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Molecular cloning and preliminary functional analysis of six RING-between-ring (*RBR*) genes in grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

Ubiquitination is a post-translational modification of proteins that is widely present in eukaryotic cells. There is increasing evidence that ubiquitinated proteins play crucial roles in the immune response process. In mammals, RING-between-RING (RBR) proteins play a key role in regulating immune signaling as the important E3 ubiquitin ligases during ubiquitination. However, the function of RBR in fish is still unclear. In the present study, six *RBR* genes (*RNF19A*, *RNF19B*, *RNF144*AA, *RNF144*AB, *RNF144B* and *RNF217*) of grass carp (*Ctenopharyngodon idellus*) were cloned and characterized. Similar to mammals, all six members of RBR family contained RING, in-between-ring (IBR) and transmembrane (TM) domains. These genes were constitutively expressed in all studied tissues, but the relative expression level differed. Following grass carp reovirus(GCRV) infection, the expression of six *RBR* genes in liver, gill, spleen and intestine significantly altered. Additionally, their expression in *Ctenopharyngodon idellus* kidney (CIK) cells was significantly increased after GCRV infection. And deficiency of *RNF144B* in CIK with small interference RNA (siRNA) up-regulated polyinosinic:polycytidylic acid poly(I:C))-induced inflammatory cytokines production, including *IFN-I*, *TNF-α*, *IL-6*, and transcription factor *IRF3*, which demonstrated that *RNF144B* was a negative regulator of inflammatory cytokines. Our results suggested that the RBR might play a vital role in regulating immune signaling and laid the foundation for the further mechanism research of RBR in fishes.

1. Introduction

Ubiquitination is a widespread and significant means of post-translational modification which reversibly regulates the stability, activity and localization of target proteins. Protein ubiquitylation also plays an important role in regulating immune responses [1]. E3 enzyme, a major enzyme in the process of ubiquitination, is widespread and grouped into three families: the homology to E6AP carboxyl terminus (HECT), the really interesting novel gene (RING) finger (RNF) and the U-box protein families [2,3]. Among them, the RING-finger (RNF) family, which was discovered late, has become a hot topic in recent years due to its large number and complex functions. Evidence suggests RNF proteins are involved in a plethora of cellular processes such as apoptosis, cell cycle regulation and viral responses [4–6]. Besides, as the important E3 ubiquitin ligases in ubiquitination, RNF proteins play a role in regulating immune signaling, such as TLR and RIG-1/MAVS signaling [7,8]. Recent study has shown that RNF2 is a negative regulator of interferon–STAT1 signaling in antiviral response [9].

In RNF protein superfamily, there are some hydrophobic regions predicted to be transmembrane (TM) domains, implying that they are embedded in the cellular membrane and directly participate in the biological processes of both the cellular membrane and membranous organelles [10–12]. According to the phylogenetic tree analysis, the transmembrane RNF Proteins are grouped into tripartite motif-containing (TRIM), PA-TM-RING, RING between RNFs (RBR) and membrane-associated RING-CH (MARCH) families [2]. In recent years, functional studies on TRIM, PA-TM-RING, and MARCH families have become more and more popular. In mammals and even in fish, most of them were involved in regulation of intracellular signaling, cell development and innate immunity [13–18]. However, there are few studies on RBR.

RBR family is characterized by the RBR signature, which consists of two RNFs linked by an in-between-ring (IBR) domain. The most studied

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RBR E3 is Parkin, because of its association with Parkinson's disease [19]. Moreover, there were also some studies on RBRs involving in susceptibility to intracellular pathogens, such as *Salmonella typhi*, *Salmonella paratyphi* and *Mycobacterium leprae* [20–22]. Even more and more evidence suggested some *RBR* members were involved in immune responses [20–24]. *RNF144*, *RNF19* and *RNF217* belong to the *RBR* ubiquitin ligase family that contains a transmembrane (TM) domain. *RNF144* comprises *RNF144A* and *RNF144B*. *RNF144B*, which was also known as *p53RFP*, induced a p53-dependent and caspase-independent apoptotic pathway [25] and was implicated in regulating inflammatory cytokines [26–29]. *RNF19B*, as a member of *RNF19*, played an important role in the survivability of chickens infected with highly pathogenic avian influenza viruses (HPAIVs) [30]. Most of the research on these five *RBR* genes just focused on mammals, such as in *Homo sapiens* [31–33], *Mus musculus* [34,35] and *Rattus norvegicus* [36,37]. However, knowledge on the six *RBR* genes among teleosts is limited, and it has only been investigated in *Danio rerio* [38,39]. The function of RBR family in fish is even less clear.

In the present study, grass carp (*Ctenopharyngodon idellus*) was served as a model to study the regulation of immune signaling by *RBR* in teleost fish. Six *RBR* members (*RNF144*AA, *RNF144*AB, *RNF144B*, *RNF19A*, *RNF19B* and *RNF217*) from grass carp were cloned and characterized. Tissue expression and responses to grass carp reovirus (GCRV) infection were examined. Moreover, deficient of *RNF144B* in *Ctenopharyngodon idellus* kidney (CIK) cells with small interference RNA (siRNA) was performed to investigate the possible roles of *RNF144B*. This study could shed new insight into the functions of RBR family in teleost fish.

2. Materials and methods

2.1. Fish sample collection, virus exposure and ethics statement

Healthy five-month-old grass carp (average mass of 18 g) were obtained from the Guan Qiao Experimental Fish Breeding Base, Institute of Hydrobiology, Chinese Academy of Sciences, China. Before GCRV challenge, the fish were kept in aerated freshwater at 28 °C for 1week for acclimatisation and fed with commercial feed (Tong Wei, China) twice a day. Water was replaced once daily. The grass carp were not subjected to further virus exposure study until no abnormal symptom was observed. For the viral challenge experiment, 80 healthy grass carp were intraperitoneally injected with GCRV-GD108(3.12×10^3 copy/μL)at a dose of 10 μL/g of fish weight. The appraisal method of GCRV types and virus drops has been described in previously published literature [40]. The injected fish were fed with commercial feed (Tong Wei, China) twice daily and carefully monitored. The temperature was maintained at 26°C-28 °C throughout the experiment.

The gill, liver, spleen, intestine, kidney, head kidney, heart, muscle, skin, blood and brain were collected from five uninfected fish and homogenised in TRIzol reagent (Invitrogen, USA) to obtain the total RNA following the manufacturer's instructions. RNA was obtained from these tissues for analysis of tissue distribution. In addition, gill, liver, spleen and intestine samples were isolated from five infected fish at 0–6 day post–infection (dpi). RNA from these tissues was obtained to analyse the response of the six *RBR* members to GCRV infection. All animal experiments were performed in conformity to the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). The protocol was approved by the committee of institute of hydrobiology, Chinese Academy of Sciences (CAS). All surgeries were performed under eugenol anaesthesia to minimise the suffering of animals.

Table 1
DNA sequences of PCR primers used in the study.

Primers	Sequences (5′→3′)	Usage
RNF144AA-1F	CAGCACTAGGAAATACTCAGACTAA	*RNF144*AA cDNA cloning
RNF144AA-1R	GAAGTGAGAGAAAAGCCCCT	
RNf144AB-1F	TTTATCTGCGACAAATGCG	*RNF144*AB cDNA cloning
RNf144AB-1R	AAACTGAGGTTATGTGATTATGCTA	
RNF144B-1F	TGGAGGATGTCACTTCACCGTT	*RNF144B* cDNA cloning
RNF144B-1R	GGGATTGGCACGTGGAAGA	
RNF144B-2F	CCACATAGGAGAACTCTTCGTAA	
RNF144B-2R	GAGAAATGCTTCCTTTGTCG	
RNF19A-1F	CCCGACAAAACAACTCCCTAA	*RNF19A* cDNA cloning
RNF19A-1R	ACAAGGTTCCTCTTGTGCTTGG	
RNF19A-2F	TTCCCTCTGCCGCCTCA	
RNF19A-2R	ACTCAGGTTGTCTCTCATTGCTC	
RNF19A-3F	CTGCGGGGAATGGAAAAG	
RNF19A-3R	TGCCCCGTCAGGAGTAAGA	
RNF19B-1F	AAGCCCTGAGAAAGGATTACGAT	*RNF19B* cDNA cloning
RNF19B-1R	TGTTATGTTCTCCCACTTGAGTCCT	
RNF19B-2F	ATGGGATCTGAAAAGGACTCTGAAT	
RNF19B-2R	AACGGGGATGCCGATAACC	
RNF19B-3F	GGCGTATGTGTATGGTGTTGTG	
RNF19B-3R	ACCTGTCCGTGCTTGTAATAGAG	
RNF217-1F	ATGGAAGATGACTCTTCCGTGC	*RNF217* cDNA cloning
RNF217-1R	GCACAGCCAGCGGCCC	
RNF217-2F	GTCGTAGCCAACAGACATCCATC	
RNF217-2R	CATCCTAAAACAAAATCTCCTCAAA	
qRNF144AA-F	GCACCCTGTGCCTGAAGCAG	qPCR
qRNF144AA-R	AGTTGGCACACAGCCTGGCA	
qRNF144AB-F	GCAGCTCTCGCTACGAACCC	
qRNF144AB-R	AGCTGGTTCGAGGCCCTCTT	
qRNF144B-F	TCTGCTGTGGCTGCAGAAGC	
qRNF144B-R	TCTTGCAGAGCATCTGAGCACA	
qRNF19A-F	GCGGGTATGCAGTCATTGCC	
qRNF19A-R	CGCTCTCCTGGCTGTAGCTG	
qRNF19B-F	GAAATGCCGTGGGCCTGGTA	
qRNF19B-R	GTGTGGGAGGCTGGAGAAGC	
qRNF217-F	TTGGAGCTGAGCAGACTGGACT	
qRNF217-R	GACACTTCAGGCCTTCGTGC	
qβ-actin-F	AGCCATCCTTCTTGGGTATG	
qβ-actin-R	GGTGGGGCGATGATCTTGAT	

2.2. Cloning cDNA of six RBR genes in grass carp

Total RNA was isolated from the tissues of healthy grass carp by using Trizol reagent (Invitrogen, USA) (as mentioned in 2.1). First-strand cDNA synthesis was carried out using DNase I (Promega, USA)-digested totlal RNA as a template and oligo (dT) primer (TOYOBO, Japan) for reverse transcription. Based on the cDNA sequences of zebrafish six RBR genes (*D.rerio RNF19A*, Accession no. NM_001326695.1; *D.rerio RNF19B,* Accession no. NM_001202440.1; *D.rerio RNF144*AA, Accession no. NM_001045209.2; *D.rerio RNF144*AB, Accession no. NM_001002727.1; *D.rerio RNF144B*, Accession no. NM_201137.2; *D.rerio RNF217*, Accession no. NM_001082853.1), specific primers (Table 1) were designed according to their deduced cDNA sequences extracted from the grass carp genome [41]. The above cDNA was used as template for PCR amplification. The cDNA sequences were amplified by PCR using the primers described in Table 1.

2.3. Sequence analysis

The *RBR* gene sequences from other species were searched from NCBI (http://www.ncbi.nlm.nih.gov/). Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) was used to predict the protein domain features, and Illustrator for Biological

Sequences (IBS) Version 1.0 (http://ibs.biocuckoo.org/) was used to draw the Schematic diagram of the protein. Multiple sequence alignments were performed using the ClustalW2.1 program (http://www.ebi.ac.uk/tools/clustalw2.1). A phylogenetic tree was constructed using neighbour-joining method with Mega5.1 software. Reliability was assessed by 1000 bootstrap replicates.

2.4. Tissue distribution and responses of six RBR genes to GCRV infection

Total RNA was extracted from 11 tissues (gill, liver, spleen, intestine, kidney, head kidney, heart, muscle, skin, blood and brain) of five uninfected grass carp and reverse transcribed to obtain cDNA. The obtained cDNA were served as template for real-time quantitative PCR (qPCR) analysis to examine the expression level of six *RBR* genes in different tissues. The qPCR reaction mixture was as follows: 0.8 μL each of sense and reverse primers, 1 μL template, 10 μL 2 × ChamQ SYBR qPCR Master Mix (Vazyme, NanJing), and 7.4 μL ddH_2O. Three replicates were conducted for each sample and *β-actin* was used as reference for normalisation. The program for qPCR was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, and 62 °C for 15 s. The expression level in the head kidney was used as baseline (1.0) for qPCR analysis. In addition, total RNA from four tissues (gill, liver, spleen and intestine) of five grass carp at 0–6 dpi with GCRV was extracted and reverse transcribed to obtain cDNA. Subsequently, the cDNA from the four tissues were served as template for qPCR by using gene-specific qPCR primers (Table 1) to measure the expression level of six *RBR* member genes in different tissues at various times at post-GCRV infection.

For qPCR data analysis, *β-actin* was used as internal control, and the expression level of the untreated groups (0 days) was set as baseline (1.0). The relative expression level of each gene was calculated using $2^{-\Delta\Delta Ct}$ methods [42]. Data were expressed as mean ± standard deviation of the three replicates. T-test was used to determine differences in expression, with p value < 0.05 indicating statistical significance.

2.5. Responses of six RBR genes in CIK cells after GCRV infection

The *Ctenopharyngodon idellus* kidney (CIK) cells kidney (CIK) cells used in the study were cultivated in M199 (Hyclone, USA) supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin at 28 °C in a humidified atmosphere with 5% CO_2。 To observe the responses of six RBR genes in CIK cells after GCRV infection, CIK cells were seeded in six-well plates overnight at 28 °C. Afterwards, cells were incubated with GCRV, which was diluted in a small volume of serum free M199. At the same time, CIKs were inoculated with serum free medium as control. After adsorption for 2 h, fresh complete growth medium was added and the cells were placed in the incubator for the following experiments. The control cell and GCRV-infected cell samples were harvested at 0, 6, 12, 24, 48, 72 and 96 h. Total RNA was extracted from the harvested cells and reverse transcribed to cDNA. The cDNA were used as a template for qPCR to determine the expression level of six *RBR* genes. Data analysis was the same as that mentioned in 2.4.

2.6. Expression of related factors in RNF144B-deficient cells stimulated by poly(I:C)

Given that *RNF144B* is an E3 ubiquitin ligase, and ubiquitination has recently been implicated in regulation of inflammation function, a potential role for *RNF144B* in inflammation activation was next investigated. Three pairs of *RNF144B* siRNA oligo fragments were transfected into cells respectively to screen the most efficient knockdown siRNA oligo fragments for subsequent experiments. SiRNA oligo fragments of *RNF144B* were transfected into CIK cells according the manufacturer's instructions of Hieff Trans™ (Yeasen; www.yeasen.com/transfection/351.htm). After transfection for 24 h, cells were stimulated with poly(I:C) (sigma, USA) at a final concentration of 20 μg/ml or phosphate-buffered saline (PBS) as control groups. Samples were collected after 8 h stimulation and total RNA was extracted and reverse transcribed to cDNA. The cDNA was used as a template for qPCR to determine the expression levels of *RNF144B* and related factors (*IFN-I*, *TNF-α*, *IL-6* and *IRF3*). The specific primers for qPCR and sequences of siRNA were listed in Table 2.

Table 2
DNA sequences of PCR primers used in the study.

Primers	Sequences (5′→3′)	Usage
qIFN-I—F	AAGCAACGAGTCTTTGAGCCT	qPCR
qIFN-I-R	GCGTCCTGGAAATGACCT	
qTNF-α-F	CGGCATTTACTTCGTCTACAGC	
qTNF-α-R	TAGGAATCGGAAATTCGCATAA	
qIL-6-F	CAGCAGAATGGGGGAGTTATC	
qIL-6-R	CTCGCAGAGTCTTGACATCCTT	
qIRF3-F	TCCAGGCCAAGCATACGAA	
qIRF3-R	CCATTTGCAACAGCCATCAT	
RNF144B-128F	GCACACUGCAGUCCUGUAATT	siRNA
RNF144B-128R	UUACAGGACUGCAGUGUGCTT	
RNF144B-475F	CCUUGGAUAGAUGGUCAUUTT	
RNF144B-475R	AAUGACCAUCUAUCCAAGGTT	
RNF144B-849F	CCUGUGCUGUGUGUGCAAATT	
RNF144B-849R	UUUGCACACACAGCACAGGTT	
Scrambled-F	UUCUCCGAACGUGUCACGUTT	
Scrambled-R	ACGUGACACGUUCGGAGAATT	

3. Results

3.1. Sequence, homology and phylogenetic analyses

Six members of the *RBR* family (*RNF19A*, *RNF19B*, *RNF*144AA, *RNF*144AB, *RNF144B* and *RNF217*) were identified in grass carp. The cDNA sequence details were presented in Table 3. Like mammals, the putative proteins of these six RBRs possessed all domains including RING, IBR and TM domains (Fig. S1). Multiple alignment analysis revealed only the structure of RING2 domains in *RNF*144AA, *RNF*144AB and *RNF144B* resembled classical RINGs. And the IBR domain in all six RBR proteins of grass carp comprises two zinc-binding structures (RINGs) in a C6HC configuration (Fig. S2).

To elucidate the molecular evolutionary relationships of all studied proteins between *C. idellus* and other species, a phylogenetic tree was constructed based on their corresponding amino acid sequences obtained from the NBCI GenBank of teleost fish (*D. rerio*, *P. formosa*, *S. salar* and *O. latipes*) and non-fish (*H. sapiens*, *B. taurus*, *M. musculus*, *G. gallus* and *X. tropicalis*). The accession numbers of the sequences used to construct the tree were presented in Table 4. As shown in Fig. 1, separate from *RNF144A-like* from *S. salar* and *RNF217* isoform X2 from *H. sapiens*, RBR proteins could be divided into four groups: the *RNF144A* group (containing *RNF144A*, *RNF144A-A* and *RNF144A-B*), *RNF144B* group (containing *RNF144B* and *RNF144B-like*), *RNF19* group (containing *RNF19B*, *RNF19B-like*, *RNF19A* and *RNF19A-like*) and *RNF217* group. *RNF*144AA, *RNF*144AB, *RNF144B*, *RNF19B* and *RNF217*

Table 3
The cDNA information for the *RBR* family.

Gene	GenBank accession no.	Full lengths	ORF lengths	Lengths of 5′UTR	Lengths of 3′UTR
*RNF*144AA	MG679802	1468 bp	882 bp	114 bp	472 bp
*RNF*144AB	MG679803	1500 bp	882 bp	123 bp	495 bp
RNF144B	MG679804	1619 bp	939 bp		680 bp
RNF19A	MG679805	2911 bp	2745 bp	77 bp	89 bp
RNF19B	MG679806	2781 bp	2109 bp	489 bp	183 bp
RNF217	MG679807	1945 bp	1629 bp	73 bp	243 bp

proteins were the most highly related to the RBR proteins of *D.rerio*, except that *RNF19A* was highly conserved in all species.

3.2. Expression of six RBR members in different tissues

QPCR analysis was performed to study the tissue distribution of the six *RBR* members (*RNF19A*, *RNF19B*, *RNF144*AA, *RNF144*AB, *RNF144B* and *RNF217*) in different tissues. As shown in Fig. 2, the six *RBR* members were constitutively expressed in all of the 11 examined tissues. *RNF19A* and *RNF19B* exhibited the highest expression level in the blood (Fig. 2A and B) and broadly expressed in other organs. *RNF144*AA and *RNF144*AB exhibited the highest expression in the brain and were also highly expressed in the muscle, spleen and heart, but were lowly expressed in the kidney and head kidney (Fig. 2C and D). *RNF144B* was widely expressed in all tissues but at low levels (Fig. 2E). *RNF217* was prevalent in the liver, kidney, gill, heart and brain and exhibited the lowest expression in blood (Fig. 2F). Overall, some RBRs clearly had a tissue-significant expression pattern.

3.3. Expression of RBR member genes in response to GCRV infection

In order to reveal the response of six *RBR* members after exposure to GCRV, The levels of mRNA were measured by qPCR analysis in primary immune organs (gill, liver, spleen and intestine) at 0–6 dpi. The expression of the six *RBRs* was significantly altered in the gill, liver, spleen and intestine following GCRV infection (Fig. 3).

Overall, *RNF19A* (Fig. 3A), *RNF19B* (Fig. 3B) and *RNF217* (Fig. 3F) showed a similar expression in the gill; that is, the expression levels of these genes were down-regulated at all time points after GCRV infection but remained at the original level at 4 dpi. However, *RNF144*AA expression was up-regulated in the entire process of infection and sharply peaked at 4 dpi (4.18-fold, $p < 0.05$) (Fig. 3C). *RNF144*AB expression was down-regulated from 1 dpi to 3 dpi, suddenly reached the peak and down-regulated again from 5 dpi to 6 dpi in the gill (Fig. 3D). In the liver, *RNF19A*, *RNF144*AB and *RNF217* expression levels were down-regulated during the infection process, but the latter two genes were highly expressed at 4 dpi and 1 dpi, respectively. *RNF19B* expression in the liver was slightly up-regulated from 1 dpi to 4 dpi and then down-

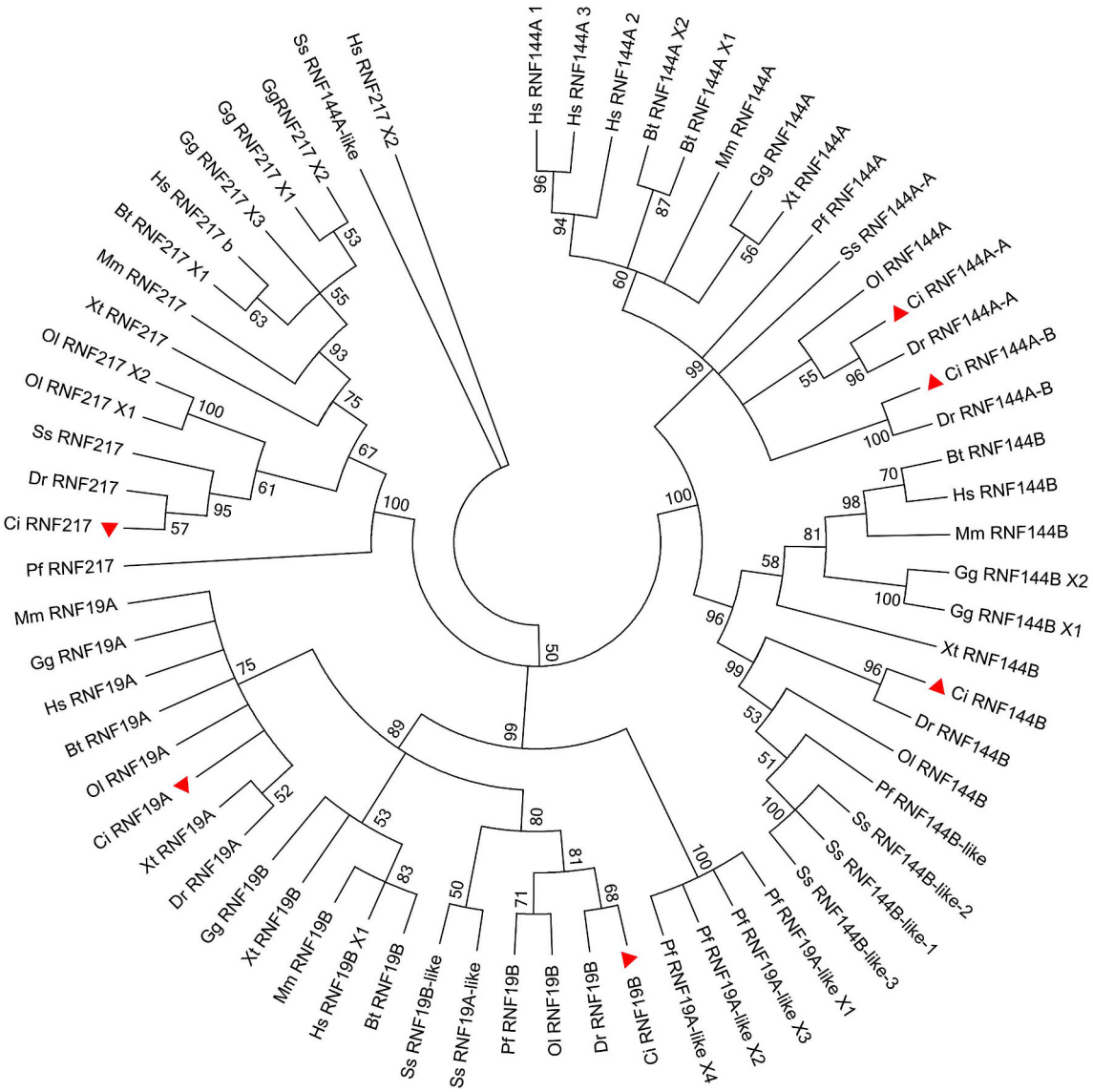

Fig. 1. Neighbour-joining phylogenetic tree analysis. A neighbour-joining tree was constructed based on the analysis of 66 RBR family protein sequences using MEGA 5.1 software. The accession numbers of the sequences used for tree construction are listed in Table 4. Members in *Ctenopharyngodon idellus* are indicated by the red triangle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

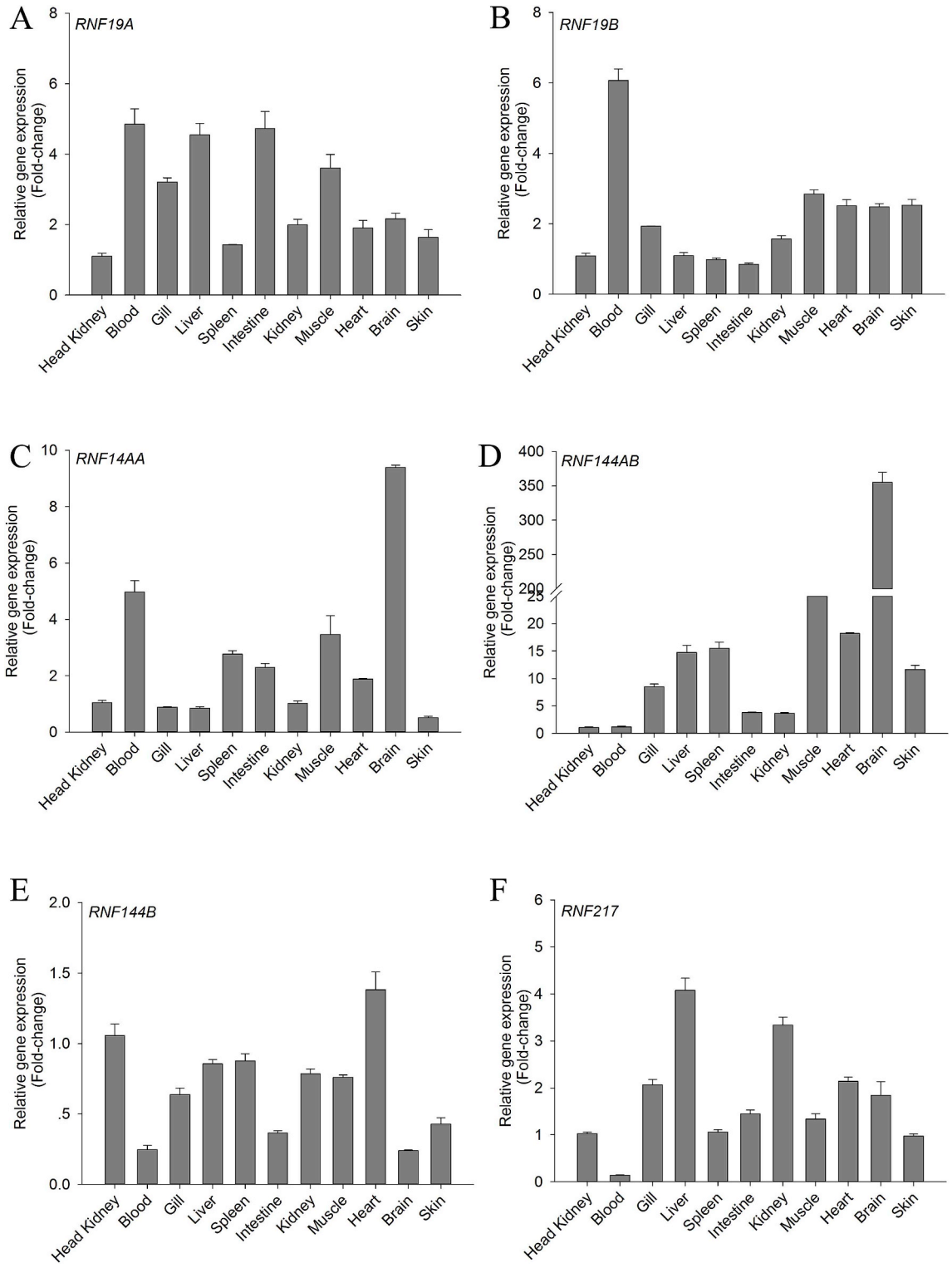

Fig. 2. Tissue distribution of *RBR* mRNA expression in healthy grass carp. The relative expression is the ratio of gene expression in different tissues relative to that in the head kidney. The *β-actin* gene was used as the internal control. The x- and y-axes indicate the different tissues of *Ctenopharyngodon idellus* and mean ± s.e. fold change of expression, respectively. (A) *RNF19A*, (B) *RNF19B*, (C) *RNF144*AA, (D) *RNF144*AB, (E) *RNF144B* and (F) *RNF217*.

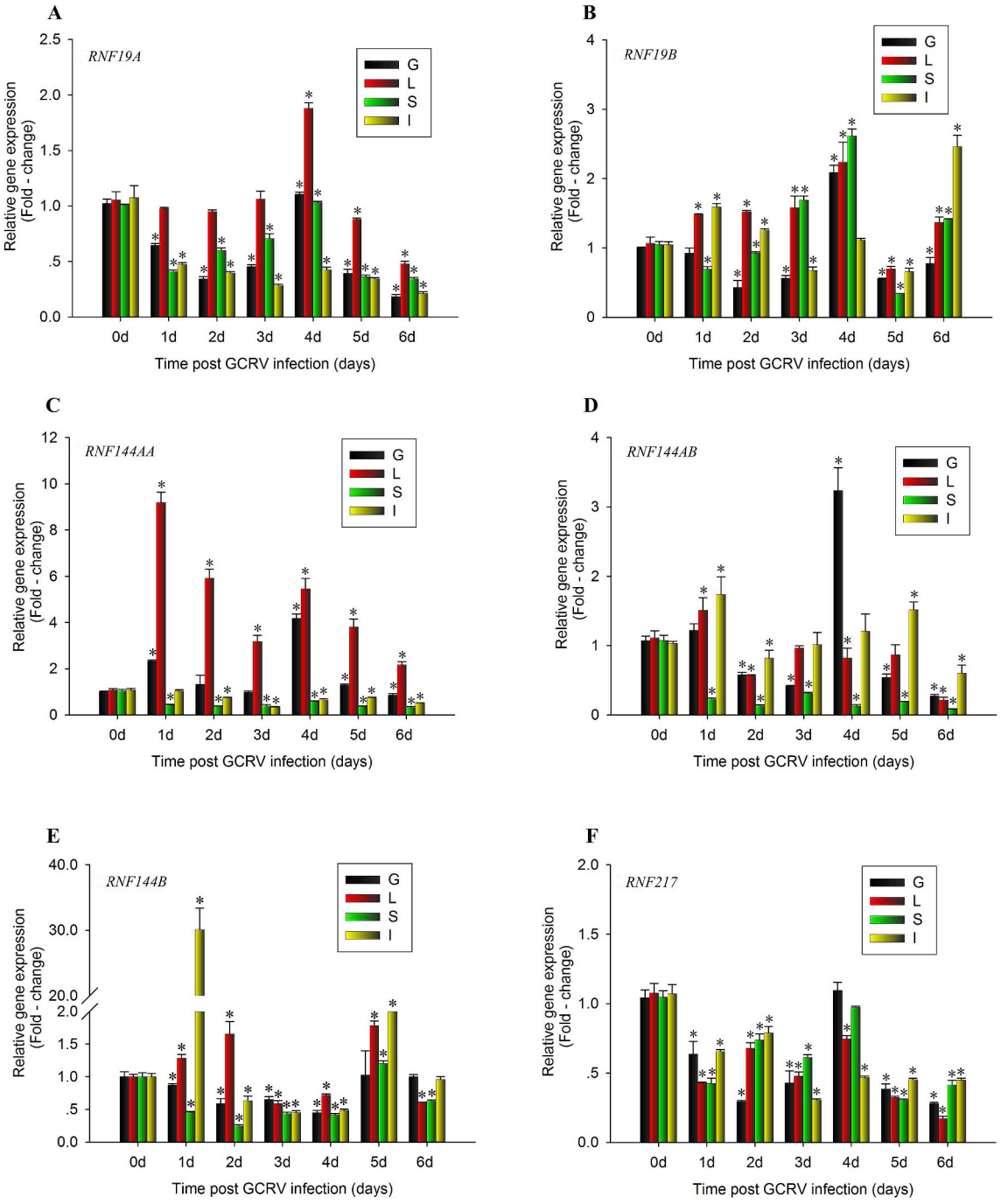

Fig. 3. Expression level of six *RBR* genes after GCRV infection. (A) *RNF19A*, (B) *RNF19B*, (C) *RNF144*AA, (D) *RNF144*AB, (E) *RNF144B* and (F) *RNF217*. RNA was isolated from gill (G), liver (L), spleen (S) and intestine (I), and subjected to qRT-PCR analysis. The relative expression is the ratio of gene expression after exposure to GCRV for 1–6 day to that in the control group (day 0) at the same tissue, normalised to the *β-actin* gene. *, significant difference between the control and treated group ($p < 0.05$).

regulated at 5 dpi and 6 dpi. *RNF144*AA was dramatically up-regulated during the entire infection process. Interestingly, the mRNA expression levels of *RNF19A*, *RNF144*AA and *RNF217* in the spleen and intestine were significantly down-regulated and maintained at low levels at all time points. *RNF144*AB expression in the spleen kept at a low level under the control (0 dpi) from 1 dpi to 6 dpi. However, the expression of *RNF144*AB in the intestine and that of *RNF19B* in the intestine and spleen fluctuated throughout the test period. The response of *RNF144B* was wavy in the liver and intestine but subtle in the gill and spleen (Fig. 3E).

The responses of RBR members *in vitro* were also observed. The mRNA expression levels of six *RBR* genes were detected in GCRV-infected CIK cells. As shown in Fig. 4, compared with the control (0 h), the expression levels of six *RBR* genes were significantly up-regulated at all time points after GCRV infection.

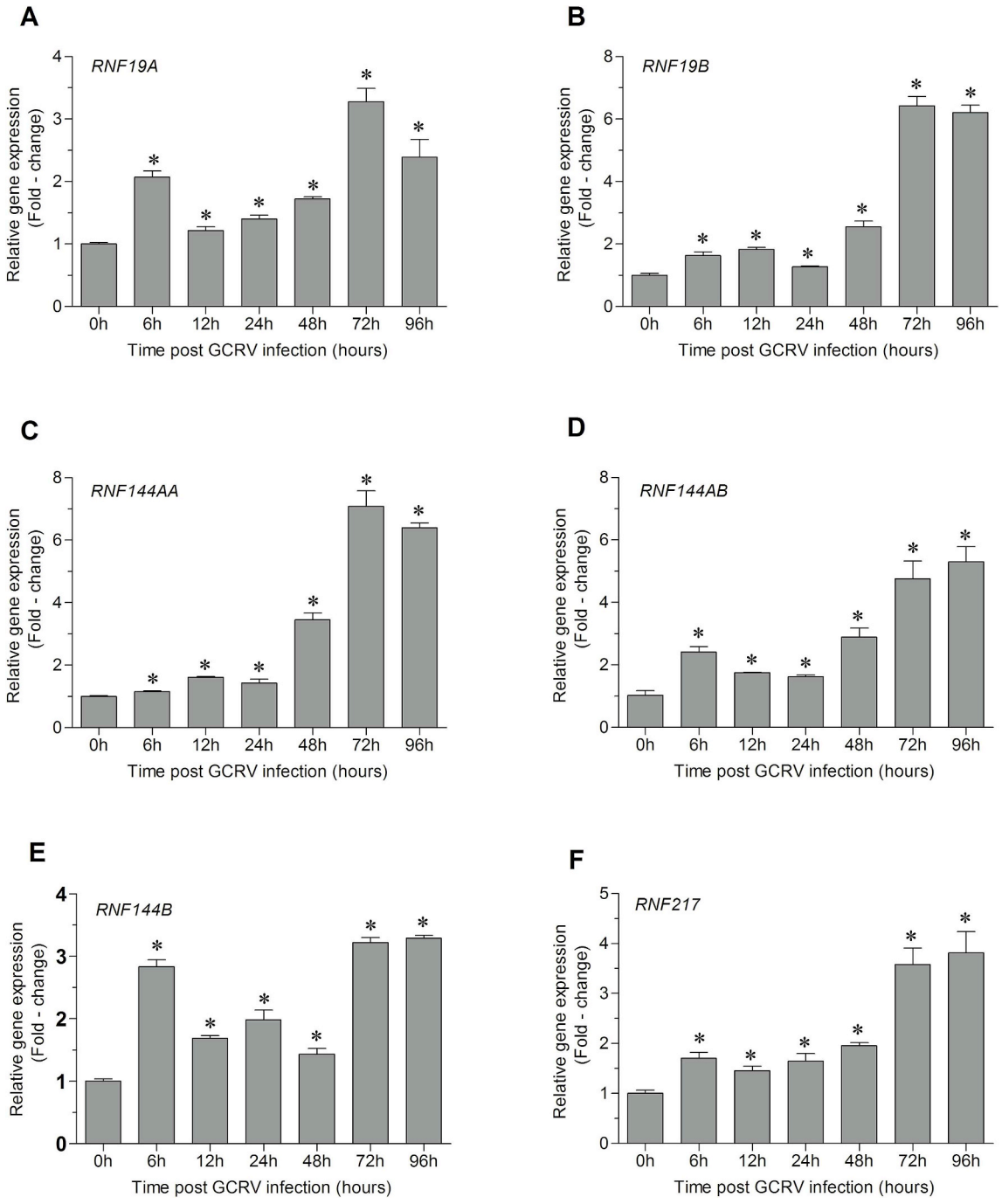

Fig. 4. Expression level of six *RBR* genes in CIK cells after GCRV infection. The expression of six *RBR* genes was measured at 0, 6, 12, 24, 48, 72, and 96 h post challenge and the mRNA levels were normalised with *β-actin* expression. Asterisk (*) marked the significant difference between experimental group and control group (0 h) ($p < 0.05$). Error bars indicated standard deviation. (A) *RNF19A*, (B) *RNF19B*, (C) *RNF144AA*, (D) *RNF144AB*, (E) *RNF144B* and (F) *RNF217*.

3.4. Responses of RNF144B-regulated genes in CIK cells after poly(I:C) stimulation

To determine the role of *RNF144B* in immune inflammation, *RNF144B* deficient CIK cells were generated using siRNA. *RNF144B* mRNA level were successfully depleted in CIK cells using three different pairs of siRNA oligo fragments (Fig. 5A). The most efficient pair of siRNA oligo fragments was used for subsequent experiments, and expression levels of *IFN-I*, *TNF-α*, *IL-6* and *IRF3* were investigated after poly(I:C) stimulation in deficient CIK cells. As shown in Fig. 5B–E, compared to the scrambled siRNA control groups, *RNF144B* depletion significantly heightened poly(I:C)-induced the expression of *IFN-I*, *TNF-α*, *IL-6* and *IRF3*. Taken together, the results suggest that *RNF144B* is a negative regulator of inflammatory cytokines.

4. Discussion

The RBR domain mediates protein-protein interactions and a subset of RBR proteins has been shown to function as E3 ubiquitin ligases. RBR proteins have attracted interest. Despite their strong persistence throughout evolution, most RBR E3s are not well understood. *RNF144A*, *RNF144B*, *RNF19A*, *RNF19B* and *RNF217* belong to the *RBR* ubiquitin ligase family that contains a transmembrane (TM) domain. However, previous studies of RBR just focused on mammals, research in

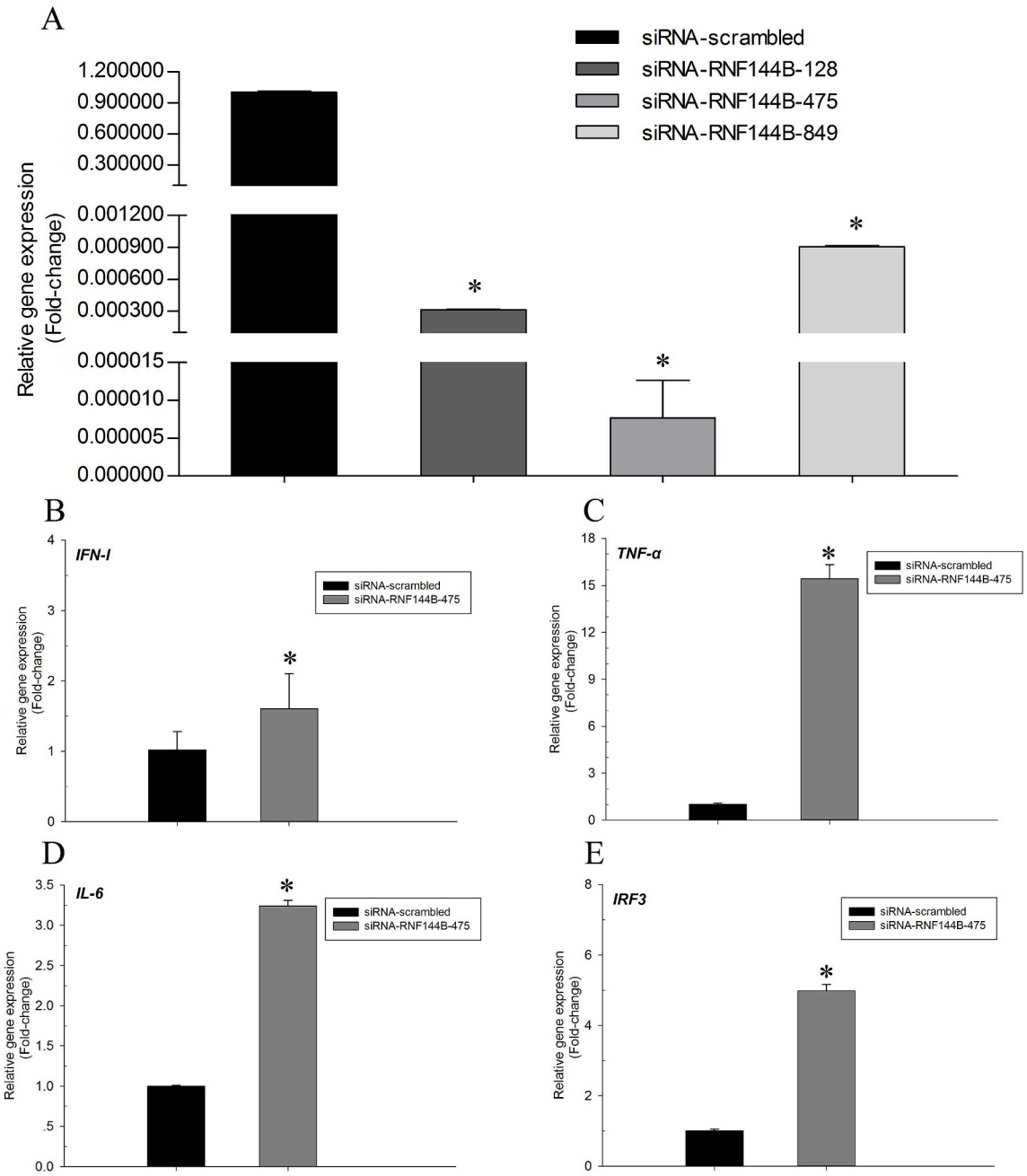

Fig. 5. Poly(I:C)-mediated expression of inflammatory cytokines in RNF144B-deficient cells. *RNF144B* deficient CIK cells were generated using siRNA, then RNF144B-deficient CIK cells were stimulated with poly (I: C) (sigma, USA) at a final concentration of 20 μg/ml or phosphate-buffered saline (PBS) as control groups. Cells were collected after 8 h stimulation, total RNA was extracted and reverse transcribed into cDNA. QRT-PCR was performed to determine the expression levels of *RNF144B* and related factors. The expression levels of these genes in the control group were set to 1. Significant difference ($p < 0.05$) between the control and treated group was indicated with asterisks (*).(A) *RNF144B*, (B) *IFN-I*, (C) *TNF-α*, (D) *IL-6*, (E) *IRF3*.

fish was limited. In the present study, six *RBR* genes were cloned and characterized in *C. idellus* for the first time.

Amino acid sequence comparison and phylogenetic analyses indicated that six *RBR* members (*RNF144*AA, *RNF144*AB, *RNF144B*, *RNF19A*, *RNF19B* and *RNF217*) were categorised into four groups: *RNF144A* group, *RNF144B* group, *RNF19* group and *RNF217* group (Fig. 1). *RNF144*AA and *RNF144*AB were only found in *D. rerio* and *C. idellus* but were within the same branch of *RNF144A* in mammals; hence, these genes in *D. rerio* and *C. idellus* may exhibit similar roles to those of *RNF144A* alone in mammals, and the difference in copy number is due to the result of genome replication during evolution. Previous researches have shown that genomic replication is ubiquitous in fish and sub-functionalized, for example, in mammals, the chemokine receptor CXCR3 exists as a single gene, while it has two isoforms, CXCR3.1 and CXCR3.2 in the teleosts: ayu (*Plecoglossus altivelis*), grass carp (*Ctenopharyngodon idellus*), and spotted green pufferfish (*Tetraodon nigroviridis*) [43,44]. Furthermore, these six RBR member proteins in *D. rerio* and *C. idellus* shared a high degree of homology probably because zebrafish and grass carp share a similar genomic evolutionary history [41]. In addition, results of protein structure prediction showed that similar to mammalian RBR, grass carp six RBR genes also encoded proteins containing a putative RING-IBR-RING domain, as well as a transmembrane domain at the C-terminus (Fig. S1). The evolutionary information of RBRs suggests a high reliability of the RBR sequences in

Table 4
GenBank accession numbers of RBR family in phylogenetic tree.

Name	Species	Accession no.	Name	Species	Accession no.
CiRNF144A-A	*C.idella*	MG679802	*HsRNF144A-1*	*H.sapiens*	NP_001336110.1
CiRNF144A-B		MG679803	*HsRNF144A-2*		NP_001336111.1
BtRNF144A-X1	*B.taurus*	XM_005213106.3	*HsRNF144A-3*		NP_001336114.1
BtRNF144A-X2		XP_010808612.1	*GgRNF144A*	*G.gallus*	XP_419938.3
DrRNF144A-A	*D.rerio*	NP_001038674.1	*XtRNF144A*	*X.tropicalis*	NP_001095278.1
DrRNF144A-B		XP_005160733.1	*OlRNF144A*	*O.latipes*	XP_004082304.1
PfRNF144A	*P.formosa*	XP_007565595.1	*SsRNF144A-A*	*S.salar*	XP_014065667.1
MmRNF144A	*M.musculus*	NP_001075446.1	*SsRNF144A-like*		XP_013993537.1
CiRNF144B	*C.idella*	MG679804	*GgRNF144B-X1*	*G.gallus*	XP_004939680.1
BtRNF144B	*B.taurus*	NP_001092498.1	*GgRNF144B-X2*		XP_015131438.1
DrRNF144B	*D.rerio*	NP_957431.2	*OlRNF144B*	*O.latipes*	XP_011483208.1
PfRNF144B-like	*P.formosa*	XP_007550574.1	*SsRNF144B-like1*	*S.salar*	XP_014056805.1
MmRNF144B	*M.musculus*	NP_001164114.1	*SsRNF144B-like2*		XP_014056802.1
HsRNF144B	*H.sapiens*	NP_877434.2	*SsRNF144B-like3*		XP_014056802.1
XtRNF144B	*X.tropicalis*	NP_001107142.1			
CiRNF19A	*C.idella*	MG679805	*MmRNF19A*	*M.musculus*	NP_038951.1
BtRNF19A	*B.taurus*	NP_001178260.1	*HsRNF19A*	*H.sapiens*	NP_001267468.1
DrRNF19A	*D.rerio*	NP_001313624.1	*GgRNF19A*	*G.gallus*	XP_418362.3
PfRNF19A-likeX1	*P.formosa*	XP_016527448.1	*XtRNF19A*	*X.tropicalis*	NP_001116899.1
PfRNF19A-likeX2		XP_016527449.1	*OlRNF19A*	*O.latipes*	XP_011483185.1
PfRNF19A-likeX3		XP_007553066.1	*SsRNF144A-like*	*S.salar*	XP_013998794.1
PfRNF19A-likeX4		XP_016527450.1			
CiRNF19B	*C.idella*	MG679806	*HsRNF19B-X1*	*H.sapiens*	XP_006710419.1
BtRNF19B	*B.taurus*	NP_001192591.1	*GgRNF19B*	*G.gallus*	XP_015153351.1
DrRNF19B	*D.rerio*	NP_001189369.1	*XtRNF19B*	*X.tropicalis*	XP_002940071.3
PfRNF19B	*P.formosa*	XP_016530847.1	*OlRNF19B*	*O.latipes*	XP_004074120.1
MmRNF19B	*M.musculus*	NP_083495.1	*SsRNF19B-like*	*S.salar*	XP_013997346.1
CiRNF217	*C.idella*	MG679807	*GgRNF217X1*	*G.gallus*	XP_015139925.1
BtRNF217	*B.taurus*	XP_015328268.1	*GgRNF217X2*		XP_015139926.1
DrRNF217	*D.rerio*	NP_001076322.1	*GgRNF217X3*		XP_015139927.1
PfRNF217	*P.formosa*	XP_007561053.1	*XtRNF217*	*X.tropicalis*	XP_004914658.1
MmRNF217	*M.musculus*	NP_001139821.1	*OlRNF217X1*	*O.latipes*	XP_020570567.1
HsRNF217b	*H.sapiens*	NP_689766.1	*OlRNF217X2*		XP_004083900.1
HsRNF217X2		XP_011533797.1	*SsRNF217*	*S.salar*	XP_014060584.1

grass carp.

Previous studies showed that in mammals, a classical zinc finger (RING) is minimally described as cysteine-rich motif and follows the general formula C–x_2 –C–$x_{(9–39)}$ –C–$x_{(1–3)}$ –H–$x_{(2–3)}$ –C–x_2 –C–$x_{(4–48)}$ –C–x_2–C, where x can be any amino acid. Typically, the sequence of each ring domain in the RBR region contains a cluster of eight cysteine and histidine residues that potentially bind metal ions [45]. There are mounting evidences suggesting that RBR structure is closely related to the function of RBR protein. The RING domain of KAP-1 played an important role in mediating transcriptional repression [46]; and the RING domains of PML could cooperate with the viral protein Z to bind with eIF4E and altered the mRNA transport and translational functions of eIF4E by altering its affinity for the 5′cap of mRNA [47,48]. Besides, many RING domains functioned as E3 ubiquitin protein ligases in the ubiquitin conjugation pathway and helped E2 ubiquitin conjugating enzymes to intended substrates [49,50]. Therefore, it is necessary to predict the protein domains of these genes and analyse the conservation degree of RBR structural sequences. The RBR domains of *RNF144*AA, *RNF144*AB, *RNF144B*, *RNF19A*, *RNF19B* and *RNF217* in *C. idellus* indicated that RING2 (the C-terminal RNF, also called C-RING) resembled a classical RING, while RING1 (the N-terminal RNF, also called N-RING) did not. That is in contrast to mammals, where N-RING is usually similar to a typical structure but C-RING is different [45]; suggesting RING2 functions as a typical RING structure in grass carp. These key differences between the sequences of the RING1 and the classical RING domain led us to speculate that the RING1 may adopt a different topology.

All of the six *RBR* members were constitutively expressed in 11 tissues examined. This finding is similar to that reported by a study on human *RBR* [51]. Interestingly, *RNF19A* and *RNF19B* exhibited the highest expression level in blood (Fig. 2A and B); meanwhile, *RNF144*AA and *RNF144*AB expression was highest in the brain (Fig. 2C and D), and *RNF217* was highly expressed in the liver (Fig. 2F), suggesting different *RBR* groups play different roles in the tissues and organs of *C. idellus*. As we all know, spleen is one of the important immune organism in fish, but surprisingly, the expression levels of *RNF19A*, *RNF144*AA and *RNF217* in the spleen and intestine were significantly down-regulated at all time points of post GCRV infection (Fig. 3). Studies in mammals suggest that *RNF19A*, *RNF144A*, and *RNF217* are involved in different ubiquitin process. For example, *RNF144A* is the first identified mammalian E3 ligase for DNA-dependent protein kinase, catalytic subunit, (DNA-PKcs) and plays roles in cell metabolism and proliferation [52]. Many researches have revealed that GCRV could induce cell apoptosis in grass carp [53–55]. Spleen is not only a hematopoietic tissue of fish but also an important peripheral immune organ. Following GCRV infection, spleen exerts an immune response against pathogens invading the body. At the moment, a large number of immune cells are in a state of stress or even apoptotic, the cell proliferation-related factors are not activated but inhibited. This may be the reason for the down-regulation of *RNF144*AA (as RNF144A in mammals) in the spleen at all time points of post GCRV infection. Since the study of *RNF19A* and *RNF217* is limited, it is difficult to

speculate the reason for their down-regulation in the spleen after GCRV infection based on the limited information. So far, previous studies have only revealed that *RNF217* regulates splicing in B cell development and promotes leukemia development [56]. *RNF19A* also known as Dorfin, which mediated calcium-sensing receptor CaR ubiquitination, contributed to maintenance of systemic Ca^{2+} homeostasis [57]. This may be the reason why *RNF19A* was highly expressed in blood.

In mammals, among the six RBR members, *RNF144B* has captured more attention in view of its involvement in immune regulation. *RNF144B* was not only a regulatory factor for Bax and apoptosis activation [33,36], but also necessary for priming of inflammasome responses in primary human macrophages [25]. Meanwhile, in this study, although all of *RNF19A*, *RNF19B*, *RNF144*AA, *RNF144*AB, and *RNF144B* responded substantially after GCRV challenge (Fig. 3); *RNF144B* respond intensely in intestine which is sensitive to GCRV [58] and forms the first protective barrier to invading pathogens. So in orders to get a better understand of the immune regulation of RBR in teleost fish, we chose *RNF144B* as a representative for further analysis. In the present study, RNF144B-deficient CIK cells were generated and stimulated with poly (I: C) to further verify the functions of *RNF144B* gene in the immune response of grass carp and its further effects on inflammation signaling. As shown in Fig. 5, RNF144B silencing significantly increased *IFN-I*, *TNF-α*, *IL-6* and *IRF3* release after poly(I:C) stimulation, suggesting that *RNF144B* acts as a negative regulator of inflammatory related factor. Moreover, the expression of *RNF144B* in liver, especially in intestine was intensely up-regulated (30.12 fold, $p < 0.05$) (Fig. 3E) at 1 dpi, revealing that immune inflammatory response occurs at the early stage of GCRV infection, and the response in the liver and intestine is more obvious. At the moment, the expression of *RNF144B* in the liver and intestine quickly increases and reduces the production of inflammatory factors. It is a pity that we did not get the antibody of RNF144B, so we could not perform further analysis at the protein level. Further evidence is required to identify the functional differences of these six RBR members in *C.idellus*.

In conclusion, six *RBR* genes from *Ctenopharyngodon idellus* were cloned and analysed in the present study. The responses of these genes to GCRV infection *in vivo* and *in vitro* were also characterized, suggesting that they might play a role in the immune system of *C. idellus*. In addition, deficiency of *RNF144B* in CIK with siRNA up-regulated polyinosinic:polycytidylic acid poly(I:C))-induced inflammatory cytokines production, revealing that *RNF144B* was a negative regulator of inflammatory cytokines and played an important role in immune responses. Therefore, the studies reported herein will provide basic information that can be used in future research on teleost RBR proteins.

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Appendix A. Supplementary data

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