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Characterization of cathepsin C from orange-spotted grouper, *Epinephelus coioides* involved in SGIV infection

Shina Wei[a], Shaowen Wang[a], Min Yang[a], Youhua Huang[a], Jingguang Wei[a], Xiaohong Huang[a], Qiwei Qin[a,b,*]

[a] College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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ABSTRACT

The lysosomal cysteine protease cathepsin C plays a pivotal role in regulation of inflammatory and immune responses. However, the function of fish cathepsin C in virus replication remains largely unknown. In this study, cathepsin C gene (Ec-CC) was cloned and characterized from orange-spotted grouper, *Epinephelus coioides*. The full-length Ec-CC cDNA was composed of 2077 bp. It contained an open reading frame (ORF) of 1374 bp and encoded a 458-amino acid protein which shared 89% identity to cathepsin C from bicolor damselfish (*Stegastes partitus*). Amino acid alignment analysis showed that Ec-CC contained an N-terminal signal peptide, the propeptide region and the mature peptide. RT-PCR analysis showed that Ec-CC transcript was expressed in all the examined tissues which abundant in spleen and head kidney. After challenged with Singapore grouper iridovirus (SGIV) stimulation, the relative expression of EC-CC was significantly increased at 24 h post-infection. Subcellular localization analysis revealed that Ec-CC was distributed mainly in the cytoplasm. Further studies showed that overexpression of Ec-CC *in vitro* significantly delayed the cytopathic effect (CPE) progression evoked by SGIV and inhibited the viral genes transcription. Moreover, overexpression of Ec-CC significantly increased the expression of proinflammatory cytokines during SGIV infection. Taken together, our results demonstrated that Ec-CC might play a functional role in SGIV infection by regulating the inflammation response.

1. Introduction

Cathepsins are a group of lysosomal proteases that consist of cysteine protease (cathepsin B, C, F, H, K, L, O, S, W and Z), aspartic protease (cathepsin D and E), and serine protease (cathepsin A and G) on the basis of amino acid residues involved in their active sites [1]. The function of individual cathepsin may vary due to different cleaving abilities. Cathepsin C is a lysosomal cysteine proteinase belonging to the papain superfamily [2]. In human, cathepsin C has a unique structure consisting of four identical subunits, each subunit is composed of three polypeptide chains: a significantly long propeptide, the heavy and the light chains [3–5]. This enzyme has numerous biological functions. In addition to a role of the lysosomal proteins and peptides degradation through autophagy or endocytosis [6,7], it is also a crucial enzyme in the activation of granule serine proteases from immune and inflammatory cells [8]. For instance, the inflammatory cells infiltration and inflammatory cytokines production have been shown to be decreased in cathepsin C knockout mouse models of abdominal aortic aneurysms, asthma, sepsis and chronic obstructive pulmonary disease (COPD) [9–12]. In invertebrates, cathepsin C is also play an important role in anti-bacterial or antiviral immune response. The expression of cathepsin C is upregulated in response to *V. anguillarum* stimulation in *Fenneropenaeus chinensis*, *Sinonovacula constricta* or *Eriocheir sinensis*, lipopolysaccharide (LPS) stimulation in *Penaeus monodon*. Moreover, knockdown of cathepsin C increase the replication of white spot syndrome viruses (WSSV) in *Fenneropenaeus chinensis* [13–16]. Although great progresses have been made in the function of cathepsin C from mammals [17,18], there are no reports on the immune function of fish cathepsin C so far.

Orange-spotted grouper, *Epinephelus coioides* is an economically important marine fish cultured in China and Southeast Asian countries. However, outbreaks of viral diseases caused by Singapore grouper iridovirus (SGIV) and nervous necrosis virus (VNN) causing large economic losses each year due to high mortality rates [19,20]. Our previous study found that grouper cathepsin B play an important role during the SGIV infection [21]. While whether cathepsin C is involved in the antiviral response in grouper still remains unclear.

In this study, a cathepsin C from grouper *E. coioides* (Ec-CC) was

* Corresponding author. College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China.
E-mail address: qinqw@scau.edu.cn (Q. Qin).

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cloned, we analyzed the expression patterns, subcellular localization and its role in SGIV infection. Our present results provide important information to better understand the biological function of cathepsin C in grouper antiviral innate immunity.

2. Material and methods

2.1. Fish, cells and viruses

Juvenile orange-spotted grouper, *E. coioides* (50–60 g) were purchased from a fish farm in Huizhou, Guangdong province, China. Fish were acclimatized in a laboratory recirculating seawater system at 25–30 °C and fed twice daily for two weeks before experimental manipulation.

The fish were anesthetized by immersion in 100 ng/ml of tricaine methanesulfonate (MS222, Sigma, USA). A series of tissue samples including liver, spleen, kidney, head kidney, heart, muscle, stomach, intestine, gill, skin and brain were removed with a pair of small forceps and immediately frozen by liquid nitrogen, followed by storage at −80 °C until used. This study involving animals was carried out in accordance with the protocols of "South China Agricultural University Experimental Animal Welfare Ethics Committee."

Grouper spleen cells (GS) were grown in Leibovitz's L15 medium that contained 10% fetal bovine serum (Invitrogen, USA) at 25 °C, respectively [22]. SGIV was kept in our laboratory. Propagation of SGIV was performed as described previously [20].

2.2. Cloning and sequencing of E. coioides cathepsin C (Ec-CC)

Total RNA was extracted from spleen of *E. coioides* tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, the quality of total RNA was detected by electrophoresis on 1% agarose gel. The RNA was used for rapid amplification of cDNA ends (RACE-PCR) (SMART RACE, Clontech) and cDNA synthesis ReverTra Ace (TOYOBO, Japan).

The first-strand cDNA was synthesized from total spleen RNA with the SMART™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol for 5′ RACE and 3′ RACE. The primers used for RACE PCR (Table 1) were designed based on the identified expressed sequence tag (EST) sequence from the transcriptome library established in our laboratory (Accession No. SRA040065.1) [23], In detail, the gene specific primer CC 5′NGSP1, CC 3′GSP1 (Table 1)and UPM (supplied by the kit) were used for the first-round touchdown PCR. The touchdown PCR was conducted at 94 °C for 3 min, followed by 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min, 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min. The product of the first round PCR was diluted 10 times, and then used as the template for the nested PCR. The nested PCR was performed with the gene specific primers CC 5′NGSP2, CC 3′GSP2 (Table 1) and NUP (supplied by the kit). The RACE PCR condition and assembly of Ec-CC cDNA were performed as described previously [21].

Table 2
GenBank accession numbers of cathepsin C used in this study.

Protein	Accession no.
Epinephelus coioides	KC832925
Stegastes partitus	XP_008299785.1
Pundamilia nyererei	XP_005720770.1
Maylandia zebra	XP_004543938.1
Haplochromis burtoni	XP_005942856.1
Oreochromis niloticus	XP_003441633.1
Fundulus heteroclitus	XP_012714842.1
Oryzias latipes	XP_004075795.1
Cynoglossus semilaevis	XP_008308025.1
Takifugu rubripes	XP_011606588.1
Salmo salar	XP_014070770.1
Danio rerio	AAH64286.1
Gallus gallus	XP_417207.3
Pongo abelii	NP_001125612.1
Callithrix jacchus	XP_002754752.1
Homo sapiens	AAL48192.1
Loxodonta africana	XP_003418602.1

Table 1
Sequences of primers used in this study.

Primers	Sequence (5′-3′)
NUP	AAGCAGTGGTATCAACGCAGAGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
CC3′GSP1	TATAAGGAGGGCATCTACCACCACAC
CC3′GSP2	GAGTGTTCCATCGAGAGCATCGCTGT
CC5′NGSP1	CCCACCATCGCAACCTTGAGAATACT
CC5′NGSP2	AGGCAAAGGAGTAGCAGCTTCCACAT
pEGFP-CC-F	CCGGAATTCTATGAAGATGAGGCTGAGCGGT
pEGFP-CC-R	CGCGGATCCCAGTTTGGGGATGGGGTTGGCTG
pcDNA-CC-F	CGGGGTACCATGAAGATGAGGCTGAGCGGTGTG
pcDNA-CC-R	CCGCTCGAGCAGTTTGGGGATGGGGTTGGCTGCT
RT-CC-F	CTCACCAACAACACTGCGAATCCCA
RT-CC-R	CATGACTCATCCACAATGCCAAAAT
RT-Actin-F	GTTTGTCTGAGCCAGTGGGAGTC
RT-Actin-R	CATGCGTTCACTGAGGGAGAGGT
RT-162-F	GCACGCTTCTCTCACCTTCA
RT-162-R	AACGGCAACGGGAGCACTA
RT-049-F	ATGTACGTATACCCCGCAAT
RT-049-R	TCATTTTTTTTGCCTAA
RT-072-F	GCACGCTTCTCTCACCTTCA
RT-072-R	AACGGCAACGGGAGCACTA
RT-TNFα-F	GTGTCCTGCTGTTTGCTTGGTA
RT-TNFα-R	CAGTGTCCGACTTGATTAGTGCTT
RT-IL1β-F	AACCTCATCATCGCCACACA
RT-IL1β-R	AGTTGCCTCACAACCGAACAC
RT-IL8-F	GCCGTCAGTGAAGGGAGTCTAG
RT-IL8-R	ATCGCAGTGGGAGTTTGCA
RT-IL6-F	CTCTACACTCAACGCGTACATGC
RT-IL6-R	TCATCTTCAAACTGCTTTTCGTG

2.3. Bioinformatics analysis

The cDNA and predicted amino acid sequences of Ec-CC were analyzed using Genetyx7.0 software. The similarity of Ec-CC with other cathepsin Cs were analyzed using the BLASTP search program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported cathepsin C amino acid sequences was performed using ClustalX2.0 and a phylogenetic tree was constructed using the MEGA 5.0 software, numbers at branch nodes represent the bootstrap majority consensus values of 1000 replicates.

2.4. Tissue distribution of Ec-CC mRNA

Total RNA was extracted from healthy orange-spotted grouper liver, spleen, kidney, head kidney, heart, muscle, stomach, intestine, gill, skin and brain with TRIzol Reagent (Invitrogen, USA) according to manufacturer's protocol. Expression levels of Ec-CC in different tissues was determined by RT-PCR using primers RT-Actin-F and RT-Actin-R to amplify the internal control β-actin (GenBank accession number AY510710) while RT-CC-F and RT-CC-R were used to amplify Ec-CC (Table 1). The PCR conditions were 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final elongation step of 10 min at 72 °C.

2.5. Expression profiles of Ec-CC after challenge with SGIV

For the virus challenge experiment, each grouper was injected with 200 μL SGIV at a dose of 2×10^4 $TCID_{50}$, and each control sample was injected with 200 μL PBS respectively. Six fish in each group were collected for Q-PCR at 0, 6, 12, 24, 36, 48, 72 and 96 h of post-injection. Expression levels of Ec-CC were examined by Q-PCR in spleen after challenging with SGIV. Q-PCR was carried out using a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland), with SYBR Green as the fluorescent dye, according to the manufacturer's protocol (TOYOBO). β-actin was amplified as an internal control using primers RT-Actin-F and RT-Actin-R, while RT-CC-F and RT-CC-R were used to amplify Ec-CC (Table 1). All primer pairs amplified a single PCR product with the expected size using the similar Tm value by agarose gel electrophoresis and melting curve analysis. The PCR amplification efficiency of each primer pair is normal and identical according to methods described previously [21]. All samples at indicated time points after infection were analyzed in triplicate wells using the cycling condition as follows: 94 °C for 5 min, followed by 40 cycles of 5 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. We analyzed the relative gene expression by the typical Ct method ($2^{-\Delta\Delta Ct}$ method) [24], then the results were calculated as the folds based on the expression level of Ec-CC in different challenged grouper relative to that in PBS injected grouper at the same time point. Data were expressed as mean ± SD, and statistical analysis were performed using SPSS software.

2.6. Subcellular localization analysis

Subcellular localization of Ec-CC was performed by EGFP fusion protein expressions in GS cells. The open reading frame (ORF) of Ec-CC was amplified by specific primers (pEGFP-Ec-CC, Table 1) and cloned into the *Eco*RI and *Bam*HI restriction enzyme sites of pEGFP-N3 vector. The recombinant plasmid constructed was verified by DNA sequencing analysis. The GS cells were seeded onto coverslips (10 mm × 10 mm) in a 24-well plate. After the cell adhering for 18 h, the GS cells were transiently transfected with pEGFP-Ec-CC or pEGFP-N3 plasmid using Lipofectamine™ 2000Reagent (Invitrogen, USA) according to the manufacture's protocol. After transfection for 24 h, GS cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min, and then stained with 6-diamidino-2-pheny-lindole (DAPI) (1 μg/ml) for 15 min. Finally, the cells were rinsed with PBS, mounted with 50% glycerol, and observed using fluorescence microscopy (Leica, Germany).

2.7. Virus infection

To detect the effects of Ec-CC on virus replication, the open reading frame (ORF) of Ec-CC was amplified by specific primers (pcDNA-Ec-CC, Table 1) and cloned into the *Kpn*I and *Xho*I restriction enzyme sites of pcDNA3.1-Flag vector. The recombinant plasmid constructed was verified by DNA sequencing analysis. The GS cells were transiently transfected with pcDNA-Ec-CC or pcDNA3.1-Flag plasmid using lipofectamine™ 2000 reagent (Invitrogen, USA) for 18 h following the manufacturer's protocol, Then the cells overexpressing pcDNA-Ec-CC or pcDNA3.1-Flag were infected with SGIV at indicated time points. At 24 and 48 h p.i., cell morphology was imaged under inverted light microscopy. In addition, virus infected cells at indicated time points were collected for RNA extraction and further Q-PCR analysis.

2.8. Q-PCR analysis of viral genes and proinflammatory cytokines

To evaluated whether Ec-CC was able to alter the transcription levels of viral genes and inflammation response *in vitro*, Q-PCR was used to detect the relative mRNA expression of SGIV genes, including an immediate-early gene (IE) ORF162 (GenBank accession number YP_164257), an early gene (E) ORF049 (GenBank accession number YP_164144), the late gene (L) major capsid protein ORF072 (GenBank accession number YP_164167) and their primers were listed in Table 1. The expression levels of proinflammatory cytokines in Ec-CC overexpressing cells, including TNF-a (GenBank accession number FJ009049), IL-6 (GenBank accession number JN806222), IL-8 (GenBank accession number FJ913064), IL-1β (GenBank accession number EF582837) were also detected and their primers were listed in Table 1. The procedures were performed as described above.

2.9. Statistical analysis

All analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The data were calculated as the folds based on the expression level of targeted genes normalized to β-actin at different time points. All data were expressed as mean relative stimulation as Means ± SD from three separate experiments with each performed in triplicate wells, and then subjected to Student's t-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Bioinformatic analysis of Ec-CC

The determined grouper cathepsin C cDNA consisted of an open reading frame of 1374 bp encoding 458 amino acids with a predicted molecular mass of 51 kDa and a theoretical isoelectric point of 6.2, the deduced amino acid sequence of Ec-CC contains a putative signal peptide of 22 residues at the N-terminal of deduced protein (Fig. 1). The full-length cDNA of Ec-CC contains a 5′-terminal untranslated region (UTR) of 131 bp, a 3′-terminal UTR of 572 bp with a 28 bp poly (A) tail and a consensus polyadenylation signal sequence AATAAA. The full-length Ec-CC cDNA sequence was deposited in Genbank with accession number KC832925. BlastP analysis showed that Ec-CC shared 89% and 72% identity with bicolor damselfish (*Stegastes partitus*) and zebrafish (*Danio rerio*), respectively. Amino acid alignment analysis showed that Ec-CC contained a putative signal peptide of 22 amino acids (position 1–22aa), a long propeptide of 204 amino acids (position 23–226aa) and a putative mature peptide region of 231 (position 227–457aa) in Ec-CC. The mature protein, comprised of a heavy chain (position 227–389aa) and a light chain (position 390–457aa), contained three catalytic active sites (Cys^{250}, His^{399} and Asn^{421}) that were highly conserved in all papain family members. The N-terminus of the propeptide and the heavy

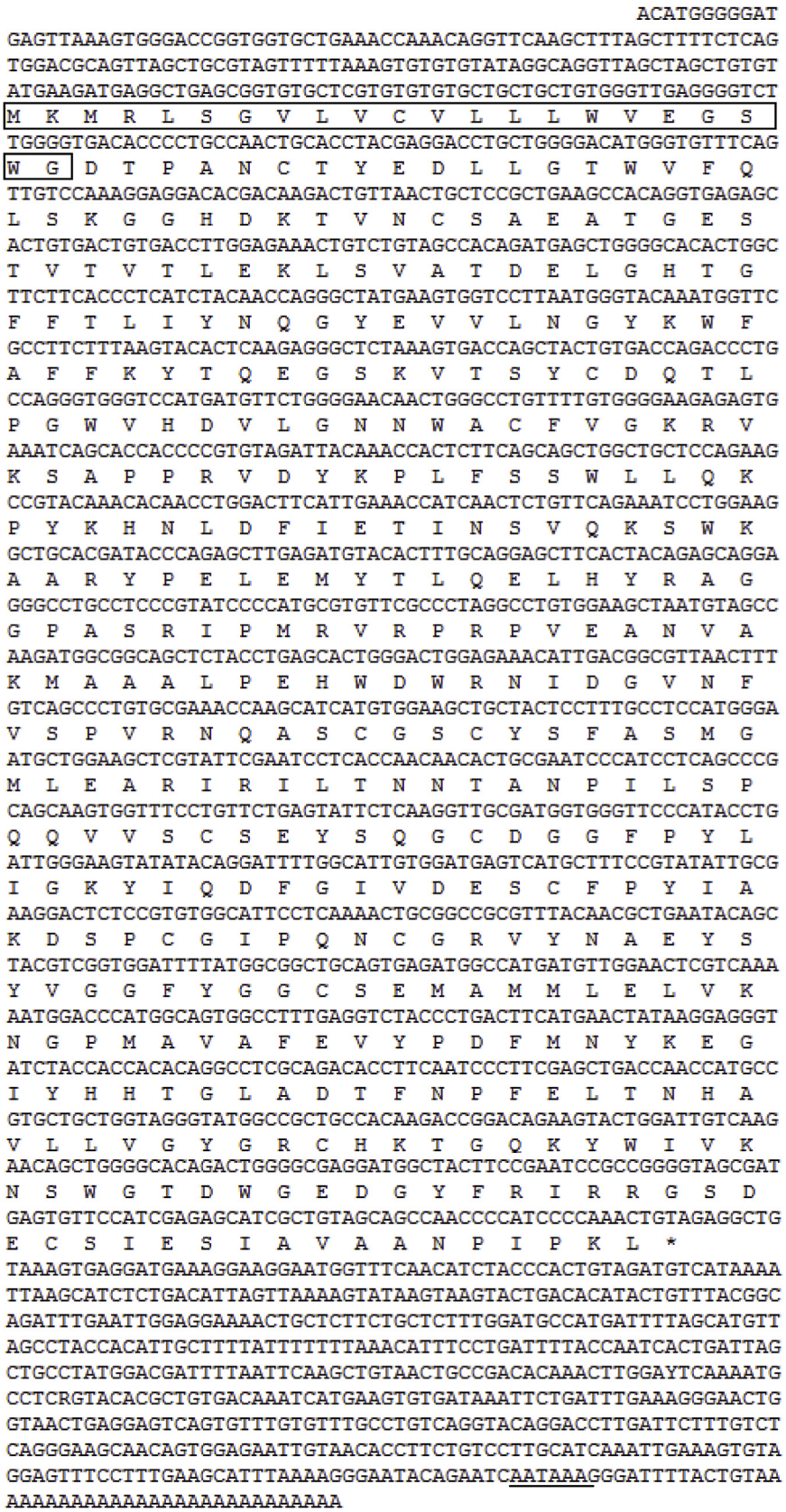


Fig. 1. The nucleotide and deduced amino acid sequences of Ec-CC. The putative signal peptide sequence is in box and the polyadenylation signal (AATAAA) is shown in underlined.

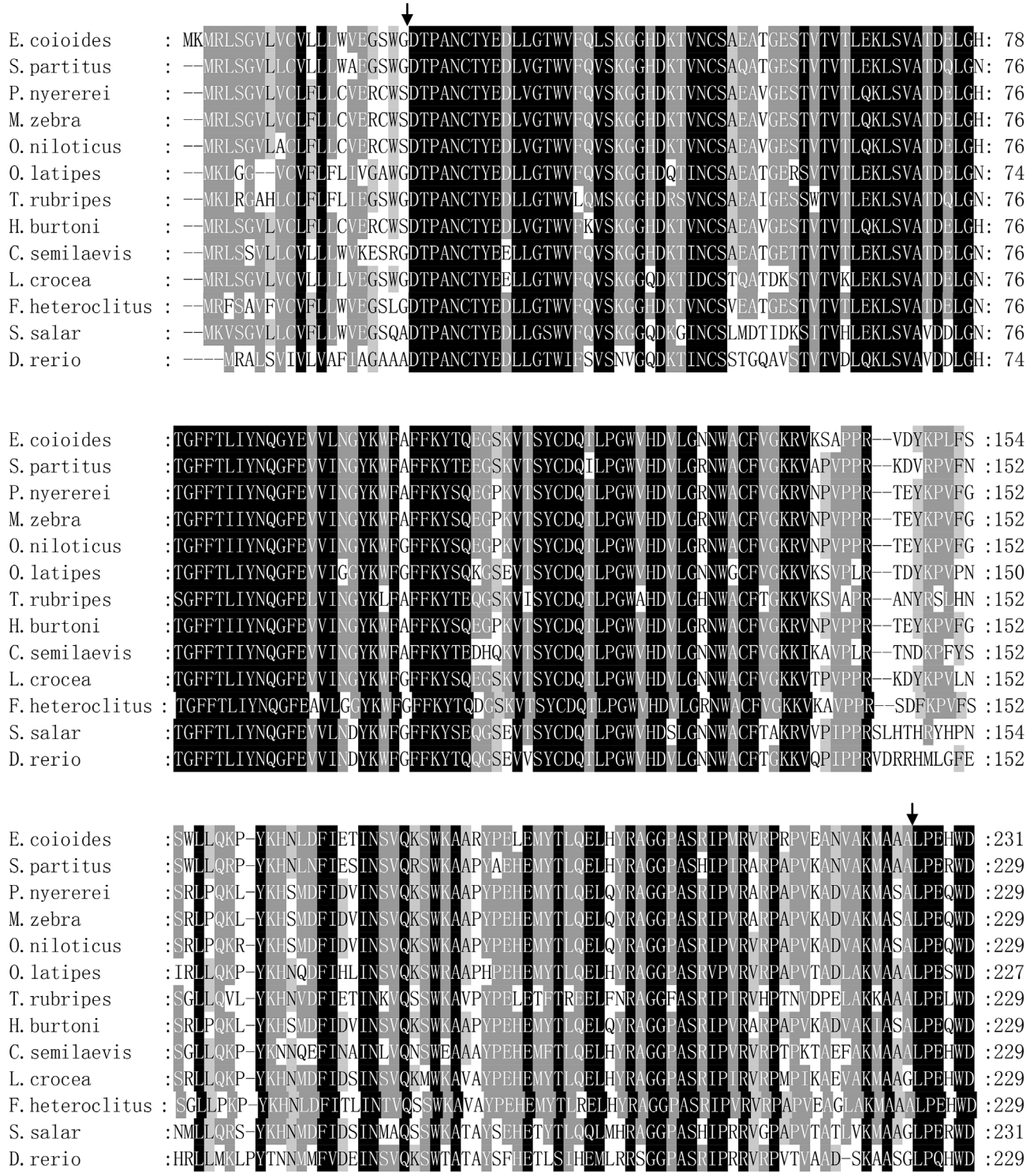


Fig. 2. Multiple alignments of Ec-CC with other known cathepsin C proteins. The conserved and identical residues are represented by black shading and conservative substitutions are represented by gray shading. The regions of the signal peptide, propeptide, and the heavy and light chains of the mature peptide are indicted by arrows. The conserved catalytic residues (Cysteine, Histidine and Asparagines) are indicated by stars.

and light chains of the mature peptide were all well conserved as compared with those of the other animals, and a high identity was detected in the heavy and light chains of the mature peptide region (Fig. 2). Phylogenetic tree was constructed based on amino acid sequences of cathepsin C from various species by the Neighbour-Jointing method. Ec-CC presented the closest distant relationship with cathepsin C of *Stegastes partitus*, followed by other fishes and mammals (Fig. 3).

3.2. Tissue distribution of Ec-CC

The RT-PCR results showed that the expression of mRNA in various normal tissues using β-actin as a reference gene. Ec-CC transcript was abundant in the spleen and head kidney, and to a less extent in the stomach, brain, skin and liver (Fig. 4A).

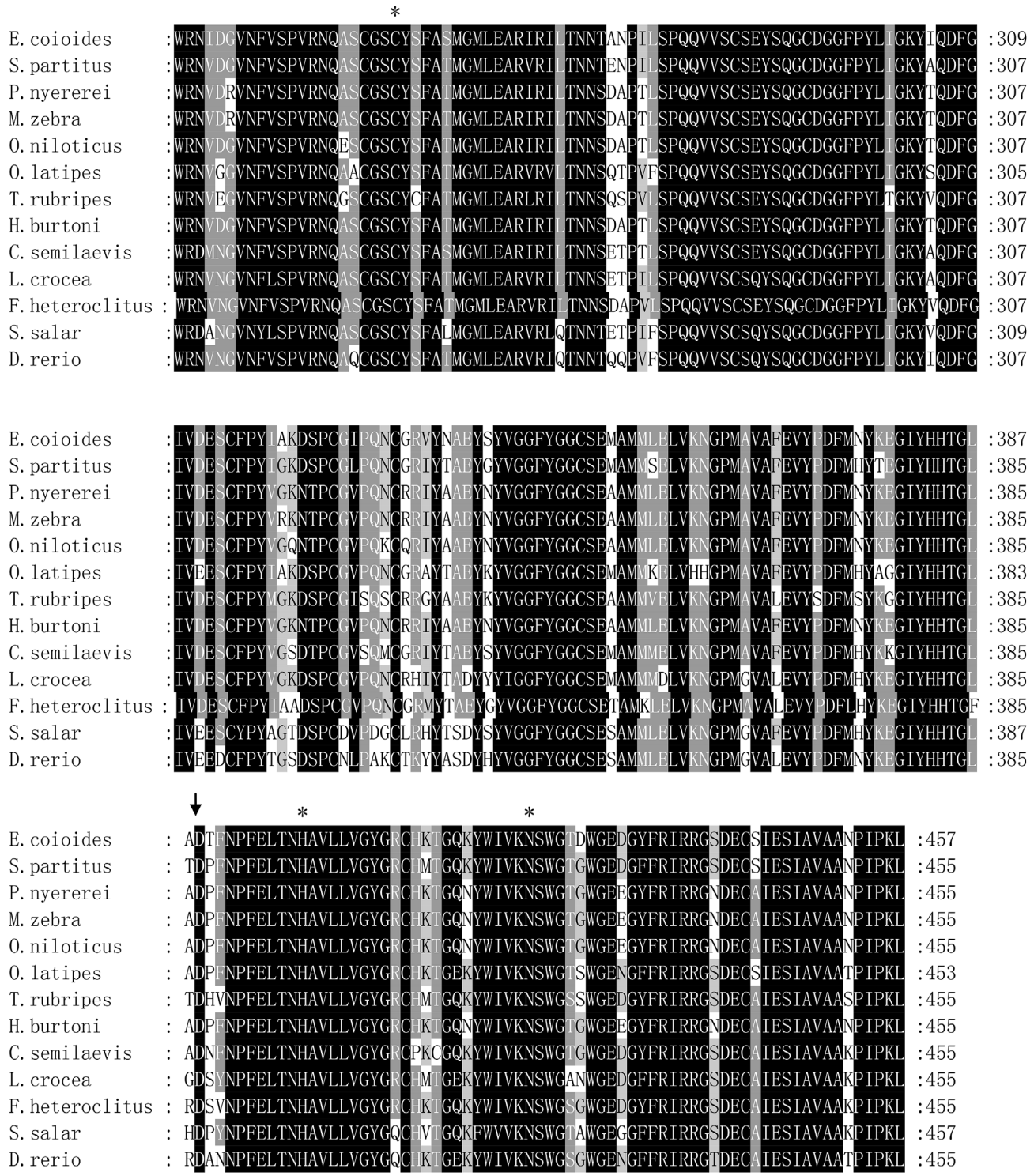


Fig. 2. (*continued*)

3.3. Expression changes of Ec-CC in grouper spleen after challenge with SGIV

To determine whether the transcription of Ec-CC was regulated after SGIV infection, the transcripts of Ec-CC were detected in spleen after SGIV challenge at indicated time points. The relative expression of Ec-CC was decreased at 6 h post infection and then increased up to the peak at 24 h post infection, suggesting that Ec-CC might be activated and play critical role during SGIV infection (Fig. 4B).

3.4. Subcellular localization of Ec-CC

The subcellular localization of Ec-CC was determined by Ec-CC-GFP fusion protein expression in GS cells. As shown in Fig. 5, the green fluorescence in Ec-CC-GFP fusion protein transfected GS cells was distributed mainly in the cytoplasm (Fig. 5, lower row). In EGFP-N3 transfected cells, the fluorescence signal was obseved in both cytoplasm and nucleus (Fig. 5, upper row).

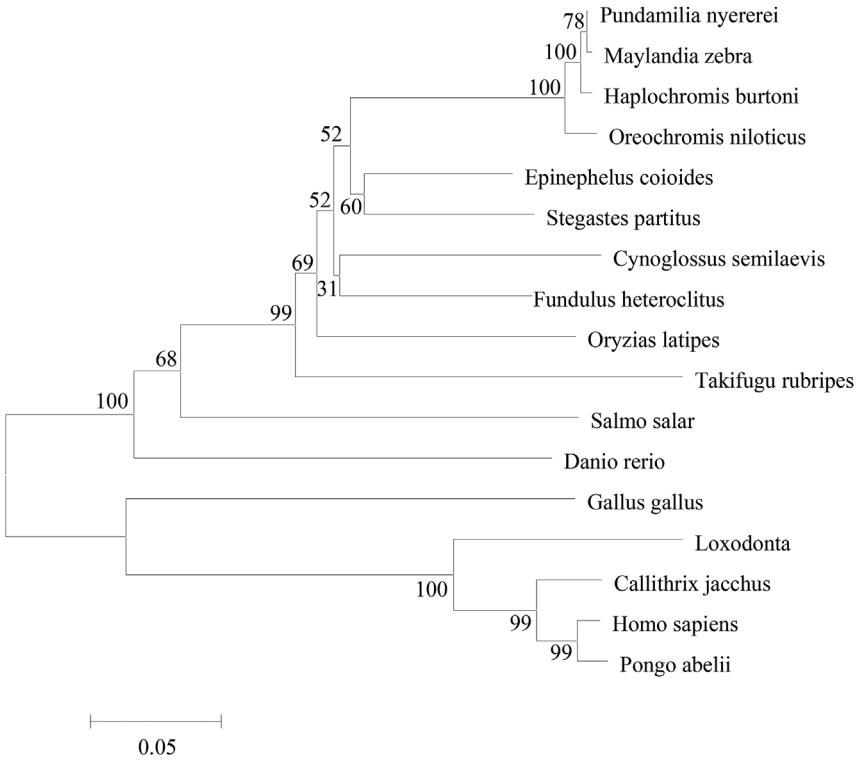
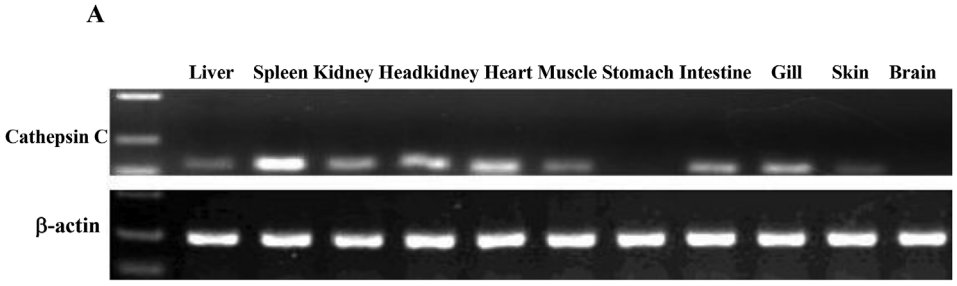


Fig. 3. Phylogenetic analysis of Ec-CC with regard to other cathepsin C gene family members. Neighbour Joining (NJ) trees were produced using MEGA 4. One thousand bootstraps were performed to determine the reproducibility of the results. The GenBank accession numbers of selected cathepisn C sequences are listed in Table 2.



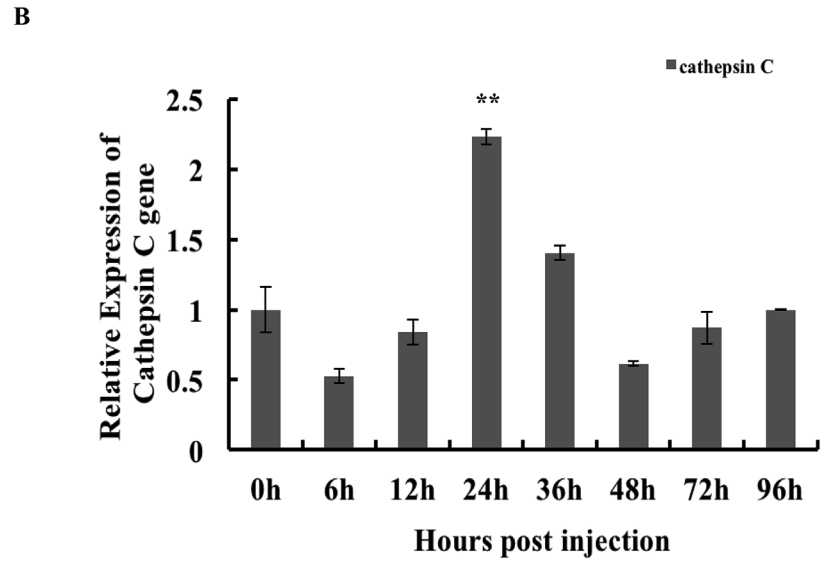


Fig. 4. Expression profile of Ec-CC. (A) Tissue distribution of Ec-CC. (B) The expression profile of Ec-CC in spleen infected with SGIV. The relative expression of Ec-CC was calculated in SGIV challenged grouper as the folds relative to that in PBS injected grouper at the same time point, and then the results were expressed as relative fold of one sample in each experiment. Vertical bars represented the means ± SD (n = 6), and significant differences of Ec-CC expression between the challenged and control samples were indicated with two asterisks (**) at $p < 0.01$.

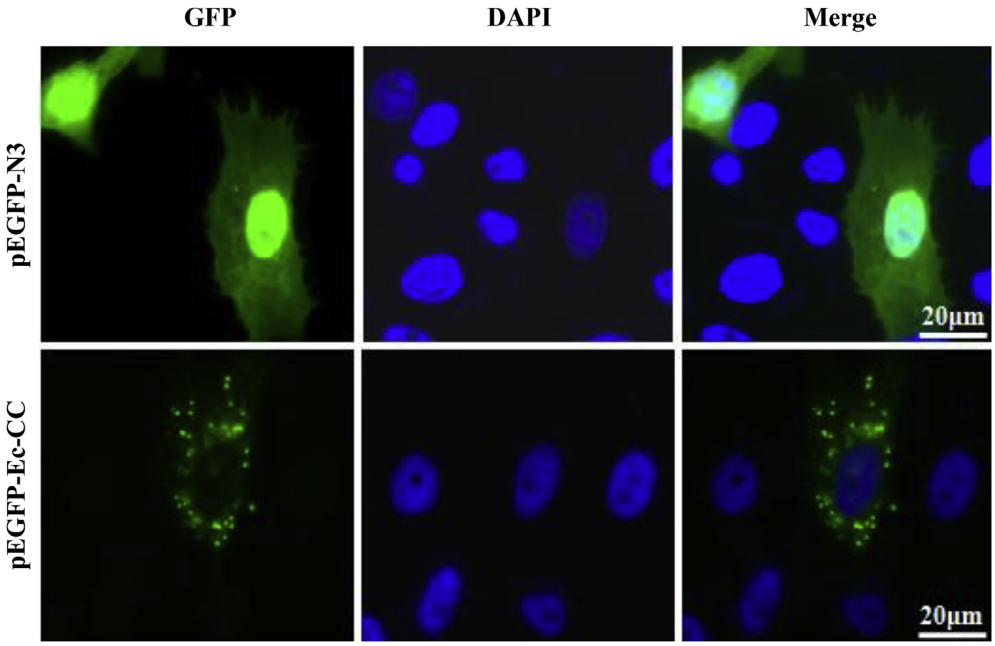


Fig. 5. Intracellular localization of grouper Ec-CC. Intracellular localization of Ec-CC by fluorescence microscopy, GS cells were transfected with pEGFP-N3 or pEGFP-Ec-CC. The localization of the nucleus was shown by DAPI staining.

3.5. Effects of overexpression Ec-CC on SGIV replication

To determine whether the Ec-CC overexpression affects SGIV replication, the GS cells were transfected with Ec-CC and then infected with SGIV. After SGIV infection, we found that the CPE progression induced by virus was inhibited significantly in Ec-CC overexpressing cells (Fig. 6A). The expression level of Ec-CC was significantly increased in Ec-CC overexpressing cells in compared with the control cells (Fig. 6B). We also detected the effects of overexpression of Ec-CC on viral gene transcription by qPCR. As shown in Fig. 6C–E, the transcripts of 3viral genes, including immediate-early gene (IE) ORF162, early gene (E) ORF049 and late gene (L) major capsid protein ORF072 were all significantly down-regulated by overexpression of Ec-CC. Thus, we proposed that Ec-CC play crucial roles during SGIV infection.

3.6. The overexpression of Ec-CC enhanced proinflammatory cytokines response during SGIV infection

To explore the potential mechanism involved in the action of Ec-CC in SGIV infections, we evaluated the roles of Ec-CC on the host inflammation response. As shown in Fig. 7, the expression level of TNF-a, IL-6, IL-8 and IL-1β were significantly increased in Ec-CC overexpressing cells in compared with the control cells. After SGIV infection, the expression level of TNF-a, IL-6, IL-8 and IL-1β were both increased significantly at 24 and 48 h post infection. Thus, we proposed that the Ec-CC overexpression increased cytokine expression protected from SGIV infections.

4. Discussion

Cathepsins are multifunctional proteins that participate in numerous physiological and pathological processes [25]. Cathepisn C is crucial for various immune diseases by activating several other proteases or driving inflammation [26]. To our knowledge, cathepisn C has been well studied in several species of regarding different types of shrimp and crab [13–16]. However, the function of cathepsin C on virus infection has yet to be reported in fish. In this study, we cloned cathepsin C gene from orange-spotted grouper, *E.coioides* and investigated its potential function properties involved in SGIV infection.

The deduced amino acid sequence of Ec-CC showed high identities with the cathepsin C of teleosts and mammals. The Ec-CC protein consisted of three functional domains: N-terminal signal peptide, long propeptide, and mature peptide. The mature peptide contained the heavy and light chains, and three amino acid catalytic residues (Cys-His-Asn) that are exist in all papain family members [27]. Furthermore, phylogenetic tree analysis revealed that Ec-CC from grouper was clustered with closer to those known fish cathepsin C proteins, following by mammals. All these data suggested that Ec-CC might perform similar functions to that from mammals. In addition, cathepisn C localization was dispersed in the cytoplasm of HaCaT cells [28]. Cathepsin C was predominantly expressed in hippocampal CA2 neurons in C57BL/6 J mice under normal conditions [29]. Consistent with this, our data showed that Ec-CC mainly localized in cytoplasm of GS cells. Therefore, all these data suggested that Ec-CC might perform similar functions to that from mammals.

Cathepsin C was expressed in various tissues, suggesting it may be involved in a multifunctional role. There are notable differences in the expression of cathepsin C among different species. For example, Human cathepsin C mRNA is highly expressed in polymorphonuclear leukocytes, alveolar macrophages, lung, kidney, and placenata, and low expression in brain and liver [30,40]. Murine cathepsin C mRNA is widely expressed in the lung, liver, spleen, and small and large intestines, and low expression in brain, heart and kidney [31]. In *Sinonovacula constricta*, cathepsin C mRNA showed the highest expression in the digestive gland [14]. In *Fenneropenaeus chinensis*, cathepsin C mRNA exhibited high expression level in the hepatopancreas, and the expression was upregulated by white spot syndrome viruses (WSSVs) [13]. Moreover, knockdown cathepsin C or injection of cathepsin C antiserum increased the replication of the WSSV. The activity of cathepsin C increased in Human Embryonic Kidney cell cultures and culture fluids infected with *Herpesvirus hominis* (*Herpes simplex*) [32]. Cathepsin C mediated resistance to ectromelia virus (ECTV) *in vivo* [33], cathepsin C can also inhibit Murine cytomegalovirus (MCMV) replication *in vivo* and involved in antiviral immune responses [34]. In this paper, Ec-CC mRNA expression was detected in all examined tissues, and up-regulated by SGIV infection in spleen tissue (Fig. 4). Given that cathepsin C is also highly expressed in leukocytes [40], we speculate that the variation of cathepisn C levels may related to changes in the proportion of

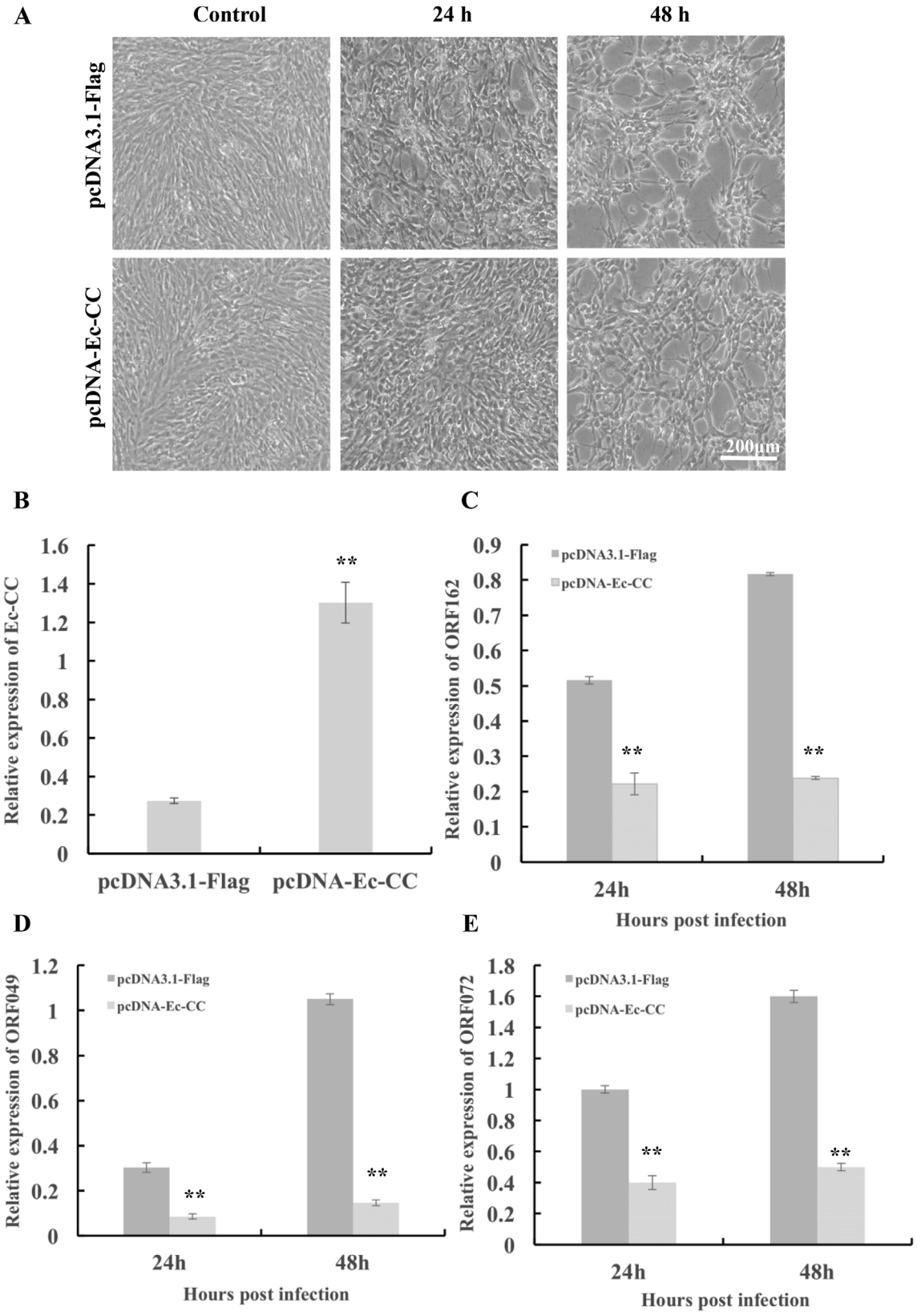


Fig. 6. Effects of Ec-CC overexpression on viral gene transcription. (A) After transfection pcDNA3.1-Flag or pcDNA-Ec-CC, cells were infected with SGIV for 24 and 48 h, respectively. Ec-CC overexpression delayed the appearance of the SGIV-induced cytopathic effect (CPE) in GS cells. (B) Detection of Ec-CC expression in empty vector or Ec-CC transfected GS cells. (C, D, E) Quantitative analysis of the SGIV ORF162, ORF049, ORF072 genes expression during SGIV infection. All data were normalized relative to β-actin and represented by means ± SD (n = 3). **, $P < 0.01$.

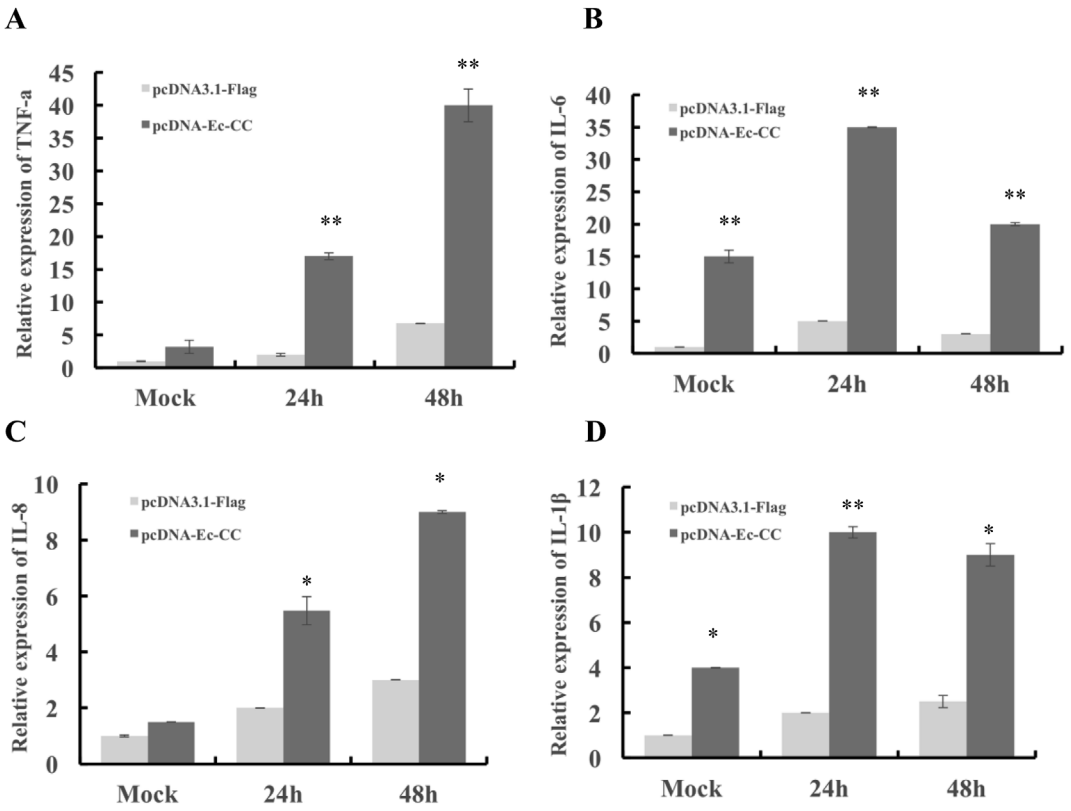


Fig. 7. The overexpression of Ec-CC increased the mRNA expression levels of proinflammatory cytokines during SGIV infection. The expression levels of proinflammatory cytokines, including TNF-a, IL-6, IL-8 and IL-1β in were examined. All data were normalized relative to β-actin and represented by means ± SD (n = 3). *, $p < 0.05$; **, $p < 0.01$.

lecukocyte population isolation from three major lymphoid tissue of fish, including head kidney, spleen and blood. We also examined the effects of Ec-CC on SGIV infection, and found that Ec-CC overexpression delayed the occurrence of CPE and inhibited the transcription of SGIV ORF162, ORF049 and ORF072 in GS cells (Fig. 6). Taken together, these results revealed that cathepsin C might play a crucial role during the SGIV infection.

Cathepsin C is a mediator in the physiological activator of serine proteases from immune inflammatory processes that cause it to be developed an attractive target for drugs to treat diseases [35]. Loss-of-function mutations in human cathepsin C gene result in a decrease in host defense and susceptibility to microbe infection, which lead to severe Papillon-Lefèvre syndrome (PLS) [36]. Previous report showed that Cathepsin C and neutrophils are crucial in the Sendai virus-induced asthma phenotype as a result of a cathepsin C-dependent neutrophil recruitment and cytokine response [10]. In addition, the inflammatory cytokines production had been shown to be reduced in cathepsin C knockout mouse models of sepsis, abdominal aortic aneurysms and chronic obstructive pulmonary disease (COPD) [37–39]. In this study, to further explore the potential roles of Ec-CC during SGIV infection, we detected the changes in the mRNA expression level of proinflammatory cytokines in Ec-CC overexpressing cells. We found that the mRNA expression level of TNF-a, IL-8 were consistently increased until 48 h post infection. However, the mRNA expression of IL-6 and IL-1β were decreased again after 24 h post infection between Ec-CC overexpressing and control cells. It has been demonstrated that proinflammatory factors including IL-6 and IL-1β could enhance the mRNA and protein expression of cathepsin C. In addition, these proinflammatory cytokines induced the extracellular release of Cathepsin C and upregulation of enzymatic activity [29]. Therefore, we speculated that proinflammatory factors mediated by cathepsin C might play more important roles in the action of cathepisn C against SGIV. Moreover, cathepsin C may further modulate proinflammatory cytokines by activating serine proteases. However, the precise mechanisms of Ec-CC affect SGIV infection need to be further explored.

In conclusion, we have identified and characterized the cathepsin C gene from *E.coioides*. The tissue distribution of Ec-CC mRNA was shown ubiquitous expression in healthy tissues, and we confirmed the up-regulation of cathepsin C in spleen after SGIV infection. Furthermore, overexpression of Ec-CC *in vitro* significantly increased the viral gene transcription and regulated inflammation response during SGIV infection. Our present results indicated firstly that Ec-CC play crucial roles in response to SGIV infection, and will contribute greatly to understanding molecular mechanisms of Ec-CC during fish virus infection.

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