Full length article

Molecular cloning, characterization and mRNA expression of six peroxiredoxins from Black carp *Mylopharyngodon piceus* in response to lipopolysaccharide challenge or dietary carbohydrate

Chenglong Wu*, Jun'e Gao, Fang Cao, Zhibin Lu, Lian Chen, Jinyun Ye

School of Life Science, Huzhou University, 759 Erhuan Road (E), Huzhou, 313000, PR China

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**A B S T R A C T**

Peroxiredoxin (Prx) belongs to a cellular antioxidant protein family that plays important roles in innate immune function and anti-oxidative capability. In the present study, six Prxs were cloned from Black carp *Mylopharyngodon piceus* (MpPrx) by homology cloning and rapid amplification of cDNA ends (RACE) techniques. There were 199, 197, 250, 260, 189 and 222 amino acids in six MpPrxs, respectively. BLAST analysis reveals that MpPrxs shares high identities and similar characteristics with other known Prxs from animals. The phylogenetic analysis evidenced three major subclasses corresponding to one-Cys-Prx (MpPrx6), typical two-Cys-Prx (MpPrx1-4) and atypical 2-Cys-Prx (MpPrx5) that reflected the present hierarchy of vertebrates and invertebrates. Although six MpPrxs are constitutively expressed in all tissues, relatively higher-level mRNA expression levels of six MpPrxs can be detected in liver, eyes, heart and adipose tissues by real-time PCR assays. The transcriptional patterns of six MpPrxs mRNA in liver were detected by real-time PCR in Black carp after lipopolysaccharide (LPS) challenge and treated with graded levels of dietary carbohydrate (CHO) (106.5, 194.3, 288.4 and 379.1 g kg⁻¹), respectively. These results showed that stimulation with LPS could induce up-expression of six MpPrxs mRNA, and the variations of MpPrx4 were more sensitive than these of other MpPrxs in the liver of Black carp. Compared with those in group with 106.5 g kg⁻¹ dietary CHO, the expression levels of MpPrx2, MpPrx3 and MpPrx6 were significantly down-regulated while MpPrx5 were significantly induced in liver of Black carp fed with adequate dietary CHO (194.3 g kg⁻¹). In addition, significant up-regulations of MpPrx2, MpPrx3 and MpPrx6 were observed in Black carp fed with excessive dietary CHO (379.1 g kg⁻¹). And MpPrx4 could be constantly induced with increasing dietary CHO contents in this study. These results indicated that MpPrxs were constitutive and inducible proteins and might play important roles in innate immune function after LPS challenge and regulating redox homeostasis in the metabolism of dietary CHO.

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1. Introduction

During physiological metabolism process and innate immune defense reaction, aerobic organisms constantly generated kinds of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide anion (O₂⁻ radicals), hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻) and peroxynitrite (ONOO⁻) [1,2]. Although ROS could play important roles in cell proliferation, differentiation, signaling transduction [3,4] and immune functions [5], excessive accumulation of ROS could cause serious oxidative damage to biological macromolecules and then induce various diseases in animals [4,6]. And oxidative stress could cause β-cell apoptosis [7], insulin resistance [8] and the development of diabetes [9]. In order to protect cells against the toxicity caused by ROS, aerobic animals have evolved protective antioxidant systems, such as superoxide dismutase (SOD), catalase (CAT) and peroxiredoxins (Prx) that have been well demonstrated to be involved in scavenging ROS, alleviating or resisting oxidative damage and then maintaining metabolic homeostasis and health [5,7,10,11].

Among them, peroxiredoxins (Prxs: EC 1.11.1.15) are a family of antioxidant enzymes which prevent oxidative damage from ROS, which have been ubiquitously discovered in a variety of prokaryotic and eukaryotic species [12,13]. Prx exhibits
thioredoxin-dependent peroxidase activity and catalyze the reduction of peroxides and alkyl peroxides using thioredoxin as the immediate reducing cofactor [14,15]. According to the number and position of conserved cysteine residues that participated in catalysis, Prxs could be categorized into three subgroups: one-Cys-Prx, typical two-Cys-Prx and atypical two-Cys-Prx [14–16]. The one-Cys-Prx contains only one conserved cysteine residue in N-terminal. The typical two-Cys-Prx contains two conserved cysteine residues in both N- and C-terminal. Although atypical 2-Cys-Prx contains only 1 N-terminal conserved residue; it requires a supplementary non-conserved cysteine residue to form intramolecular disulfide bridge for its catalytic activity [15]. Previous studies have demonstrated the importance of Prxs to the anti-oxidative or immune response as well as the role in protecting animals against pathogen invasions [17–20], food stress [19] and chemical challenges [21]. In addition, higher Prxs levels have positive relationships with suppressing oxidative stress, improving glucose tolerance and insulin sensitivity in human and other mammals [8,22–26].

Black carp Mylopharyngodon piceus is an important freshwater-cultured carnivorous species in China due to its high market value and high market demand [27,28]. However, Black carp culture has suffered serious problems of mortality from malnutrition [27–30] and bacterial disease outbreaks [31]. And their results have shown that both the growth and oxidative/ redox states could be regulated when there were higher contents of cottonseed meal or carbohydrate (CHO) in the feed of Black carp [28,30]. In addition, previous studies also found that oxidative stress could be induced and then the innate immunity were decreased in other fish after infected by parasite [19], bacteria [32–35] and virus [36–39]. Therefore, it is necessary and important to enhance the anti-stress abilities and innate immunity of Black carp. And many studies have proved that nutritional method is a useful and important way to alleviate stresses, improve animal’s antioxidant abilities and enhance the animal’s innate immunity [40]. Although many studies have reported that the antioxidant and immune roles of Prxs in different fish species, there is no report on molecular characterization and corresponding functions of Prxs in Black carp up to now. Considering disease prevention and health aspects of Prxs, it would be useful to investigate the associated roles of Prxs in the innate immune system and metabolic system of Black carp. Therefore, the aims of the present study were to clone and characterize the full-length cDNA sequence of six Prxs from Black carp. Furthermore, to evaluate the expression levels of in various tissues of Black carp, as well as the immune responses to lipopolysaccharide (LPS) challenge and regulative characterization of Prxs in Black carp fed with different levels of dietary CHO, respectively.

2. Materials and methods

2.1. Experimental animals

Healthy Black carp were obtained from a spawning in June 2013 at Fengyi Fisheries Co., Zhejiang, China. Prior to initiation of the experiment, Black carp were acclimated to laboratory conditions (pH 7.1 ± 0.3 at 28 ± 1.5 °C) in 500 L tanks for 2 weeks. And then these Black carp were fed with an artificial feed made by our own lab and daily ration was equal to 3% w/w of the body weight. To minimize possible stress factors, 30 animals per 500 L were maintained during the experiment. Further, laboratory environmental settings were also uniformly maintained during the experiment.

2.2. Immune challenge and dietary CHO induction experiments

To determine the immune responses of six MpPrxs, lipopolysaccharide (LPS) (Sigma, USA) was used as immune-stimulant in time course experiments. For LPS challenge, sixty Black carp (initial body weight: 5.7 ± 0.1 g) were intraperitoneally injected with LPS (5 µg mL⁻¹) suspended in phosphate buffered saline (PBS; 100 µL animal⁻¹). Sixty healthy Black carp were injected with the same amount of PBS and then kept separately as a control group. The liver and serum samples (n = 6 for each time frame) were excised at 0, 2, 4, 8, 12, 24 and 48 h from the challenge and the control group, respectively.

Black carp juveniles (average weight: 1.5 ± 0.05 g) were used to evaluate transcriptional expression levels of MpPrxs after the Black carp were fed with artificial diets containing four levels of dietary CHO (100, 200, 300 and 400 g kg⁻¹). The compositions of the experimental diets are presented in Table 1. And dextrin was used as the sources of dietary CHO. All ingredients were ground into fine powder through 246 µm mesh. All the ingredients were thoroughly mixed with menhaden fish oil, and water was added to produce stuff dough. The dough was then pelleted into proper size (1.8 × 2.0 mm) with an experimental feed mill and dried for 24 h in a ventilated oven at 40 °C. Final CHO concentrations in the feeds were detected to be 106.5, 194.3, 288.4 and 379.1 g kg⁻¹ determined by the phenol-sulfuric acid method after boiling these samples in 0.5 mol/L H₂SO₄ for 1 h [41]. All the diets were sealed in sample bags and stored at −20 °C until use. Prior to initiation of the feeding experiment, all Black carp were acclimated to laboratory conditions and fed the CHO-free diet (0 g kg⁻¹) for 2 weeks. Then four hundred and eighty Black carp juveniles were assigned to a flow-through system using a completely randomized design with three quadruplicate treatments. Each replicate was stocked with 30 Black carp juveniles in acrylic tank (500 L). Each diet was fed to satiation to Black carp twice daily (08:00 and 17:00) for 9 weeks. After feeding, excessive or uneaten diets were removed and weighted to calculate the feed intake at two ours later. Every morning, feces were also removed to maintain water quality. During the experimental period, water temperature ranged from 27.5 to 33.0 °C, pH 6.9–7.3, and dissolved oxygen was not less than 4.5 mg L⁻¹. There were negligible levels of free ammonia and nitrite.

2.3. Sample collection, RNA extraction and cDNA synthesis

Five healthy Black carp were used to isolate and examine the tissue-specific expression levels of MpPrxs. After being anesthetized with ice, the liver, gills, brain, blood, heart, spleen, intestine, head kidney, kidney, skin and muscle were collected. All tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analyses. And all the serum samples were centrifuged at 3000 g for 10 min at 4 °C and the supernatant was collected and then stored at −80 °C for further serum immune index’s analysis. Total RNA was extracted using Trizol Reagent (Invitrogen, USA), spectrophotometrically quantified, and electrophoresed on a 1% denaturing agarose gel to test the integrity. For each reverse transcription (RT) reaction, 3 mg of total RNA was firstly treated with RNase-Free DNase (Takara, Japan) to remove DNA contaminant, and then subjected to cDNA synthesis by SuperScript™ II RT reverse transcriptase (Takara, Japan) in 25 µL volume according to manufacturer’s instructions. Generally, an Oligo (dT)-adaptor primer (Table 1) was used as RT primer to introduce an adaptor. All primers except especially specified were synthesized by Biosune Co. (Shanghai, China).
Both directions with primers M13-47 and M13-48 (Table 2) and these resulting sequences were verified and subjected to cluster analysis in NCBI.

After transforming into the competent cells of Escherichia coli DH5α, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates and confirmed by PCR. Positive clones were sequenced in both directions with primers M13-47 and M13-48 (Table 2) and these resulting sequences were verified and subjected to cluster analysis in NCBI.

To get the 3'-end and 5'-end of the MpPrxs cDNA, gene-specific primers MpPrxs (Table 2) were designed based on the fragment obtained from these homology-based clonings using Primer Premier 5.0, respectively. The 3'-end of MpPrxs were cloned by using specific primer MpPrx 02F and reverse primer AAP. The 5'-end of MpPrxs was cloned by using specific primer MpPrx 02Rs and primer UPM in the first round PCR and MpPrx 02Rs and primer NUP in the second round PCR using 5'-RACE system kit (Invitrogen), respectively. All the PCR products were performed and sequenced following the procedures described above. All the resulting sequences were verified and subjected to cluster analysis.

2.4. Cloning of the six MpPrxs cDNA

The polymerase chain reaction (PCR) to get the fragment of MpPrxs was conducted on an Eppendorf Mastercycler gradient (Eppendorf, German) with degenerate primers MpPrx 01F and 01R (Table 2) designed by using Primer Premier 5.0 (www.premierbiosoft.com) after multiple alignment and phylogenetic comparison by using the Clustal W 1.83 multiple sequence alignment program. These PCR fragments were subjected to electrophoresis on a 1.5% agarose gel for length difference and cloned into the pMD-18T vector (Takara). After transforming into the competent cells of the pMD-18T vector (Takara). After transforming into the competent cells of the pMD-18T vector (Takara). After transforming into the competent cells of the pMD-18T vector (Takara). After transforming into the competent cells of the pMD-18T vector (Takara).

2.5. Sequence analysis and phylogenetic analysis

The cDNA sequence and deduced amino acid sequence of MpPrxs were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The signal peptide was predicted with the SignalP 4.1 server (http://www.cbs.dtu.dk/service/SignalP). The protein domains were revealed by the PROSITE program (http://www.expasy.org/prosite/) and the SMART version 4.0 (http://smart.embl-heidelberg.de/). In addition, the ProtParam program (http://www.expasy.ch/tools/protparam.html) was used to compute physical and chemical parameters of amino acid sequence.

The ClustalW Multiple Alignment program was used to create the multiple sequence alignment. An unrooted phylogenetic tree

### Table 1

Nucleotide sequences of the primers used in this work.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Sequence information</th>
</tr>
</thead>
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<tr>
<td>Olgo(dT)-adaptor</td>
<td>GCCACCCGGCTGCACTAGTACT2s</td>
<td>3' RACE primer</td>
</tr>
<tr>
<td>AAP</td>
<td>GCCACCCGGCTGCACTAC</td>
<td>5' RACE primer</td>
</tr>
<tr>
<td>UPM</td>
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</tr>
<tr>
<td>NUP</td>
<td>AACAGTTGCTATACGCGAGTACGGG</td>
<td>5' RACE primer</td>
</tr>
<tr>
<td>M13-48 (forward)</td>
<td>GACGGGATACCAATTTACACGAGG</td>
<td>Vector primer</td>
</tr>
<tr>
<td>M13-47 (reverse)</td>
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</tr>
<tr>
<td></td>
<td>02F GTGCTCCTTGATTCCTCCTCTG</td>
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<tr>
<td></td>
<td>02R TAGCAAGTGGATCAAGGACGGCG</td>
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</tr>
<tr>
<td></td>
<td>03F CTCATGGATATCCCCACTCTTG</td>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>02F TACGGGATACCAATTTACACGAGG</td>
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</tr>
<tr>
<td></td>
<td>02R TACGGGATACCAATTTACACGAGG</td>
<td>5' RACE primer</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>02F AACAAACGGAGACGGCGCCAGG</td>
<td>3' RACE primer</td>
</tr>
<tr>
<td></td>
<td>02R GCGCGTCCCATCGGGAGACTTCTCGGGACT</td>
<td>5' RACE primer</td>
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<tr>
<td></td>
<td>03F ACTCTCTCCAGGGTCCTCA</td>
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<tr>
<td></td>
<td>03R CATCCGGGCTTTCGGGCTT</td>
<td>Real-time PCR primer</td>
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<td>Prx6</td>
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<td>01R GTCACTGGATCTCCTGCTT</td>
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<td>02F TACGGGATACCAATTTACACGAGG</td>
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<tr>
<td></td>
<td>02R ACTCTCTCCAGGGTCCTCA</td>
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<tr>
<td></td>
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<td>Real-time PCR primer</td>
</tr>
</tbody>
</table>

A,g,R; R,A,C = M; A,T,W; C,T,Y = g,t,K; A,T,C,H = H; g,T,C,B = B; g,A,T,D = D.
was added as template in a 1/C14 position of MpPrxs transcripts by real-time PCR and Ct o9 5/C14 C at the end of reaction. Fluorescent data were acquired during each fluorescent dye.

ANOVA analysis was performed using F-test. The significance level was 0.05 and statistical significance was considered when p < 0.05 [41].

3. Results

3.1. Sequence analysis of the MpPrxs

In the present study, six Prxs were cloned from Black carp (MpPrx) by degenerate primers and RACE techniques. The complete cDNA sequence of six MpPrxs cDNA were 1006, 1073, 981, 953, 935 and 945 bp, respectively. As shown in Table 2, there were 199 (MpPrx1), 197 (MpPrx2), 250 (MpPrx3), 189 (MpPrx4), 189 (MpPrx5) and 222 (MpPrx6) amino acids in length, respectively. The calculated molecular weight of MpPrxs are 22.2, 21.9, 27.3, 29.3, 20.1 and 25 kDa with a theoretical isoelectric point of 6.32, 5.9, 8.9, 7.0, 5.96, 8.36 and 5.8 determined by the ProtParam program, respectively. All these Black carp Prx sequences were introduced in GenBank with accession numbers KP192121 (MpPrx1), KP192122 (MpPrx2), KP192123 (MpPrx3), KP192124 (MpPrx4), KP192125 (MpPrx5) and KP192126 (MpPrx6), respectively.

The amino acid sequence analysis results showed that there were all two classical consensus Val−Cys−Pro (VCP) motifs containing catalytically active Cys residues in MpPrx1-4. And these two VCP motifs were surrounded by two consensus motifs FFYPLDFTFVCP-TEI and GEVCPA, respectively (Fig. 1). And the conserved GGLG and two classical consensus Val Pro (VCP) motifs containing catalytically active Cys residues in MpPrx1-4. And these two VCP motifs were surrounded by two consensus motifs FFYPLDFTFVCP-TEI and GEVCPA, respectively (Fig. 1).

3.2. Homology analysis and phylogenetic analysis of the MpPrxs

BLAST homology analysis yielded close matches of MpPrxs with many other Prxs in fish and other vertebrates. To determine the position of MpPrxs gene in evolution, BLAST results from fish, mammals and invertebrates were analyzed to construct a phylogenetic tree. Based on the phylogenetic analysis, mammalian Prxs and MpPrxs can be classified into six subclasses, named as Prx1, Prx2, Prx3, Prx4, Prx5, and Prx6 (Fig. 2). In addition, MpPrxs could be classified into three subclasses, namely as cys-Prx, typical two-Cys-Prx and atypical 2-Cys-Prx. As expected, MpPrx1-4 belonged to typical two-Cys-Prx, MpPrx5 belonged to one-Cys-Prx and MpPrx6 belonged to atypical 2-Cys-Prx. Furthermore,
there are three subclusters in the Prx5 family. In addition, invertebrate Prx5s from Giant freshwater prawn Macrobrachium rosenbergii, Bay scallop Argopecten irradians and Zhikong scallop Chlamys farreri, appeared to fit more closely to fish Prx5s than mammalian Prx5 subclusters. And the vertebrate Prx5s were diverged from mammalian and fish Prx6 subclusters. Therefore, phylogenetic analysis provided evidence that the MpPrxs had been derived from a common ancestor with other animal and mammal Prxs.

3.3. Expression of MpPrx gene in different tissues

The tissue specific mRNA expression was determined by conducting real-time PCR assay with MpPrx gene specific primers, and the β-actin gene was used as an internal control. Constitutive MpPrxs mRNA expression was detected in all tissues obtained from healthy Black carp without any treatment (Fig. 3). The relative MpPrx1 mRNA expression was significantly higher in liver, kidney, heart, blood and brain than that in gill, muscle, skin and spleen which showed tissue specific variation of MpPrx1 (p < 0.05). And the higher levels of MpPrx2 were observed in liver, heart and blood than that in kidney and spleen (p < 0.01). Relatively higher mRNA expression levels of MpPrx3 were detected in liver, muscle, heart, skin and eye, and lower expression in spleen, kidney, intestine and gill (p < 0.01). The relative MpPrx4 mRNA expression was significantly higher in liver, adipose tissue and eye than that in other tissues (p < 0.05). And the higher mRNA expression levels of MpPrx5 were shown in liver, adipose tissue, eye and brain. Similarly, the higher mRNA expression levels of MpPrx6 were detected in liver, adipose tissue, eye, brain and heart by real-time PCR assays (p < 0.01).

3.4. Expression of MpPrxs in response to LPS challenge

Real-time quantitative PCR was used to investigate the mRNA levels of MpPrx genes in the liver of Black carp after LPS challenge (Fig. 4). The expression levels of MpPrx1 mRNA were significantly up-regulated in the liver at 2, 4, 8, 12, 24 and 48 h post-challenge; values were 4.95, 2.86, 1.14, 0.54, 1.24 and 0.38-fold higher than those of the control group, respectively (Fig. 4A). And LPS challenge could up-regulate the expression levels of MpPrx2 mRNA in the liver of Black carp. The expression levels of MpPrx2 mRNA were significantly up-regulated at 2, 4, 8, 24, and 48 h post-challenge, with values that were 5.11, 6.42, 2.22, 3.37, 1.18 and 1.1-fold higher than those of the control group (p < 0.05) (Fig. 4B). The expression levels of MpPrx3 mRNA were significantly up-regulated in the liver after LPS challenge compared with those of the control group, and the highest level was reached at 4 h post-challenge (Fig. 4C). The amount of MpPrx4 transcripts were drastically induced by LPS, with maximum induction folds reaching 41.27-fold higher at 4 h post-challenge (Fig. 4D). As shown in Fig. 4E, moderate up-regulation of MpPrx5 transcripts was detected in liver at 2 h (0.86-fold), 4 h (1.33-fold), 8 h (0.99-fold), 12 h (0.31-fold), 24 h (0.55-fold) and 48 h (0.84-fold) after the post LPS challenge. The amount of MpPrx6 transcripts firstly increased at 2 h post-injection (1.12-fold compared to time 0 h) and reached to the maximum value at 8 h (1.93-fold, p < 0.05), and then decreased to the original level at 24 h and 48 h post-challenge (p < 0.05) (Fig. 4F).

3.5. Measurement of transcripts and activities of LYZ in response to LPS challenge

The amount of LYZ transcripts were firstly increased at 2 h post-injection and reached to the maximum value at 4 h, and then decreased at 24 h post-challenge (p < 0.05) (Fig. 5A). And the expression levels of LYZ mRNA were significantly up-regulated in the liver of Black carp at 2, 4, 8, 12, 24 and 48 h post-challenge; values were 0.72, 4.62, 2.7, 3.57, 0.96 and 0.4-fold higher than those of the control group, respectively. Similarly, the activities of LYZ in the serum were also constantly increased and reached to highest level at 24 h post-challenge (Fig. 5B). In addition, LYZ activities in the serum after LPS challenge were all significantly higher than these in control groups (p < 0.05).

Fig. 1. Amino acid sequence alignment between six Prxs of Black carp Mylopharyngodon piceus. The signal peptide sequence is double underlined. The conserved cysteine residues are boxed and VCP motifs are underlined. Conserved ‘GGCLC’ and ‘XF’ motifs are in gray boxes. The peroxidase catalytic center (PVCTTE) was in gray and double underlined.
3.6. Expression of MpPrxs in response to different levels of dietary CHO

The relative expression levels of the MpPrx response to dietary CHO are presented in Fig. 6. Although MpPrx1 mRNA levels significantly decreased in liver of Black carp fed with 379.1 g Kg⁻¹ dietary CHO compared with groups fed with 288.4 g kg⁻¹ (Fig. 6A), there were no significant differences between groups fed 106.5, 194.3 and 379.1 g kg⁻¹ dietary CHO (p > 0.05). MpPrx2 mRNA levels were firstly decreased to the lowest level in groups fed with 194.3 g kg⁻¹ dietary CHO and then increased to the highest level in groups fed with 379.1 g kg⁻¹ dietary CHO, and no significant differences could be observed between groups fed with 106.5 and 288.4 g kg⁻¹ dietary CHO (Fig. 6B). Similar results were also shown in MpPrx3, and transcriptions of MpPrx3 gene were significantly 0.54- and 1.13-fold higher in Black carp fed with dietary CHO at 106.5 g kg⁻¹ and 379.1 g kg⁻¹ (p < 0.05) than that in 194.3 g kg⁻¹ dietary CHO (Fig. 6C). However, the transcriptions of MpPrx4 were steadily increased and reached to the highest level in the liver of groups fed with 379.1 g kg⁻¹ dietary CHO (p < 0.05) (Fig. 6D). And the mRNA levels of MpPrx5 were firstly increased to the highest level at 194.3 g kg⁻¹ (p < 0.01), and then decreased with the increasing content of dietary CHO (Fig. 6E). Similar to MpPrx2 and MpPrx3, MpPrx6 mRNA levels were also firstly decreased to the lowest level at 194.3 g kg⁻¹ and then increased with increasing content of dietary CHO (p < 0.05), and there was no significant difference between groups fed with 194.3 and 288.4 g kg⁻¹ dietary CHO (Fig. 6F).

4. Discussion

Peroxiredoxins, a ubiquitous peroxide family and functioning in the elimination of hydrogen peroxide by using thioredoxin as the electron donor, have been demonstrated in a variety of prokaryotic and eukaryotic species [12]. In the present study, six cDNA sequences of peroxiredoxins (MpPrxs) were successfully cloned from Black carp and verified by homology PCR approach and RACE techniques.

In the predicted protein sequences of MpPrx1-4, there are two highly conserved canonical peroxidatic and resolving cysteine residues. The former cysteine residue was embedded within the highly conserved F-motif (FTFVCPTEI) and the latter was adjacent to a highly conserved hydrophobic region (VCPAG/SW) (Fig. 1). These signature features are highly conserved amongst all typical 2-Cys Prxs from all phyla studied to date, including plants, bacteria, fungi, fish, terrestrial vertebrates and mammals [14,19,35,47–49]. As “sensitive” typical 2-Cys Prxs in eukaryotic species, MpPrx1–4 possess two highly conserved CGLG and YF or MF motifs [15,47], which contribute to the regulatory activity for H₂O₂-mediated signal transduction [50]. These two conserved motifs can hinder disulphide bond formation between Cys–S₉SH and Cys–S₉SH, which makes Cys–S₉SH longer lived and therefore be more susceptible to attack by a second peroxide molecule [49]. In addition, there was a predicted signal peptide in the N-terminal of MpPrx4 predicted protein sequence, which indicates MpPrx4 is a secretory characteristic protein and might function within the extracellular [15,51,52]. Two cysteines (C₇₄ and C₇₉) in MpPrx5 were found to be highly conserved in vertebrate Prxs through multiple sequence alignment analysis, which are critical for enzyme function by serving as a catalytic site and a resolving residue [53]. Distinct from other categories of Prxs, atypical 2-Cys Prxs have a C-terminal resolving cysteine located within the same polypeptide chain and form an intramolecular disulfide bond when reaction between the peroxidatic cysteine and the resolving cysteine occurs [54–57]. In addition, only the C₄₇ was conserved with respective Cys belongs to all Prx6 members although there are four cysteines in MpPrx6. Moreover, The AhpC domain, Prx domain, peroxidase catalytic center and the catalytic triads in MpPrx6 also classified it as a member of 1-Cys Prx [15,18,57,58].

To develop the phylogenetic relationship of MpPrxs, an unrooted phylogenetic tree was constructed using 58 vertebrate Prx counterparts. The phylogenetic analysis results evidenced that Prxs of all selected species clustered into three groups that were consistent with typical 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx (Fig. 2). Similar clustering result of Prx genes was also previously described in mammals [59], invertebrates [53,57,58,60] and other teleost fish species, such as gilthead seabream Sparus aurata [19], turbot Scophthalmus maximus [32], ayu Plecoglossus altivelis [33], golden pompano Trachinotus ovatus [35], common carp Cyprinus carpio [39], spotted green pufferfish Tetraodon nigroviridis [48], yellowtail kingfish Seriola lalandi [52], Atlantic salmon Salmo salar [61], channel catfish Ictalurus punctatus [62] and rainbow trout Oncorhynchus mykiss [63,64]. Interestingly, the Prx2 branch of fish was related to the monophyletic Prx1 cluster rather than to Prx2 cluster of mammals, which is consistent with results in gilthead seabream [19]. More importantly, MpPrxs in this study can be classified into six subclusters, named as Prx1, Prx2, Prx3, Prx4, Prx5, and Prx6 family members, respectively, just like these in gilthead seabream [19]. Therefore, the phylogenetic analysis provides evidence that the MpPrxs have been derived from a common ancestor with other Prx family proteins from invertebrates, teleost fish and mammals.

Real-time PCR analysis demonstrated that six MpPrxs were expressed in all tested tissues, which suggests that MpPrxs might play diverse roles in different tissues of Black carp. As the metabolic organ and constantly metabolizing various materials that contains levels of nutrients and toxins, the liver is prone to be exposed to oxidative by-products of metabolic activity and it is the main defense line against oxidative stress caused by toxins, excessive or deficiency nutrients [19,48,65]. Meanwhile, the liver is also closely mediated with the immune response, such as synthesizing a variety of cytokines, identifying and removing pathogens by phagocytosis [66,67]. The highest expression in liver suggested that six MpPrxs are constitutive proteins that might play crucial roles in fish metabolism, antioxidative and immune defense. In addition, the intestine is vulnerable to oxidative stress on account of the constant exposure to ROS induced by oxidized food debris, transition metals, bacterial metabolites, etc [68]. Thus the high expression level of MpPrx1 in intestine suggested that MpPrxs might also act as antioxidant in fish as in mammals [19,48]. Higher expression levels of MpPrx1, 3, 4, 5 and 6 were also found in Black carp brain and eyes that are extremely vulnerable to oxidative damage, which suggested MpPrxs expression patterns could contribute to protect them against oxidative insults [19]. Furthermore, both MpPrx2 and MpPrx3 have higher expression levels in the heart compared with other MpPrxs, which indicated that MpPrx2 and MpPrx3 might play important roles in protecting organs involving energy metabolism from oxidative stress. Interestingly, our results also shown that MpPrxs have higher expression levels in adipose tissues of Black carp, which is contrary to the results in gilthead seabream [19]. Except as the main endocrine tissues, adipose tissue has been considered to dispose the glucose from circulation of the blood system and then to store amounts of lipid, including unsaturated fatty acids [69]. Therefore, the higher levels of MpPrxs in adipose tissue indicated that Prxs might play critical roles in protecting the unsaturated fatty acids from oxidation. The innate immune system is the most important defense line to protect the host from pathogen infection or diseases [67], and their transcript profiles are associated with kind of pathogen-associated pattern molecules. As a powerful stimulator, LPS is known to
Fig. 2. Phylogenetic relationship between the amino acid sequences of MpPrxs and other 52 available Prxs from invertebrates, fish and mammals. Human Homo sapiens (Prx1: NP_002565; Prx2: NP_005800; Prx3: NP_006784; Prx4: NP_006397; Prx5: NP_036226; Prx6: NP_004896); Rat Rattus norvegicus (Prx1: AAH58450; Prx2: AAH58481; Prx3: EDL94585; Prx4: AAH59122; Prx5: AAH78771; Prx6: NP_446028); Common carp Cyprinus carpio (Prx1: BAA32086; Prx2: AAH58481; Prx3: EDL94585; Prx4: AAH59122; Prx5: AAH78771); Zebra fish Danio rerio (Prx1: NP_001013489; Prx2: AAH76347; Prx3: AAH5443; Prx4: AAH5755; Prx5: AAH53617); Catfish Ictalurus furcatus (Prx1: AAU29515; Prx3: ADI78064; Prx4: AAH78771); Flounder Paralichthys olivaceus (Prx1: AAY25400); Sea bream Sparus aurata (Prx1: ADI78064; Prx2: ADI78065; Prx3: ADI78066; Prx4: ADI78067; Prx5: ADI78068; Prx6: ADI78069); Zebra fish Danio rerio (Prx1: NP_001013489; Prx2: AYY21814; Prx3: ABC59169); Black carp Mylopharyngodon piceus (Prx1: AAY21814; Prx2: ABC59169); Spotted green pufferfish Tetraodon nigroviridis (Prx1: AAY21814; Prx2: ABC59169); Black carp Mylopharyngodon piceus (Prx1: ALD62537; Prx2: ALD62538; Prx3: ALD62539; Prx4: ALD62540; Prx5: ALD62541; Prx6: ALD62542); Giant freshwater prawn Macrobrachium rosenbergii (Prx5: AET34923); Chinese mitten crab Eriocheir sinensis (Prx6: AC35639). The tree is based on an alignment corresponding with 58 representative complete Prxs sequences using ClustalW and MEGA (5.0) and the neighbour-joining distance method with the Kirumma two parameter.

Fig. 3. MpPrxs mRNA expression levels were detected in different tissues of Black carp without any treatments by real time-PCR assay and expressed relative to β-actin levels. MpPrxs transcript levels were detected in Adipose tissue (A), blood (Bl), brain (Br), eye (E), gill (G), Heart (H), intestine (I), kidney (K), liver (L), muscle (M), skin (Sk) and spleen (Sp). All values represent the mean ± S.E.M. (n = 4). Bars bearing different letters are significantly different (p < 0.05; Tukey’s test).
stimulate immune cells through the activation of transcription factors resulting in increased proinflammatory responses [70–72]. Meanwhile, LPS can stimulate macrophages to release important cytokine mediators as well as to produce large amounts of nitric oxide (NO) and reactive oxygen species (ROS). Previous studies have shown that Prx 1 gene expression was activated by LPS challenge in mouse [73]. In the present study, all the expression levels of MpPrxs were up-regulated after 12 h post-LPS challenge (Fig. 4), which indicated the higher expression of MpPrxs might serve as an adaptive mechanism to protect cells from oxidative damage induced by LPS [74]. Except as two important antioxidant enzymes, Prx1 and Prx2, known as natural killer enhancing factor (NKEF)-A and NKEF-B, can enhance the cytotoxicity of natural killer cells which are components of the innate immune system [75]. Other researchers have demonstrated that NKEF could be up-regulated by purified pathogen-associated molecular patterns, such as CpG ODNs and poly I:C [76], LPS [77], bacteria [32–35] and virus [36–39]. Therefore, the higher expression of Prx1 and Prx2 after LPS stimulation also indicated their innate immune functions in Black carp.

In addition, it has demonstrated that Prx3 knockdown mice showed the LPS induce oxidative stress [78,79] and an aberrant mitochondrial redox state [80]. In this study, MpPrx3 expression levels could be increased by LPS stimulation compared with that the negative groups, which is similar with previous results in rock bream (Oplegnathus fasciatus) after the challenge of bacteria, rock bream iridovirus (RBIV) and LPS [81]. Similarly, Gilthead sea bream Prx3 was also significantly up regulated after parasitic exposure [19]. Considering these results, it can be concluded that higher MpPrx3 transcription can serve as a major antioxidant enzyme to protect cells against oxidative stress induced by LPS and involve in immune functional role [81–83]. Moreover, higher levels of MpPrx4 were also shown after LPS challenge, which is consistent with other results in mammals, fish and invertebrates infected by
some viral and bacteria [17,84–86]. Most importantly, the variations of MpPrx4 transcription were the biggest among the six MpPrx5s (Fig. 4), which indicated that MpPrx4 was an inducible and sensitive acute-phase protein to LPS stimulation and it could be involved in immune response against bacteria infection [87]. Previous studies have found that the mRNA level of AOE8166, a mammalian Prx5 gene, was increased in rat lung after acute inflammation induced by LPS [54]. Similar up-regulation of MpPrx5 transcripts after LPS stimulation had been found in this study. Thus, it could be inferred that the enhanced expression level of MpPrx5 might be an indicator for the oxidative stress resulting from inflammation induced by LPS. Previous studies have found that Prx6 could be induced in aquatic organisms under various stress factors such as environmental pollutants [88], thermal [89], poly I:C [20], bacteria [18,58] and virus [18,20]. And the recombinant Prx6 could exhibit thiol-dependent antioxidant activity and then protect cells from H2O2-induced oxidative damage or apoptosis [18,20,21]. Furthermore, as a key immune defense effector and index, LYZ has been widely used to reflect the immune defense abilities in animal [40,44,67]. In present study, the transcriptional levels and activities of LYZ in Black carp were constantly increased after LPS challenge compared with these in control groups infected with PBS (Fig. 5), which is consistent with the results in channel catfish infected with Aeromonas hydrophila [90]. And the higher LYZ level has demonstrated the better innate immunity in Black carp [44,90]. Taken together, the higher transcriptional inductions of MpPrx6 indicate that it has anti-oxidative property and is likely to be involved in both cellular maintenance and protective response during innate immune defense.

It is well-known that liver plays a key role in regulating intermediary metabolism in response to nutritional status [65,91–93]. During oxidative phosphorylation in the cellular metabolism, O2− was generated via the reaction of O2 and electrons that escaping from the mitochondrial electron transport complexes I and III [94]. And O2− could be converted to H2O2 by SOD in mitochondria and cytoplasm, respectively. Our previous study has found that there were higher SOD activities in Black carp fed with excessive dietary CHO (379.1 g kg−1), which indicated that amounts of H2O2 were generated and then caused oxidative stress [44]. H2O2 can play important roles in regulating energy metabolism and glucose homeostasis [8,22]. And H2O2 could be efficiently removed by peroxiredoxins due to their high affinity toward H2O2 [12,95]. So the higher levels of MpPrx1 might play roles in removing H2O2 and to maintaining redox homeostasis in the liver of Black carp fed with 288.4 g kg−1 dietary CHO, and the lower level of MpPrx1 might suggested strong oxidative stress was caused by 379.1 g kg−1 dietary CHO. And previous studies also proved that the elevation of ROS was associated with decreased antioxidant capacity in the islet β-cells [96,97], insulin resistance [26,98] and the development of diabetes mellitus [99]. Thus, higher Prx2 and Prx3 expression in some Black carp fed with 379.1 g kg−1 dietary CHO indicated that high levels of Prx2 and Prx3 could protect the cell against oxidative stress via reducing ROS and then improve glucose tolerance and insulin sensitivity [8,22,23]. And the lower levels of MpPrx2 and MpPrx3 might be due to the better cellular redox homeostasis state in Black carp fed with 194.3 g kg−1 dietary CHO.

In addition, the levels of MpPrx4 are constantly increased with increasing of dietary CHO, which is consistent with results in both human patients and animal models of type 2 diabetes [26,98]. Since Prx4 can protect against the metabolic abnormalities leading to type 2 diabetes in mammals, so the up-regulated MpPrx4 levels could suppress oxidative stress and systemic inflammatory signaling, and regulate insulin sensitivity in Black carp [24–26]. And the existence of a dynamic balance of ROS and NO is relevant in the control of contraction in the mitochondria [99]. Optimal and/or low levels of ROS are required for the generation of dynamic balance while low NO limits the balance. Although O2− could be catalysed by Mn-SOD, O2− is more efficient to react with NO and be converted to peroxynitrite (ONOO−) in mitochondria [100]. Our previous study has found that both NO levels and total nitric oxide synthase (t-NOS) activities were higher in groups fed with 194.3 and 288.4 g kg−1 dietary CHO than that in 106.5 and 379.1 g kg−1 dietary CHO [44], which suggested the higher ONOO− contents might be shown in mitochondria. Because Prx5 has more efficient reaction with ONOO− compared with other Prx isoforms [101–103], so the higher Prx5 levels might have pivotal roles in regulating the cellular redox state and acting as important actor in redox signaling during the glucose metabolism in Black carp fed with 194.3 and 288.4 g kg−1 dietary CHO. However, the dynamic balance between O2− and NO could be perturbed due to increased levels of O2− and then the H2O2 contents were also be increased, which resulted to oxidative stress in cells [99]. Moreover, Da Silva-Azevedo et al. have confirmed that Prx6 could be significantly up-regulated in the extensor digitorum longus muscle (EDL) of neuronal nitric oxide synthase (nNOS)-knockout mice [104]. Similarly, elevated levels of H2O2 have also been shown to induce the up-regulation of Prx6 in murine hepatocytes, which contributes physiologically to the homeostasis of ROS [105]. Combined with these results, it is suggested that higher levels of MpPrx6 might play important roles in removing H2O2 and maintaining dynamic balance in fish fed with 106.5 and 379.1 g kg−1 dietary CHO.

In conclusion, six peroxiredoxin (Prx) genes were cloned and
characterized from Black carp. The deduced amino acid sequences showed high identity to the sequence of Prxs from other fish and mammals, which indicated they might also have similar functions. The higher mRNA expressions of MpPrxs were found in liver, eyes and adipose tissues. Variations of expression profiles shown MpPrxs might play important role in the innate immune function after LPS challenge and the redox signaling and homeostasis in Black carp fed with different dietary CHO contents.

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Fig. 6. Relative MpPrxs mRNA levels in liver of Black carp treated with different content of dietary carbohydrate, respectively. MpPrxs mRNA levels were evaluated by real-time quantitative PCR and expressed relative to β-actin levels. All values represent the mean ± S.E.M. (n = 4 replicates, and 4 Black carp per replicate). Bars bearing different letters are significantly different (p < 0.05; Tukey’s test).

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