www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18: 529-537 (2018) DOI: 10.4194/1303-2712-v18_4_04

RESEARCH PAPER

Molecular Characterization of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMGR) in Grass Carp *Ctenopharyngodon idellus* and Its Regulation by Oxidized Fish Oil

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Received 07 January 2017
Accepted 03 August 2017

Abstract

3-Hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR) is a rate-limiting enzyme for cholesterol biosynthesis. The aim of this study was to identify HMGR molecular characterization in grass carp (*Ctenopharyngodon idellus*), and to evaluate regulation of its expression by oxidized fish oil. Specific primers were designed for 3'RACE and 5'RACE to gain HMGR cDNA sequence of grass carp and to clone full-length HMGR cDNA. This cDNA covered 3591 bp, with 2526 bp for the open reading frame (ORF), 77 bp for 5'-untranslated region (UTR) and 988 bp for 3'UTR. ORF encoded a protein consisting of 841 amino acids. The result revealed that HMGR mRNA is predominantly expressed in liver. Multiple alignments and phylogenetic analysis indicated that grass carp HMGR has a high degree of conservation with other fish species. A 10-week feeding trial was conducted to investigate effects of offering diets containing fresh and oxidized fish oil on HMGR expression in liver and intestine of grass carp. The results showed that HMGR expression in fish fed the diet containing oxidized fish oil was significantly higher than fish fed the fresh fish oil after 10, 20, 30 and 70 days of feeding. Serum diamineoxidase (DAO) activity of fish was also increased by oxidized fish oil.

Keywords: HMGR, gene cloning, grass carp, oxidized fish oil.

Introduction

Cholesterol acts as an important precursor for several physiological compounds such as steroid hormones, corticoids and bile acids (Sheen, 2000; Chen *et al.*, 2012a). Although it is not an essential nutrient for fish, it plays important physiological roles. Growth promoting effect of dietary cholesterol has been documented in fish by administration of plant protein or plant oil based diets (Deng *et al.*, 2012; Norambuena *et al.*, 2013). Moreover, it has been reported that intestine damage caused by oxidized fish oil is associated with impairment of cholesterol metabolism (Huang *et al.*, 2015). 3-Hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR) is a rate-limiting enzyme for cholesterol biosynthesis, catalyzing conversion of HMG-CoA to mevalonic acid (Norambuena *et al.*, 2013). It has been reported that HMGR is a good marker of stress response and tissue damage in fish because of its conversed N- and C-terminus portions (Chen *et al.*, 2012a; Gornati *et al.*, 2005). However, to date there is very little available information on fish HMGR.

The aim of the present study was to identify molecular characterization of HMGR cDNA of grass carp (*Ctenopharyngodon idellus*), and to elucidate alterations of HMGR mRNA expression in liver and intestine following feeding grass carp with oxidized fish oil. The results can have important implications for understanding cholesterol metabolism and tissue damage caused by oxidized fish oil in fish.

Materials and Methods

Sample Collection

Fish was obtained from a fish hatchery of Jiaxing (Zhejiang province, China). Fish (weight: 32 ± 1 g) were starved 24 h and then euthanized by 100 mg/L MS-222 (tricaine methanesulfonate; Sigma, USA). Heart, liver, spleen, kidney, muscle, intestine and skin were collected and flash frozen in liquid nitrogen and stored at −80°C for using.

Molecular Cloning

Total RNA was extracted from liver using RNAiso Plus (Takara Co. Ltd, Japan). To avoid genomic DNA amplification, RQ1 Rnase-free Dnase was used to treat RNA samples. Then, the isolated

RNA was later determined by absorbance measures at 260 and 280 nm. According to the high-throughput sequencing in our previous study, partial HMGR fragment was obtained. The gene-specific primers were designed for 3' RACE and 5' RACE (Table 1) according to the sequence information of this fragment. Briefly, outer and inner primers of the 5' and 3' (HMGR-5'outer, HMGR-5'inner, HMGR-3'outer and HMGR-3'inner) were designed for amplification of cDNA ends. The 5'-RACE and 3'-RACE were carried out using a SMART RACE cDNA Amplification Kits (Clontech) according to the manufacturer's instructions as follows: denaturation for 30 s at 94°C, annealing for 30 s at 62°C and extension for 3 min at 72°C. PCR products were cloned into pMD19-T vector for sequencing.

Sequence Analysis

The sequence was predicted by similarity with those of other HMGR in the database using the multiple sequence alignment of BLAST (http://www.ncbi.nlm.nih.gov/blast) and ClustalW (http://www.ebi.ac.uk/clausalw/), and the phylogenetic tree of HMGR gene among animal species was founded by MEGA (6.0) software (http://www.megasoftware.net). ExPASy Proteomics Server (http://www.expasy.org/tools/ protparam.html) was used to calculate physical and chemical parameters for the deduced amino acid sequence.

Quantitative Real-Time PCR (qPCR)

RNA was isolated from samples and the purity and concentration of RNA were measured using a ND-2000 spectrophotometer (NanoDrop 2000, Wilmington, DE, USA). RNA integrity was confirmed by running 1 μg RNA on an ethidium-bromide stained 1.5% agarose gel with 1X TAE buffer. For each sample, 3-μg (0.15 μg/μl) RNA was reverse-transcribed into cDNA using a RevertAid First-Strand Synthesis System (Thermo Scientific, Waltham, MA, USA) for reverse transcription PCR according to the manufacturer's protocol. The qPCR was performed in a total volume of 20 μL, containing 1 μL of each primer (10 μM), 9 μL of the diluted first strand cDNA product and 10 μL of AceQ® qPCR SYBR® Master Mix (Nanjing, China). The qPCR program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 15s and an extension at 60°C for 60s. At the end of each PCR reaction, melting curve analysis will be performed to confirm that only one PCR product was present in these reactions. Expression levels genes were normalized to β-actin using the $2^{-\Delta\Delta CT}$ method. Each sample was analyzed via qPCR in triplicate.

Study of Oxidized Fish Oil on HMGR mRNA Expression

In order to understand effect of oxidized fish oil on HMGR expression, the study designed as the follows:

Feeding trial and sampling

Fish juveniles were obtained from fish Hatchery and transported to Soochow University aquaculture laboratory. Fish were acclimated to experimental conditions for two weeks in an indoor recirculating

Table 1. The primers used for cloning and expression analysis

Primers	Primer sequence(5'-3')
Primers used for RACE	
HMGCR-F1	CATCAGTGTCCCAAAGTACAAGAG
HMGCR-R1	CGGCAGAGCGTCATTCAGT
HMGCR-3'outer	ACCTTCGTCAGCTCGGCTCCAAA
HMGCR-3'inner	GACGCTCTGCCGTTCTTCCTGTTG
HMGCR-5'outer	GAACGGCAGAGCGTCATTCAGTCC
HMGCR-5'inner	GCAACCGTTCCCACGATCACCTC
SMART II A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN (N = A, C, G, or T; V = A, G, or C)
5'-RACE CDS Primer	$(T)_{25}$VN (N = A, C, G, or T; V= A, G, or C)
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Long	CTAATACGACTCACTATAGGGC
Short NUP	AAGCAGTGGTATCAACGCAGAGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Long	CTAATACGACTCACTATAGGGC
Short NUP	AAGCAGTGGTATCAACGCAGAGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Long	CTAATACGACTCACTATAGGGC
Short NUP	AAGCAGTGGTATCAACGCAGAGT
Primers for real-time PCR	
HMGR-F	CAACAGGAAGAACGGCAGAG
HMGCR-R	CATCAGTGTCCCAAAGTACAAGAG
Actin-F	CGTGACATCAAGGAGAAG
Actin-R	GAGTTGAAGGTGGTCTCAT

aquaculture system. After the acclimation, 150 fish (average weight, 1.85±0.02 g) were randomly distributed into six 100-l circular tanks at a density of 25 fish per tank. Triplicate groups of fish were fed for 70 days with one of two experimental diets (Fresh fish oil and oxidized fish oil). During the feeding period, fish were reared under the following conditions: water temperature: 25-27°C; DO: >8 mg/l; pH: 7.2-7.6; photoperiod: 12:12 h (light: dark). At 10, 20, 30 and 70-day of feeding trial, serum, liver and intestine samples were collected for expression analyses.

Measurement of Serum Diamineoxidase (DAO)

At 10, 20, 30 and 70-day of feeding trial, serum samples were collected for analyzing DAO activity. The activity was measured by commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing China).

Histology Study of Liver

Liver samples for light microscopy observation were fixed in 10% buffered formalin, according to Lu *et al*. (2017). Samples were dehydrated in a graded ethanol series, embedded in paraffin, and sectioned with a rotary microtome. The sections were then stained with hematoxylin–eosin for observation.

Determination of HMGR mRNA Expression in Liver and Intestine

At 10, 20, 30 and 70-day of feeding trial, liver and intestine samples were collected for analyzing HMGR mRNA expression. The methods for total RNA extraction, reverse transcription and qPCR were same with 2.2 and 2.4 sections.

Statistical Analysis

Statistical analysis was performed using SPSS 21.0 for Windows. One-way analysis of variance (ANOVA) was used to analyze differences among treatments. Duncan's test was used for the multiple comparisons. The level of significance was set at $P<0.05$. All data were presented as means ± S.E. (standard error of the mean).

Results

The full-length cDNA of HMGR from liver of grass carp (GenBank accession number: KT781905) was obtained. This full-length cDNA covered 3591 bp with 2526 bp of the open reading frame (ORF), 77 bp of 5'-untranslated region (UTR) and 988 bp of 3'UTR. The complete ORF encoded a protein consisting of 841 amino acids with a calculated molecular mass (MM) of 91.4 kDa and isoelectric point of 6.27. Within this protein, hydrophobicity analysis predicts the presence of 5 trans-membrane helices spanning residues 20-37, 57-79, 91-113, 157-179 and 192-214. Furthermore, the N-terminal domain contained a sterol-sensing domain (SSD) locating at residues 61-218. The C-terminal catalytic domain contained an active site (His^{820}) and one phosphorylation site (Ser^{826}) (Figure S1). The amino acid sequence had a high similarity to HMGR of other species (Figure 1).

The phylogenetic analysis among seven fish species and two endotherm based on HMGR amino acid sequences was shown in Figure 2. Tree topology showed that *Ctenopharyngodon idellus* and *Astyanax mexicanus* were in the same group forming a clade, which was distinguished from *Homo sapiens*, *Sus scrofa* and other fish species. *Danio rerio* and *Gobiocypris. rarus* share 100% identical residues.

HMGR was expressed in heart, liver, spleen, kidney, muscle, intestine, and skin (Figure 3). The transcription in liver was the highest, followed by kidney, heart and muscle, intestine, skin and spleen.

HMGR expression of liver and intestine in fish following fresh and oxidized fish oil administration are shown in Figure 4. After 10, 20, 30 and 70 days of feeding, HMGR expression with oxidized fish oil was significantly ($P<0.05$) higher than that of the fresh fish oil group.

Liver of fish fed diet with fresh fish oil had a normal structure (Figure 5) while several abnormalities were found in the group received diet with oxidized fish oil, including lipid vacuolization, irregularly arranged and stricture sinus.

After 10, 20, 30 and 70 days of feeding, serum diamineoxidase (DAO) activity of fish fed oxidized fish oil was significantly higher than that of fish fed fresh fish oil (Figure 6).

Discussions

The full-length cDNA of HMGR was cloned in this study. The result showed 2526 bp-open reading frame (encoding 841 amino acids) including a putative signal peptide of 39 amino acids showing some differences with other fish species. HMGR contains 884 amino acids in *D. rerio* and 885 amino acids in *S. scrofa*. Grass carp HMGR lacks 52 amino acids fragment compared with other animals. Extensive mutagenesis of protein residues often alters the sensitivity to substrate and/or catalytic activity (Pan *et al*., 2002), which implies that grass carp HMGR has different characteristics. According to the alignment of HMGR amino acid sequences between grass carp and other organisms, the N- and C-terminus portions are conserved. The most conserved region contained Sterol-sensing domain (SSD) profile (61-218) and hydroxyl-methylglutaryl-coenzyme A reductases family profile (418-825). Sterol regulatory element-binding proteins have sterol-sensing domain and control cholesterol synthesis (Radhakrishnan, Ikeda, Kwon, Brown, & Goldstein, 2007). Phosphorylation and glycosylation can regulate

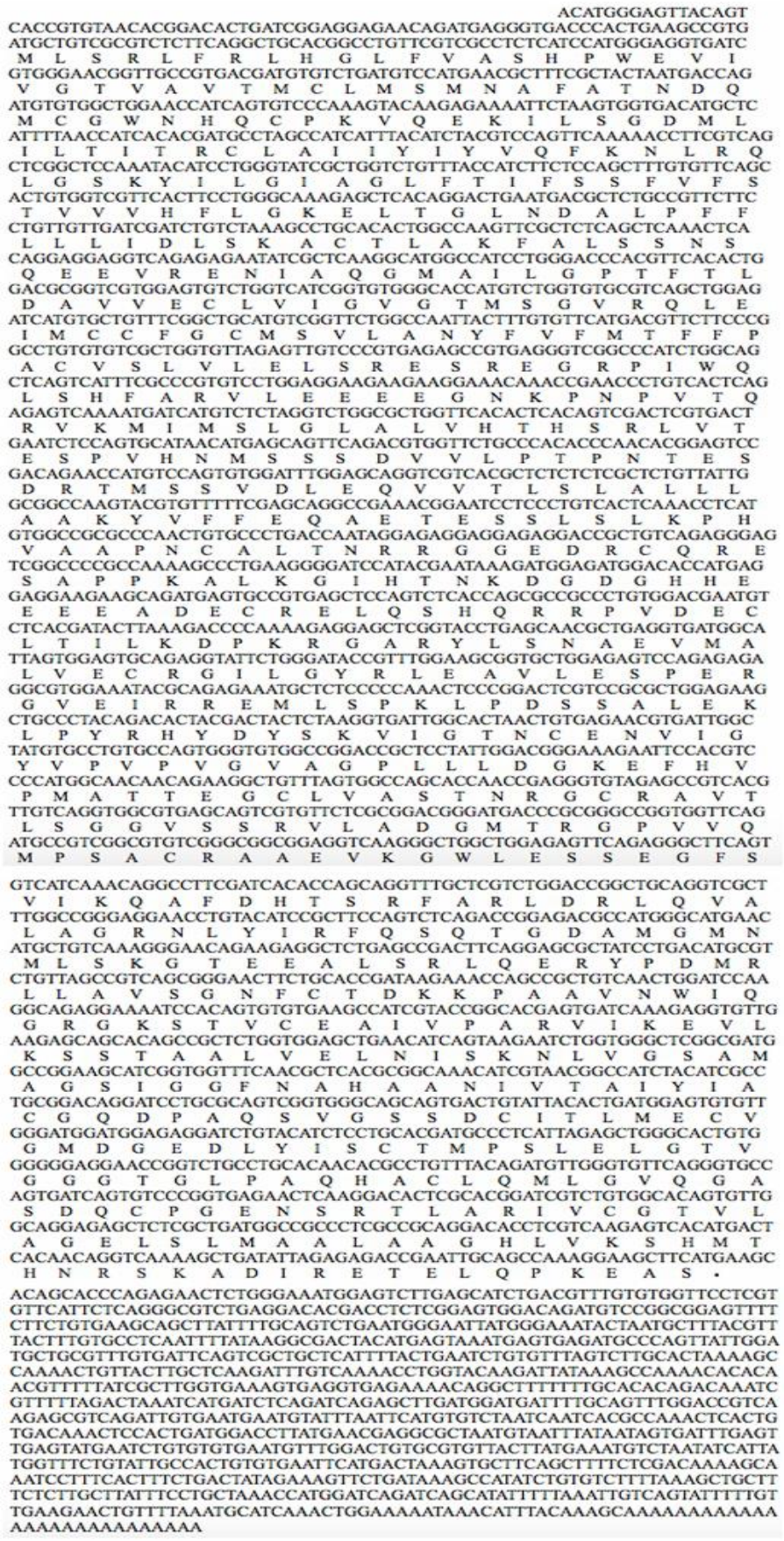

Figure S1. The nucleotide sequence of HMGR gene full-length cDNA and the deduced amino acid sequence of grass carp.

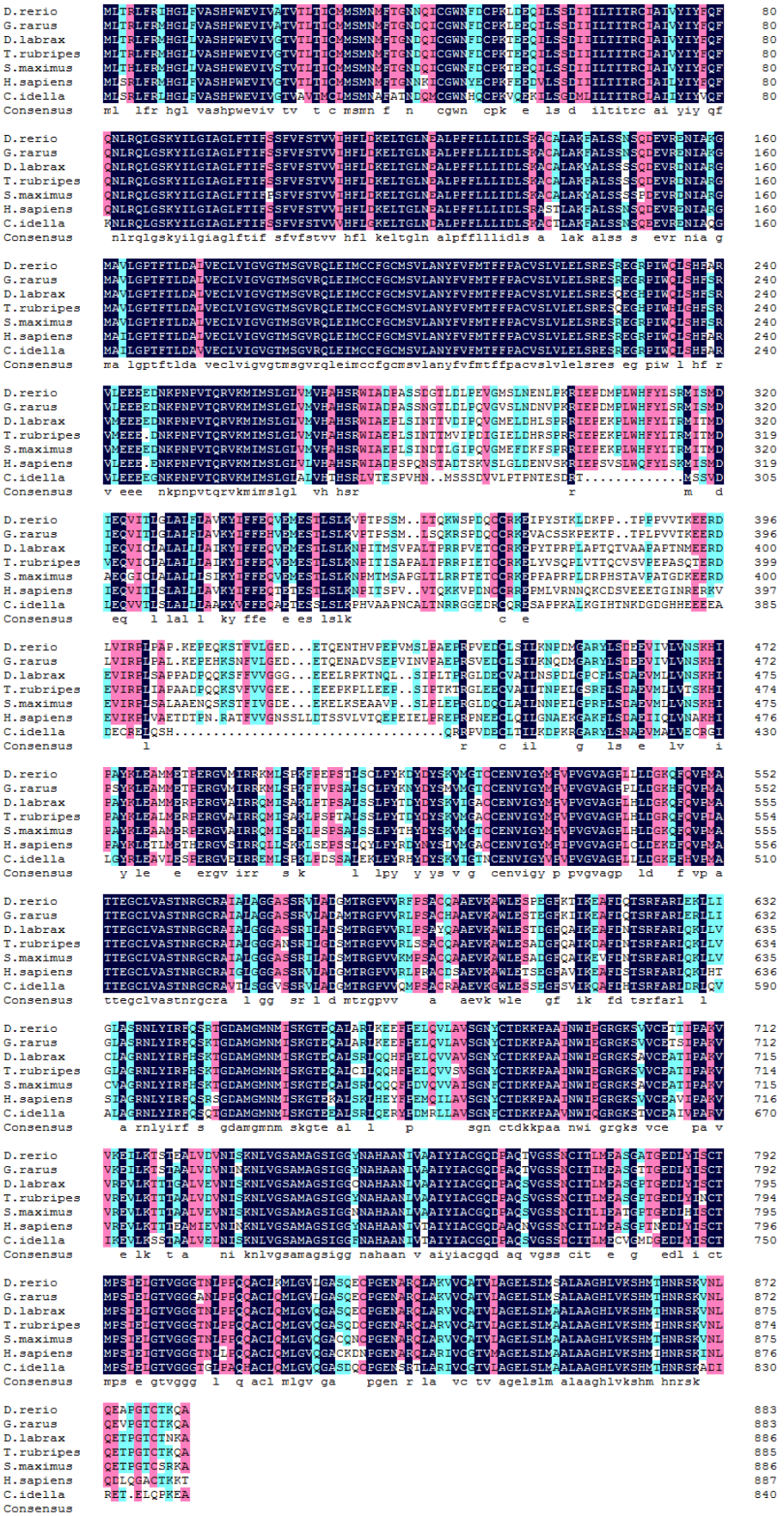

Figure 1. ClustalW alignment of the deduced amino acid sequence of HMGR gene from grass carp and other organisms. Identical amino acids are marked in blue.

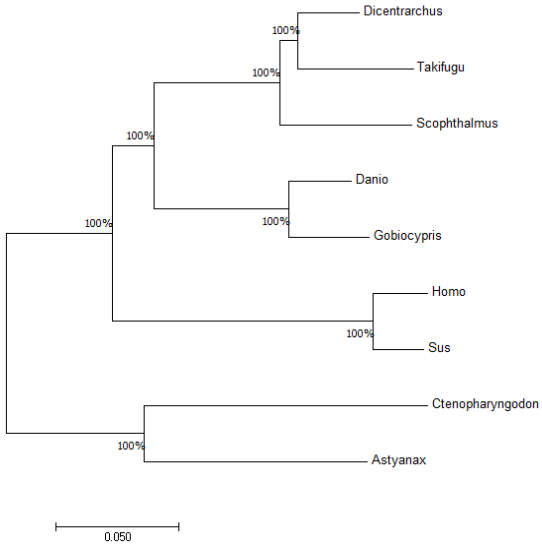

Figure 2. Phylogenetic tree based on HMGR amino acid sequences made with MEGA 3.1 software using the neighbor-joining method.

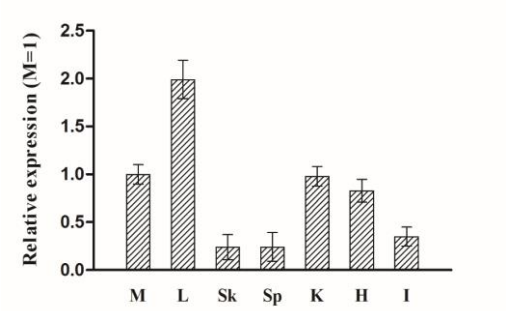

Figure 3. The expression of HMGR mRNA in heart (H), liver (L), spleen (Sp), kidney (K), muscle (M), intestine (I), and skin (Sk).

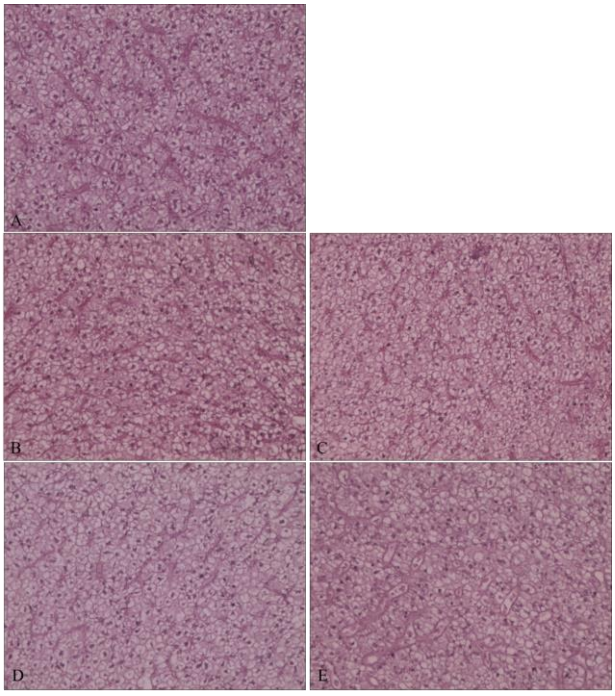

Figure 4. Liver and intestine HMGR mRNA expression of grass carp fed diets with fresh or oxidized fish oil. Bars assigned with asterisk are significantly different ($P < 0.05$). White bars present the fresh fish oil group and black bars are the oxidized fish oil group.

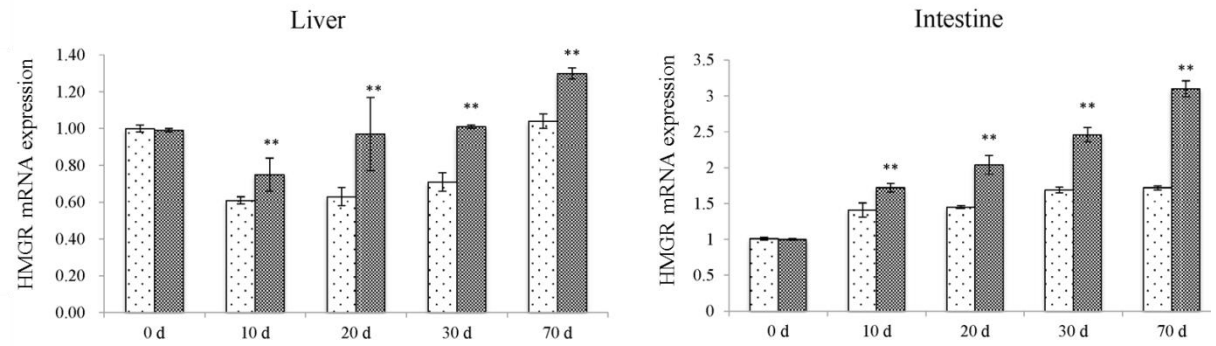

Figure 5. Photomicrographs (magnification × 200) of liver tissue of grass carp. Sampling time point and group: (A) 70 days, the fresh fish oil group, (B) 10 days, the oxidized fish oil group, (C) 20 days, the oxidized fish oil group, (D) 30 days, the oxidized fish oil group, (E) 70 days, the oxidized fish oil group.

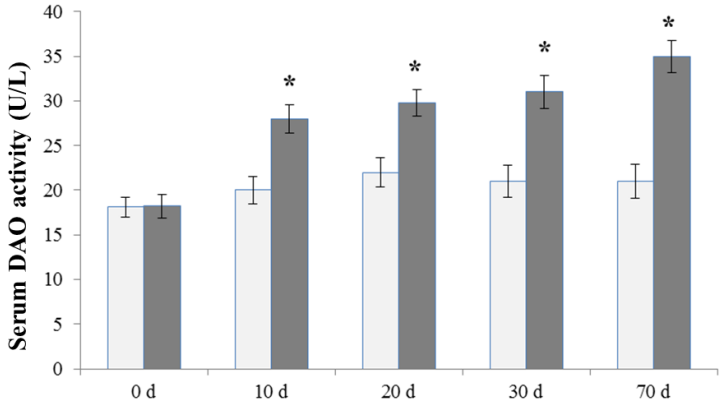

Figure 6. Serum DAO activity of grass carp fed diets with fresh fish oil and oxidized fish oil. Bars assigned with asterisk are significantly different (P<0.05). White bars present the fresh fish oil group and gray bars are the oxidized fish oil group.

proteins function (Clarke & Hardie, 1990). In this study, grass carp HMGR amino acid sequence also contained phosphorylation (Ser^{826}) and N-glycosylation sites ($Asn^{282,\ 686,\ 824}$). HMGR has a conserved phosphorylation site (Ser^{872}) (Clarke & Hardie, 1990) which is primarily phosphorylated by AMP-activated protein kinase (AMPK) (Istvan & Deisenhofer, 2001). Therefore, grass carp HMGR is a downstream target gene of AMPK.

The trans-membrane domain of protein can serve as a membrane receptor for membrane-anchored protein and ion channel protein (Chen *et al*., 2012a). Grass carp HMGR is also a trans-membrane protein (the trans-membrane domain at the N-terminal 20-37, 57-79, 91-113, 157-179 and 192-214, respectively). There are 8 trans-membrane helices of HMGR protein in mammals (Olender & Simon, 1992).

In this study, tissue-specific expression of grass carp HMGR was detected. The result showed that HMGR mRNA was expressed in all examined tissues. The transcription in liver was the highest, followed by kidney, heart, muscle, intestine, skin and spleen, respectively. This further confirmed that liver is the major site of cholesterol synthesis (Romsos, Allee, & Leveille, 1971).

Fish oil is widely used in aquaculture feeds as a rich source of polyunsaturated fatty acids (PUFA) (Izquierdo *et al.*, 2003). However, it is very susceptible to oxidizing, and its oxidization results in formation of primary and secondary products such as lipid hydroperoxides, ketones and aldehydes. These oxidation products negatively affect feeds nutritive value and suppress fish growth (Huang & Huang, 2004; Peng *et al.*, 2009). It has been reported that oxidized fish oil influences cholesterol metabolism leading to cholesterol shortage in cell (Ren & Huo, 2000). The result of current study showed that oxidized fish oil up-regulated HMGR expression. However, cholesterol biosynthesis is a rather expensive metabolic exercise as it requires 18 acetyl-CoA, 18 ATP, 16 NADPH and 4 O_2 molecules to produce 1 molecule of cholesterol (Lunt & Heiden 2011). This is the reason for suppression of fish growth performance by oxidized fish oil. Also, oxidized fish oil could cause oxidative damage in animals (Yuan *et al.*, 2014; Chen *et al.*, 2012b; Lu *et al.*, 2017; Yang *et al.*, 2014). In the present study, profound alterations in liver histology of grass carp were noticed after feeding oxidized fish oil. Due to the conserved role of HMGR, its expression can be a

good marker for detecting fish health (Gornati *et al.*, 2005). HMGR expression could be increased by pathological conditions such as tumors (Hentosh, Yuh, Elson, & Peffley, 2001) and pentachlorophenol (Svensson *et al.*, 2003; Deng *et al.*, 2014). Moreover, enhanced expression of heat shock protein (HSP) and HMGR has been shown after heat stress (Zager & Johnson, 2001) and crowding stress (Gornati *et al.*, 2005). In the present study, up-regulated expression of HMGR by oxidized fish oil was associated with the histological damage of liver.

Serum diamineoxidase (DAO) is a useful marker of intestinal barrier function and its elevated activity is indicative of intestine damage (Li *et al.*, 2000). Increased DAO activity in grass carp fed the oxidized fish oil may imply the damage of intestine. Moreover, DAO activity showed a similar trend with HMGR expression probably indicating that HMGR expression could be used as a marker of tissue damage.

Molecular cloning and characterization of HMGR as well as its tissue-specific mRNA expression were first described in freshwater fish. Multiple alignments and phylogenetic analysis indicated that grass carp HMGR had a high degree of conservation with other fish species. The transcription in liver was the highest, followed by kidney, heart and muscle. Oxidized fish oil induced up-regulation of HMGR expression in liver and intestine. Serum diamineoxidase (DAO) activity of fish was also increased by oxidized fish oil.

Acknowledgments

This works was funded by National Nature Science Foundation of China (No. 31172417/C1904).

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