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Cloning and expressions of *chop* in loach (*Misgurnus anguillicaudatus*) and its response to hydrogen peroxide (H_2O_2) stress

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Abstract C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (*chop*) which plays an important role in endoplasmic reticulum stress-induced apoptosis was investigated here by RACE and qPCR in an aquaculture animal for the first time. The full-length cDNA sequence of loach (*Misgurnus anguillicaudatus*) *chop* was 2533 bp, encoding 266 amino acids. The expression level of loach *chop* changed during different early life stages, with the highest expression at the 8-cell stage. Among different tissues, loach *chop* predominantly was expressed in gill, spleen, and gonad. We performed a hydrogen peroxide (H_2O_2, a common-used disinfectant) stress trial to explore the role of loach *chop*, with three different concentrations (0 μM, 50 μM, and 100 μM) of H_2O_2. The 100-μM dose was lethal for half the population but the other concentrations did not result in mortality. The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) in loach gill, liver, and spleen decreased with extended stress time and increased H_2O_2 concentration. The expression levels of gill *chop* in loaches from the 100-μM group were significantly higher than those from the other two treatment groups at 12 and 24 h of exposure. *atf4* and *bax*, two proapoptotic genes, were significantly upregulated in gills of loaches from the 100-μM group compared to the other two groups 18 h and 24 h after treatment. *bcl2*, an antiapoptotic gene, presented an opposite trend. These results indicated a close relationship between H_2O_2 stress and fish apoptosis with loach *chop* playing an important role in H_2O_2 stress response.

Keywords *Misgurnus anguillicaudatus* · *Chop* · Gene cloning · Spatiotemporal expressions · Hydrogen peroxide stress

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Introduction

C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (*chop*), also known as the growth arrest and

DNA damage-inducible gene 153 (GADD153), was considered a master proapoptotic molecule of endoplasmic reticulum (ER) stress-induced apoptosis (Li et al. 2014). Although ubiquitously expressed at very low levels under normal conditions (Ariyama et al. 2008), *chop* was a highly stress-inducible expressed gene, markedly following the ER stress (Zhang et al. 1999; Cheng et al. 2009; Pirot et al. 2007). Cells responded to ER stress by activating a series of signaling pathways, collectively termed the unfolded protein response (UPR) pathway (Walter and Ron 2011). There were three branches of the UPR pathway: inositol requiring enzym1 (IRE1), prospective evaluation of radial keratotomy (PERK), and activating transcription factor 6 (ATF6). All triggered apoptosis when the ER stress was severe, prolonged, or chronic (Zhang et al. 2016; Deegan et al. 2015; Shang and Lehrman 2004). The apoptotic program was activated by the elevated expression of many kinds of transcription factors including *chop*, X-box binding protein 1 (XBP1), specificity protein 1 (SP1), and SP2 (Rutkowski et al. 2006).

chop was mainly expressed in the PERK pathway. When the organisms were exposed to the ER stress, the expression level of *chop* was significantly increased (Cao et al. 2012). PERK could upregulate the translations of transcription factor-4 (*atf4*) (B'chir et al. 2013), by phosphorylating its substrate eIF2α. *atf4* was a member of C/EBP family, and it elevated expression of its key downstream target gene *chop* when stress cannot be alleviated, resulting in the onset of apoptosis (Su and Kilberg 2008). In the PERK branch, *chop* was suggested to facilitate apoptosis by repressing the transcriptional expression of the antiapoptotic gene B-cell lymphoma-2 (*bcl2*) in response to ER stress (McCullough et al. 2001). Another widely accepted mechanism of *chop*-induced apoptosis was the induced overexpression of proapoptotic gene BCL2-associated X (*bax*), which may function as an executioner in ER stress-mediated apoptosis (Zou et al. 2012). *chop*-mediated apoptosis enhanced by ER stress played a significant role in the pathophysiology of many mammalian diseases (Gopalan et al. 2013; Oyadomari et al. 2002; Lindholm et al. 2006; Thorp et al. 2009; Gotoh et al. 2010; Lee et al. 2012), affecting the survival of organisms. So far, many studies on functions of *chop* had been carried out in mammals (Nishimiya et al. 2012; Pereira et al. 2006). However, no information on the cloning and roles of *chop* in cultured aquatic species was available.

The water environment changes were easy to cause ER stress responses in aquaculture animals (Chen et al. 2015; Zhu et al. 2013; Ji 2014), which had many adverse effects (Cripps and Bergheim 2000), including poor growth, great incidence of disease, increased mortality, and low production (Barton and Iwama 1991). At present, due to the impact of the 2019-nCoV epidemic, hydrogen peroxide (H_2O_2) was widely used as an environmental disinfectant (Kirst and Juergenlomann 2000). To understand the impact of H_2O_2 on aquaculture animals, loaches (*Misgurnus anguillicaudatus*) were used in this study. The loach, belonging to the family Cobitidae, was one of the most commercially cultured species in several Eastern Asian countries including Korea, Japan, and China (Okada et al. 2020). In this study, we first cloned the *chop* gene in loaches, and then monitored its expression profiles in different tissues and early life stages. In addition, a H_2O_2 stress experiment was performed to study the stress response in loach in order to explore the role of *chop*. The results would further our understanding of the H_2O_2 stress responses and the role of *chop* in fish.

Materials and methods

Fish and sample collections

Loaches were obtained from Baishazhou fish market (Wuhan, China). All loaches were subjected to a ploidy analyzer (Partec, Germany) to determine their ploidy level. Diploid loaches were used and divided into four groups (1 = cloning group, 2 = *chop* expression group, 3 = RNA extraction group, 4 = H_2O_2 stress group). The first group (15 g ± 0.2) was used for cloning *chop*. Three female and three male adult loaches of the second group (15 g ± 0.2) were used for determining expression levels of *chop* in ten tissues including liver, gill, brain, spleen, heart, intestine, muscle, gonad, kidney, and skin. Each fish served as an experimental unit and $n = 6$ (3 females + 3 males). A pair of parents from the third group (20 g ± 0.2) was used for reproduction, and embryos were collected at different early life stages including oosperm, 2-cell stage, 8-cell stage, 32-cell stage, blastula stage, gastrula stage, neurula stage, tail-bud forming stage, muscle effect stage, heart-beating stage, and hatching stage. All samples were frozen at – 80 °C prior

to RNA extraction. The fourth group (5 g ± 0.2) contained 270 individuals and was used for H_2O_2 stress experiment.

Cloning the full-length cDNA of *chop* in loach

Total RNA was isolated from the liver tissues by using RNA isoPlus (TaKaRa, Japan). Quantities and qualities of isolated RNAs were measured by electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Following the method of the SMART™ RACE cDNA amplification kit (Clontech, USA), the RNA obtained was completely reverse-transcribed into cDNA, which was used to clone the gene.

A pair of universal amplified primers was designed (Table S1), depending on the multiple sequence alignments of four species including zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), roughskin sculpin (*Trachidermus fasciatus*), and yellow catfish (*Pseudobagrus fulvidraco*). The PCR program was set as follows: initial denaturing for 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 18 s at 72 °C, and extra elongation for 5 min at 72 °C. The PCR products were separated on 2% agarose (Sangon, China) and then purified with a TaKaRa Agarose Gel DNA Purification Kit ver.2.0 (TaKaRa). After purification, the DNA fragments were ligated into PMD19 (TaKaRa) and colonies were randomly screened, and the positive clones sequenced (*Escherichia coli* was used for cloning) (Invitrogen, China).

Sequence and phylogenetic analysis

Similarity analysis of all the sequences was conducted by BLAST program at the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of loach *chop* were translated into amino acid sequences by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Multiple alignments of the deduced amino acid sequences were conducted by BioEdit software. Other vertebrate *chop* amino acid sequences for alignments and constructing phylogenetic tree were obtained from NCBI and the identities of these sequences were blasted by Blastp (NCBI). The phylogenetic tree of amino acid sequence of *chop* was constructed by MEGA6.0 program using the neighbor-joining method (NJ, bootstrap method: 1000 replications, Arizona State University, USA). The evolutionary distance between loach *chop* and orthologous sequences was calculated by using p-distance and gaps were removed by pairwise detection, using default parameters.

H_2O_2 stress experiment

Two hundred seventy loaches were stocked in nine 30-L white tanks (10 L water volume and 25 ± 0.5 °C water temperature) at a stocking density of 30 fishes/tank. Loaches were respectively treated with 0 μM, 50 μM, and 100 μM H_2O_2 with triplicate for 24 h (Park et al. 2012). During the experiment, there was no feeding and human interference to prevent introducing other stressors. The number of dead individuals in each tank was recorded every 6 h. The data were input into GraphPad Prism 6.0, and the survival rate curves were generated.

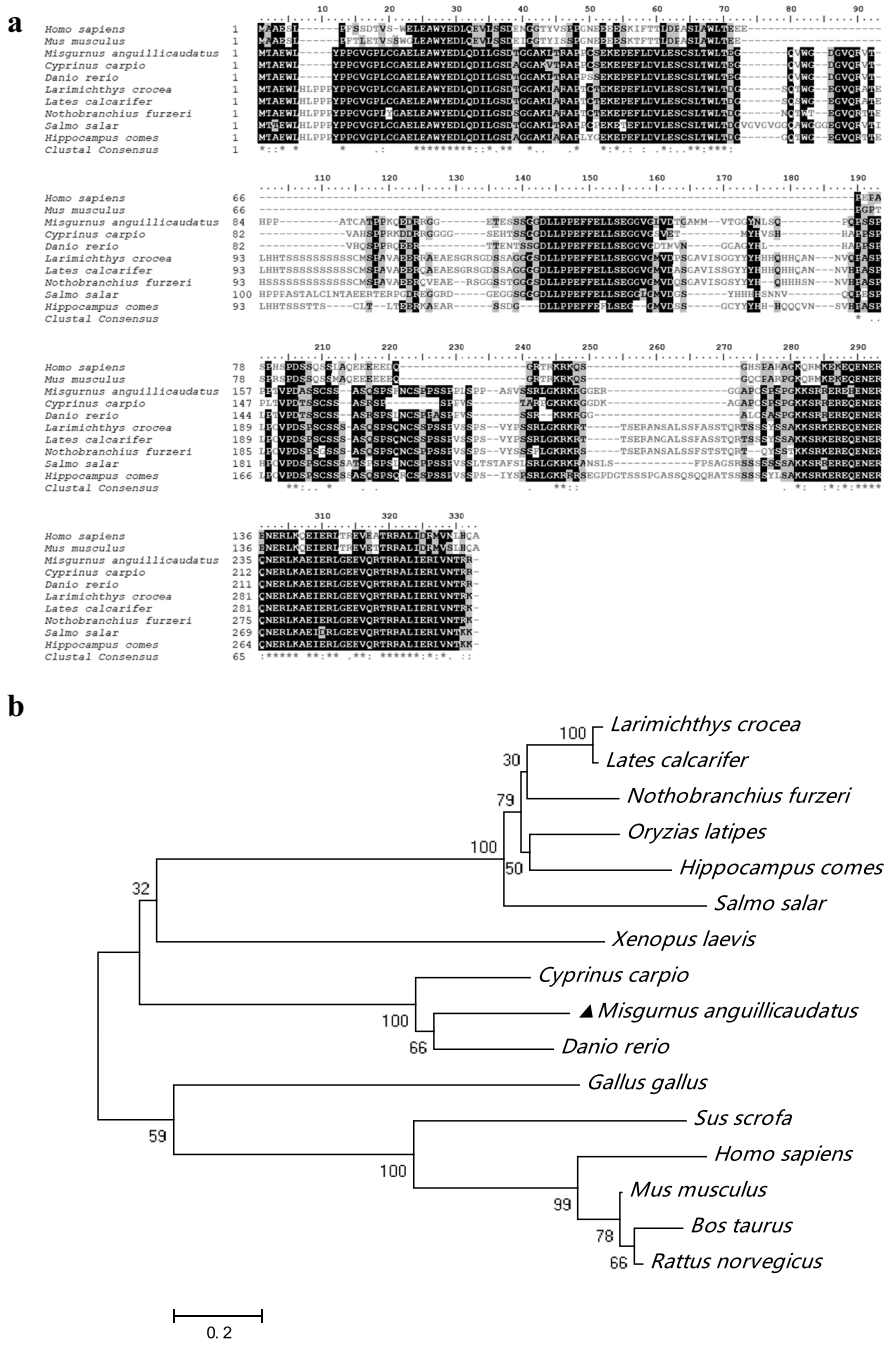
Gill, liver, and spleen tissues were collected from three loaches per tank at 6-, 12-, 18-, and 24-h H_2O_2 stress exposure. All tissues were frozen in liquid nitrogen and stored at – 80 °C for determining catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activities. In addition, gills were also used for detecting expression levels of *chop* and its related genes (*atf4*, *bcl2*, and *bax*).

Real-time quantitative PCR

Quantitative PCR (qPCR) was performed using a MiniOpticon real-time detector (Bio-Rad, Hercules, CA, USA). Specific primers of the target genes and reference genes (*β-actin* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase)) are summarized in Table S1. The relative expressions of the target genes were calculated with the comparative Ct method ($2^{(-\Delta\Delta Ct)}$). All the procedures were based on the methods from our laboratory described by Cui et al. (2018).

Determination of antioxidant enzyme activities

The activities of SOD, GPX, and CAT were estimated by using the commercially available kits (Nanjing Jiancheng Bioengineering Institute, China). The examinations of the three antioxidant enzyme activities were carried out according to the manuals of the kits.



Statistical analysis

The data were expressed as the means ± SD (standard deviation) or SE (standard error). One-way analysis of variance (ANOVA) was conducted by using Tukey's post hoc test in SPSS statistical package version 25.0 (SPSS Inc., USA). Alpha of 0.05 was used to determine significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Gene cloning of *chop* and its sequence analysis in loach

The full-length of loach *chop* cDNA was 2533 bp, containing 801-bp open reading frame (ORF) encoding 266 amino acids, a 217-bp 5′-untranslated region (UTR) and a 1519-bp 3′-untranslated region.

◂**Fig. 1** Alignment and phylogenetic tree of the deduced amino acid sequences of *chop* in *Misgurnus anguillicaudatus* and other species. **a** Alignment of the deduced amino acid sequences of *chop*. The same and similar amino acids were respectively highlighted by black and gray. Black was used to label amino acids with a similarity more than 75% and light gray was to those with a similarity merely more than 50%. **b** Phylogenetic tree of the deduced amino acid sequences of *chop*. The horizontal branch length was proportional to the amino acid substitution rate at each position (the scale was 0.2). The GenBank accession numbers for animals involved in the figure, except for *Misgurnus anguillicaudatus*, were set out as follows: *Homo sapiens* (AAB22646.1), *Mus musculus* (BAE20435.1), *Cyprinus carpio* (KTF75003.1), *Danio rerio* (XP_005166228.1), *Larimichthys crocea* (XP_010730414.2), *Lates calcarifer* (XP_018534443.1), *Nothobranchius furzeri* (XP_015814628.1), *Salmo salar* (XP_014021417.1), *Hippocampus comes* (XP_019716650.1), *Bos taurus* (NP_001071631.1), *Gallus gallus* (AJA72779.1), *Oryzias latipes* (BAL14286.1), *Rattus norvegicus* (NP_001103456.1), *Sus scrofa* (NP_001138317.1), *Xenopus laevis* (NP_001082635.1)

Deduced amino acid sequence of loach *chop* shared high similarity with those of many other species. The putative results indicated that several completely conserved residues might be functionally important (Fig. 1a). The resulting phylogenetic tree of *chop* amino acid sequences demonstrated that the *chop* in loach was grouped into distinct clade similar to higher vertebrate species, and the deduced amino acid sequence of *chop* in loach displayed high similarity to those in *D. rerio* and *C. carpio* (Fig. 1b).

Spatial and temporal expression profiles of *chop* in loach

The expression levels of loach *chop* in different tissues were measured (Fig. 2a). The highest expression of *chop* was found in gill, the second highest in spleen, and the third highest in gonad. The expression levels of loach *chop* in different early life stages were measured as well (Fig. 2b). The expression of *chop* was highest at the 8-cell stage, followed by muscle effect stage and blastula stage (ANOVA, F (between group df 10, within group df 22) = 459.944, $p = 0.000$). There were no significant differences in *chop* expression level among oosperm, 2-cell, 32-cell, gastrula, neurula, tail-bud forming, heart-beating, and hatching stages ($p = 1$). In addition, Tukey's HSD for multiple comparisons found that *chop* expression was significantly different between 8-cell stage and 32-cell stage ($p = 0.000$, 95% CI = [lower bound 915.9650, upper bound 1061.0893]).

H_2O_2 stress experiment

Survival

Mortality was first observed at 12 h of H_2O_2 stress in the 100-μM group. The percentages of death at 12, 18, and 24 h of the 100-μM group were 10%, 25%, and 15%, respectively. After 24 h of H_2O_2 stress, 50% of mortality was observed in the 100-μM group, while no deaths were numbered in other experimental groups (Fig. 3).

The antioxidant enzyme activities

As shown in Fig. 4a, at each time point of H_2O_2 stress, gill CAT activities of loaches from the 100-μM group

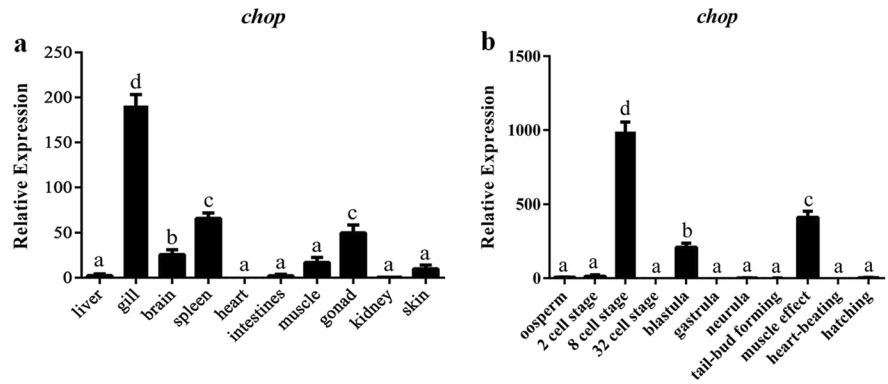


Fig. 2 Expression levels of *chop* in ten different tissues (namely, liver, gill, brain, spleen, heart, intestine, muscle, gonad, kidney, and skin) (**a**) and in eleven different early life stages (namely, oosperm, 2-cell stage, 8-cell stage, 32-cell stage, blastula stage, gastrula stage, neurula stage, tail-bud forming stage, muscle effect stage, heart-beating stage and hatching stage) (**b**) of *Misgurnus anguillicaudatus*, determined by real-time quantitative PCR. Significant differences ($P < 0.05$) were represented by different small letters

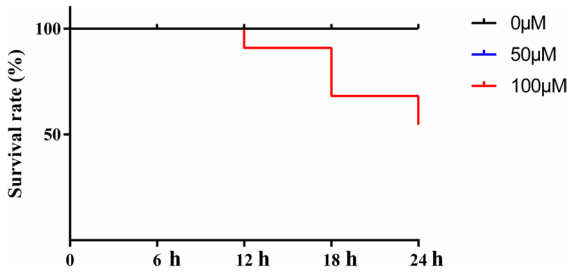


Fig. 3 Survival rates of the loaches under H_2O_2 stress of different concentrations (0 μM, 50 μM, and 100 μM). A total of 10%, 25%, and 15% loaches were dead at the 12, 18, and 24 h of 100 μM H_2O_2 stress, respectively

were significantly lower than those from the 0-μM and 50-μM groups (6 h: ANOVA, F (2, 6) = 93.280, P = 0.000; 12 h: ANOVA, F (2, 6) = 779.163, P = 0.000; 18 h: ANOVA, F (2, 6) = 2339.967, P = 0.000; 24 h: ANOVA, F (2, 6) = 490.376, P = 0.000). Between 12 and 24 h of H_2O_2 stress, loaches from the 50-μM and 100-μM groups presented significantly lower hepatic CAT activities, compared with the control group (12 h: ANOVA, F (2, 6) = 1987.985, P = 0.000; 18 h: ANOVA, F (2, 6) = 4366.507, P = 0.000; 24 h: ANOVA, F (2, 6) = 3504.436, P = 0.000) (Fig. 4b). Figure 4c shows that spleen CAT activities in the 100-μM group were the lowest. The trends of SOD and GPX activities were similar to the CAT activity trend (Fig. 4d–i).

Expression levels of chop and its related genes in gill

Expression levels of *chop* and its related genes *atf4*, *bcl2*, and *bax* in gills of loaches from the 0-μM, 50-μM, and 100-μM groups were analyzed (Fig. 5). The expression levels of *chop* from the 50-μM and 100-μM groups were significantly higher than those from the 0-μM group at 12 (ANOVA, F (2, 6) = 107.228, P = 0.000), 18 (ANOVA, F (2, 6) = 60.674, P = 0.000), and 24 h (ANOVA, F (2, 6) = 191.329, P = 0.000) of H_2O_2 stress (Fig. 5a). The

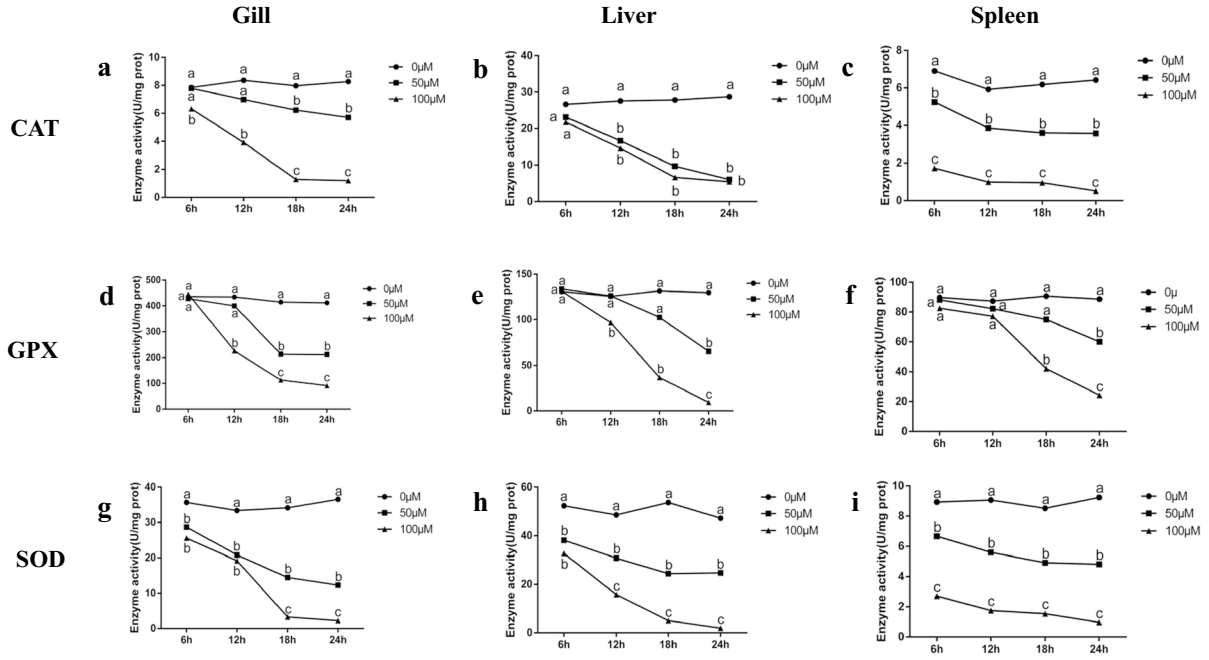


Fig. 4 Determinations of three antioxidant enzyme activities in *Misgurnus anguillicaudatus* under H_2O_2 stress of three different concentrations (0 μM, 50 μM, and 100 μM). Catalase (CAT) activities in gills (**a**), livers (**b**), and spleens (**c**) of the loaches at the 6, 12, 18, and 24 h of H_2O_2 stress; glutathione peroxidase (GPX) activities in gills (**d**), livers (**e**), and spleens (**f**) of the loaches at the 6, 12, 18, and 24 h of H_2O_2 stress; superoxide dismutase (SOD) activities in gills (**g**), livers (**h**), and spleens (**i**) of the loaches at the 6, 12, 18, and 24 h of H_2O_2 stress. Significant differences (P < 0.05) for each antioxidant enzyme activity at each time point among the three different stress groups were represented by different small letters

expression level of *chop* from the 100-μM group was the highest at 24 h of H_2O_2 stress, 10 times of that compared to the 0-μM group.

There were no significant differences in the expression levels of *atf4* and *bax* (two proapoptotic genes) among the three groups (0 μM, 50 μM, and 100 μM) at the 6 h ($p = 0.299$ and 0.259) and 12 h ($p = 0.773$ and 0.505) of H_2O_2 stress (Fig. 5b, d). Similar to *chop*, the expression levels of *atf4* and *bax* from the 100-μM group were the highest at the 24 h of H_2O_2 stress. However, Fig. 5c shows that the expression profile of *bcl2* was opposite to those of *chop*, *atf4*, and *bax*.

Discussion

This study cloned the *chop* gene from an aquaculture animal (namely *M. anguillicaudatus*) for the first time. The multiple alignments and phylogenetic tree of the deduced amino acid sequences of *chop* genes showed that the loach *chop* kept relatively conservative sequences and structural homology compared to other vertebrates. This suggested that *chop* functions were stable during vertebrate evolution (Luethy et al. 1990; Lee et al. 2011). The expression profiles of loach *chop* were also determined. The results showed that loach *chop* was highly expressed during certain embryogenesis periods, consistent with *Xenopus* (Iijima et al. 2003). In addition, the tissue expression analysis showed that *chop* was predominantly expressed in the gill and spleen of loach. It had been previously reported that gills and spleens were parts of the immune system, responding to the organism stress (Dautremepuits et al. 2009; Kocabas et al. 2002). The elevated loach *chop* expression in gills and spleens might indicate its immunological functions related to stress response (Uehara et al. 2014).

To explore the effects of H_2O_2 stress in fish, we treated the loaches with different concentrations of H_2O_2. Our results confirmed that activities of antioxidant enzymes in the loaches had a highly negative

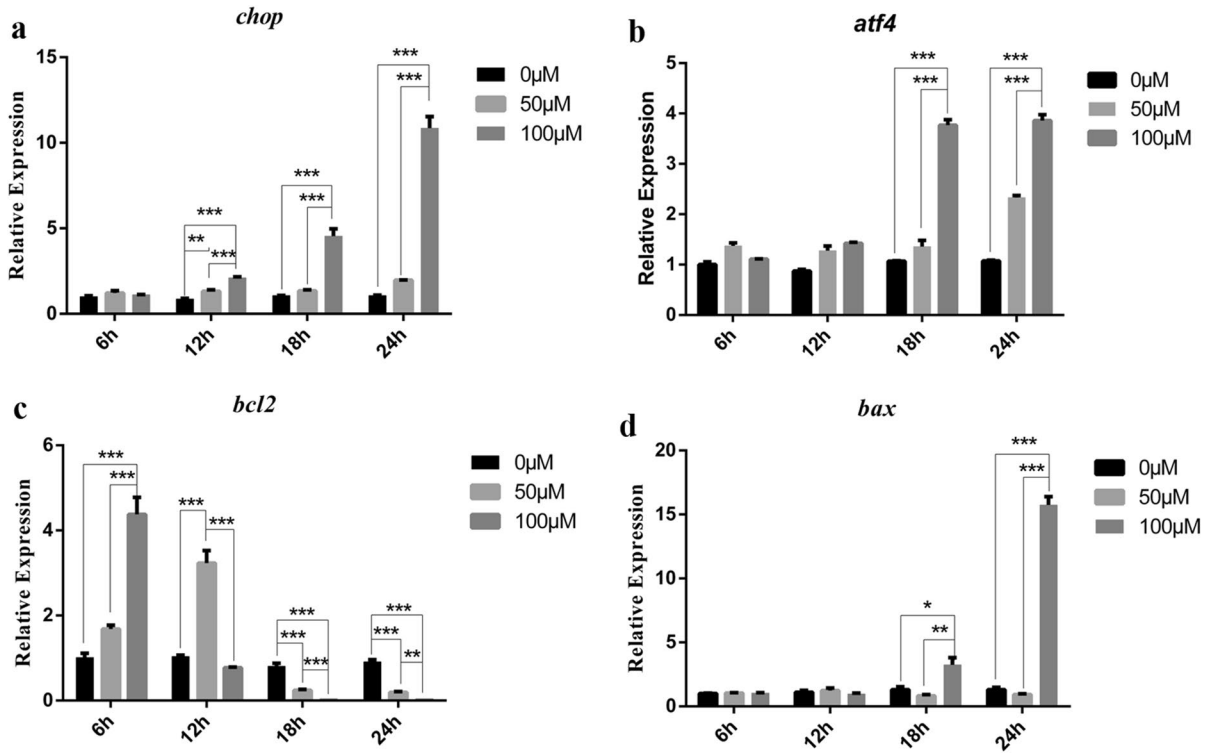


Fig. 5 Expression levels of *chop* (**a**) and its related genes (*atf4* (**b**), *bcl2* (**c**), and *bax* (**d**)) in gills of *Misgurnus anguillicaudatus* under H_2O_2 stress of three different concentrations (0 μM, 50 μM, and 100 μM). The values were expressed as the means ± SEs. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *chop*, C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene; *atf4*, activation of transcription factor-4; *bcl2*, B-cell lymphoma-2; *bax*, BCL2-associated X

correlation with the prolongation of H_2O_2 stress and the increase of H_2O_2 concentrations, similar to the results of other stress studies. For example, when oxidative stress occurred, the activities of SOD and CAT in gills of zebrafish significantly dropped (Jin et al. 2015). Analogous results were also found in mice liver and spleen (Meng et al. 2004; Matsumoto et al. 2002). In addition, oxidative stress reduced GPX activities in gills and livers in black porgy (Chen et al. 2009), and in spleens of zebrafish (Komoike and Matsuoka 2013). In summary, H_2O_2 appeared to induce stress responses in the loach due to decreased antioxidant enzymes expressions.

In the H_2O_2 stress experiment, we analyzed the expression levels of *chop* and its related genes *atf4*, *bcl2*, and *bax* in gills of the loach. We showed that the expression level of *atf4* in the loach surged under high concentration H_2O_2 stress. The expression of loach *chop* was significantly upregulated with increased concentrations and the prolongation of H_2O_2 stress. These results were consistent with previous studies (Teijido and Dejean 2010). When the apoptotic pathway was turned on, under the regulation of a series of upstream factors, the mRNA transcription of *atf4* increased (Cripps and Bergheim 2000). Afterwards, expression of its key downstream transcriptional target *chop* elevated (Barton and Iwama 1991), resulting in the repression of the antiapoptotic gene *bcl2* and the overexpression of proapoptotic gene *bax* (Luethy et al. 1990). A reasonable conclusion was that *chop* might promote apoptosis in the loach under H_2O_2 stress. However, the specific functions of *chop* warranted further studies.

In conclusion, we identified and characterized full-length cDNA of loach *chop* for the first time. Expression profiles of *chop* in different tissues and different early life stages suggested potential immunological functions in loach. The *chop* expression levels in loaches treated with various concentrations of H_2O_2 indicated that loach *chop* expression was sensitive to H_2O_2 stress and that the stress induced apoptosis in loach. Finally, this study provided critical information for further investigation of *chop* functions in fish under H_2O_2 stress.

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Author contribution Xiaojuan Cao and Zhiying Jia designed the experiment. Hui Li, Minxin Kang, Shouxiang Sun, and Jian Gao carried out the experiments. Hui Li and Minxin Kang analyzed the data. The article was written by Hui Li with input from all authors. Xiaojuan Cao contributed to the revision of the manuscript. All authors reviewed and approved the paper.

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Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Ethics approval This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to minimize suffering of the loaches.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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