antiviral protein induced by interferon, has antiviral activity against both DNA and RNA viruses, including hepatitis C virus (HCV) [12], West Nile virus (WNV) [13], human immunodeficiency virus 1 (HIV-1) [14], chikungunya virus (CHIKV) [15], dengue virus 2 (DENV-2) [16], herpes simplex virus 1 (HSV-1) [17],

influenza A virus (IAV) [18], Japanese encephalitis virus (JEV) [19], zika virus (ZIKV) and tick-borne encephalitis virus (TBEV) [11].

Viperin was firstly identified as a human cytomegalovirus (HCMV)-inducible gene in primary fibroblasts [20]. Although the expression level of viperin in normal cells was low, it can be induced by IFN, and poly(I:C), bacteria and viruses [20–24]. The induced viperin exerted crucial roles during the different stage of virus life cycle. For example, viperin was demonstrated to suppress HCV replication by affecting the interaction between vesicle-associated membrane protein-associated protein subtype A (VAP-A) and nonstructural protein 5A (NS5A), thus preventing the formation of HCV replication complexes [25]. In addition, viperin also could interact with farnesyl diphosphate synthase (FPPS), an enzyme involved in the synthesis of multiple isoprenoid, to disrupt lipid rafts and inhibit the release of the virus [18].

Although great progress has been made in elucidating the antiviral

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action of viperin against viruses in mammals, limited literature focused on the function of viperin in fish, including red drum (*Sciaenops Ocellatus*) [24], crucian carp (*Carassius auratus*) [26,27], tilapia (*Oreochromis niloticus*) [28], and rock bream (*Oplegnathus fasciatus*) [29]. Groupers, *Epinephelus* spp. are important economic fish species in China and Southeast Asian countries. While the outbreak of virus diseases which evoked by Singapore grouper iridovirus (SGIV) and red spotted grouper nervous necrosis virus (RGNNV) have caused great economic losses to grouper aquaculture industry in recent years [30–32]. To elucidate the potential mechanism underlying the regulatory roles of grouper immune genes against virus infection, multiple genes related to interferon and apoptotic signaling pathway were identified and their actions on grouper virus infection were dissected [33–43]. For example, grouper mitochondrial antiviral signaling protein (MAVS), melanoma differentiation-associated gene 5 (MDA5) and stimulator of interferon genes (STING) exerted different antiviral effects against SGIV and RGNNV infection in grouper cells [36,37,42]. Besides, TANK-binding kinase-1 (TBK1), IRF7 and TRIM25 played crucial roles during SGIV and RGNNV infection [35,40,43]. As an important IFN-induced protein, the detailed function of viperin in response to grouper virus infection still remained uncertain.

In this study, a viperin homolog from orange spotted grouper (EcViperin) was cloned and characterized. The subcellular localization was detected and the roles of EcViperin during SGIV replication were evaluated. Meanwhile, the effects of EcViperin on host interferon and inflammatory response were investigated. Our data will provide new insights into understanding the function of fish viperin against virus infection.

## 2. Materials and methods

### 2.1. Fish, cells and virus

Orange-spotted groupers, *E. Coioides* (50–60 g) used in the study were purchased from Hainan Province, China, then kept in a laboratory recirculating seawater system before use. Grouper spleen (GS) cells used in this study were grown in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco) at 28 °C [44]. The SGIV stocks was prepared and stored at –80 °C as described previously [45].

### 2.2. Cloning of EcViperin and sequence analysis

The full length of EcViperin was cloned by PCR amplification and sequenced based on the EST sequences of EcViperin from grouper spleen transcriptome [46]. The primers were listed in Table 1. The sequence of EcViperin was analyzed using BLAST program (<http://www.ncbi.nlm.nih.gov/blast>), and the conserved domains or motifs were predicted using SMART program (<http://smart.embl-heidelberg.de/>). Amino acid sequence alignment was carried out using ClustalX1.83 software and edited by GeneDoc program. The Neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 6.0 software.

### 2.3. Expression patterns for EcViperin in grouper

To determine the tissue distribution pattern of EcViperin in healthy orange-spotted grouper, total RNA from 12 tissues of 3 healthy groupers, including head kidney, kidney, liver, spleen, brain, gill, heart, muscle, intestine, fin, skin and stomach, was extracted using the SV Total RNA Isolation Kit (Promega) and the relative expression level of EcViperin was detected by quantitative real-time PCR (qRT-PCR) as described in the following.

To examine the expression profiles of EcViperin in response to virus infection, GS cells were infected with SGIV and harvested at 4, 8, 12, 18, 24, 36 h post infection (p.i.) for RNA extraction and further qRT-PCR analysis.

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

Primer names	Sequence (5'-3')
EcViperin-ORF-F	ATGCGATACTGGTCAGGTC
EcViperin-ORF-R	TCACCACCTCCAGCCTCAT
EcViperin-C1-EcorI-F	CGGGAATTCATGCGATACTGGTCAGGTC
EcViperin-C1-BamHI-R	CGCGGATCCCCACTCCAGCCTCATGTCCG
EcViperin-RT-F	GTGTCAGCATCGTCAGCAA
EcViperin-RT-R	CCGAGTTGATTTTGAAGGC
Actin-RT-F	TACGAGCTGCCTGACGGACA
Actin-RT-R	GGCTGTGATCTCCTTCTGCA
SGIV MCP-RT-F	GCA CGCTTCTCACCTTCA
SGIV MCP-RT-R	AACGGCAACGGGAGCACTA
SGIV VP19-RT-F	TCCAAGGGAGAAAACGTAAAG
SGIV VP19-RT-R	GGGGTAAGCGTGAAGAC
SGIV LITAF-RT-F	GATGCTGCCGTGTGAAGTC
SGIV LITAF-RT-R	GCACATCCTTGGTGGTGTG
SGIV ICP-18-RT-F	ATCGGATCTACGTGGTTGG
SGIV ICP-18-RT-R	CCGTGCTGGTGTCTATTC
EcIRF3-RT-F	GACAACAAGAACGCCCTGCTAA
EcIRF3-RT-R	GGGAGTCCGCTTGAAGATAGACA
EcIRF7-RT-F	CAACACCGGATACAACCAAG
EcIRF7-RT-R	GTTCCTCAACTGCTACATAGGG
EcISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC
EcISG15-RT-R	GTGCTGTGGCAGTGACGTTGTAGT
EcIFP35-RT-F	TTCAGATGAGGAGTTCTCTCTTGTG
EcIFP35-RT-R	TCATATCGGTGCTCGTACTTTTCA
EcMX 1-RT-F	CGAAAGTACCGTGGACGAGAA
EcMX 1-RT-R	TGTTTGATCTGCTCCTTGACCAT
EcTRAF6-RT-F	CCCTATCTGCTTATGGCTTTGA
EcTRAF6-RT-R	ACAGCGGACAGTTAGCGAGAGTAT
EcTNF $\alpha$ -RT-F	GTGTCCTGCTGTTTGCTTGTA
EcTNF $\alpha$ -RT-R	CAGTGTCCGACTTGATTAGTGCTT
EcIL-1 $\beta$ -RT-F	AACCTCATCATCGCCACACA
EcIL-1 $\beta$ -RT-R	AGTTGCCTCACAAACCGAACAC
EcIL-6-RT-F	GGTTGGTCCAAGGTGTGCTTA
EcIL-6-RT-R	CTGGGATGTCGAGGTCCTT

### 2.4. Plasmid construction and cell transfection

To illustrate the function and subcellular localization of EcViperin *in vitro*, the full length of EcViperin (aa1-361) was cloned into pEGFP-C1 using the primers in Table 1. The recombinant plasmid (pEGFP-EcViperin) was subsequently confirmed by DNA sequencing.

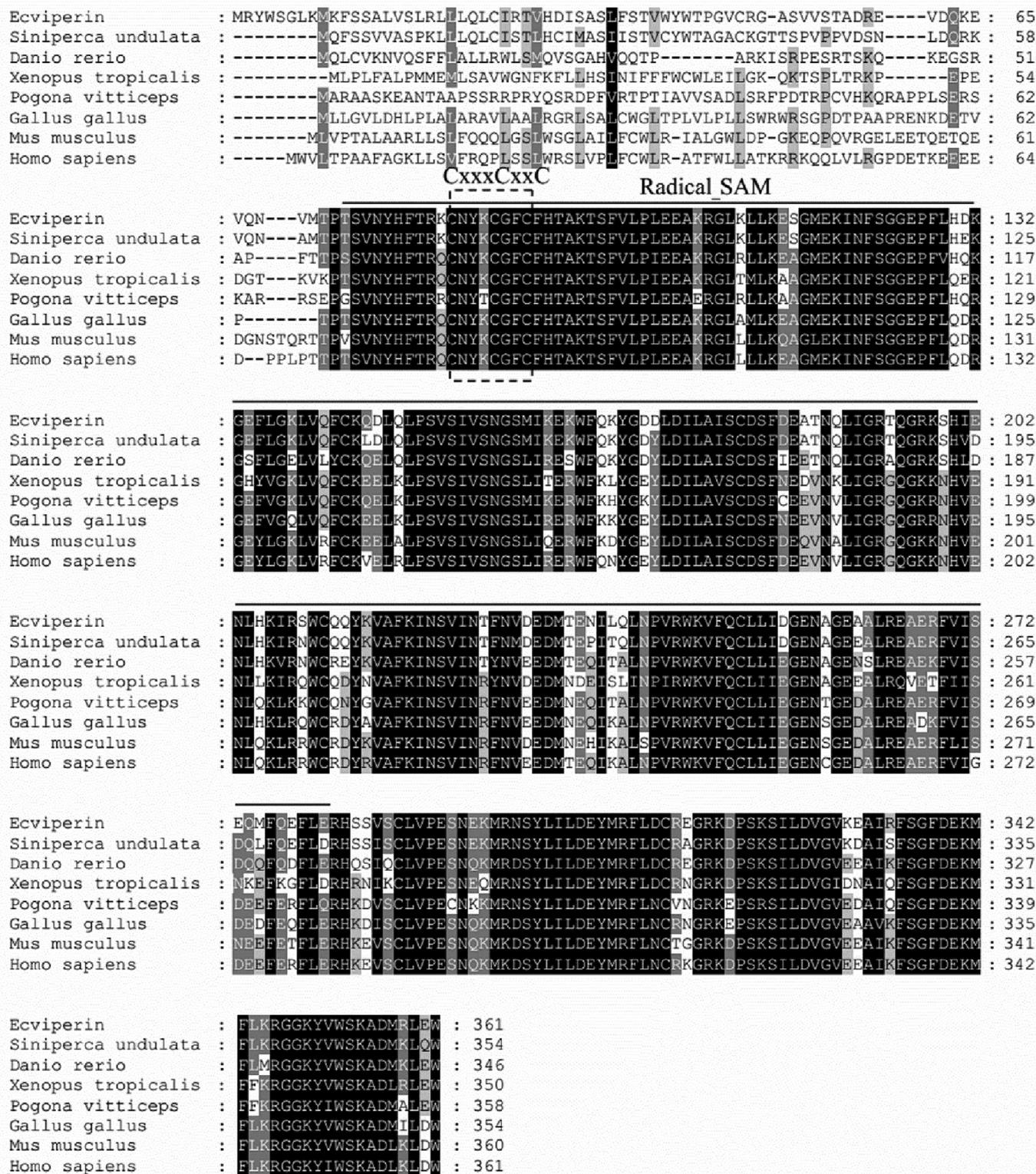
Cell transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) as described previously [47]. Briefly, GS cells were seeded in 24-well plates or 6-well plates at 60–70% confluence for 18–24 h, then incubated with the mixture of Lipofectamine 2000 and plasmids for 6 h. After replacing with the fresh normal medium, cells were cultured at 28 °C for further study.

### 2.5. Fluorescent microscopy

To observe the subcellular localization of EcViperin, pEGFP-C1 or pEGFP-viperin was co-transfected with pDsRed2-ER into GS cells as described above, respectively. Cells were fixed with 4% paraformaldehyde, and stained with 4,6-diamidino-2-phenylindole (DAPI) at 48 h post-transfection. Fluorescence was observed under fluorescence microscopy.

### 2.6. Virus infection assay

To evaluate the effects of EcViperin on virus infection, GS cells overexpressing pEGFP-C1 or pEGFP-viperin were infected with SGIV at a multiplicity of infection (MOI) of 2. At indicated time points, the cell morphology was observed under a phase contrast microscopy. Meanwhile, mock and virus-infected cells were harvested for RNA extraction and qRT-PCR analysis.



**Fig. 1.** Amino acid alignment of viperins from different species. Radical-SAM domain and CxxxCxxC motif were indicated by the straight line and the dotted box, respectively. The accession numbers were listed as follows: *Epinephelus coioides*, EU926745.1; *Siniperca undulata*, ABO48457.1; *Danio rerio*, ABJ97316.1; *Xenopus tropicalis*, XP\_002935073.1; *Gallus gallus*, ATU83332.1; *Mus musculus*, AAL50054.1; *Homo sapiens*, AAL50053.1.

**2.7. Reporter gene assay**

To examine the effects of Ecviperin on the activity of interferon and NF-κB promoter, luciferase plasmids including ISRE-Luc, IFN1-Luc and NF-κB-Luc were used in this study. In brief, GS cells were co-transfected with 150 ng ISRE-Luc (IFN1-Luc or NF-κB-Luc) and 800 ng pEGFP-

Ecviperin or pEGFP-C1, respectively. A total of 40 ng SV40 was included to normalize the luciferase activities. Then cells were harvested to measure the luciferase activities by Dual-Luciferase® Reporter Assay System (Promega) at 48 h post-transfection according to the manufacturer's instructions.

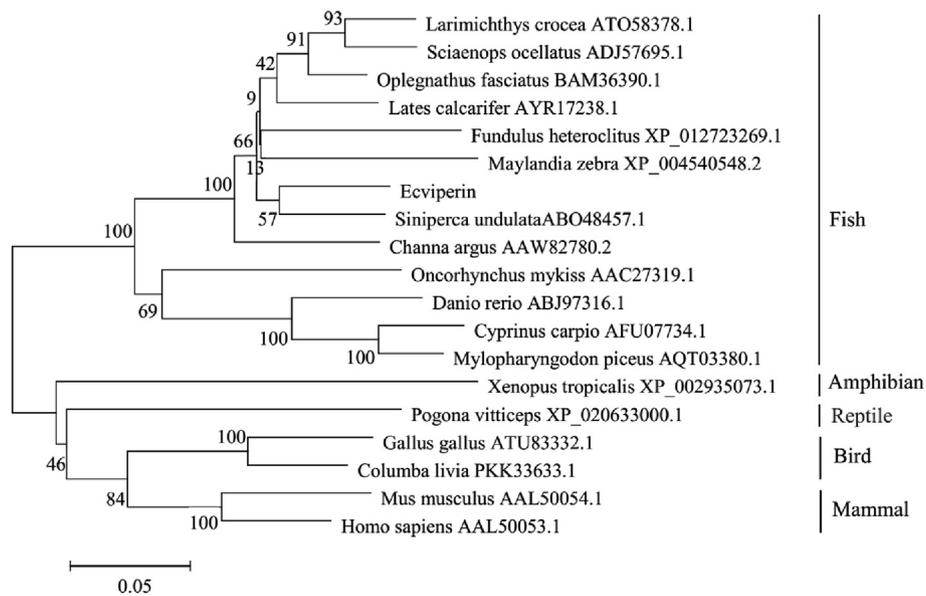


Fig. 2. The phylogenetic analysis of viperins. A neighbor-joining tree was constructed based on the protein sequences of viperin-like genes from different species using MEGA 6.0 software. Numbers at the nodes denote the bootstrap values of 1000 replicates. Scale represents the numbers of substitutions per 1000 bases.

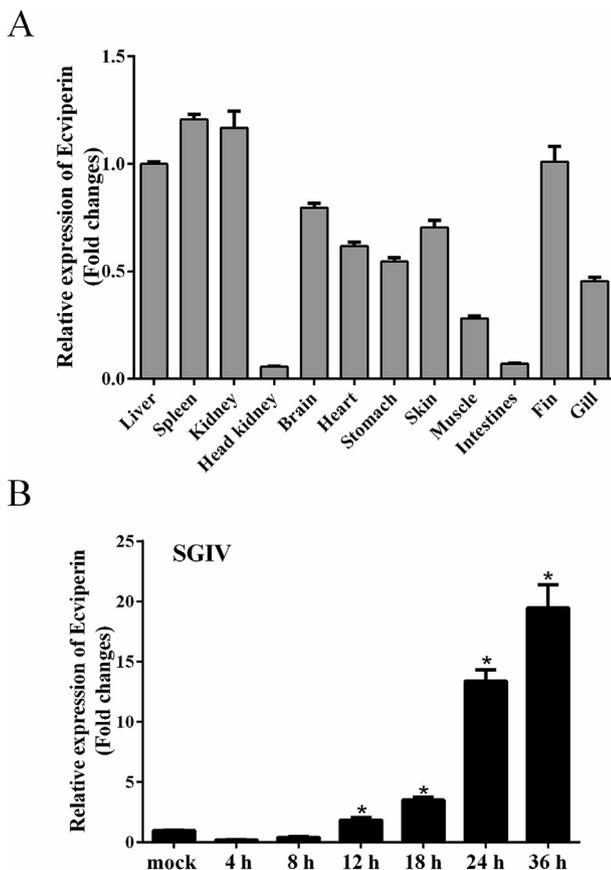


Fig. 3. The expression patterns of Ecviperin in healthy and SGIV-infected cells. (A) The expression level of Ecviperin in different tissues from healthy groupers. (B) After infection with SGIV, the expression levels of Ecviperin in GS cells were detected using qRT-PCR. The error bars represent standard deviations of triplicates and \* indicates that the means were statistically significantly at  $P < 0.05$ .

## 2.8. Quantitative PCR

In order to examine the transcriptional expression level of host or virus genes, qRT-PCR was performed in a Applied biosystems QuantStudio 3 Real Time Detection System (Thermofisher, USA). Each assay was carried out in triplicate with the following cycling conditions: 95 °C for 1 min for activation, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. The used primers were listed in Table 1. The expression levels of target genes were normalized to  $\beta$ -actin and calculated with the  $2^{-\Delta\Delta CT}$  method. The data were represented as mean  $\pm$  SD.

## 2.9. Statistical analysis

Statistics were carried out using SPSS version 20 by one-way ANOVA. Differences were considered statistically significant when  $P < 0.05$  (\*).

## 3. Results

### 3.1. Sequence characterization of Ecviperin

Based on the EST sequences from grouper spleen transcriptome, the full length of open reading frame (ORF) of viperin was amplified by PCR. BLASTN analyses indicate that the obtained sequence was identical to *E. Coioides* viperin (EU926745.1) and designated as Ecviperin. Further analysis shows that Ecviperin encoded a 361-aa protein which shared 87% and 69% identity with *S. undulata* (ABO48457.1) and *H. sapiens* (AAL50053.1), respectively. Amino acid alignment indicates that Ecviperin contained a Radical-SAM domain (aa73-281) with the three-cysteine motif CxxxCxxC (Fig. 1). The phylogenetic analysis reveals that Ecviperin shows the nearest relationship to *S. undulata*. Besides, all the viperins from different fish species are clustered into one group which is separated from other groups, including bird, reptile, mammal and amphibian (Fig. 2).

### 3.2. Tissue distribution and expression profiles of Ecviperin

The transcription level of Ecviperin in different tissues from healthy grouper were detected by qRT-PCR. As shown in Fig. 3A, Ecviperin expression was detected in all tissues, with the predominant expression

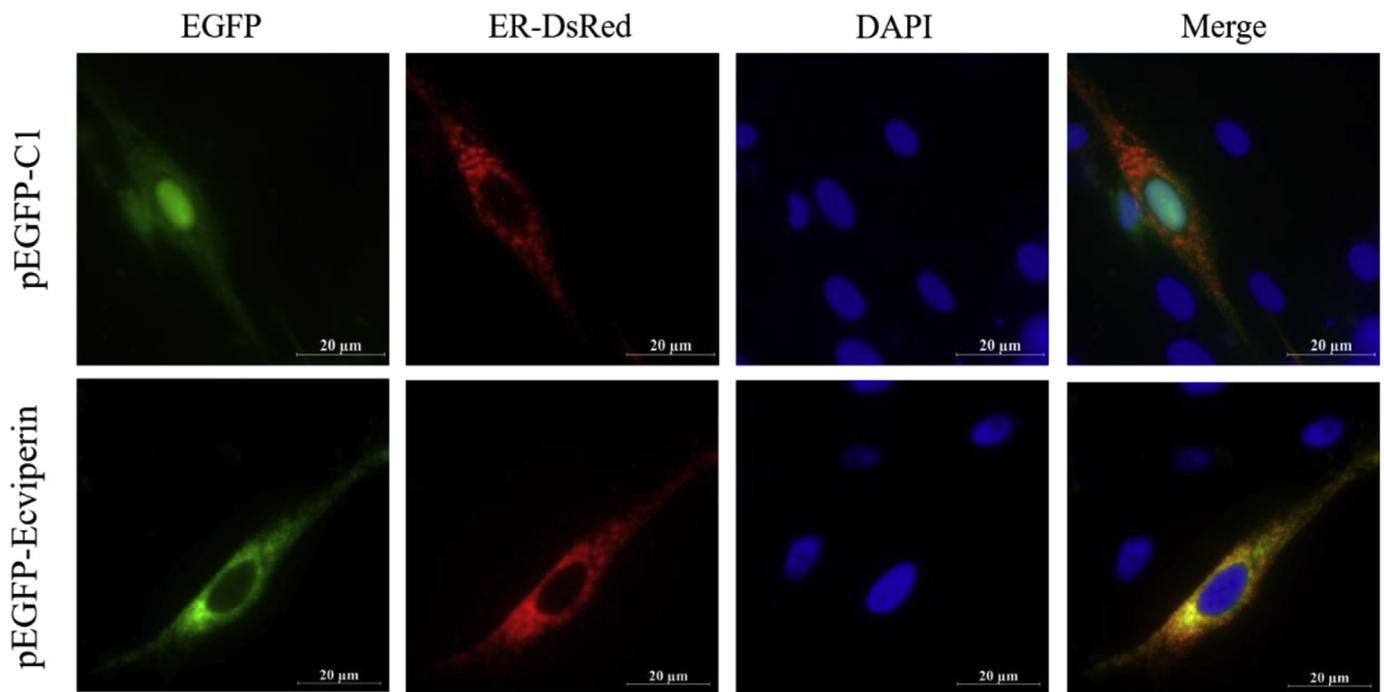


Fig. 4. Subcellular localization of Ecviperin in grouper cells. GS cells were transfected with pEGFP-C1, pEGFP-Ecviperin and pDsRed2-ER plasmid, then stained with DAPI. Samples were observed under fluorescence microscopy.

in kidney, spleen, liver and fin.

To determine the expression level of Ecviperin during SGIV infection, the transcript of Ecviperin in SGIV-infected GS cells was examined by qRT-PCR. The results showed that the transcription level of Ecviperin decreased from 4 h p.i. to 8 h p.i., while gradually increased from 12 h p.i., and reached a peak up to 19.5-folds at 36 h p.i. compared to that of mock cells (Fig. 3B), suggesting that Ecviperin might play an important role in response to fish DNA virus infection.

### 3.3. Subcellular localization of Ecviperin

In order to explore the subcellular localization of Ecviperin *in vitro*, pEGFP-C1 and pEGFP-Ecviperin was co-transfected with pDsRed2-ER plasmids into GS cells, respectively. As shown in Fig. 4, the green fluorescence in pEGFP-Ecviperin transfected cells was observed in the cytoplasm and co-localized with the red fluorescence of ER. However, in pEGFP-C1 transfected cells, the green fluorescence was distributed throughout the cytoplasm and nucleus. Thus, Ecviperin was speculated to encode an ER-localized protein.

### 3.4. Ecviperin overexpression inhibited SGIV replication *in vitro*

To investigate the effects of Ecviperin on fish virus replication, we examined the severity of virus-induced cytopathic effect (CPE) and the transcription level of virus gene in Ecviperin overexpressed cells. Firstly, we evaluated the transcription level of Ecviperin in transfected cells to clarify the successful overexpression of Ecviperin in grouper cells. The expression of Ecviperin was significantly increased up to 1493.6 folds compared with the control vector cells (Fig. 5A). As shown in Fig. 5B, the severity of CPE induced by SGIV was obviously weakened in Ecviperin overexpressed cells. Consistently, the overexpression of Ecviperin significantly reduced the transcription level of SGIV major capsid protein (MCP), VP19, infected cell protein 18 (ICP-18) and lipopolysaccharide-induced TNF- $\alpha$  factor (LITAF) genes (Fig. 5C). Thus, the results indicated that Ecviperin overexpression inhibited the replication of SGIV.

### 3.5. Ecviperin overexpression differently regulated interferon and inflammatory response

To clarify the effect of Ecviperin on host interferon and inflammation response, we examined the expression of interferon signaling molecules and pro-inflammatory cytokines in Ecviperin overexpressed cells. As shown in Fig. 6, compared with control cells, the expression level of several interferon related molecules, including IRF3, IRF7, ISG15, MXI, IFP35 and TRAF6 were all significantly increased in Ecviperin overexpressed cells. Moreover, the expression of TNF- $\alpha$  and IL-6 were significantly increased, while that of IL-1 $\beta$  was significantly decreased in Ecviperin overexpressed cells compared with control cells (Fig. 7). Together, the ectopic expression of Ecviperin *in vitro* could differently regulate the interferon and inflammatory response.

### 3.6. Ecviperin enhanced ISRE, IFN-1 and NF- $\kappa$ B promoter activity

Using dual-luciferase reporter assay system, we evaluated the promoter activity of IFN-1, ISRE and NF- $\kappa$ B in Ecviperin overexpressed cells. As shown in Fig. 8, the overexpression of Ecviperin significantly increased the luciferase activity of ISRE, IFN-1 and NF- $\kappa$ B promoter compared to the control cells, respectively. Thus, it was proposed that Ecviperin enhanced interferon and NF- $\kappa$ B promoter activity and regulated gene transcription.

## 4. Discussion

Viperin is an important ISG, and suppresses a broad spectrum of viruses at different life cycle stages. However, its roles during fish virus infection still remained largely unknown. Here, we cloned a viperin homolog from grouper and demonstrated the effects of Ecviperin on SGIV infection.

Sequence analysis showed that Ecviperin encoded a 361-aa protein and shared the highest (87%) identity with *Siniperca undulata*. Amino acid alignment analysis indicated that Ecviperin contained a variable N-terminal domain, a highly conserved C-terminal domain, and a conserved middle radical SAM domain (aa73-281) with the three-cysteine

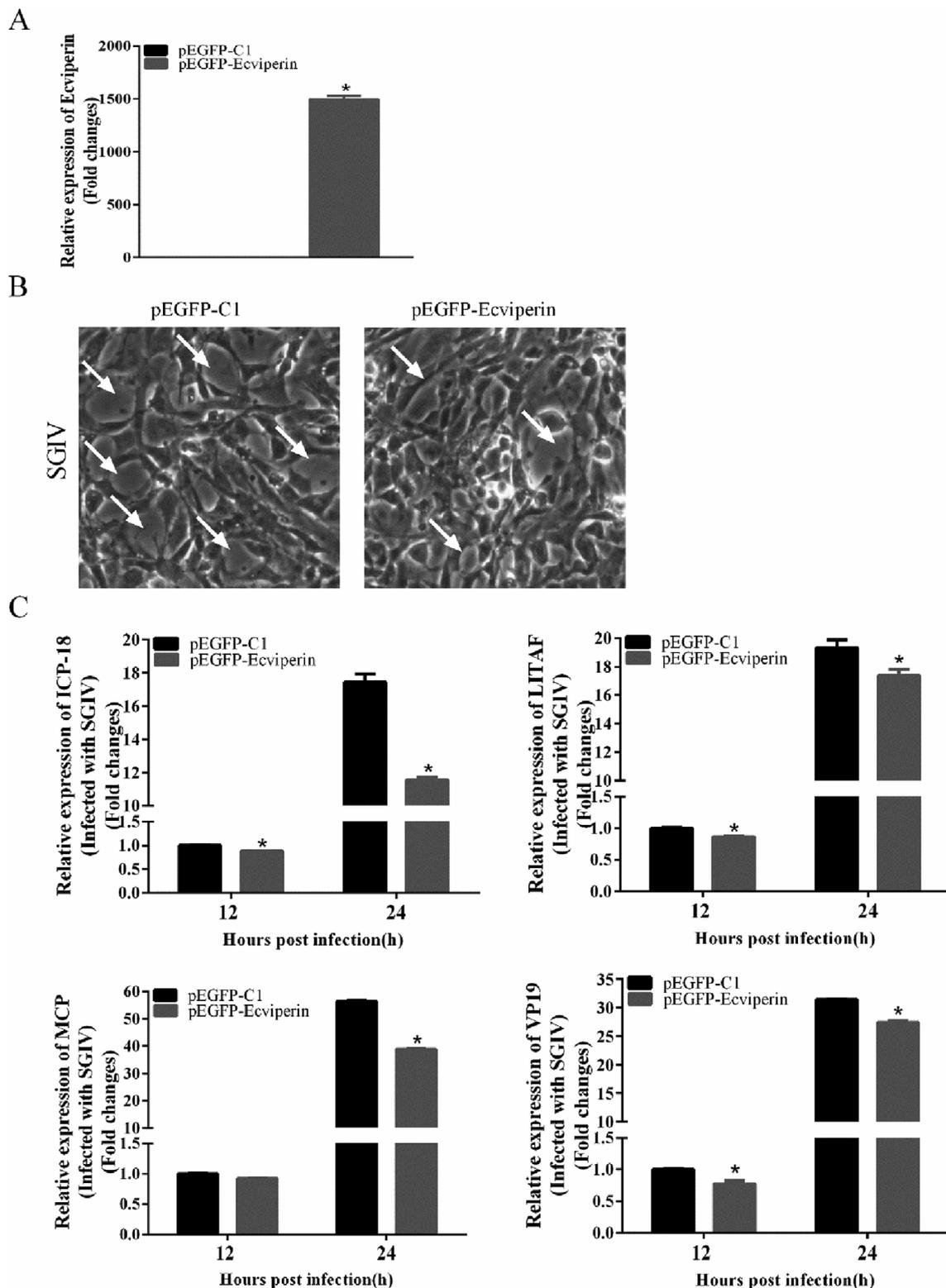


Fig. 5. Overexpression of Ecviperin significantly inhibited SGIV replication. (A) The transcription level of Ecviperin in Ecviperin-overexpressing cells. (B) Ecviperin overexpression weakened the severity of CPE induced by SGIV in GS cells. The white arrows indicated the severity of SGIV infection induced CPE which were characterized by cell rounding and aggregation of cells. (C) The relative expression level of MCP, VP19, ICP-18 and LITAF of SGIV in infected Ecviperin overexpressed cells. The error bars represent standard deviations of triplicates and \* indicates that the means were statistically significantly at  $P < 0.05$ .

motif CxxxCxxC, which was consistent with those of other teleosts [23,26,48]. In addition, Ecviperin was significantly induced by SGIV infection, which was also similar to the previous studies [20,21,48], suggesting that fish viperin might play an important role in innate immune response against viruses infection. Similar to localization of

crucian carp (*Carassius auratus*) viperin [26], Ecviperin was also found to co-localize with ER, suggesting that fish and mammalian viperins might exert their physiological function in the same cellular compartment.

Increased evidences have demonstrated that overexpression of

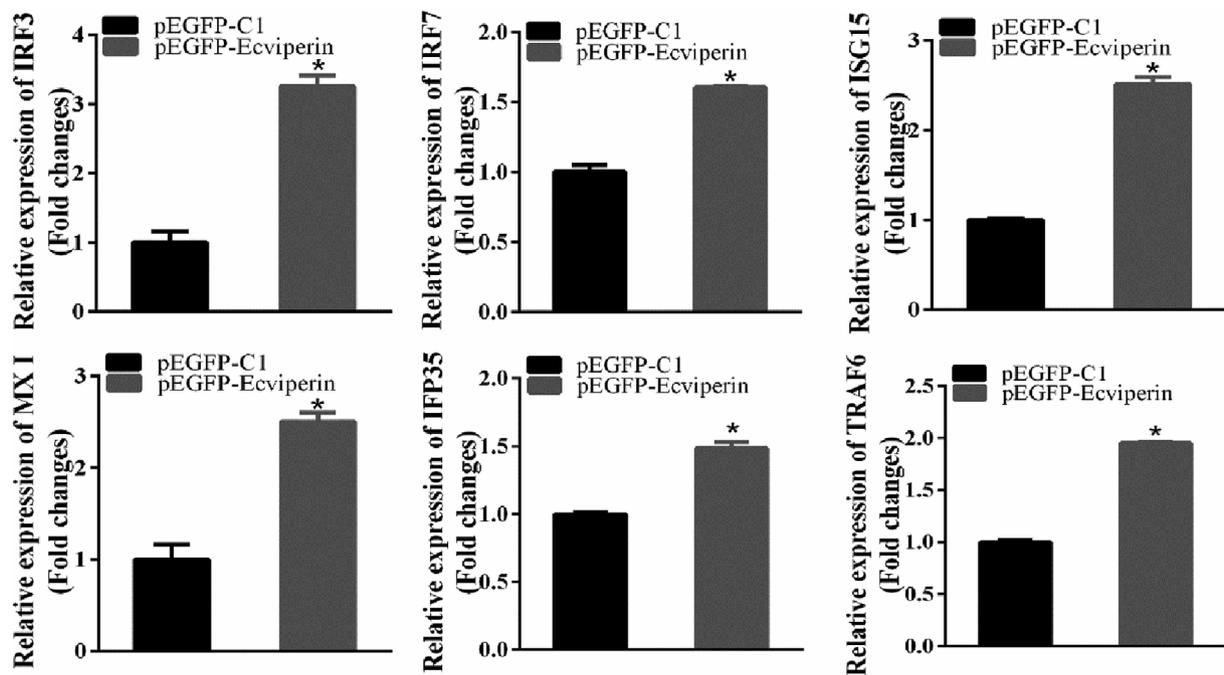


Fig. 6. Overexpression of Ecviperin positively regulated the expression level of interferon related signaling molecules. The relative expression level of interferon signaling molecules including IRF3, IRF7, ISG15, IFP35, MX1 and TRAF6 in pEGFP-Ecviperin or pEGFP-C1 transfected cells were determined by qRT-PCR. The error bars represent standard deviations of triplicates and \* indicates that the means were statistically significantly at  $P < 0.05$ .

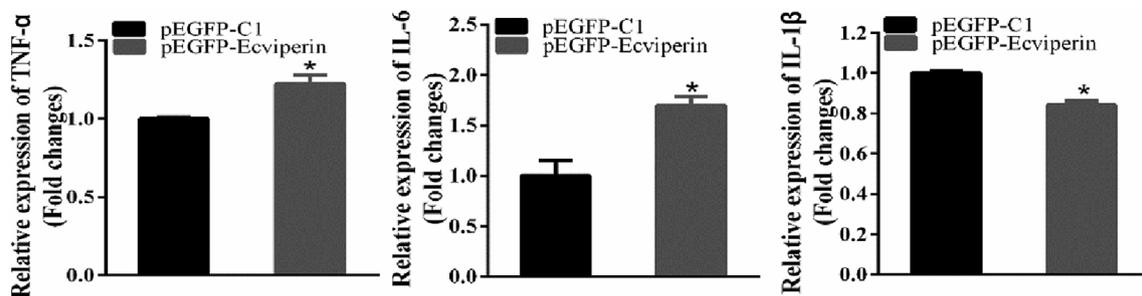


Fig. 7. Overexpression of Ecviperin differently regulated the expression level of proinflammatory cytokines. The relative mRNA expression level of proinflammatory cytokines including TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in pEGFP-Ecviperin or pEGFP-C1 transfected cells were detected by qRT-PCR. The error bars represent standard deviations of triplicates and \* indicates that the means were statistically significantly at  $P < 0.05$ .

viperin exerted broad antiviral roles against some viruses. For example, overexpression of viperin limited HCV replication [49,50]. Viperin inhibited DENV-2 early RNA production but not entry, and knockdown of viperin increased DENV-2 replication *in vitro* [51]. Similarly, the RNA synthesis of TBEV was inhibited in viperin-expressing cells [52]. In addition, a study completed by Szretter et al. [13] indicted that mice lacking viperin gene were more susceptible to WNV infection. In our study, the ectopic expression of Ecviperin significantly weakened the severity of CPE induced by SGIV infection. Moreover, Ecviperin overexpression inhibited the transcription of viral genes, suggesting that Ecviperin exerted antiviral function against fish DNA virus.

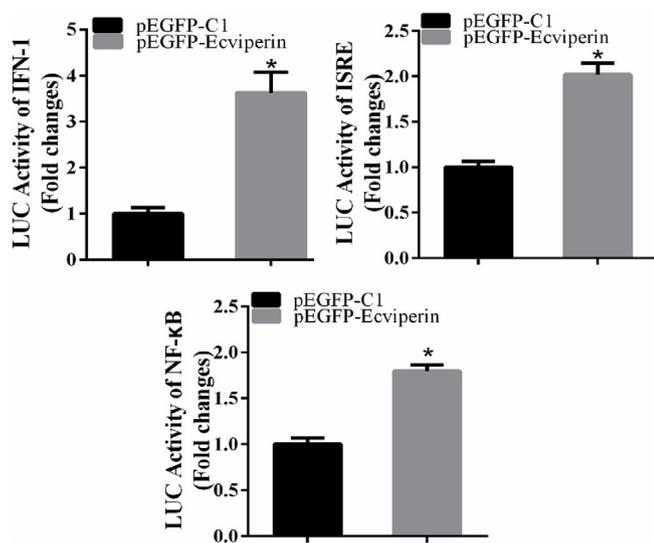
Viperin is identified as one of ISGs, that can be induced by both IFN-dependent and independent pathways [19,53,54]. In the present study, Ecviperin overexpression significantly increased the transcriptional level of several interferon related cytokines, including IRF3, IRF7, ISG15, IFP35, MX1 and TRAF6. Further analysis showed that Ecviperin enhanced IFN-1, ISRE and NF- $\kappa$ B promoter activity. Consistently, it has been reported that viperin promoted TLR7-and TLR9-dependent production of type I IFN, and facilitated the ubiquitination of signal mediator IRAK1 to induce the nuclear translocation of IRF7 [55]. In Ecviperin overexpressing cells, inflammatory cytokines, such as TNF- $\alpha$  and IL-6 were both induced, while the expression of IL-1 $\beta$  was

significantly decreased. Together, the ectopic expression of Ecviperin *in vitro* could positively regulate the interferon immune response and differently regulate the expression of pro-inflammatory cytokines to exert its antiviral function.

In summary, a novel viperin homolog from orange spotted grouper (*E. Coioides*) (Ecviperin) was cloned and characterized in this study. Ecviperin encoded a cytoplasmic protein and co-localized with ER. The transcription of Ecviperin was induced by SGIV infection. *In vitro*, the ectopic expression of Ecviperin decreased the SGIV replication due to positively regulate the interferon immune response. Moreover, Ecviperin differentially regulated the transcriptional expression of pro-inflammatory factors. Our results firstly demonstrated that the regulatory roles of grouper viperin in innate immune response during DNA virus infection and will contribute to understanding the function of fish viperin in response to viruses.

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**Fig. 8.** Overexpression of Ecviperin enhanced interferon and NF- $\kappa$ B promoter activity. GS cells were co-transfected with IFN-1-Luc/ISRE-Luc/NF- $\kappa$ B-Luc, and Ecviperin, then the IFN-1, NF- $\kappa$ B and ISRE promoter activity were determined using reporter gene assay. The error bars represent standard deviations of triplicates and \* indicates that the means were statistically significantly at  $P < 0.05$ .

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