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# Molecular characterization of three novel perforins in common carp (*Cyprinus carpio* L.) and their expression patterns during larvae ontogeny and in response to immune challenges

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## Abstract

**Background:** In the host immune system, perforin is a cytotoxic effector molecule that eliminate virus-infected and malignant cells. Moreover, some recent studies also imply the involvement of perforin in antibacterial immunity. Common carp (*Cyprinus carpio* L.), one of the most economically important fish species in China, has a high susceptibility to viruses and bacteria. Thus far, in common carp, no data are available regarding the identification and immunologic function of the perforin.

**Results:** In the present study, the cDNA and genomic DNA sequences of three perforin isoform genes were cloned and characterized in common carp, named CcPRF1, CcPRF2 and CcPRF3. Amino acid sequences of the three CcPRFs were quite different, with identities ranged from 37.3 to 39.5%. Phylogenetic analysis showed that three CcPRFs, each in a separate sub-branch, possessed closer evolutionary relationship with other teleost perforins, especially with cyprinid fishes, than higher vertebrates. Expression analysis revealed that each CcPRF gene was differentially expressed in all of the nine tested tissues. During larvae ontogeny, each CcPRF displayed a distinct expression pattern, while with a common expression peak at 22 days post hatching (dph). Moreover, in vivo or in vitro, after stimulation with polyI:C, LPS and *Aeromonas hydrophila*, each CcPRF was induced significantly, with differential expression dynamics.

**Conclusions:** Our findings suggest that perforin might play significant roles in larval immune system and in the immune defense of common carp against viral and bacterial pathogens. Meantime, the differential expression dynamics seem to imply possible different cellular locations or functional differences across various CcPRF isoforms.

**Keywords:** Common carp (*Cyprinus carpio* L.), Perforin, Evolutionary relationship, Expression pattern

## Background

In mammals, natural killer (NK) cells and CD8<sup>+</sup> cytotoxic T lymphocytes (CD8<sup>+</sup> CTLs) play important roles in the immune-cytotoxic system against virus-infected and tumor cells. In this process, it has been confirmed that perforin plays a crucial role [1]. Moreover, some

recent studies pointed also to the role of perforin in resisting bacterial pathogens [2, 3]. Until now, in addition to these two cytotoxic cells, some studies reported the expression of perforin in other types of cell, including NKT cells [4], effector-like  $\gamma\delta$  T cells [5] and some CD4<sup>+</sup> T lymphocytes [6].

Perforin is a 60–70 kDa pore-forming glycoprotein, which belongs to the membrane-attack-complex/perforin (MACPF) family [7, 8]. Mature perforin molecule has three domains: an N-terminal MACPF domain, an intermediate epidermal growth factor (EGF)-like domain

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and a C-terminal calcium-binding (CalB) domain. Pore formation of perforin depends on these two characteristic domains, in which MACPF is related to pore-forming [9, 10] and the CalB motif is related to calcium-dependent membrane binding to target cells [11]. Under normal circumstances, perforin is stored in cytoplasmic secretory granules of NK cells and CTLs. After these cytotoxic cells are activated, monomer perforin polymerizes to form a channel of 5–20 nm in diameter in target cell membranes, leading to the disruption of cell membrane and subsequently the death of target cells [12]. Additionally, the pores formed by perforin allow granzymes (Gzms), serine proteases stored in cytoplasmic granules of NK/CTL cells with perforin, into the cytosol of target cells [13], which will induce the activation of proapoptotic pathways and DNA degradation.

Perforin was first characterized as a lytic pore-forming protein isolated from CTLs in 1985 [14]. In humans and mice, perforin is a single-copy gene, and presently its immunological function has been well studied. In teleosts, until now, the perforin genes have been characterized in 6 species, including Japanese flounder (*Paralichthys olivaceus*) [15, 16], channel catfish (*Ictalurus punctatus*) [17], rainbow trout (*Oncorhynchus mykiss*) [18], gibel carp (*Carassius auratus langsdorffii*) [19], rock bream (*Oplegnathus fasciatus*) [20, 21] and zebrafish (*Danio rerio*) [22]. Moreover, different with humans and mice, there are more than one perforin genes in fish genomes [19, 22], although only one isoform was reported in some teleosts to date [16–18, 20].

Common carp (*Cyprinus carpio* L.) is one of the most important aquaculture fish species in China and has a high susceptibility to viruses and bacteria. In common carp, although there were data suggesting the existence of a perforin/Gzm pathway like mammals [23], no data are currently available regarding the identification and immune function of the perforin. In the present study, three perforin isoform genes were cloned and characterized from common carp, named *CcPRF1*, *CcPRF2* and *CcPRF3* according to their amino acid identities with crucian carp perforins. We studied their evolution and analysed their constitutive expression patterns in healthy carps and during larvae ontogeny. Moreover, gene expression patterns of the three *CcPRFs* upon polyinosinic-polycytidylic acid (polyI:C) and *Aeromonas hydrophila* (*A. hydrophila*) stimulation were determined in vivo. Meanwhile, we isolated leukocytes from the head kidney and peripheral blood of common carp, and analysed polyI:C and lipopolysaccharides (LPS)-induced expression of *CcPRFs* and *CD8 $\alpha$*  gene in vitro.

## Methods

### Fish rearing and sample collection

Healthy common carps, with a body weight of  $80 \pm 5$  g, were obtained from the Fresh Water Fishery Research

Institute of Shandong Province, China. Before the start of experiments, fish were acclimated to the laboratory environment in a recirculating freshwater system at 20 °C for 2 weeks and fed once daily with commercial carp pellets. Four healthy fish were euthanatized by anesthesia in 100 mg/L MS222 (Sigma, USA), and the tissue samples, including those of the liver, spleen, head kidney, foregut, hindgut, gill, skin, brain and muscle, were isolated for RNA extraction.

To investigate gene expression patterns of three *CcPRFs* during embryonic and early larval stages, four pairs of parent fish were selected for artificial propagation, which was performed based on a previous study [24]. Fertilized eggs were incubated in a water tank at 28–30 °C with enough oxygen. At 2 days post hatching (dph), the larvae started to be fed with aseptic soymilk. During 9 dph–22 dph, the juvenile fish were fed with fine-grained fish meal. At various developmental stages with blastula stage (1 days post fertilization, 1 dpf), optic primordium appearance (2 dpf), 1 dph, 2 dph, 4 dph, 8 dph, 14 dph and 22 dph, five embryos or larvae were sampled randomly for RNA extraction.

### RNA extraction and cDNA preparation

Total RNA was extracted from the tissue, embryo and larva samples using the RNAsimple Total RNA Kit (Tiangen, China). The concentration and quality of total RNA was measured using a spectrophotometer. The RNA template was subjected to reverse transcription into first-strand cDNA using the FastQuant RT Kit (with gDNase) (Tiangen, China).

### cDNA and genomic DNA cloning of *CcPRF1*, *CcPRF2* and *CcPRF3* genes

To clone the cDNA sequences of perforin from common carp, three pairs of primers, PRF1-F/PRF1-R, PRF2-F/PRF2-R and PRF3-F/PRF3-R, were designed based on the conserved regions of published perforin cDNA sequences from other fish species. The first-strand cDNA synthesized by spleen-derived RNA served as a template to amplify the corresponding cDNA sequences of *CcPRF1*, *CcPRF2* and *CcPRF3*, and a 405-bp cDNA fragment of *CcPRF1*, a 611-bp of *CcPRF2* and a 1367-bp of *CcPRF3* were obtained. PCR was performed using the following steps: denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, with a final extension step of 72 °C for 10 min. Next, the full-length cDNA sequences of *CcPRF1*, *CcPRF2* and *CcPRF3* were obtained by the rapid amplification of the cDNA ends (RACE) method with a 5'-Full RACE and 3'-Full RACE Core Set Kit (TaKaRa, Japan). The procedure was carried out as described in the user manual, and two rounds of PCR were performed to amplify the 5' and

3' flanking regions. The primers used for cDNA cloning are shown in Additional file 1: Table S1.

To obtain the DNA sequences of three *CcPRF* genes, genomic DNA was purified from the spleen of common carp with the Genomic DNA Kit (Tiangen, China). Based on *CcPRF1*, *CcPRF2* and *CcPRF3* cDNA sequences, three, four and three primer pairs were designed respectively, and then their PCR products were analysed and sequenced. The primers used for DNA cloning are shown in Additional file 2: Table S2.

All PCR products were analysed by 1% agarose electrophoresis, and the anticipated fragments were purified using the PCR purification kit (Tiangen, China). These fragments were then ligated into the pMD18-T vector (TaKaRa, Japan), transformed into competent *Escherichia coli* cells DH-5 $\alpha$  and sequenced by BGI China.

#### **PolyI:C and *A. hydrophila* stimulation in vivo**

The protocols for the immune challenges were carried out as described in previous studies. PolyI:C (Sigma, USA) was suspended in sterile phosphate-buffered saline (PBS) at a final density of 1.6 mg/ml, and twenty-one fish (body mass 80  $\pm$  5 g) were injected intraperitoneally with polyI:C at 500  $\mu$ l per fish [25–27]. *A. hydrophila* was incubated in Luria-Bertani medium at 28 °C overnight under continuous shaking. *A. hydrophila* was inactivated in 0.5% formaldehyde at 4 °C overnight and was subsequently suspended in sterile PBS at a final density of 2.0  $\times$  10<sup>8</sup> CFU/ml. Twenty-one carps whose body weight was 80  $\pm$  5 g were injected intraperitoneally with 500  $\mu$ l of inactivated *A. hydrophila* [28–30].

For the polyI:C-challenged carps, at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 120 h post injection (hpi), three carp individuals were euthanized, and the liver, spleen, head kidney, foregut, hindgut and skin were sampled and kept in liquid nitrogen for total RNA extraction. For *A. hydrophila*-challenged carps, at 3, 6, 12, 24, 72 and 168 hpi, three fish were dissected, and the same tissues as those of polyI:C-stimulated group were selected for analysis. In addition, three un-stimulated fish served as control group.

#### **Isolation and stimulation of peripheral blood leukocytes (PBLs) and head kidney leukocytes (HKLs)**

The method for isolating common carp PBLs was described in previous reports [31]. Heparinized blood samples were taken from the caudal blood vessel and diluted with an equal volume of complete media (incomplete L-15 medium (Gibco, USA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). Then, the resulting diluted blood was placed onto 65% percoll density cushions (Sigma, USA) and centrifuged at 2500 rpm for 30 min at normal temperature. The layer of PBLs was collected and washed three times with

PBS. The method for isolating common carp HKLs was performed based on previously described protocols [32]. Healthy common carp, with a body weight of 80 g, was obtained from the Fresh Water Fishery Research Institute of Shandong Province and raised in a recirculating freshwater system. The tissue head kidney was collected from freshly killed carp under sterile conditions, pressed with a plunger through a 100- $\mu$ m sterile nylon mesh and rinsed with incomplete Leibovitz's L-15 medium (Gibco, USA). Cell isolation was performed using a 34/51% non-continuous Percoll gradient (Sigma, USA). After 30 min of centrifugation at 1500 rpm, the cell layer present in interphase was collected and then washed three times with PBS. Finally, the cells isolated from heparinized blood and head kidney were individually resuspended in the complete media and cultured in 24-well plates at 25 °C.

After incubation overnight, drug treatment of polyI:C and LPS (10  $\mu$ g/ml) (Sigma, USA) was performed in these two groups. At 3 h, 6 h, 12 h and 24 h post stimulation, the cells were harvested for RNA extraction. Additionally, the un-stimulated cells served as control group.

#### **Real-time PCR**

Gene expression analyses of *CcPRF1*, *CcPRF2*, *CcPRF3*, and CD8 $\alpha$  were performed on a LightCycler<sup>®</sup> 96 Real-time PCR System (Roche, Switzerland) using *TransStart<sup>®</sup>* Tip Green qPCR SuperMix (Transgen, China). The program was as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 58 °C for 15 s and 72 °C for 10 s. Finally, melt curve analysis was carried out to verify the presence of one single PCR product at the end of the assay. Each sample was analysed in triplicate, and the 40S ribosomal protein S11 gene served as a house-keeping gene to normalize the mRNA expression. The relative gene expression was determined using the 2<sup>- $\Delta\Delta$ CT</sup> method [33], and the primers used in Real-time PCR are shown in Additional file 3: Table S3.

#### **Bioinformatics and statistical analyses**

The putative open reading frames (ORFs) and protein prediction were analysed with EditSeq within DNASTAR. The protein domains were predicted using the simple modular architecture research tool (SMART) (<http://smart.embl-heidelberg.de/>). Phylogenetic trees were generated with MEGA 6.0 using the neighbor-joining (NJ) method, in which the Jones-Taylor-Thornton (JTT) model was used as an amino acid substitution model. The protein tertiary structures were predicted by SWISS-MODEL (<https://swissmodel.expasy.org/>). GeneBank accession numbers used in phylogenetic and homology analyses are shown in Additional file 4: Table S4 and Additional file 5: Table S5.

The differences in relative gene expression between the stimulated group and control group (0 h) were conducted using two-way analysis of variance (ANOVA) in



Graphpad Prism 5.0 and were considered significant when  $p < 0.05$ .

**Results**

**Characterization of three CcPRF cDNA sequences**

The full-length cDNA sequences of CcPRF1, CcPRF2 and CcPRF3 were 1869 bp, 1971 bp and 1966 bp (Fig. 1a, b, c), and their GenBank accession numbers were MH271082, MH271083 and MH271084, respectively. CcPRF1 comprised a 60-bp 5'-untranslated region (UTR), an ORF of 1767 bp encoding 588 amino acids and a 42-bp 3'-UTR. CcPRF2 comprised a 40-bp 5'-UTR, an ORF of 1677 bp encoding 558 amino acids and a 254-bp 3'-UTR. CcPRF3 was composed of a 66-bp 5'-UTR, an ORF of 1728 bp encoding 575 amino acids and a 172-bp 3'-UTR. Protein domain prediction showed that each CcPRF contained a signal peptide domain, a MACPF domain and a CalB domain (Fig. 1d). The locations of the MACPF domain for CcPRF1, CcPRF2 and CcPRF3 were residues 163–365, 154–354 and 160–

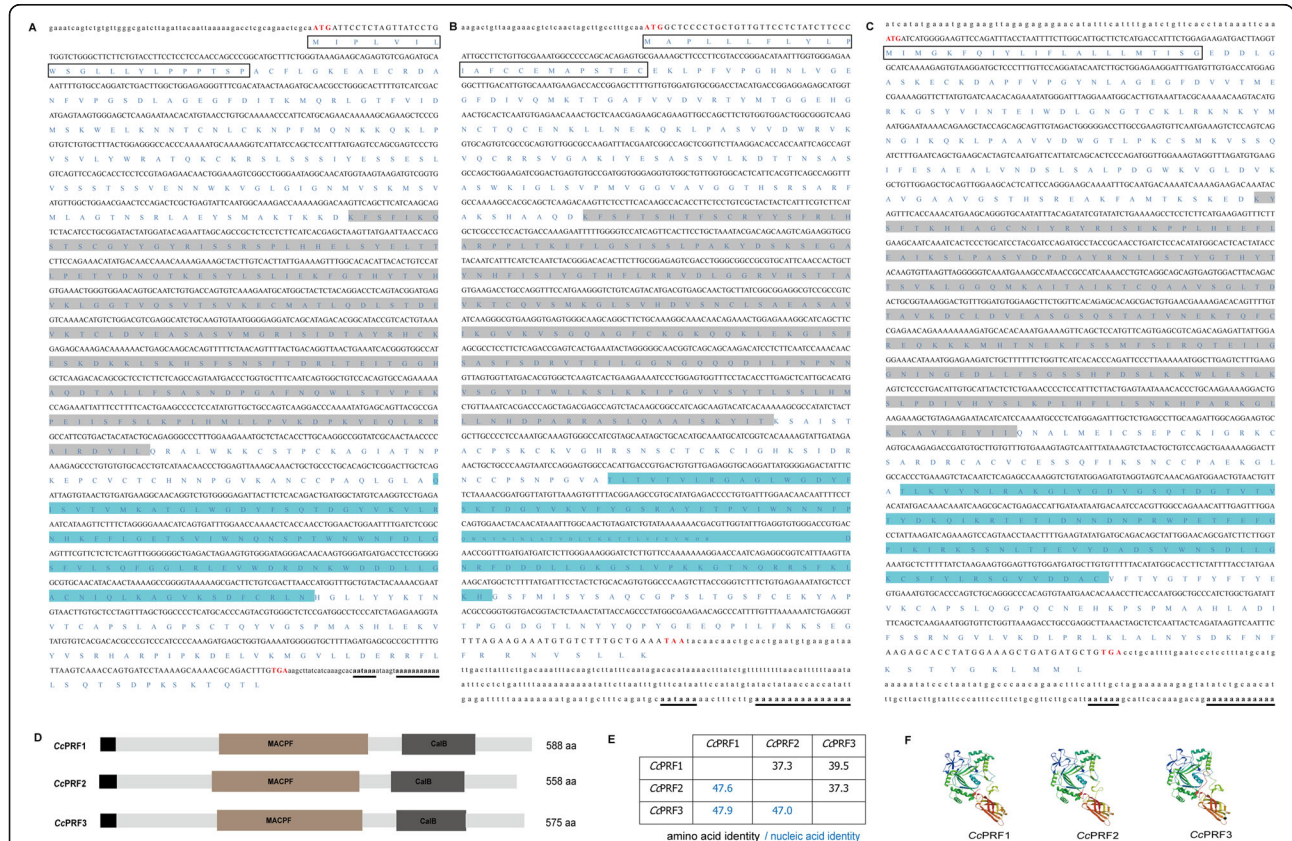
358, respectively, and the CalB domains were located at residues 412–511, 398–497 and 405–499, respectively.

**Genomic structures of three CcPRFs**

CcPRF1 and CcPRF2 genes contained five exons and four introns, which were 3003 bp and 6046 bp, respectively. CcPRF3 DNA was 2761 bp, comprising four exons and three introns. The sizes of exons and introns and their position in DNA were indicated in Fig. 2, and the exon-intron splice junctions followed the AG/GT rule (Table 1). In addition, the specific exon and intron sequences of CcPRF1, CcPRF2 and CcPRF3 are listed in Additional file 6; Additional file 7 and Additional file 8, respectively.

**Phylogenetic and homology analyses**

Phylogenetic analysis of the MACPF family proteins (perforin, C6, C7, C8, C9) and the MACPF-domain containing protein (macrophage-expressed gene 1, Mpeg-1) showed that the three CcPRFs and the perforins of other



**Fig. 1** Molecular characteristic of the three CcPRF genes. **a-c** cDNA and deduced amino acid sequences. CcPRF1, CcPRF2 and CcPRF3 are shown in **a**, **b** and **c**, respectively. The translation start codon ATG and termination codons TGA or TAA are shown in red. The signal peptides are boxed, and the MACPF and CalB domains are highlighted in light gray and blue, respectively. In addition, the polyadenylation signal (aataaa) and poly (a) tail are in bold and underlined. **d** Domain structures of three CcPRF proteins. The signal peptide (black), MACPF (brown) and CalB (darkgray) are depicted in different colors, and the numbers refer to the length of the amino acid sequences. **e** Amino acid (black) and nucleic acid (green) identities among the three CcPRFs. **f** 3-Dimensional protein structures were predicted using the SWISS-MODEL server, with selection of the model based on the best C-score



**Fig. 2** Schematic diagrams of exon-intron arrangement of various vertebrate perforin genes. Exons are shown by boxes, and introns are shown by straight or interrupted lines. The filled boxes show the coding region, while the empty boxes show the untranslated region. The number of nucleotides in each exon and intron is shown above or below the corresponding element. The sequences selected are human (gene ID 5551), mouse (gene ID 18646), rat (gene ID 50669), Japanese flounder (refer to [16]), channel catfish (gene ID 108280710), grass carp (GenBank EF635861), zebrafish (gene ID: 569443, 559849, 103,909,237, 559,384, 100,000,903, 795,573)

species were clustered in one branch, where fish perforins were separated from the cluster of other species, including the mammals, birds and amphibians (Additional file 9: Figure S1). While for common carp, the three CcPRFs belonged to three different sub-branches, in which each CcPRF was phylogenetically closest to the perforins of other cyprinid fish, such as the gimbuna crucian carp, zebrafish and grass carp (Fig. 3).

Homology analysis showed that CcPRF1 shared the highest amino acid identities with crucian carp PRF1 (86.9%), grass carp PRF1 (83%) and zebrafish PRF1.9 (81%), CcPRF2 with gimbuna crucian carp PRF2 (89.8%) and zebrafish PRF1.3 (76.6%), and CcPRF3 with gimbuna crucian carp PRF3 (77.8%) and zebrafish PRF1.2 (71.0%).

The amino acid identities of three CcPRFs with the human, mouse, chicken and green anole perforin proteins ranged from 29.2 to 36.8% (Table 2). Additionally, as shown in Fig. 1e, each CcPRF had less than amino acid 40.0% identities (37.3–39.5%) with the other two isoforms.

**Tissue distribution of three CcPRFs in healthy common carps**

Each CcPRF was expressed in all the nine tested tissues, including the liver, spleen, head kidney, foregut, hindgut, gill, skin, brain and muscle, with different expression levels in various tissues (Fig. 4a). Moreover, in each tissue, the three CcPRFs also showed differential expression profiles (Fig. 4b).

**Table 1** Intron-exon junctions and flanking sequences of the CcPRF1, CcPRF2 and CcPRF3

Exon no.	Position in DNA	Exon size (bp)	Splice donor	Splice acceptor	Intron size (bp)
CcPRF1-1	1-52	52	<b>g</b> taagtgaaa	atctcag <b>cag</b>	443
2	496-1033	538	<b>g</b> taagactta	ctgtctt <b>cag</b>	89
3	1123-1261	139	<b>g</b> taagccaaa	aatctgt <b>tag</b>	83
4	1345-1562	218	<b>g</b> tttgtgctt	tttctaca <b>ag</b>	530
5	2093-3003	911			
CcPRF2-1	1-27	27	<b>g</b> taaatatca	tctcttt <b>cag</b>	1127
2	1155-1670	516	<b>g</b> taagtcttg	ctttcat <b>cag</b>	2475
3	4146-4416	271	<b>g</b> tgagtgggc	gggcgtg <b>ag</b>	391
4	4808-5406	599	<b>g</b> taagtgggt	cattttac <b>ag</b>	97
5	5504-6046	543			
CcPRF3-1	1-69	69	<b>g</b> taagaaact	ttcactac <b>ag</b>	603
2	673-838	166	<b>g</b> ttcttatgt	gagcgaaa <b>ag</b>	85
3	924-1275	352	<b>g</b> ttattttc	ttctttat <b>ag</b>	119
4	1395-2761	1367			

Bold text indicates the invariant nucleotides of the exon-intron boundaries

Specifically, the highest expression of CcPRF1 mRNA was detected in the spleen, hindgut, gill, skin and brain, followed by the head kidney and muscle, and very low expression in the foregut and liver. CcPRF2 was mainly distributed in the skin, brain and muscle, with a moderate level of expression observed in the spleen, foregut, hindgut and gill, while low gene expression in the head kidney and liver. For CcPRF3, the mRNA expression was predominantly detected in the hindgut, spleen, head kidney, gill and muscle, followed by the brain and skin, and the lowest level in the foregut and liver.

Overall, in healthy carps, gene expression of CcPRF2 predominated in most of the tested tissues, including the muscle (94.1%), skin (93.1%), brain (89.6%), foregut (88.6%), liver (66.7%), hindgut (48.3%) and head kidney (46.9%). In the spleen and gill, CcPRF1 mRNA levels was the highest. In contrast, CcPRF3 mRNA expression was obviously lower than CcPRF1 and CcPRF2, with the proportion of 23.7%, 13.6%, 5.4%, 4.9%, 3.0%, 1.8%, 0.5% and 0.6% in the head kidney, hindgut, spleen, foregut, gill, muscle, skin and brain, respectively. Moreover, in the nine tested tissues, the brain and skin showed the highest cumulative expression levels of the three CcPRFs.

#### Ontogeny of three CcPRF genes in common carp larvae

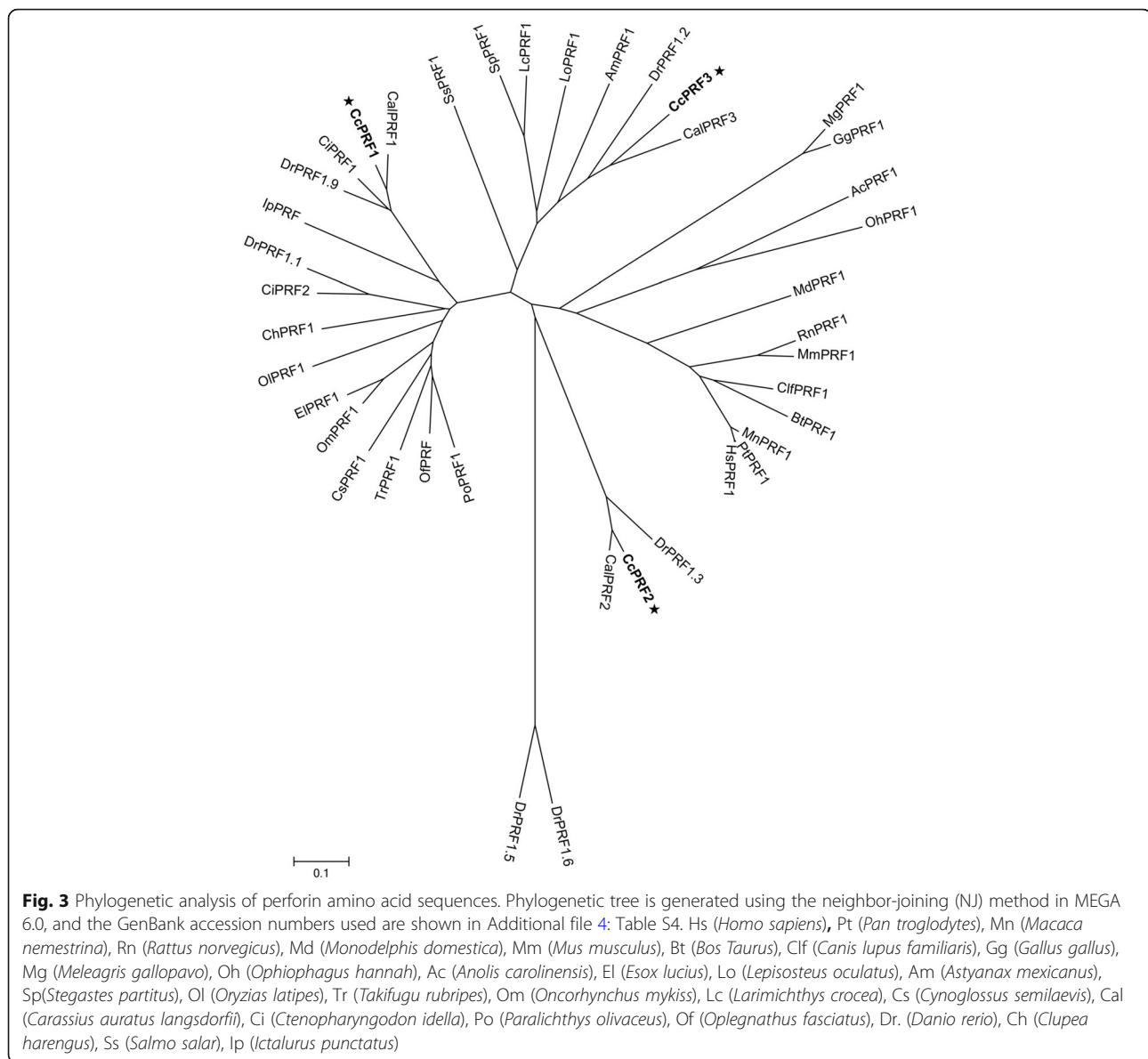
As shown in Fig. 5, we analysed gene expression patterns of the three CcPRFs from blastula stage to 22 dph. The results showed that CcPRF1 had two expression peaks, with the first at 4 dph and the second at 22 dph. As for CcPRF2, after fertilization, its gene expression was up-regulated at embryonic stage and continued to

increase up to 22 dph. While for CcPRF3, it displayed one expression peak at 22 dph. Obviously, the three CcPRFs displayed differential expression patterns in larvae ontogeny, while with a common expression peak at 22 dph.

#### Expression profiles of three CcPRFs upon polyI:C and A. hydrophila in vivo

After polyI:C stimulation, CcPRF1 gene expression was detected highest at 2 dpi in the liver, spleen, head kidney, foregut and hindgut, with 3.7-fold, 5.4-fold, 1.5-fold, 3.2-fold and 8.3-fold higher expression than the control group, respectively (Fig. 6a,  $p < 0.05$  or  $p < 0.01$ ). CcPRF2 mRNA level reached peak values at 2 dpi in the liver, spleen and hindgut, with 2.1-fold, 2.2-fold and 5.8-fold up-regulation, respectively, as compared with the control group (Fig. 6b,  $p < 0.05$  or  $p < 0.01$ ). In the head kidney, mRNA expression of CcPRF2 began to increase at 2 dpi and reached a peak at 5 dpi (2.9-fold,  $p < 0.05$ ). With regard to CcPRF3, the mRNA expression of CcPRF3 never increased significantly in the analysed tissues, with the exception of the skin (7.5-fold,  $p < 0.05$ ) and head kidney (2.0-fold,  $p < 0.05$ ) (Fig. 6c). Additionally, in the skin, CcPRF1 and CcPRF2 mRNA levels decreased significantly within 12hpi, and soon afterwards return to the basic expression level (Fig. 6a, b).

After *A. hydrophila* stimulation, mRNA level of each CcPRF was significantly up-regulated in all the analysed tissues, with large fold-change higher expression than that in the control (Fig. 7a, b, c,  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.005$ ). Moreover, similar to polyI:C-stimulated group, the three CcPRFs showed different expression patterns in *A. hydrophila*-stimulated group.



**Fig. 3** Phylogenetic analysis of perforin amino acid sequences. Phylogenetic tree is generated using the neighbor-joining (NJ) method in MEGA 6.0, and the GenBank accession numbers used are shown in Additional file 4: Table S4. Hs (*Homo sapiens*), Pt (*Pan troglodytes*), Mn (*Macaca nemestrina*), Rn (*Rattus norvegicus*), Md (*Monodelphis domestica*), Mm (*Mus musculus*), Bt (*Bos Taurus*), Clf (*Canis lupus familiaris*), Gg (*Gallus gallus*), Mg (*Meleagris gallopavo*), Oh (*Ophiophagus hannah*), Ac (*Anolis carolinensis*), El (*Esox lucius*), Lo (*Lepisosteus oculatus*), Am (*Astyanax mexicanus*), Sp (*Stegastes partitus*), Ol (*Oryzias latipes*), Tr (*Takifugu rubripes*), Om (*Oncorhynchus mykiss*), Lc (*Larimichthys crocea*), Cs (*Cynoglossus semilaevis*), Cal (*Carassius auratus langsdorffii*), Ci (*Ctenopharyngodon idella*), Po (*Paralichthys olivaceus*), Of (*Oplegnathus fasciatus*), Dr (*Danio rerio*), Ch (*Clupea harengus*), Ss (*Salmo salar*), Ip (*Ictalurus punctatus*)

### Expression profiles of CcPRFs and CD8 $\alpha$ in response to polyI:C and LPS in vitro

To further identify the immunologic function of perforin in common carp, we assayed gene expression of three CcPRFs and CD8 $\alpha$  upon polyI:C and LPS in PBLs and HKLs.

In PBLs, after polyI:C stimulation, mRNA expression of CcPRF1, CcPRF2 and CcPRF3 reached peak values at 24 hpi, 12 hpi and 6 hpi, respectively, with 2.7-fold, 1.5-fold and 1.4-fold up-regulation, respectively, as compared with the control group (Fig. 8a,  $p < 0.05$  or  $p < 0.01$ ). In response to LPS, CcPRF1 mRNA level reached the highest level at 24 hpi with 1.7-fold higher expression than the control group (Fig. 8c,  $p < 0.001$ ). Gene expression of CcPRF2 and CcPRF3 was up-regulated to the highest level at 6 hpi, with a 3.7-fold and 25.2-fold higher expression than that of the

control group, respectively (Fig. 8c,  $p < 0.01$  or  $p < 0.005$ ). In HKLs, gene expression of CcPRF1, CcPRF2 and CcPRF3 was up-regulated by polyI:C and increased to peak values at 24 hpi, with 3.8-fold, 5.5-fold and 3.0-fold higher expression than the control (Fig. 8b,  $p < 0.01$  or  $p < 0.005$ ). Upon LPS stimulation, CcPRF1, CcPRF2 and CcPRF3 mRNA expression reached the highest level at 24 hpi as well, with 11.0-fold, 18.4-fold and 5.8-fold higher expression than the control group (Fig. 8d,  $p < 0.005$ ).

As for CD8 $\alpha$ , in PBLs, polyI:C induced the gene expression of CD8 $\alpha$ , with 1.5-fold (6 hpi) higher expression than the control group (Fig. 9a,  $p < 0.05$ ). In response to LPS, CD8 $\alpha$  mRNA expression was increased significantly, with 12.8-fold (12 hpi) higher expression than the control group (Fig. 9c,  $p < 0.01$ ). While in HKLs,

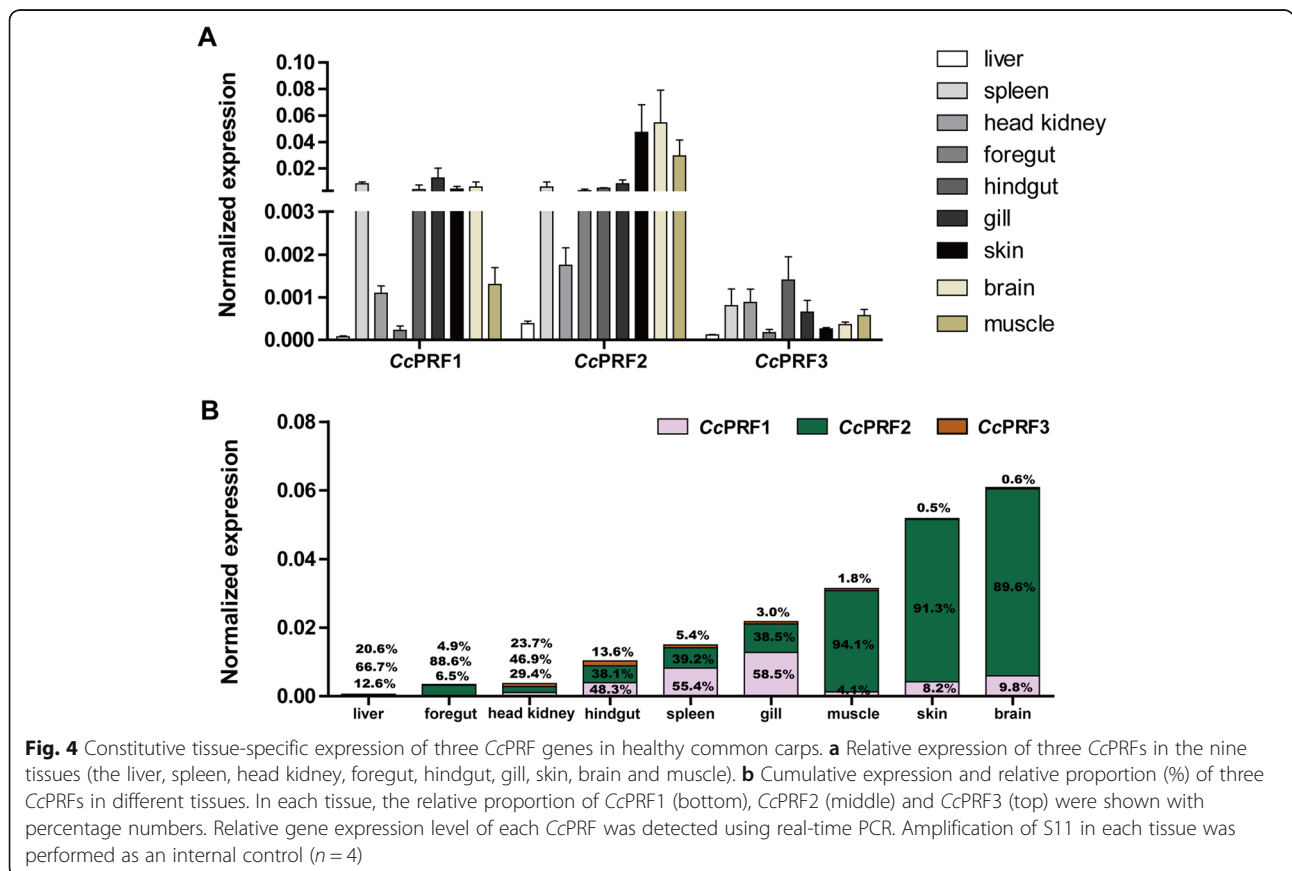
**Table 2** Percent identity of perforin proteins between common carp and other species

Species	Name	CcPRF1	CcPRF2	CcPRF3
Human	PRF1	36.1	36.7	36.2
House mouse	PRF1	34.7	36.3	36.6
Chicken	PRF1	28.9	31.7	30.8
Green anole	PRF1	33.5	33.2	32.1
Fugu rubripes	PRF1	49.3	36.6	38.2
Japanese flounder	PRF1	51.0	37.8	38.0
Atlantic salmon	PRF1	41.1	36.3	43.3
Zebrafish	PRF1.9	81.0	39.0	39.5
	PRF1.2	38.0	37.1	71.0
	PRF1.3	36.2	76.6	36.7
Ginbuna crucian carp	PRF1	86.9	36.2	37.6
	PRF2	36.0	89.8	36.5
	PRF3	37.5	37.6	77.8
Grass carp	PRF1	83.0	37.9	38.4
	PRF2	53.7	35.6	38.9

upon polyI:C challenge, the gene expression of CD8 $\alpha$  reached the highest level at 24 hpi, in which time point the expression level was 28.6-fold higher than the control (Fig. 9b,  $p < 0.05$ ). In response to LPS, CD8 $\alpha$  mRNA levels reached the highest at 6 hpi, with 9.7-fold higher expression than the control (Fig. 9d,  $p < 0.05$ ).

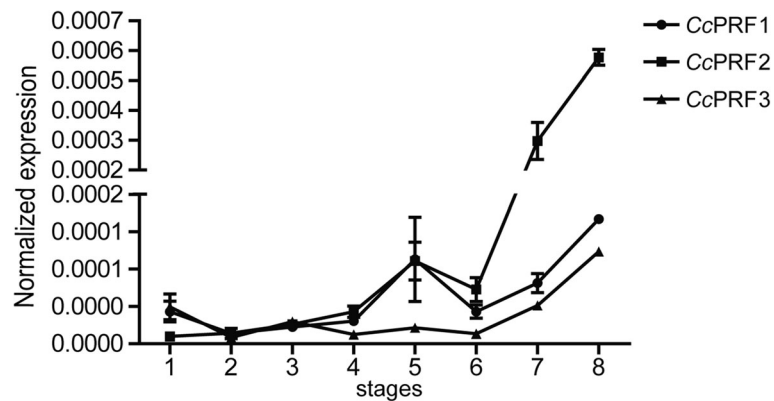
**Discussion**

In the present study, we cloned and confirmed three perforin isoform genes from common carp, named CcPRF1, CcPRF2 and CcPRF3. The three CcPRFs were quite different with each other, with nucleic acid identities of ORF ranged from 47.0 to 47.9% and amino acid identities from 37.3 to 39.5% (Fig. 1e). Nevertheless, in the phylogenetic tree of perforin, C6, C7, C8, C9 and Mpeg-1, the same evolutionary branch of CcPRFs with other species' perforins strongly implied that the three CcPRFs all belong to the perforin superfamily (Additional file 9: Figure S1). The three CcPRF proteins were all comprised of a signal peptide, a middle MACPF domain and a C-terminal CalB domain, exhibiting the same domain architecture as mammalian orthologs [34, 35] although the three CcPRFs only shared 34.7–36.7% amino acid identities with human and mouse perforins



**Fig. 4** Constitutive tissue-specific expression of three CcPRF genes in healthy common carps. **a** Relative expression of three CcPRFs in the nine tissues (the liver, spleen, head kidney, foregut, hindgut, gill, skin, brain and muscle). **b** Cumulative expression and relative proportion (%) of three CcPRFs in different tissues. In each tissue, the relative proportion of CcPRF1 (bottom), CcPRF2 (middle) and CcPRF3 (top) were shown with percentage numbers. Relative gene expression level of each CcPRF was detected using real-time PCR. Amplification of S11 in each tissue was performed as an internal control ( $n = 4$ )





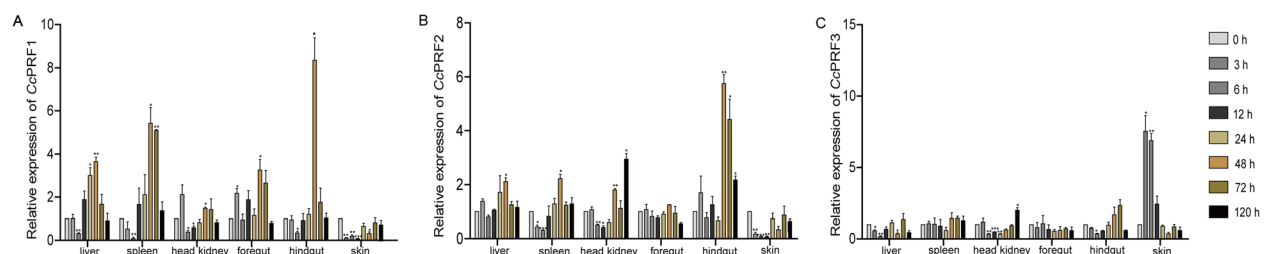
**Fig. 5** The mRNA expression patterns of three CcPRF genes during larval ontogeny. Each CcPRF is shown with a distinct line graph. Abscissa values (1–8) are indicated as 8 developmental stages with (1) blastula stage; (2) optic primordium appearance; (3) 1 dph; (4) 2 dph; (5) 4 dph; (6) 8 dph; (7) 14 dph; (8) 22 dph. The relative expression of each CcPRF is normalized to S11 gene and displayed as the means  $\pm$  SD ( $n = 5$ )

(Table 2). Thus, the consistency of CcPRF protein domains with the typical structure of perforin protein implied that CcPRFs might play the similar role as do the other species' perforins [36].

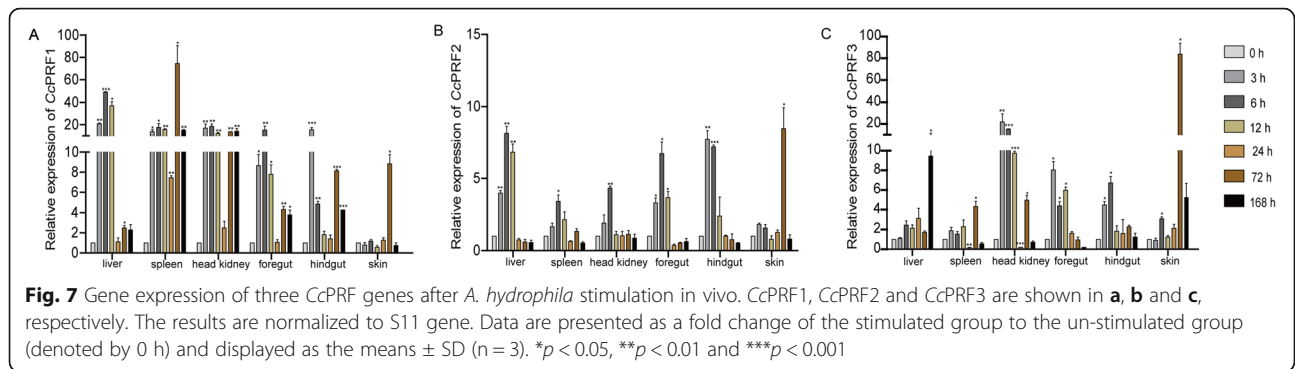
The intron-exon arrangement of perforin gene in humans, mice and rats was conserved, in which they were all comprised of three exons and two introns, and the sizes of the exons located in their ORFs were 536/539 and 1129 bp (Fig. 2). However, these characteristics were quite different from that of the three CcPRF genes and other fish perforins, which was in line with the previous suggestion of different genomic structures of the perforin between teleosts and mammals [16]. On the other hand, for CcPRF1, its intron-exon arrangement was similar to that of zebrafish PRF1.9 and grass carp, channel catfish and Japanese flounder PRF1, particularly showing more similarities with grass carp, channel catfish and zebrafish, with the same exon sizes (530, 139, 218, 880), than Japanese flounder. CcPRF2 genomic DNA comprised five exons and four introns, and the sizes of the exons located in its ORF were 503, 271, 599 and 304. Similarly, for zebrafish PRF1.3 gene, the size of the first exon located in its ORF was 503 bp as well. And for CcPRF3, the size of the last exon located in

ORF was 1207 bp, which was the same as that of zebrafish PRF1.2 gene. Notably, all these similarities were in line with the phylogenetic relationship of perforin in fish species (Fig. 3). Moreover, although intron/exon arrangement of zebrafish PRF1.5 and PRF1.6 was largely different from that of the three CcPRFs, the exons of these two zebrafish perforins had highly similarities with each other, which was in accordance with their very close evolutionary relationship. Thus, taken together all these results implying that intron-exon organization of perforin gene was in accordance with the evolutionary relationship and species evolution.

Similar to the previous report that under basal conditions 6 zebrafish perforin genes were all expressed in the liver, spleen, kidney, intestine, gill and muscle [22]. Each CcPRF gene was expressed in the nine analysed tissues (the liver, spleen, head kidney, foregut, hindgut, gill, skin, brain and muscle) (Fig. 4). However, it was reported that perforin gene was not able to be detected in the liver, stomach, brain, muscle, fin and eye of Japanese flounder [15, 16] and the liver of trout [18]. Although the tissue distribution pattern of perforin was inconsistent in fish species, its gene expression was always detected in the pivotal immune-related tissues (the head kidney, spleen,



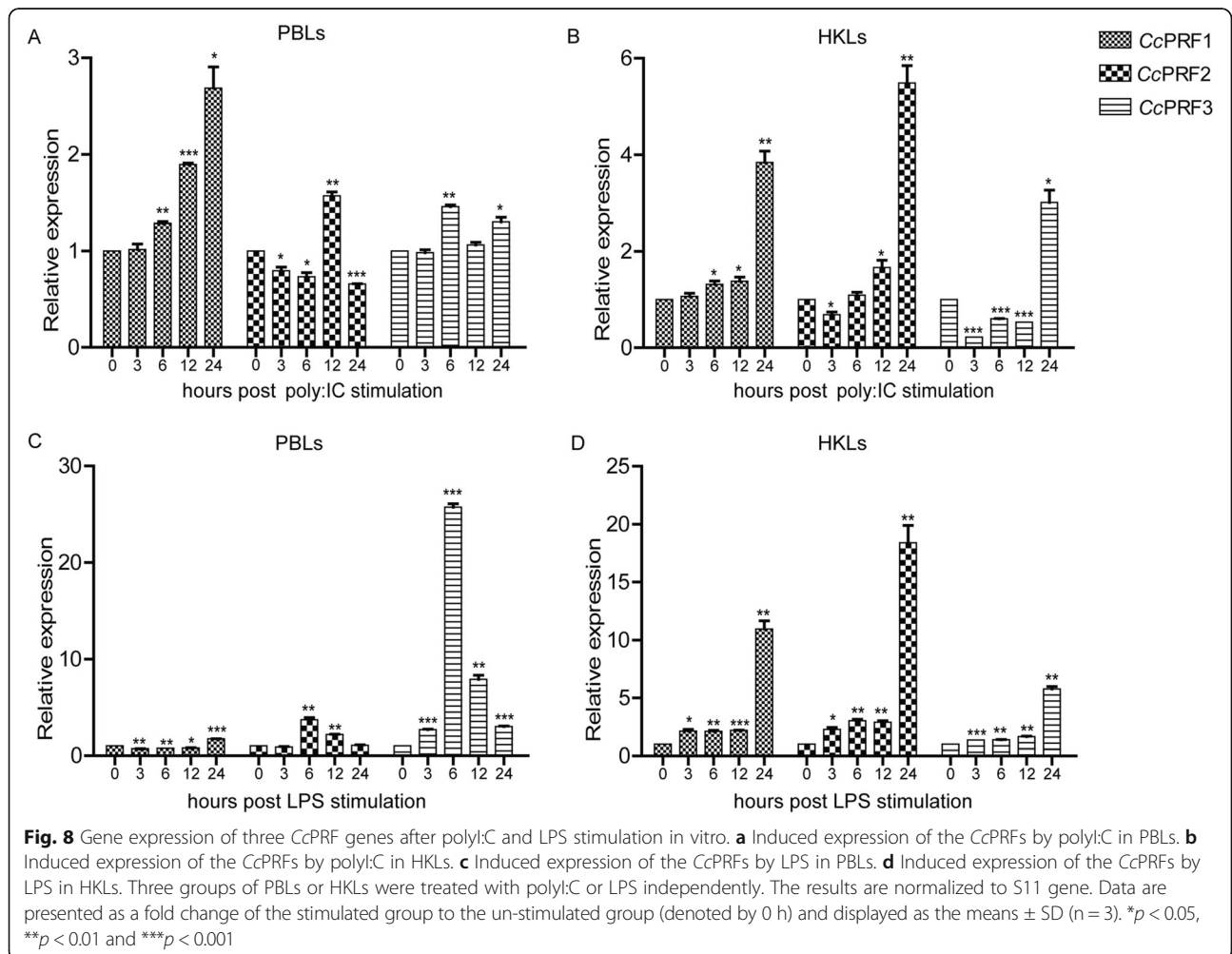
**Fig. 6** Gene expression of three CcPRF genes after poly:I:C stimulation in vivo. CcPRF1, CcPRF2 and CcPRF3 are shown in a, b and c, respectively. The results are normalized to S11 gene. Data are presented as a fold change of the stimulated group to the un-stimulated group (denoted by 0 h) and displayed as the means  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

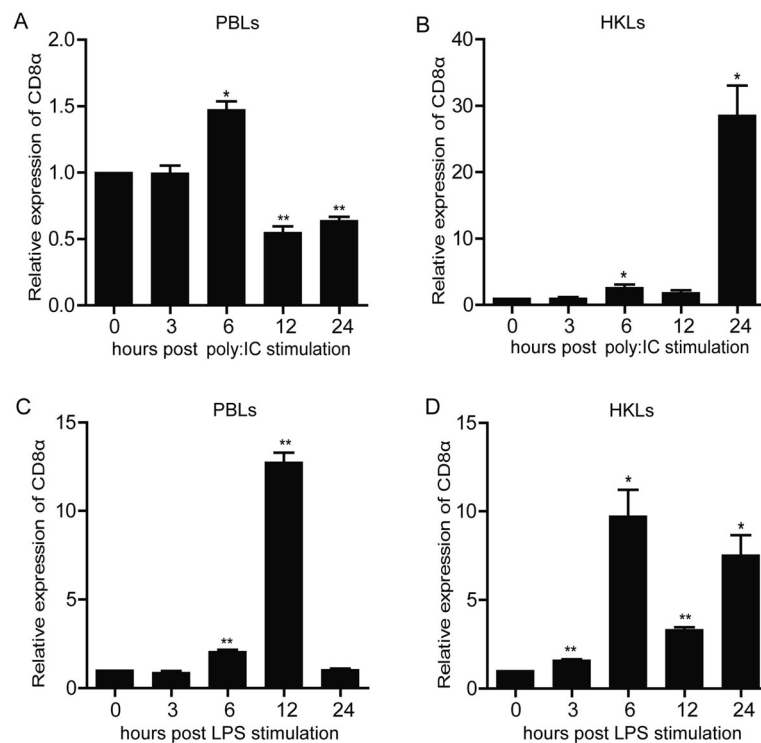


intestine, gill and skin), at least implying the potential role of fish perforin in the immune system.

Previously, it was shown that the innate immune system is a fundamental defense weapon of fish during early stages of development [37–39]. In the common carp, it was reported that innate immune factors such as C3 and  $\alpha 2$ -macroglobulin were present before hatching and these factors were involved in the immune defense

response during ontogeny of carp [38, 40]. Additionally, in the zebrafish, it was demonstrated that immune system of the larvae was completely mature between 4 and 6 weeks post fertilization [41]. In accordance with this time period, during the larvae ontogeny of zebrafish, expression peaks were observed around the fourth week of development (23 dpf and 29 dpf) in several perforin genes [22]. Similarly, in the early development stages of





**Fig. 9** Gene expression of CD8 $\alpha$  after polyI:C and LPS stimulation in vitro. **a** Induced expression of CD8 $\alpha$  by polyI:C in PBLs. **b** Induced expression of CD8 $\alpha$  by polyI:C in HKLs. **c** Induced expression of CD8 $\alpha$  by LPS in PBLs. **d** Induced expression of CD8 $\alpha$  by LPS in HKLs. Three groups of PBLs were treated with polyI:C or LPS independently. The results are normalized to S11 gene. Data are presented as a fold change of the stimulated group to the un-stimulated group (denoted by 0 h) and displayed as the means  $\pm$  SD ( $n=3$ ). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

common carp larvae, in spite of differential expression patterns for the three *CcPRFs*, they showed a common expression peak at 22 dph (Fig. 5). Moreover, in the larval development of Japanese flounder, perforin gene was significantly expressed during nephron formation [15]. Thus, all the results indicated that together with other immune-related factors, fish perforin may play a crucial role in the larval immune system.

PolyI:C is a mimetic of virus-derived double-stranded RNA, and is usually designed to mimic viral infections [42, 43]. In mammals, polyI:C or virus was able to induce the expression of perforin in cytotoxic cells. In the mouse hepatic and splenic NK cells [44, 45] and human NK cells isolated from peripheral blood [46], stimulation with polyI:C increased gene expression or protein secretion of the perforin. Using perforin-deficient mice, it was demonstrated that CD8<sup>+</sup> T cell-mediated protection against viral infection, such as Ebola virus, West Nile virus and influenza virus, was dependent on perforin [47–49]. In addition, in fish, some studies have reported gene expression of perforin in response to viral stimulation. In the kidney of rock bream injected with rock bream iridovirus (RBIV) or megalocytivirus, significant up-regulation of perforin gene expression was observed at 4 or 8 dpi [20, 21]. In the rainbow trout RTS11 cell

line, infection with viral hemorrhagic septicaemia virus (VHSV) up-regulated perforin gene expression [50]. In the zebrafish kidney, in vivo stimulation with spring viremia of carp virus (SVCV) increased mRNA levels of multiple perforins including PRF1.1, 1.3, 1.5, 1.6 and 1.9 at 6 hpi, and polyI:C induced PRF1.6 expression at 3 hpi [22]. Similarly, in common carp, mRNA levels of the three *CcPRFs* were significantly up-regulated in response to polyI:C in vivo and in vitro (Figs. 6 and 8a, b). However, in contrast to SVCV, polyI:C was not able to increase mRNA levels of zebrafish perforins within 24 hpi, with the exception of PRF1.6, in the kidney [22], which was consistent with the in vivo results in common carp. Additionally, rock bream perforin gene was not induced by virus shortly post infection, until a few days later [20, 21]. Moreover, unlike trout RTS11, in the trout HKLs, no significant up-regulation of perforin expression was observed after incubation for 24 h with VHSV [50]. These data suggested that perforin expression was induced by viral stimulation possibly in a time- and cell population-dependent manner, which may partly explain why in common carp the induction of perforin by polyI:C had the relative tissue-specificity. Nevertheless, in PBLs and HKLs, three *CcPRF* mRNAs were all up-regulated by polyI:C (Fig. 8a, b). Collectively, all these

data implied that perforin might play significant roles in the protective host immune response of common carp to virus.

To date only few studies investigated the modulation of perforin gene expression in response to bacterial pathogens, especially the extracellular bacteria. In human NK cells, extracellular mycobacteria induced mRNA and protein production of the perforin, which eliminated the bacteria by damaging cell wall structures [3]. In the fish rohu, stimulation with Gram-negative bacterium *A. hydrophila* significantly up-regulated expression of perforin gene, particularly in the spleen and gill tissues [51]. Additionally, in the sponge, a cDNA clone resembling human perforin displayed strong antibacterial activity [52]. Here, in common carp, after stimulation of *A. hydrophila*, mRNA levels of the three *CcPRFs* was remarkably enhanced in a variety of immune-related tissues (Fig. 7). Meanwhile, in vitro, stimulation with LPS also increased gene expression of the carp perforins (Fig. 8c, d). Similarly, in the zebrafish adult kidney, significant up-expression of PRF1.1, 1.2, 1.6 and 1.9 was observed over the time course post injection with LPS [22]. LPS is a major component shared in the outer membrane of Gram-negative bacteria, and previous reports showed that LPS was the main virulence factor responsible for the dominant response to Gram-negative bacteria infection [53, 54]. So, based on these data indicating possible relevance of LPS in *A. hydrophila*-induced up-regulation of *CcPRFs*. Taken together, all these results suggested potential involvement of perforin in the immune defense of common carp against bacteria.

In mammals, polyI:C or virus was capable of augmenting antigen-specific responses in CD8<sup>+</sup> T cells, with inducing perforin expression [55–58]. Moreover, in fish species, a recruitment of CD8 $\alpha$ <sup>+</sup> cells was found in the liver of rainbow trout in response to VHSV, meantime with an up-regulation of CD8 $\alpha$  and perforin genes observed [59]. Similarly, in rock bream, upon RBIV infection, CD8 $\alpha$  gene expression was increased, accompanied by the induction of perforin at the same time point [21]. As for LPS, via an indirect pathway involving antigen presenting cells (APCs), it is able to promote proliferation and activation of murine CD8<sup>+</sup> T cells [60, 61]. Additionally, in humans, LPS could directly activate naive CD8<sup>+</sup> T cells via TLR4 on their cell membranes, and afterwards these activated cells secreted perforin in response to LPS [2]. In line with these findings, in common carp, gene expression of CD8 $\alpha$  (a main marker of teleost CTLs [62]) was increased by polyI:C and LPS in vitro, coincident with the induced expression pattern of perforin. So, based on all these observations, it was inferred that, in common carp, polyI:C and LPS may induce immune activation of CD8<sup>+</sup> T cells, after which

this type of cells may, at least in part, contribute to the production of perforin in response to immune stimulation.

Besides, it was worth noting that tissue distribution pattern was different for each *CcPRF* isoform, where *CcPRF2* showed broad expression across various tissues, and the other two were more tissue-specific. Additionally, differential expression dynamics were also detected during the larvae ontogeny of common carp (Fig. 5) and in response to immune stimulation (Figs. 6 and 7). As previously reported, the similar phenomenon has been observed in other fish species. In the zebrafish, 6 identified perforin isoforms had diverse constitutive expression patterns in healthy organisms and in larvae ontogeny. And as mentioned above, in the zebrafish kidney, 6 perforins displayed differential response dynamics upon LPS, polyI:C and SVCV [22]. Moreover, in crucian carp CTLs from allo-sensitized fish kidneys, PRF1 mRNA was increased, while PRF2 and PRF3 not changed significantly [19]. These observations raise an interesting question of why fish possess multiple perforin isoforms with different expression dynamics. One explanation is that fish perforins have broader cell distribution and roles or more complex regulatory patterns than in mammals. For instance, perforin is essentially restricted to NK cells and CTLs in mammals, while in fish perforin may be present in more types of immune cells with each isoform being restricted to a particular type. Alternatively, all perforin isoforms may be present in the same cell type, with each responding to distinct signals. Actually, in one previous study, it has been found that multiple isoforms of fish perforins were present at distant loci [63], implying that these perforin genes were likely to be controlled by different regulatory elements. Thus, these facts implied possible different cell locations or functional differences for multiple fish perforin genes, which probably evolved as a strategy to expand immune recognition capabilities in fish and compensate for their limitations of adaptive immunity [64].

## Conclusions

In the present study, three perforin isoform genes, named *CcPRF1*, *CcPRF2* and *CcPRF3*, were identified and investigated for the first time in common carp. Evolution analysis shows that *CcPRF* genes have closer evolutionary relationship with other fish perforins than those of mammals, in regard to both amino acid identities and genomic organization. The constitutive expressions of three *CcPRFs* in early life stages of development imply possible relevance of perforin to the immune system of common carp larvae. Moreover, the significant up-regulation of multiple *CcPRF* genes indicate that perforin might play significant roles in the immune defense



of common carp against viral and bacterial pathogens. Meanwhile, the differential expression dynamics were observed across three CcPRF genes, implying possible different cellular locations or functional differences for various perforin isoforms. These observations could provide some clues for preventing carp infection by pathogenic microorganisms present in the aquatic environment.

## Additional files

**Additional file 1: Table S1.** Primers used for cDNA cloning. (DOCX 15 kb)

**Additional file 2: Table S2.** Primers used for genomic DNA cloning. (DOCX 14 kb)

**Additional file 3: Table S3.** Primers used for Real-time PCR. (DOCX 14 kb)

**Additional file 4: Table S4.** GenBank accession numbers for perforin proteins. (DOCX 17 kb)

**Additional file 5: Table S5.** GenBank accession numbers of Mpeg-1, C6, C7, C8 and C9 proteins. (DOCX 15 kb)

**Additional file 6:** Intron and exon sequences of CcPRF1. (DOCX 13 kb)

**Additional file 7:** Intron and exon sequences of CcPRF2. (DOCX 14 kb)

**Additional file 8:** Intron and exon sequences of CcPRF3. (DOCX 13 kb)

**Additional file 9: Figure S1.** Phylogenetic analysis of perforin, Mpeg-1, C6, C7, C8 and C9 polypeptides. This tree is generated using the neighbor-joining (NJ) method in MEGA 6.0. GenBank accession numbers used are shown in Additional file 4: Tables S4 and Additional file 5: Table S5, and the species abbreviations in Fig. 3. (TIF 29791 kb)

**Additional file 10:** ARRIVE (Animal Research: Reporting of in Vivo Experiments) checklist.

## Abbreviations

*A. hydrophila*: *Aeromonas hydrophila*; ANOVA: analysis of variance; APCs: antigen presenting cells; CalB: calcium-binding; CTLs: cytotoxic T lymphocytes; dpf: days post fertilization; dph: days post hatching; EGF: epidermal growth factor; FCS: fetal calf serum; Gzm: granzyme; HKLs: head kidney leukocytes; hpi: hours post injection; JTT: Jones-Taylor-Thornton; LPS: lipopolysaccharides; MACPF: membrane-attack-complex/perforin; Mpeg-1: macrophage-expressed gene 1; NK: natural killer; ORF: open reading frame; PBLs: peripheral blood leukocytes; PBS: phosphate-buffered saline; PolyI:C: polyinosinic-polycytidylic acid; RACE: rapid amplification of the cDNA ends; RBIV: rock bream iridovirus; SMART: simple modular architecture research tool; SVCV: spring viremia of carp virus; UTR: untranslated region; VHSV: viral hemorrhagic septicaemia virus

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## Availability of data and materials

The dataset supporting the conclusions of this article is available in the GenBank and the accession numbers are MH271082, MH271083 and MH271084.

## Authors' contributions

TL and HL participated in the design of the study, performed the experiments, collected data and drafted the manuscript. LW and YHZ participated in the cloning studies. XYG and XZC helped with the animal experiments. FMZ and GWY performed the statistical analysis. WJW conceived the study and participated in its coordination. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSDNA2017005). Additionally, the ARRIVE (Animal Research: Reporting of in Vivo Experiments) checklist has been displayed in Additional file 10.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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